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AVSUNVIROIDAE FAMILY: VIROIDS CONTAINING HAMMERHEAD RIBOZYMES

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I. INTRODUCTION

A recent review in this series stated that the word "big" is, perhaps, one of the first to come to mind when considering coronaviruses (Lai

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and Cavanagh, 1997). Moving to the other end of the genome size scale, the word "small" is, almost certainly, the first to come to mind when considering viroids. Viroids, independently replicating single-stranded circular RNAs of 246–399 nucleotides (nt) able to infect certain monocot and dicot plants, are currently the lowest well-characterized step of the biological scale. On this basis, viroids have an obvious academic interest, but additionally most of them incite specific diseases in economically important plants such as potato, tomato, cucumber, hop, citrus, grapevine, coconut palm, fruit trees (apple, pear, plum, peach, avocado), and ornamentals (chrysanthemum and *Coleus*). Viroids, therefore, are also agriculturally relevant.

A series of pioneering works (Diener and Raymer, 1967; Diener, 1971b, 1972) led to the discovery of the first member of the group, potato spindle tuber viroid (PSTVd). Those results, supported by others obtained subsequently with citrus exocortis viroid (CEVd), the second member of the group (Semancik and Weathers, 1972a, 1972b), allowed establishment of the viroid concept on firm experimental grounds. Viroids differ from viruses not only in their genome size, but in other fundamental aspects, prominent among which is the lack of messenger activity of both viroid RNAs and their complementary strands. Clearly these observations, like any negative results, should be regarded with care, but the available evidence strongly supports this view: (1) Viroid RNAs are not translated in different *in vitro* (Davies *et al.,* 1974; Hall *et al.,* 1974) and *in vivo* (Semancik *et al.,* 1977) systems; (2) viroid-infected plants have increased accumulation levels of some proteins, but they are pathogenesis-related (PR) proteins coded by the host and not by the viroid genome (Conejero and Semancik, 1977; Conejero *et al.,* 1979); and (3) detailed analysis of viroid sequences have failed to reveal either standard AUG initiation codons (Gross *et al.,* 1978), or conserved open reading frames of a certain length between closely related members of the group (Haseloff and Symons, 1981). Viroids and viruses also have distinct evolutionary origins with a proposal tracing the origin of the former back to the precellular RNA world (Section VIII.B).

Up to 27 different viroid species have been identified and sequenced so far (Flores *et al.,* 1997, 2000). Most of them share a model of five structural–functional domains within the proposed rod-like or quasi-rod-like secondary structure of minimal free energy: C (central), P (pathogenic), V (variable), and TR and TL (terminal right and left, respectively) (Keese and Symons, 1985). The C domain contains a central conserved region (CCR) formed by two sets of conserved nucleotides located in the upper and lower strands, with those of the

upper strand flanked by an inverted repeat. On the basis of the presence of a CCR and the absence of hammerhead self-cleaving domains (see later), 24 viroids are classified within the first family, *Pospiviroidae,* a name derived from that of the type member, PSTVd (Elena *et al.,* 1991; Flores *et al.,* 2000). The remaining three viroids, avocado sunblotch viroid (ASBVd) (Symons, 1981; Hutchins *et al.,* 1986), peach latent mosaic viroid (Hernández and Flores, 1992), and chrysanthemum chlorotic mottle viroid (Navarro and Flores, 1997), do not have a CCR but both polarity strands are endowed with the ability to self-cleave through hammerhead ribozymes. They form the second family, *Avsunviroidae,* whose name also derives from the type member, ASBVd (Flores *et al.,* 2000). This classification is also supported by using phylogenetic reconstructions derived from the whole viroid sequences (Elena *et al.,* 1991; Hernández and Flores, 1992; Flores *et al.,* 2000).

This review focuses on the second viroid family, whose members are also referred to as hammerhead viroids taking into account their most outstanding feature. If the word "small" is the first to come to mind when considering viroids, perhaps the second word is "hammerhead," because this class of ribozymes, which on the basis of its structural simplicity has an enormous biotechnological potential, was initially described in ASBVd (Hutchins *et al.,* 1986), as well as in a viroid-like satellite RNA (Prody *et al.,* 1986). The *Avsunviroidae* family, therefore, presents many interesting features and deserves to be treated individually and in depth.

II. History

A. Avocado Sunblotch Viroid

A disease of the avocados (*Persea americana* Mill.) called sunblotch was first reported in California, although considered to be a physiological stress caused by sunburn (Coit, 1928) or a genetic disorder (Horne, 1929). Sometime later, the infectious nature of the avocado sunblotch (ASB) disease was realized when it was shown that it could be transmitted from affected scions to healthy rootstocks (Horne and Parker, 1931). Ten years later graft transmissibility of the disease was well established and the causal agent considered to be a virus (Horne *et al.,* 1941). Seed transmission was also demonstrated (Wallace and Drake, 1962). Nevertheless, efforts to isolate and characterize the postulated virus were unproductive. For example, virions were never found in electron microscope studies of tissue extracts or thin sections of diseased tissue (Desjardins *et al.,* 1980).

A series of experimental results published in 1979–1980, pointed out the possibility that the causal agent of the ASB disease could be a viroid rather than a virus. Analysis of partially purified nucleic acid extracts of ASB-affected avocados by gel electrophoresis showed the existence of a low molecular weight (8S) RNA, which was not present in similar extracts of healthy plants (Dale and Allen, 1979; Thomas and Mohamed, 1979). Although it was suggested that the 8S RNA species might be a viroid and the causative agent of the disease, infectivity data using this RNA as inoculum were not provided. Examination of purified preparations of the ASB-associated RNA by electron microscopy under partly denaturing conditions showed a mixture of circles and rod-like structures, whereas under completely denaturing conditions, mostly circular molecules were seen (Palukaitis *et al.,* 1979). Therefore, this RNA appeared to be a single-stranded covalently closed circular molecule with a high secondary structure content, in line with its presumed viroid nature. Mechanical transmission of the ASB-associated RNA was accomplished later on using fractionated extracts from fruits with typical ASB symptoms (Desjardins *et al.,* 1980). They showed that the infectious agent was a low molecular weight nucleic acid more concentrated in the high-speed supernatant than in the pellet of an extract prepared with a method noninvolving phenol. Moreover, the agent showed some sensitivity to RNase and a pronounced thermal stability. At the same time, other results showed that the ASB-associated low molecular weight RNA species was soluble in 2*M* LiCl, resistant to RNase digestion in high salt buffers and more sensitive at low salt concentrations (Mohamed and Thomas, 1980). All these properties provided additional support to the viroid hypothesis, which gained an almost definitive momentum when healthy avocado seedlings mechanically inoculated with the purified ASB-associated RNA developed the characteristic disease symptoms (Allen *et al.,* 1981; Utermohlen *et al.,* 1981). The size of the avocado sunblotch viroid (ASBVd) was estimated in 70–72 kDa on the basis of its electrophoretic mobility in denaturing and nondenaturing polyacrylamide gels. However, the lack of sequence similarity between ASBVd and other known viroids, and the ability of ASBVd dimeric transcripts of both polarities to self-cleave *in vitro* to unit-length strands through hammerhead structures—a property shared by some viroid-like satellite RNAs but not by typical viroids—left open for some time the possibility that ASBVd might be a viroid-like satellite RNA. This is especially the case because avocado trees are often infected by what appeared to be one or more seed-transmissible viruses that could potentially act as helper viruses (Sänger, 1988). This possibility was

dismissed when a second viroid, whose polarity strands were also able to self-cleave through hammerhead structures, was discovered (Hernández and Flores, 1992).

Sunblotch is an economically important disease occurring in many of the avocado-producing countries of the world. It reduces the crop yield and lowers the fruit quality. First detected in the United States (Whitsell, 1952), the disease was also reported in other important avocado growing areas such as South America, Australia, South Africa, and the Mediterranean basin including Israel and Spain (Zentmeyer, 1959; Dale *et al.,* 1982; Desjardins, 1987; López-Herrera *et al.,* 1987). The disease agent is transmitted by grafting, including root grafting that can occur naturally (Whitsell, 1952; Wallace, 1958) and, as indicated previously, mechanically, emphasizing the need to disinfect pruning tools (Desjardins *et al.,* 1980). The rate of seed transmission is important, especially in symptomless carrier trees (Wallace and Drake, 1962), which are of special concern for disease control (Section IV.B). The viroid is also pollen-transmitted in field trees at a rate of 1–4%, and both symptomatic and symptomless carrier trees can serve as pollen donors (Desjardins *et al.,* 1984). The sanitary status with respect to ASBVd was originally evaluated by graft transmission to suitable indicator avocado seedlings, but this indexing system is slow because the characteristic symptoms of ASB disease can take up to 2 years to develop in grafted seedlings (Wallace and Drake, 1962). Faster results can be obtained using molecular approaches that include, in order of increasing sensitivity, polyacrylamide gel electrophoresis (Da Graça and Moon, 1983), liquid or dot-blot hybridization with labeled cDNA or cRNA probes (Palukaitis *et al.,* 1981; Allen and Dale, 1981; Rosner *et al.,* 1983; Bar-Joseph, *et al.,* 1985), and RT-PCR (reverse transcription-polymerase chain reaction) using ASBVd-specific primers (Schnell *et al.,* 1997). A particular problem to circumvent is that large variations, up to 10,000-fold, in the ASBVd accumulation levels between different infected trees and even between branches of the same tree, have been found (Palukaitis *et al.,* 1981).

B. Peach Latent Mosaic Viroid

The peach latent mosaic (PLM) disease was initially identified in France in the course of an indexing program aimed at certifying the absence of any virus or virus-like pathogens in the peach material introduced in this country (Desvignes, 1976, 1980). It was estimated that about 30% of the peach cultivars newly imported from the United States, China, and Japan were affected by the PLM disease that was

graft-transmissible. Its high incidence converts this malady into one of the most important affecting peach nowadays. PLM is an epidemic disease that under natural conditions annually spreads in the orchards by up to 5% to any trees surrounding an infected one. The agent of PLM disease does not seem to be transmitted by mites or pollen, but it can be transmitted by the aphid *Myzus persicae,* which appears as a possible vector in the field (Flores *et al.,* 1998).

The causal agent of PLM remained unknown for a long time and was presumed to have a viral nature, as no mycoplasms, fungi, or bacteria could be isolated from infected tissues. However, a synergism with other biotic and abiotic stresses was observed; the PLM-affected trees showed a decrease in resistance to bacteriosis and virosis, as well as to low and high temperatures. Because symptoms of PLM disease may be confused with those caused by other pathogens, a specific biological test was developed in France to detect the inciting agent of the disease (Section VI.A). Some of the characteristics of this agent, although initially suspected to be a virus, suggested the possibility of a viroid etiology. Among those characteristics were, particularly, the heat resistance of the agent and the lack of success in purifying and identifying the presumed viral particles. Another interesting observation was that the minimum contact time required by the inciting agent of the PLM disease to move from the infected piece of tissue serving as inoculum into the receptor plant, a parameter that appears to increase with the size and complexity of the pathogen, was significantly shorter (1–2 days) than that required for different viruses affecting peach (3–4 days) (Boyé and Desvignes, 1986). Initial attempts to determine the nature of the PLM agent were unsuccessful (Monsion *et al.,* 1988), but they established that the infectivity was associated with a low molecular weight nucleic acid fraction, an observation consistent with the involvement of a viroid. Further studies led to the detection of a viroid-like RNA in nucleic acid extracts from peach samples infected with severe and latent isolates of the PLM agent (Flores and Llácer, 1988). Such an RNA was absent in extracts from healthy controls, supporting the association of the PLM disease with the viroid-like molecule. The association was reinforced when the same viroid-like RNA was isolated from trees of 20 peach varieties naturally infected by the PLM agent, but not from the same varieties PLM-free after a thermotherapy treatment.

Confirmation of the viroid etiology of PLM disease was obtained when peach seedlings GF-305 inoculated with a purified preparation of the PLM-associated viroid-like RNA exhibited the characteristic symptoms of the disease. Moreover, an RNA with identical properties

to that used in the inoculum was recovered from these symptom-showing plants (Flores *et al.,* 1990). In consequence, such RNA was named peach latent mosaic viroid (PLMVd). A low accumulation of PLMVd in the infected tissue was observed and, indeed, the amount of viroid RNA purified after two steps of polyacrylamide gel electrophoresis (PAGE) did not exceed 1–2 μg per kg of fresh peach leaves (Flores *et al.,* 1990). The viroid is well distributed in different organs of the infected trees: young shoots, old branches, bark, leaves, roots, fruits, main veins, and stem wood (Flores *et al.,* 1992). However, in agreement with the lack of seed transmission of this pathogen reported previously (Desvignes, 1986), PLMVd has not been detected in seeds.

Molecular characterization of PLMVd involved the preparation of cDNA clones (Hernández and Flores, 1992), which led to the development of a procedure for the rapid, sensitive, and reliable detection of the viroid based on molecular hybridization with specific labeled probes (Ambrós *et al.,* 1995; Loreti *et al.,* 1995). This technique enabled the exploration of the relationships between PLM disease and other maladies affecting peach whose etiological agent was unknown. The PLM disease had been related to peach mosaic (PM), peach calico (PC), and peach blotch (PB) diseases known in the United States for more than 50 years (Hutchins, 1932; Pine, 1976), and to peach yellow mosaic (PYM) disease reported in Japan (Kishi *et al.,* 1973). These disorders share some common characteristics: (1) similar symptoms, (2) the fact that peach is the principal natural host and transmission attempts to herbaceous plants have generally failed, (3) cross-protection between strains of different severity, and, (4) heat resistance. On this basis, it was presumed that they were caused by the same pathogen (Desvignes, 1986). Indeed, once PLMVd clones were available, hybridization experiments showed that PLMVd cRNA probes recognized different PLMVd isolates, as well as isolates from Japanese peach cultivars displaying the characteristic symptoms of PYM disease (Ambrós *et al.,* 1995). These results demonstrated that both PLM and PYM maladies are caused by the same pathogen, PLMVd, confirming previous conclusions based on cross-protection bioassays (Desvignes, 1976, 1986). However, RT-PCR experiments with primers derived from the PLMVd reference sequence showed that PLMVd does not share any similarity with the PM disease agent (Shamloul *et al.,* 1995), which presumably is caused by a virus related to cherry mottle virus (James and Howell, 1998; Gispert *et al.,* 1998). Testing a direct involvement of PLMVd in PC and PB diseases has not been possible because the typical isolates initially described are not available any longer.

From the first detection of PLMVd in peach samples introduced in France (Flores and Llácer, 1988; Flores *et al.,* 1990), the viroid has been reported worldwide in peach trees from Europe, Asia, North and South America, and Australia (Albanese *et al.,* 1992; Shamloul *et al.,* 1995; Skrzeczkowski *et al.,* 1996; Hadidi *et al.,* 1997; Flores *et al.,* 1998; Di Serio *et al.,* 1999). Moreover, a frequent occurrence of PLMVd has been observed in some studies based on an extensive sampling of a given area. For instance, of 50 peach trees of different American varieties grown in Valencia (Spain), 88% were PLMVd-infected (Flores *et al.,* 1992). A more recent study in this same area revealed that 82% of 134 peach and nectarine cultivars, most of them of American origin, were PLMVd-infected, although these high figures may also in part reflect bad nursery practices (Badenes and Llácer, 1998). Similarly, a comprehensive survey of commercial peach and nectarine cultivars collected from Virginia, Colorado, Oregon, California, and Washington showed that from a total of 1000 trees from the field and 291 trees from greenhouses, 50% were infected with PLMVd (Skrzeczkowski *et al.,* 1996).

Owing to the widespread distribution of PLMVd in germplasm, mainly from American and Japanese peach varieties, some work aimed at eliminating the viroid by *in vitro* micrografting, with or without a previous thermotherapy treatment, has been done. Although the effectiveness of the technique seems to be influenced by the variety and the size of the excised apex, a significant fraction of viroid-free plants has been obtained (Desvignes, 1986; Barba *et al.,* 1995). PLMVd can be detected in the greenhouse by a cross-protection bioassay in GF 305 peach seedlings (Section VI.A) and, as mentioned, by several standard laboratory approaches. Combination of rapid PLMVd diagnostic methods with techniques for obtaining viroid-free plants will contribute to control the dissemination and damage caused by this pathogen.

C. Chrysanthemum Chlorotic Mottle Viroid

The chrysanthemum chlorotic mottle (CChM) disease was first reported 30 years ago in commercial greenhouses of the cultivar Yellow Delaware in the state of New York (Dimock and Geissinger, 1969). Soon afterward it was described as induced by a graft-transmissible agent (Dimock *et al.,* 1971). The specific CChM symptoms did not resemble those of other chrysanthemum diseases known to be incited by a fungus, bacterium, or nematode. Attempts to isolate fungi and bacteria from diseased plants were negative, suggesting the involve-

ment of a virus-like pathogen. Mechanical inoculations of healthy chrysanthemum plants of the Yellow Delaware and Deep Ridge cultivars with sap from leaves expressing the typical symptoms of the CChM disease resulted in infection and conspicuous symptoms development, thus discarding ideas of a nutritional imbalance and reinforcing the presumable virus etiology. However, parallel sap inoculations to several herbaceous plants used as diagnostic hosts for three known chrysanthemum viruses were negative, indicating that they were not involved in the CChM disease.

Soon after the description of the CChM disease, biological evidence was presented that supported the involvement of a small-sized RNA consistent with that expected for a viroid. The causual agent was (1) extremely unstable in slightly acidic buffers but considerably more stable in alkaline buffers, (2) sensitive to RNase but not to DNase, and (3) not sedimentable in centrifugal fields up to 120.000 *g*. Rate-zonal centrifugations in sucrose density gradients showed a predominant species sedimenting between 6 and 14S (Romaine and Horst, 1975). In accordance with this proposed viroid etiology, electron microscope studies of thinly sectioned tissues and sap preparations failed to reveal virus-like particles (Dimock *et al.*, 1971). However, repeated attempts to identify a differential RNA associated with the agent of the CChM disease by PAGE were unsuccessful. This could be explained in the case of 5% nondenaturing gels because the infectivity comigrated with the cellular 7S RNA, but gels of lower porosity also failed to reveal a distinct RNA species, and assays to recover infectivity from denaturing gels were also negative (Kawamoto *et al.*, 1985). In cases where such an RNA was reported, it was most probably a contamination of chrysanthemum stunt viroid (CSVd), another viroid already identified in the same host plant. Furthermore, the causal agent of the CChM disease displayed some unusual properties compared with typical viroids, prominent among which was its insolubility in 2 *M* LiCl (Kawamoto *et al.*, 1985), raising some intriguing questions about its structure. Reinforcing the unique nature of this RNA were the results of coinoculation experiments showing that CSVd, CEVd, and a mild and a severe strain of PSTVd, but not the agent of CChM disease, exhibited cross-protection in a variety of combinations (Niblett *et al.*, 1978). Because cross-protection in viruses only occurs between strains of the same or closely related viruses, these results suggested that the agent of CChM disease might be different from the other three known viroids. In any case, although CChM was one of the first diseases presumed to be induced by a viroid, the molecular structure of its causal agent remained an enigma for more than 20 years.

In 1997, by using a combination of two consecutive PAGE steps, the first under nondenaturing gels and the second in long denaturing gels, an RNA was identified that replicated and induced the typical symptoms of the CChM disease when inoculated to chrysanthemum plants (Navarro and Flores, 1997). These results, together with the lack of virus-like particles in the infected tissue (Dimock *et al.,* 1971), showed that this RNA was a viroid, CChMVd, and not a viroid-like satellite RNA functionally dependent on a helper virus. The very low levels at which the linear, and particularly the circular, forms of CChMVd accumulate in infected tissue explain why previous attempts to identify this pathogenic RNA failed.

CChMVd can be detected by bioassay on sensitive chrysanthemum cultivars such as Bonnie Jean, Yellow Delaware, and Deep Ridge grown under defined conditions in the greenhouse or, preferably, in growth chambers (Romaine and Horst, 1975; Navarro and Flores, 1997). The viroid can also be detected by molecular techniques such as dot-blot hybridization (De la Penña *et al.,* 1999) and by RT-PCR with specific primers (De la Peña and Flores, unpublished data).

III. Molecular Structure and Taxonomy

A. Sequence

The primary structures of ASBVd, PLMVd, and CChMVd show limited and scattered regions of similarity between them and the other 24 viroids belonging to the *Pospiviroidae* family. However, the three members of the *Avsunviroidae* family have in both polarity strands the conserved residues and structural elements characteristic of the hammerhead structures present in a number of self-cleaving RNAs (Section III.C).

The 247 nt reference sequence of ASBVd (GenBank accession number J02020) is composed of 68 A (27.5%), 43 C (17.4%), 51 G (20.6%), and 85 U (34.4%), with a G+C content of 38% (Symons, 1981). The 337 nt reference sequence of PLMVd (GenBank accession number M83545) has 80 A (23.7%), 86 C (25.5%), 91 G (27.0%), and 80 U (23.7%), with a G+C content of 52.5% (Hernández and Flores, 1992; Ambrós *et al.,* 1998). The 399 nt reference sequence of CChMVd (GenBank accession number Y14700) consists of 87 A (21.8%), 109 C (27.3%), 112 G (28.1%), and 91 U (22.8%), with a G+C content of 55.4% (Navarro and Flores, 1997). ASBVd, therefore, has an unusually low G+C content compared with PLMVd and CChMVd, as well as with the members of the other family, *Pospiviroidae*. Numerous sequence variants of ASBVd (Pallás

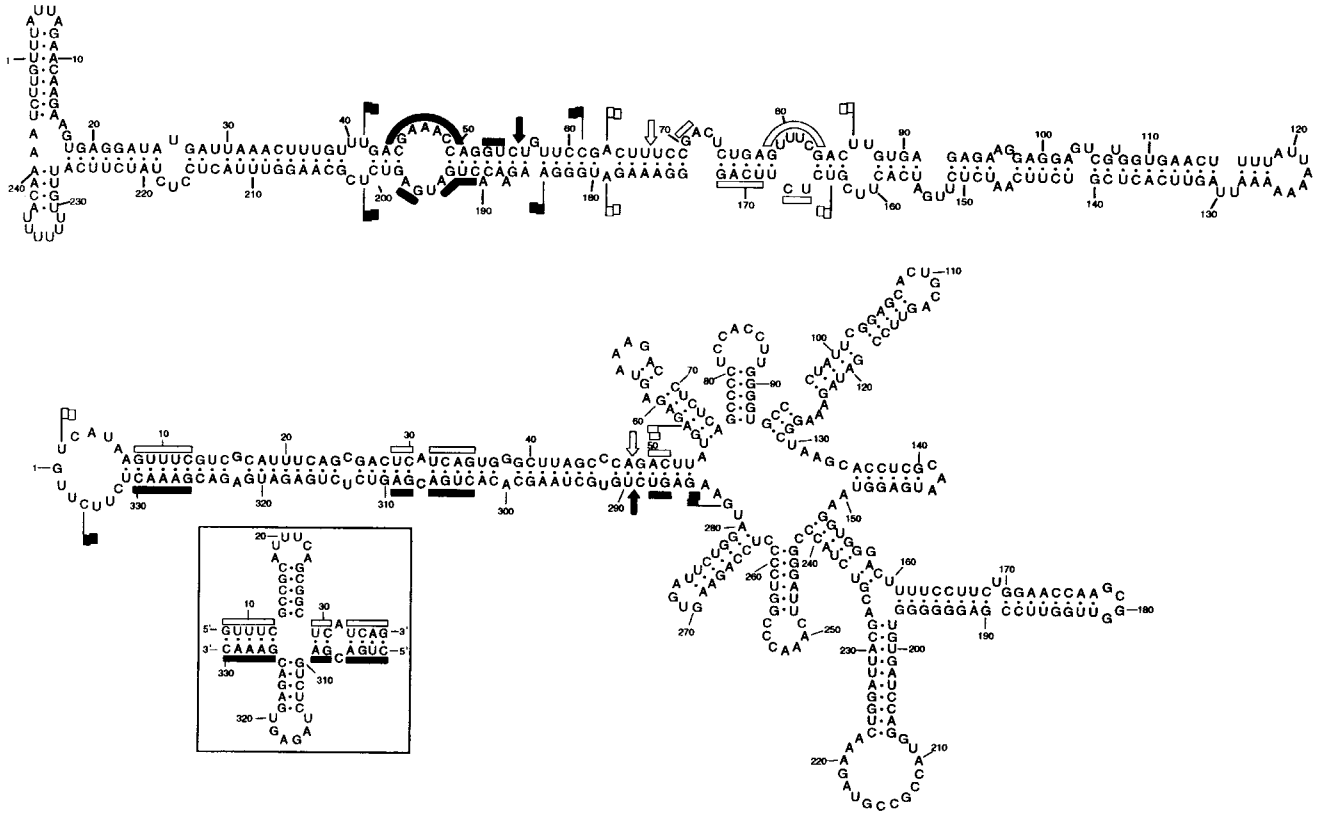
et al., 1988; Rakowski and Symons, 1989; Semancik and Szychowsky, 1994), PLMVd (Hernández and Flores, 1992; Shamloul *et al.,* 1995; Hadidi *et al.,* 1997; Ambrós and Flores, 1998; Ambrós *et al.,* 1998, 1999), and CChMVd (Navarro and Flores, 1997; De la Peña *et al.,* 1999) have also been reported. It is interesting to note that in terms of size ASBVd and CChMVd are located in the lower and upper limits of the viroid scale, respectively, excluding variants of two members of the group with sequence duplications.

B. Proposed Secondary Structure: Rod-Like vs. Branched Conformation

Study of the secondary structures of viroids has been approached on three different levels. *In silico,* studies have determined, with the aid of computer programs such as the well-known MFold (Zuker, 1989), the conformation with the lowest free energy content. *In vitro* studies have inferred viroid conformation in solution by analyzing purified preparations of viroid RNA with a panel of biophysical and biochemical techniques. Examples of biophysical techniques are electrophoresis in polyacrylamide gels, dye binding, hydrodynamic studies, temperature-jump kinetics, and electron microscopy. Examples of biochemical techniques are probing of the primary and secondary RNA structure with base-specific RNases (Riesner *et al.,* 1979; Sänger *et al.,* 1976; Sogo *et al.,* 1973). *In vivo,* studies have assessed how the sequence variability observed for a defined viroid is accommodated by the proposed *in silico* and *in vitro* structures (Section VII.A).

Computer-predicted secondary structures for the three members of the *Avsunviroidae* family show highly base-paired conformations. For PLMVd (Hernández and Flores, 1992; Ambrós *et al.,* 1998) and CChMVd (Navarro and Flores, 1997; De la Peña *et al.,* 1999), these conformations are branched (Fig. 6.1), in contrast to the rod-like or quasi-rod-like structures obtained with the same type of program for typical members of the *Pospiviroidae* family. For ASBVd, a rod-like model of secondary structure was originally proposed (Symons, 1981). Updated computer programs generate a quasi-rod-like structure containing two hairpins at the left-hand terminal domain of the molecule (Fig. 6.1).

The secondary structure predicted for ASBVd contains 70.4% of the residues paired, with 50.6% A-U, 39.1% G-C, and 10.3% G-U pairs (Fig. 6.1). The branched secondary structure proposed for PLMVd has 70.6% of the residues paired, including 38.6% A-U, 49.6% G-C, and 11.8% G-U pairs (Fig. 6.1) (Ambrós *et al.,* 1998), and the branched



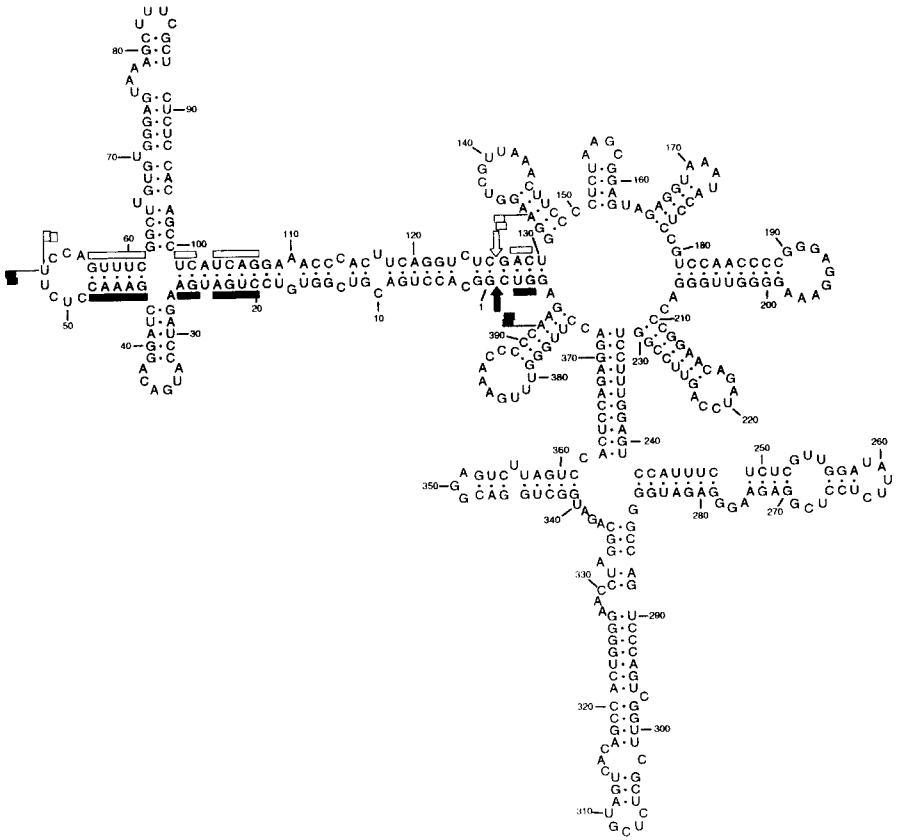


FIG 6.1. From top to bottom, primary and proposed secondary structures of minimum free energy of the reference sequences of ASBVd (Symons, 1981), PLMVd (Hernández and Flores, 1992; Ambrós *et al.*, 1998), and CChMVd (Navarro and Flores, 1997; De la Peña *et al.*, 1999). Regions involved in forming hammerhead structures are flanked by flags, conserved residues present in most natural hammerhead structures are indicated by bars, and self-cleavage sites by arrows with solid and open symbols referring to plus and minus polarities, respectively. Numbering is marked every 10 residues. *Inset:* Alternative cruciform conformation resembling that of CChMVd, which can adopt the hammerhead arm of PLMVd.

secondary structure proposed for CChMVd has 69.7% of the residues paired with a distribution of 35.2% A-U, 56.1% G-C, and 8.6% G-U pairs (Fig. 6.1) (De la Peña *et al.,* 1999). The very different computer-predicted conformations for ASBVd on the one side and for PLMVd and CChMVd on the other side have *in vitro* support: ASBVd is soluble in 2 *M* LiCl, as also are a set of representative members of the *Pospiviroidae* family, whereas PLMVd and CChMVd are insoluble (Navarro and Flores, 1997). Because the solubility of similar sized RNAs in high salt concentrations is presumably governed by conformation rather than by sequence, these results show that the *in vitro* foldings of CChMVd and PLMVd are different from those of other viroids. The most stable secondary structures provide a likely interpretation for this different behavior. Viroids having a compact rod-like or quasi-rod-like structure, including ASBVd, are soluble in 2 *M* LiCl, whereas viroids with a branched conformation are insoluble under the same conditions (Navarro and Flores, 1997). Analysis of sequence variability gives strong support for the *in vivo* existence of such branched conformations in PLMVd (Ambrós *et al.,* 1998, 1999) and, particularly, in CChMVd (De la Peña *et al.,* 1999) (Section VII.A).

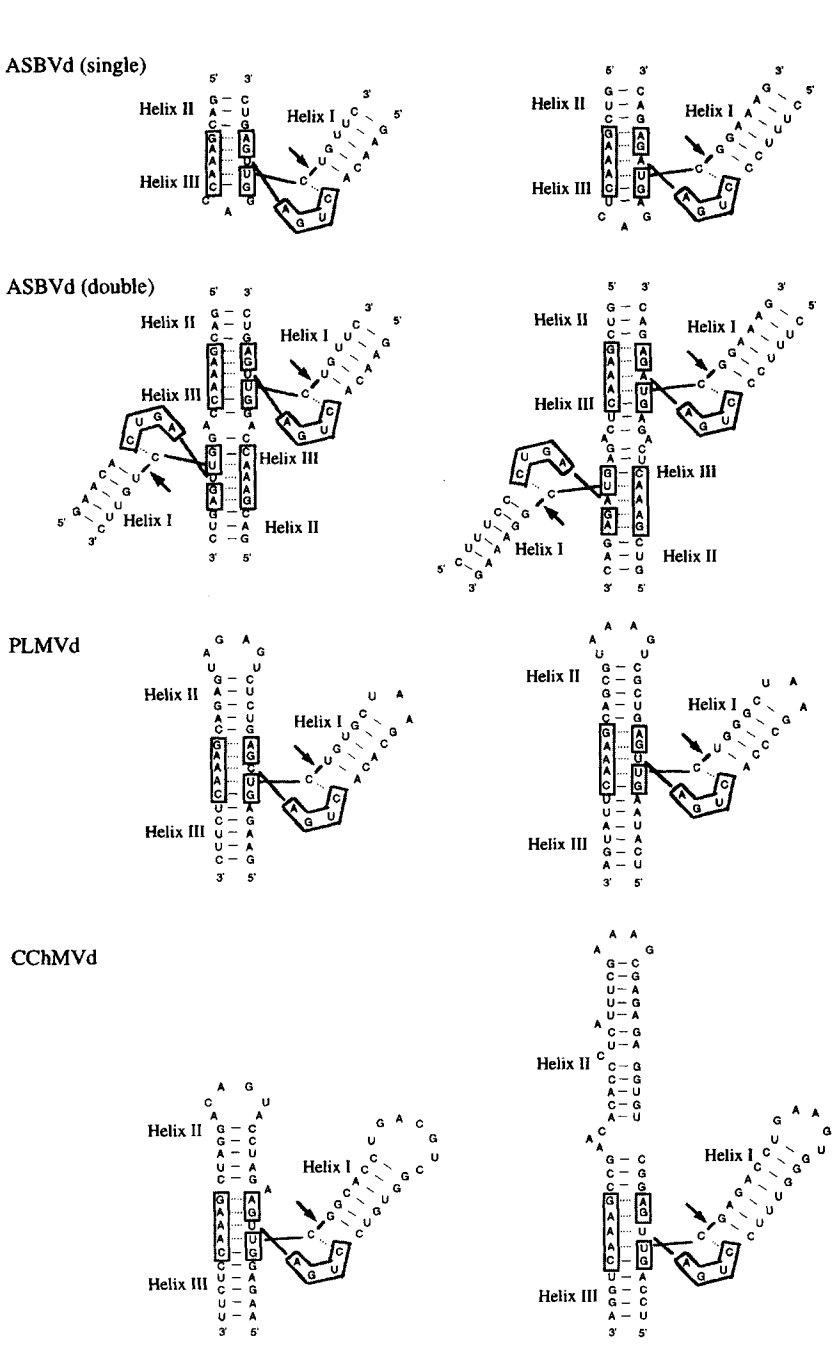
C. Hammerhead Structures

Plus and minus strands of the three members of the *Avsunviroidae* family can adopt hammerhead structures containing the 11 conserved residues and the adjacent helices, which have also been found around the *in vitro* self-cleavage sites of a group of small RNAs, which includes nine viroid-like satellite RNAs of plants (Bruening, 1989; Keese and Symons, 1987; Collins *et al.,* 1998). Hammerhead structures can be formed by the plus strands of three satellite RNAs of nepoviruses: those of tobacco ringspot virus (sTRSV), *Arabis* mosaic virus (sArMV), and chicory yellow mottle virus (sCYMoV). These three satellite RNAs contain hairpin ribozymes in their minus polarity strands (Bruening *et al.,* 1991). Five satellite RNAs of sobemoviruses can also adopt hammerhead structures: the satellite RNA of lucerne transient streak virus (sLTSV) in both polarity strands and those of *Solanum nodiflorum* mottle virus (sSNMoV), velvet tobacco mottle virus (sVTMoV), subterranean clover mottle virus (sSCMoV), and rice yellow mottle virus (sRYMV) in the plus strand. Finally, the satellite RNA of barley yellow dwarf virus (sBYDV), a luteovirus, can also adopt hammerhead structures in both plus and minus strands (Miller *et al.,* 1991). Hammerhead structures have been also reported in the strands of both polarities of two other small circular RNAs: one from

cherry whose biological nature remains to be determined (Di Serio *et al.,* 1997), and another from carnation with the remarkable property of having a homologous DNA counterpart (Hernández *et al.,* 1992; Darós and Flores, 1995) (Section VIII.A). Besides the aforementioned plant RNAs, sequences able to form hammerhead structures have been found in the transcripts of the DNA satellite 2 of six species in four different families of caudate amphibians (Epstein and Gall, 1987; Zhang and Epstein, 1996), and in a satellite DNA of three *Schistosome* species (Ferbeyre *et al.,* 1998).

The six hammerhead structures of members of the *Avsunviroidae* family have been shown to be functional *in vitro* and at least some of them also *in vivo* (Section V.C). They have been represented (Fig. 6.2) according to the three-dimensional structure determined by X-ray crystallography (Pley *et al.,* 1994a), and their helices are named following an established convention (Hertel *et al.,* 1992). ASBVd hammerhead structures have short helices III of two base-pairs with a loop of three bases in the plus polarity, and of three base-pairs with a loop of three bases in the minus polarity (Fig. 6.2). The existence of weak helices III with short closing loops implies that they are thermodynamically unstable, especially that of plus polarity. Alternative and more stable hammerhead structures were proposed for ASBVd, which involve the interaction of the sequences corresponding to two single hammerhead domains to form double-hammerhead structures with extended helices III (Fig. 6.2) (Forster *et al.,* 1988). Experimental data support the idea that a double-hammerhead structure is involved in the self-cleavage of the dimeric plus ASBVd RNA during *in vitro* transcription, as well as after purification of the primary transcript and incubation under standard conditions, whereas the dimeric minus ASBVd RNA self-cleaves by a double-hammerhead structure during transcription but by a single-hammerhead structure after purification (Davies *et al.,* 1991). As in most other natural hammerhead structures, a C residue precedes the self-cleavage sites of plus and minus ASBVd hammerhead structures. Between the conserved CUGA and GA sequences, they have U and A residues in the plus and minus polarity, respectively, conforming also to other natural hammerhead structures where the residue in this position is U or C, and exceptionally A, but never G (Bruening, 1989; Keese and Symons, 1987).

PLMVd plus and minus hammerhead structures have very stable helices III and short loops capping helices I and II (Fig. 6.2). The residue preceding the self-cleavage sites is C, and the residue between the conserved CUGA and GA sequences is C and U in the plus and minus polarity, respectively. Both CChMVd hammerhead structures


PLUS POLARITY
MINUS POLARITY
ASBVd (single)
ASBVd (double)
PLMVd
CChMVd
Helix I
Helix II
Helix III

also have stable helices III and short loops closing helices I and II (Fig. 6.2). However, helix II of the minus CChMVd hammerhead structure is unusually long and composed of shorter helices separated by bulging residues. Moreover, the plus CChMVd hammerhead structure has a bulging A residue in the junction between helix II and the conserved core sequences, a situation reported previously only in the plus hammerhead structures of sLTSV (Forster and Symons, 1987) and sArMV (Kaper *et al.,* 1988), although in these cases the extra residue is U. In both CChMVd hammerhead structures, a C residue precedes the self-cleavage sites and the residue between the conserved CUGA and GA sequences is a U. The region formed by helix I and its capping loop shows extensive sequence identity between both PLMVd hammerhead structures (Hernández and Flores, 1992), and a similar situation is observed between the two hammerhead structures of CChMVd (Navarro and Flores, 1997). Helices III of the plus hammerhead structures of CChMVd and PLMVd also share some sequence similarity (Fig. 6.2).

D. Avsunviroid and Pelamoviroid Genera

The family of hammerhead viroids, *Avsunviroidae,* is presently formed by ASBVd, PLMVd, and CChMVd. No extensive sequence similarities exist between them, apart from the conserved residues forming the core of their hammerhead structures, a peculiarity distinguishing this family from the other family, *Pospiviroidae,* characterized by presenting a set of conserved sequence motifs outstanding among which is a CCR (Section I). In any case, PLMVd and CChMVd are more closely related considering their G+C content, predicted secondary structures of lowest free energy, and morphology of their hammerhead structures, as well as on a physicochemical criterion such as their insolubility in 2 *M* LiCl (Navarro and Flores, 1997). On this basis PLMVd and CChMVd have been grouped in one genus. Because PLMVd is the type species of this genus and conforming to previous rules (Elena *et al.,* 1991), the name *Pelamoviroid* has been proposed.

FIG 6.2. Schematic representation of the hammerhead structures of ASBVd, PLMVd and CChMVd according to the three-dimensional folding obtained for this type of ribozyme by X-ray crystallography (Pley *et al.,* 1994a). Conserved residues present in most natural hammerhead structures are boxed and arrows indicate self-cleavage sites. Watson–Crick basepairs and noncanonical interactions are denoted with continuous and broken lines, respectively. For ASBVd, single- and double-hammerhead structures are represented (Forster *et al.,* 1988).

ASBVd remains a unique viroid forming a monospecific genus, *Avsunviroid* (Flores *et al.,* 2000).

IV. Biological Properties

A. Host Specificity

The only known natural host of ASBVd is avocado *(Persea americana)* (Desjardins, 1987). Because this plant has been found to be naturally infected by PSTVd (Querci *et al.,* 1995) and can also be infected by heterologous grafting with a citrus viroid (Hadas *et al.,* 1992), the specificity of the interaction ASBVd–avocado is apparently not caused by the avocado restricted ability to replicate viroids but rather from the inability of ASBVd to be recognized and multiplied by other plants. In line with this, ASBVd has been experimentally transmitted only to other species of the family *Lauraceae,* to which avocado belongs, including *Cinnamomum camphora, C. zeylanicum,* and *Ocotea bullata* (Da Graça and van Vuuren, 1980).

PLMVd has been naturally found only in peach *(Prunus persica)* and experimentally transmitted to peach hybrids (almond × peach and plum × peach) but not to other *Prunus* species (Desvignes, 1982, 1986). This latter result conflicts with reports indicating that PLMVd can also infect other *Prunus* species apart from peach (Hadidi *et al.,* 1997; Faggioli *et al.,* 1997). Attempts to transmit PLMVd to typical herbaceous hosts of other viroids, including *Gynura aurantiaca,* chrysanthemum, tomato, and cucumber, have been unsuccessful (Flores *et al.,* 1990).

Of 51 species and cultivars tested as potential hosts, including 9 chrysanthemum species and 35 genera, CChMVd was only infectious to two species of chrysanthemum, *Chrysanthemum moriflorum,* now *Dendranthema grandiflora,* and *C. zawadskii,* and noninfectious to 11 species of plants known to support replication of other viroids (Horst, 1987).

From these observations, the common trend emerges that the three members of the *Avsunviroidae* family are host-specific. The situation within the other viroid family, *Pospiviroidae,* is different. Some members such as hop stunt viroid (HSVd) have a wide host range (including hop, cucumber, citrus, grapevine, peach, plum, pear, apricot, pomegranate, banana, raspberry, hibiscus, and croton), but others such as coconut cadang–cadang viroid (CCCVd) have a narrow host range restricted to species of the *Palmae* family (Flores *et al.,* 2000).

B. Symptomatology

Symptoms induced by ASBVd may be observed in different organs of the infected plants, but the viroid may also replicate without apparent damage in the so-called symptomless carrier trees (Desjardins, 1987). Leaf symptoms are sporadic and in more dramatic situations are expressed as intense chlorotic areas associated with the vascular tissue. In other cases, symptoms are expressed as a variegated pattern affecting part or the complete leaf surface, which usually is accompanied by distortions. Stem symptoms, which are frequently the first to appear, include streaks ranging in color from white to yellow and orange, which sometimes are depressed. Bark cracking is also commonly observed in ASBVd-infected trees (Whitsell, 1952), which usually present a stunted appearance. Symptomatic fruits display crater-shaped yellow or pink areas that can cover most of the fruit surface, rendering them unmarketable. The period between inoculation and the onset of symptom development can be shortened by growing the plants at high temperature (Da Graça and Van Vuuren, 1981), but other reports point out additionally that there is the need for continuous light (Desjardins, 1987). Occasionally, some ASBVd-infected trees appear to recover (symptomless carriers) and although they appear visually healthy, they usually have a reduced fruit yield. Interestingly, there is an increase in the rate of seed transmission of the viroid in these symptomless carriers, and progeny seedlings are symptomless carriers themselves and cannot be induced to develop symptoms (Desjardins, 1987).

The term *latent* in the name of the disease incited by PLMVd refers to the observation that most of the natural infections do not induce symptoms in leaves and, when observed, they are unstable and frequently revert to a symptomless condition. The most conspicuous PLMVd symptoms become visible under field conditions 2 years after planting with infected material. These symptoms are delays in foliation, flowering, and ripening; deformations of fruits that usually present cracked sutures and enlarged stones; bud necrosis; and rapid aging of the trees. A yellow mosaic or blotch is very rarely observed on the infected leaves, which in some cases completely covers the leaf area (calico symptoms) (Desvignes, 1986). In the greenhouse, PLMVd natural isolates are divided into severe or latent strains, depending on whether or not, they induce leaf symptoms on seedlings of the peach indicator GF-305 (Desvignes, 1976, 1980).

Symptoms of the CChM disease initially described in natural infections included mild mottling of young leaves, general chlorosis of new

leaves often following mottling of early-formed leaves, dwarfing of leaves, flowers and the entire plant, and delay in blossom development (Dimock *et al.,* 1971). However, because expression of symptoms was erratic, a detailed study with plants experimentally inoculated by implantation of infected tissue was carried out. This study revealed that temperature had a pronounced effect on symptom expression. Under optimal temperature (24° to 27°C) and relatively high light intensities, cuttings of the chrysanthemum cultivars Bonnie Jean and Deep Ridge reacted uniformly to the time (12–15 days) and intensity of symptoms that followed a defined sequence: mild mottling, marked mottling with sharply defined yellow and dark-green areas in the next leaves, and complete chlorosis in the subsequently developing leaves (Dimock *et al.,* 1971; Horst, 1987).

Some foliar symptoms induced in their hosts by ASBVd, PLMVd, and CChMVd are similar, particularly the intense chlorosis that sometimes covers the complete leaf area. This may be related to the chloroplastic localization of ASBVd and PLMVd (Section IV.C), and presumably of CChMVd. Another intriguing common feature of the symptom expression patterns caused by the three members of the *Avsunviroidae* family is the frequent reversion of symptomatic plants to a nonsymptomatic condition, at least as leaves are concerned. This reversion, already mentioned in relation to ASBVd and PLMVd, has also been observed in CChMVd-infected plants that may at one time be chlorotic, and then 1 or 2 weeks later appear momentarily recovered, and finally exhibit severe symptoms (Dimock *et al.,* 1971). These fluctuating patterns may be the consequence of changes in the composition of the corresponding quasispecies distributions of viroid sequences (Section VII.A).

C. Subcellular Interactions and Cytopathic Effects

Experiments combining *in situ* hybridization with electron microscopy have shown that ASBVd accumulates in the chloroplast (Lima *et al.,* 1994), and more specifically in the thylakoid membranes (Bonfiglioli *et al.,* 1994). This is in sharp contrast to PSTVd, the type species of the *Pospiviroidae* family, which accumulates in the nucleus (Diener, 1971a; Harders *et al.,* 1989; Woo *et al.,* 1999), as do other members of this family (Bonfiglioli *et al.,* 1996). Circumstantial evidence supporting a chloroplastic localization for PLMVd has been obtained because this viroid, unlike PSTVd, is not imported into the nucleus of permeabilized tobacco protoplasts (Woo *et al.,* 1999). More recent results show that PLMVd accumulates predominantly in the

chloroplast (Bussière *et al.,* 1999). Finding a viroid in a subcellular compartment does not necessarily imply that replication takes place there, but data on the localization of viroid complementary strands support that PSTVd replicates in the nucleus (Spiesmacher *et al.,* 1983), and ASBVd (Bonfiglioli *et al.,* 1994; Navarro *et al.,* 1999) and PLMVd (Bussière *et al.,* 1999) in the chloroplast. Therefore, although still incomplete, the emerging evidence strongly suggests that the replication and accumulation site is another demarcation criterion for members of both viroid families.

Ultrastructural studies on the cytopathic alterations caused by members of the *Avsunviroidae* family have been reported only for ASBVd. Paramural bodies and grossly disorganized chloroplasts have been observed in the yellow regions of symptomatic avocado leaves, whereas in the green adjacent areas of the same leaves, the chloroplasts displayed a normal appearance; mitochondria and nuclei appeared unaffected (Da Graça and Martin, 1981). In addition to these alterations, bodies composed of large whorls of membrane have been also found in the discolored areas of young leaves (Desjardins, 1987) that resemble those detected previously in PSTVd-infected tomato leaves (Hari, 1980). In other studies carried out with avocado leaves completely chlorotic since emergence, some chloroplasts exhibited gross malformations while others looked similar to proplastids (Desjardins, 1987). Considering the nature of the macroscopic symptoms and that the chloroplast is where replication and accumulation of ASBVd take place, it is not surprising that the cytopathic effects are essentially concentrated in this cellular organelle.

V. Replication Cycle

A. Symmetric Rolling Circle Model

Viroids and viroid-like satellite RNAs, including the human hepatitis delta virus (HDV) RNA, are assumed to replicate through a rolling circle mechanism with exclusively RNA intermediates. All these small pathogenic RNAs have a circular structure, or they go through a circular intermediate during replication. A homologous DNA has never been detected in infected tissues (Zaitlin *et al.,* 1980; Branch and Dickson, 1980), despite some initial reports indicating the contrary. The only exception is the carnation small viroid-like RNA, which has a homologous DNA counterpart (Daròs and Flores, 1995). Initial studies by Northern blot hybridization showed that in infected tissues, in addition to the most abundant infectious RNA to which by convention

is assigned the (+) polarity, other viroid-specific RNAs were also present. These were presumed to play a replicative intermediate role and included CEVd RNAs of the complementary (–) polarity (Grill and Semancik, 1978), and (–) PSTVd RNAs of longer-than-unit length forming part of double-stranded complexes (Branch and Robertson, 1981; Rohde and Sänger, 1981; Owens and Diener, 1982). Oligomeric (+) RNAs were also detected in ASBVd-infected tissue (Bruening *et al.*, 1982). The longer-than-unit nature of the postulated replicative intermediates suggested they resulted from a rolling circle mechanism operating on the initial circular template, and a model of this class was first presented for PSTVd (Branch and Robertson, 1981). The model, similar to that proposed for the replication of the single-stranded circular DNA of the bacteriophage ΦX174 (Gilbert and Dressler, 1968), was originally conceived to explain the replication of some RNA viruses (Brown and Martin, 1965). It was soon expanded to include all viroid-like RNAs and two alternative pathways, asymmetric and symmetric, were considered depending on whether one or two rolling circles operated, respectively (Branch and Robertson, 1984).

Experimental data support that members of the *Pospiviroidae* family follow the asymmetric pathway (Branch *et al.*, 1988; Feldstein *et al.*, 1998), whereas members of the *Avsunviroidae* family follow the alternative symmetric pathway. In this symmetric variant of the rolling circle model (Fig. 6.3), the monomeric (+) circular RNA serves as the template for an RNA polymerase that, after several RNA-RNA transcription rounds, leads to a linear (–) oligomer. This RNA, remarkably, self-cleaves through a hammerhead ribozyme contained in the RNA itself (Sections III.C and V.C). The monomeric (–) linear RNA is then ligated to the circular form that in the second half of the cycle, symmetric to the first half, serves as the template for synthesis of the linear (+) oligomer, which after self-cleavage and circularization gives the final product:—the monomeric (+) circular RNA.

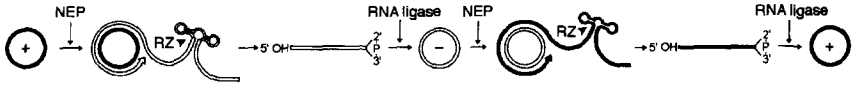


FIG 6.3. Symmetric variant of the rolling circle model proposed for the replication of members of the *Avsunviroidae* family. Solid and open symbols refer to plus and minus polarities, respectively. Self-cleavage sites determined by hammerhead ribozymes (RZ) are denoted by arrowheads, and the 5′-hydroxyl and 2′,3′-cyclic phosphate of the resulting linear monomeric RNAs are also indicated. The enzymatic activities catalyzing RNA polymerization and RNA ligation are only tentative. NEP is an abbreviation for nuclear encoded polymerase, a chloroplastic RNA polymerase.

Northern blot analysis of nucleic acids preparations from ASBVd-infected avocado using probes of both polarities showed the existence of a ladder of viroid-specific RNAs of multimeric size, ranging from the monomer to the octamer for the (+) polarity, but only to the monomer and the dimer for the (–) polarity (Hutchins *et al.,* 1985). In PLMVd-infected peach, monomers and dimers of both polarities have been also detected (Bussière *et al.,* 1999), but in CChMVd-infected chrysanthemum only monomers have been observed (Navarro and Flores, 1997). The oligomeric RNAs most probably accumulate *in vivo* owing to their inefficient self-cleavage. In this respect it is worth mentioning that ASBVd hammerhead ribozymes are efficient *in vitro* only when acting as double-hammerhead structures, particularly that of (+) polarity (Davies *et al.,* 1991), in contrast to those of PLMVd and CChMVd that are active as single-hammerhead structures (Hernández and Flores, 1992; Beaudry *et al.,* 1995; Navarro and Flores, 1997). For ASBVd, monomers and dimers are present in both linear and circular forms (Hutchins *et al.,* 1985; Daròs *et al.,* 1994), indicating that dimeric linear RNAs can also be ligated to their circular counterparts to possibly serve as templates in the rolling circle replication. In infected tissue, ASBVd (+) strands are more concentrated than (–) strands, and for monomers and dimers the circular forms are more abundant than the linears (Hutchins *et al.,* 1985; Daròs *et al.,* 1994). However, in PLMVd-infected peach both (+) and (–) strands accumulate *in vivo* at comparable concentrations, but the linear is more abundant than the circular (Bussière *et al.,* 1999). An intermediate situation is observed in CChMVd: (+) strands accumulate *in vivo* at higher levels than (–) strands, as in ASBVd; but the linear monomers are more abundant than the corresponding circular forms, as in PLMVd (Navarro and Flores, 1997).

The key molecule that demarcates the two pathways of the rolling circle model is the viroid monomeric (–) circular RNA, which is an intermediate only in the symmetric pathway where it acts as the initial template of the second half of the replication cycle. The presence of this molecule in ASBVd-infected avocado was proposed in the first Northern blot hybridizations (Bruening *et al.,* 1982; Hutchins *et al.,* 1985), although because these experiments were made under denaturing conditions where the monomeric (+) and (–) circular RNAs comigrate, it was difficult to conclude the existence of the (–) form in the presence of a high excess of the (+) form owing to the extensive self-complementarity of the viroid molecule. More conclusive evidence supporting the existence of the monomeric (–) circular RNA was obtained when nucleic acid preparations from ASBVd-infected tissue were sepa-

rated under nondenaturing conditions and then subjected to Northern blot hybridization (Daròs *et al.,* 1994). These conditions allowed the detection of two multistranded complexes containing monomeric circular ASBVd RNAs of both polarities in similar proportions.

The ASBVd multistranded complexes are most likely replicative intermediates in the symmetric rolling circle mechanism (Daròs *et al.,* 1994; Navarro *et al.,* 1999). They are resistant to RNase degradation in high ionic strength, indicating a high content of double-stranded RNA. The major constituents of both complexes are the monomeric circular and linear ASBVd RNAs of both polarities, but the slower migrating complex also contains multimeric ASBVd RNAs of both polarities (Navarro *et al.,* 1999). Therefore, they seem to be composed by the monomeric circular (+) and (–) templates bound to the nascent ASBVd RNAs of the complementary polarity. Detection of the PLMVd monomeric (–) circular RNA in infected tissue does not present technical problems because an important imbalance between (+) and (–) strands does not exist (Bussière *et al.,* 1999). This is not the situation with CChMVd in which results indicating the *in vivo* existence of the viroid monomeric (–) circular forms (Navarro and Flores, 1997) need further confirmation.

B. Initiation and Elongation of RNA Strands

As indicated previously (Section IV.C), ASBVd replicates and accumulates in the chloroplast. The available genetic and biochemical evidence supports the existence of two DNA-dependent RNA polymerases with different specificity in plastids (see for a review Stern *et al.,* 1997). One has a multisubunit core similar to that of the eubacterial RNA polymerase and, because the homologs of the genes for the α, β, and β′ subunits of the *Escherichia coli* RNA polymerase have been identified in the plastid genome (Sugiura, 1992), it has been termed the plastid-encoded polymerase (PEP). A set of σ-like factors encoded in the nucleus provides PEP with the specificity to recognize promoters similar to those of *Escherichia coli* RNA polymerase with –35 and –10 consensus sequences (Tanaka *et al.,* 1996, 1997). Additionally, plastids contain a single-subunit nuclear-encoded polymerase (NEP) similar to bacteriophage T3, T7, and SP6 RNA polymerases (Lerbs-Mache, 1993) that initiates transcription from nonconsensus promoters (Allison *et al.,* 1996; Kapoor *et al.,* 1997; Vera and Sugiura, 1995; Vera *et al.,* 1996). The gene presumably coding for NEP, *rpoZ,* has been recently identified in *Arabidopsis thaliana* together with another one, *rpoY,* both of which have similarities with those encoding the RNA

polymerases from bacteriophages such as T3, T7, and SP6, and the RNA polymerase from yeast mitochondria. Moreover, the putative transit peptides coded by *rpoZ* and *rpoY* are capable of targeting fusion proteins to chloroplasts and mitochondria, respectively (Hedtke *et al.,* 1997). In line with this view, the structure of a NEP promoter from tobacco recently characterized contains a core sequence motif also present in plant mitochondrial promoters (Liere and Maliga, 1999).

Therefore, at least two plastid RNA polymerases exist, posing the question about which enzyme catalyzes the polymerization of ASBVd strands. A similar question regarding which of the three nuclear RNA polymerases is involved in the synthesis of PSTVd and other closely related members of the *Pospiviroidae* family as CEVd and HSVd, which replicate and accumulate in the nucleus, has been tackled by studying the effects of α-amanitin on this process. Experiments with *in vivo* (Mühlbach and Sänger, 1979) and *in vitro* (Flores and Semancik, 1982; Schindler and Mühlbach, 1992) approaches have revealed that replication of HSVd, CEVd and PSTVd is affected by the low concentrations of α-amanitin that typically inhibit DNA-dependent RNA polymerase II. This strongly suggests that an RNA polymerase II-like enzyme, acting on an RNA template, is involved in the synthesis of these viroids. On the other hand, high concentrations of this inhibitor have no effects on synthesis of ASBVd strands by a cell-free system from ASBVd-infected avocado leaves, indicating the involvement of a different RNA polymerase (Marcos and Flores, 1992). Recently, by using an improved version of this system derived from purified chloroplasts of ASBVd-infected avocado leaves, which are able to catalyze *in vitro* the transcription of viroid-specific and chloroplastic RNAs, it has been possible to analyze the effects of tagetitoxin, which inhibits PEP but not NEP (Mathews and Durbin, 1990; Kapoor *et al.,* 1997; Liere and Maliga, 1999). Concentrations of tagetitoxin that prevented transcription of series of chloroplastic genes did not affect synthesis of ASBVd strands, thus suggesting that a NEP-like enzyme is the RNA polymerase required in ASBVd replication, although the participation of another tagetitoxin-resistant RNA polymerase from the chloroplast cannot be excluded (Navarro, Vera and Flores, 2000).

Another question is whether viroid strands have specific promoters for the initiation of transcription, or whether this process is initiated randomly, a possible alternative from a theoretical perspective considering that the circular nature of the template enables its complete transcription no matter where it starts. Attempts to tackle this ques-

tion by primer extension approaches have failed to provide a clear answer because the extensions do not end at only one point but at several, which might alternatively correspond to the initiation site, the site where the oligomeric viroid RNAs are cleaved to give rise to the monomeric linear RNAs, or to other sites resulting from the *in vivo* or *in vitro* degradation of the circular forms. By using a different experimental approach based on the *in vitro* capping of linear viroid strands with [α^{32}-P]GTP and guanilyltransferase, which exclusively results in labeling those RNAs with a 5′-triphosphate or diphosphate, combined with RNase protection assays, it has been possible to map unambiguously the initiation sites of ASBVd strands (Navarro and Flores, 2000). The initiation site of synthesis of the ASBVd (–) strands is located in the A+U-rich right terminal loop (Fig. 6.1), and a structurally similar A+U rich terminal loop in the secondary structure proposed for the monomeric (–) circular RNA contains the initiation site of the (+) ASBVd strand. In fact, a short segment of the ASBVd molecule harbors in the (+) and (–) polarities both initiation sites. These sites are flanked by sequences bearing some similarity to those of the two known NEP promoters (Liere and Maliga, 1999), which are solely composed by a short motif (15 to 19 nt) placed immediately upstream of the transcription start site.

C. Self-Cleavage of Oligomeric RNAs: Role of Hammerhead Ribozymes

Data obtained for the three members of the *Avsunviroidae* family indicate that the hammerhead structures are operative not only *in vitro* but also *in vivo,* mediating the excision of monomers from the replicative oligomeric intermediates. A line of evidence comes from the characterization of the linear monomeric ASBVd and CChMVd RNAs of one or both polarities isolated from viroid-infected tissues (the "native" linear forms); a significant proportion had the same 5′ termini as those produced in the corresponding *in vitro* self-cleavage reactions (Marcos and Flores, 1993; Navarro and Flores, 1997; Navarro and Flores, 2000). Furthermore, two linear (+) and (–) ASBVd RNAs of subgenomic length (137 nt and approximately 148 nt, respectively) and one linear (+) ASBVd RNA of supragenomic length (383–384 nt) have been also detected in ASBVd-infected avocado (Darós *et al.,* 1994). The two linear (+) strands have the same 5′- and 3′-terminal sequences, with the supragenomic species being a fusion product of the monomeric and subgenomic (+) ASBVd RNAs. The 3′ termini of the (+) subgenomic and supragenomic ASBVd strands, which at least in the

former has an extra nontemplate C residue, could represent a site of premature termination of the (+) strands. The 5′ termini of the subgenomic and supragenomic (+) ASBVd strands and the 5′ terminus of the subgenomic (–) ASBVd RNA are identical to those produced in the *in vitro* self-cleavage reactions of (+) and (–) dimeric ASBVd RNAs, respectively.

The *in vivo* role of the hammerhead ribozymes is also supported by the sequence heterogeneity patterns found in members of the *Avsunviroidae* family. Although many sequence variants have been reported for ASBVd (Symons, 1981; Pallás *et al.,* 1988; Rakowski and Symons, 1989; Semancik and Szychowski, 1994), PLMVd (Hernández and Flores, 1992; Shamloul *et al.,* 1995; Ambrós *et al.,* 1998, 1999) and CChMVd (Navarro and Flores, 1997; De la Peña *et al.,* 1999), the nucleotide changes detected in the regions corresponding to the hammerhead structures do not affect their stability, either because they are located in loops or because the mutations are compensatory. In one particular variant of PLMVd in which a mutation in the hammerhead catalytic pocket was observed, the RNA still showed significant *in vitro* self-cleavage, the corresponding cDNA was infectious, and the mutation was retained in a fraction of the progeny, indicating a sequence flexibility of the hammerhead structure higher than anticipated (Ambrós and Flores, 1998). Therefore, preservation of catalytically active domains in both polarity strands seems to be a structural constraint limiting the genetic divergence of the hammerhead viroids. Indirect evidence on the key role of hammerhead structures in the replication of PLMVd and CChMVd was also obtained from bioassays with individual cDNAs clones, by which a correlation was established between the infectivity of distinct sequence variants and the extent of their self-cleavage during *in vitro* transcription (Ambrós *et al.,* 1998; De la Peña *et al.,* 1999).

Ribozymes, like enzymes, need to be regulated. They must be active at some stage of the replication cycle and inactive at others. Because the ASBVd double-hammerhead structures (Section III.B) can be formed only in longer-than-unit intermediates (Forster *et al.,* 1988), they may provide a mechanism to prevent self-cleavage of circular monomeric RNAs. In contrast, self-cleavage of PLMVd and CChMVd RNAs most probably occurs through single-hammerhead ribozymes (Hernández and Flores, 1992; Beaudry *et al.,* 1995; Navarro and Flores, 1997). In these two cases, the adoption of very stable conformations that do not contain the hammerhead structures seems to protect the circular monomeric RNAs against self-cleavage (Hernández and Flores, 1992; Navarro and Flores, 1997). Thus, the catalytic structures

may form during replication and promote self-cleavage before RNA synthesis has been completed, and the most stable conformations can be adopted. Remarkably, in PLMVd and CChMVd the residues involved in forming plus and minus hammerhead ribozymes and their corresponding self-cleavage sites are opposite each other and contained within a region of the predicted branched secondary structures, which represents approximatley one third of the whole molecule (Fig. 6.1).

D. Circularization

Two different mechanisms have been proposed for the last step of the replication cycle. Because in the *Avsunviroidae* family cleavage of oligomeric viroid RNAs to the monomeric linear forms is autocatalytic and mediated by hammerhead ribozymes, the ligation may also occur autocatalytically in the absence of proteins (self-ligation). Support for this view comes from *in vitro* experiments in which linear monomeric PLMVd RNA resulting from self-cleavage, self-ligated to a certain extent after incubation (Lafontaine *et al.,* 1995). However, most of the phosphodiester bonds produced in this reaction are 2′,5′ (Côte and Perrault, 1997) and not the 3′,5′ typically found in RNA, making questionable whether this is actually the pathway operating *in vivo.* On the other hand, *in vitro* self-ligation has also been observed in the case of PSTVd (Baumstark *et al.,* 1997), indicating that it is not restricted to hammerhead viroids.

In an alternative view, circularization is assumed to be catalyzed by a host enzyme. Although *in vitro* experiments have shown that different RNases, including one of fungal origin, are able to catalyze both the cleavage and ligation of longer-than-unit PSTVd transcripts to the infectious monomeric circular form (Tsagris *et al.,* 1987; Tabler *et al.,* 1992), this mechanism appears unlikely *in vivo* considering the nonspecific nature of some of the RNases involved and the fact that cleavage is autocatalytic in the *Avsunviroidae* family. An RNA ligase similar to the well-characterized RNA ligase from wheat germ (Konarska *et al.,* 1981) appears to be a more likely candidate. This enzyme requires in the RNAs to be ligated 5′-OH and 2′,3′-cyclic phosphodiester termini, the same resulting in hammerhead-mediated RNA self-cleavage, and has a loose specificity regarding the RNAs that accepts as substrates (it can act in either intramolecular or intermolecular ways). In fact, linear PSTVd monomeric RNA isolated from infected tissue can be efficiently converted to circular forms by *in vitro* incubation with the RNA ligase from wheat germ (Branch *et al.,* 1982). Moreover, the identification of a 2′-phosphomonoester, 3′,5′-phosphodiester bond at a

unique site in a viroid-like satellite RNA that exactly coincides with the self-cleavage site determined by the hammerhead structure contained in this RNA (Kiberstis *et al.*, 1985), is a strong argument in favor of a mechanism like that catalyzed by the wheat RNA ligase, which leaves a 2′-phosphomonoester signature at the ligation site. In line with this view, deletion of the residue located 3′ to the self-cleavage site of the plus hammerhead structure in some PLMVd and CChMVd variants with a much reduced RNA self-cleavage and infectivity has been assumed to be a cloning artifact introduced by the reverse transcriptase because of the extra 2′-phosphomonoester at the nucleotide preceding the self-cleavage/ligation site (Ambrós *et al.*, 1998, 1999; De la Peña *et al.*, 1999). That a 2′-phosphomonoester represents a severe impediment for the reverse transcriptase comes from experiments in which ASBVd monomeric linear forms resulting from *in vitro* self-cleavage were ligated in the presence of the wheat germ RNA ligase and then reverse transcribed. An abrupt stop was observed at the self-cleavage/ligation site (Navarro and Flores, unpublished data). In contrast, such a stop is not detected in reverse transcriptions using the circular ASBVd RNA isolated from infected tissue, suggesting that in avocado a phosphatase catalyzes the hydrolysis of most of the 2′-phosphomonoester groups. A corollary of all this is that, because ASBVd replicates in the chloroplast, an RNA ligase must exist in this organelle. Preliminary evidence that requires further confirmation suggests that this is indeed the case (Navarro, Daròs, and Flores, unpublished data).

In addition to the enzyme catalyzing RNA ligation, the viroid RNA itself must adopt an appropriate conformation that brings its two termini in close proximity. The most stable secondary structures proposed for ASBVd, PLMVd, CChMVd (Fig. 6.1), or other similar structures with close free energy values fulfill this criterion and therefore are good candidate substrates for the RNA ligase. It is pertinent to note here that the balance between the viroid RNA molecules with conformations that promote self-cleavage (containing the hammerhead structures) and those favoring ligation will critically influence the *in vivo* ratio of circular to linear forms.

VI. Pathogenesis

A. Cross-Protection

Cross-protection phenomena have been known for some time in different plant viruses and used for establishing proximity relationships, as well as for practical purposes (for a review see Matthews, 1991).

Because of these phenomena, plants infected with a latent or nonsymptomatic strain of a pathogen become protected (do not develop the characteristic symptoms) against the challenge inoculation by a severe strain of the same or very similar agent. Renewed interest in these phenoma has resulted from experiments showing that transgenic plants expressing the coat protein gene of a virus, or other sequences derived from the viral genome, may be resistant to infection with the intact virus (for a review see Lomonossoff, 1995). Cross-protection has also been reported in the *Pospiviroidae* family between strains of the same viroid (Fernow, 1967) and closely related viroids (Niblett *et al.*, 1978). Interestingly, in this latter study the lack of cross-protection between the agent of CChM disease and PSTVd, CEVd and CSVd, established a first borderline clearly separating the former from the three other viroids.

Within the *Avsunviroidae* family, although mild and severe strains of ASBVd have not been identified, the different symptomatology observed in the ASB disease has been suspected as coming from variations in the molecular structure of the viroid. This situation, together with the long time needed for symptom expression, has so far precluded the description of cross-protection phenomena associated with this viroid, if they exist at all. However, phenomena of this kind were observed in PLMVd and CChMVd, even before they were characterized as viroids, and used for applied purposes. In fact, biological detection of PLMVd in the greenhouse was (and still is) performed in GF-305 peach seedlings by a cross-protection assay (Desvignes, 1976). In brief, the peach seedlings are first inoculated by chip budding with material from the trees to be analyzed (which even if infected do not usually display foliar alterations, the other symptoms appearing at least 2 years after planting), and approximately 2 months later they are challenge-inoculated with a severe PLMVd strain. Plants already infected with a latent strain of PLMVd do not present the characteristic leaf symptoms induced by the severe strain. Severe strains are relatively stable (some inoculated plants revert to a symptomless condition) and must be maintained in the greenhouse by periodically inoculating new indicator plants with symptomatic tissue. The duration of the bioassay is approximately 3 months, and it has enabled the selection and diffusion in France of PLMVd-free peach cultivars for the last 25 years (Desvignes, 1986).

The existence of an infectious but nonsymptomatic strain of CChMVd (CChMVd-NS) was postulated to explain why some plants of a chrysanthemum cultivar sensitive to the disease were unable to develop the characteristic symptoms when inoculated with extracts

from the symptomatic strain (CChMVd-S) (Horst, 1975). This was assumed to result from a cross-protection effect, and the existence of a transmissible agent that protected against that of the CChM disease was shown by inoculation experiments performed in a cross-protection format (Horst, 1975). However, direct physical evidence in support of this contention has remained elusive until recently, when Northern blot hybridizations with a probe derived from the CChMVd-S RNA provided the first direct proof for the existence of a CChMVd-NS RNA in the protected chrysanthemum plants (De la Peña *et al.,* 1999). The intensity of the hybridization signals generated by CChMVd-S and -NS RNAs was similar, indicating that their associated phenotypes were not the consequence of different accumulation levels in the infected tissue, but rather of subtle variations in the structure of both RNAs.

In summary, cross-protection events have been shown to exist in species of both *Pospiviroidae* and *Avsunviroidae* families. Considering the different properties of these families, which most likely include the subcellular site of replication and accumulation, it can be anticipated that distinct underlying mechanisms must necessarily be involved.

B. Molecular Determinants

Although specific ASBVd sequence variants have been associated with distinct foliar symptoms in avocado, it has not been possible to clearly assign symptomatology to a given variant with the ASBVd–avocado system. This problem occurs because it is difficult to produce successful infections by inoculating mechanically nucleic acid preparations, and also because of the long assay period (1 or more years) usually required for the symptoms to develop. By contrast, successful mechanical inoculation of PLMVd on the peach indicator GF-305 with RNA (Flores *et al.,* 1990) or cDNA preparations (Ambrós *et al.,* 1998) is possible, and the time elapsed between inoculation and the onset of symptoms is relatively short (8–12 weeks). Moreover, as indicated previously, severe and latent strains of PLMVd that induce symptomatic and symptomless infections, respectively, on GF-305 peach seedling grown in the greenhouse have been reported. Altogether, these properties made the PLMVd–peach system in principle attractive to identify the molecular determinants of pathogenicity in a hammerhead viroid. However, PLMVd isolates have turned out to be extremely complex populations of sequence variants (Ambrós *et al.,* 1998), and inoculations with specific cDNA clones of this viroid lead to the rapid accumulation of genomic heterogeneity in their progenies

(Ambrós *et al.,* 1999). This complexity complicates the analysis, and although sequence comparison between the two closest characterized PLMVd variants inciting different host responses has shown that 12 nucleotide changes are sufficient to restore pathogenicity to variants that give rise to symptomless infections, a clear association of the pathogenic effect of some PLMVd variants to a discrete structural motif is not yet possible (Ambrós *et al.,* 1998).

The recent identification and molecular characterization of CChMVd (Navarro and Flores, 1997), whose natural host chrysanthemum is also a convenient experimental host easy to multiply by cuttings and with a quick symptom development time (10–12 days), and the existence of latent or nonsymptomatic strains of this viroid, have made it possible to map its pathogenicity determinant (De la Peña *et al.,* 1999). To address this question, a series of cDNA clones from a CChMVd nonsymptomatic strain (CChMVd-NS) were prepared by RT-PCR using two pairs of adjacent primers derived from the sequence of the symptomatic CChMVd RNA (CChMVd-S). Analysis of the CChMVd-NS clones revealed a size and sequence similar to those of the CChMVd-S strain. Some of the mutations observed in CChMVd-NS molecular variants were previously identified in CChMVd-S RNA, but others were never found in this RNA. Bioassays in chrysanthemum showed that cDNA clones containing the CChMVd-NS specific mutations were infectious, but nonsymptomatic. Moreover, when the contribution of each mutation to the nonsymptomatic phenotype was assessed independently, only the tetranucleotide at positions 82–85 was directly involved (Fig. 6.4). Indeed, changing by site-directed mutagenesis the GAAA82-85 of a typical CChMVd-S variant into UUUC (the characteristic sequence of CChMVd-NS variants) led to a symptomless infection without detectable alterations of the accumulation level of the viroid progeny (De la Peña *et al.,* 1999). Therefore, a change affecting only 1% of the CChMVd sequence involves a dramatic effect in its biological properties.

The pathogenicity determinant is located in a tetraloop capping the stem of a hairpin in the computer-predicted branched conformation for CChMVd RNA (Fig. 6.4). This is also the conformation probably existing *in vivo* because, as indicated previously, it accommodates the sequence heterogeneity found in CChMVd variants (De la Peña *et al.,* 1999). That the CChMVd mutation UUUC82-85 to GAAA actually maps at a tetraloop is also supported by the concurrent change of four consecutive nucleotides. The GAAA tetraloop is a member of the GNRA tetraloop family (where R stands for a purine and N for any base) found frequently in different RNAs (Woese *et al.,* 1990) and

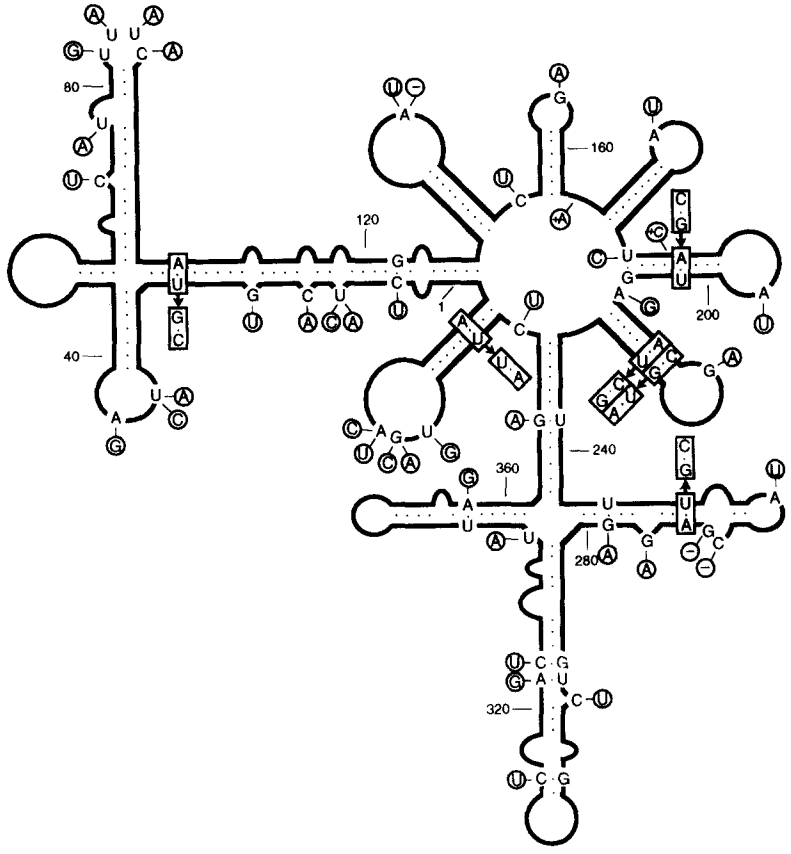


FIG 6.4. Accommodation of the sequence heterogeneity found in variants characterized so far from CChMVd-S and -NS strains in the secondary structure predicted for CChMVd RNA. Insertions and deletions are denoted by symbols + and –, respectively. For other details of the CChMVd reference variant see Fig. 6.1. (Reprinted with permission from De la Peña *et al.*, 1999. Copyright 1999 National Academy of Sciences U.S.A.)

endowed with an unusual high thermodynamic stability resulting from an array of heterogeneous hydrogen bonds (Heus and Pardi, 1991). It has been proposed that these tetraloops play an important function in RNA folding (Uhlenbeck, 1990), in RNA-RNA tertiary interactions (Pley *et al.*, 1994b), and as protein binding sites (Gluck *et al.*, 1992). A GAAA tetraloop has also been implicated in processing of PSTVd RNA (Baumstark *et al.*, 1997), but a similar role for the CChMVd tetraloop at positions 82–85 can be dismissed because processing in this viroid is mediated by hammerhead ribozymes and does not

occur in this region (Navarro and Flores, 1997). For a comparison, mutations of three-four nucleotides having profound effects on pathogenicity (Schnoölzer *et al.,* 1985; Visvader and Symons, 1986) have been mapped in PSTVd and closely related species of the same genus at a "virulence modulating (VM) region" within a domain of the rod-like structure that has been termed the *pathogenic domain* (Keese and Symons, 1985). The VM region comprises nonadjacent nucleotides of the upper and lower strands of the rod-like structure having a molecular structure different from the pathogenicity determinant of CChMVd.

VII. Genetics

A. Quasi-Species Distributions

As found for other RNA pathogens, viroids propagate as a population of similar but nonidentical sequences fitting the quasispecies concept defined by Eigen (1993). A main factor leading to the detected sequence heterogeneity in viroid isolates is the error-prone nature of the RNA polymerases involved in their replication. For RNA viruses, mutation rates of about 10^{-3} to 10^{-5} substitutions per nucleotide per round of replication have been estimated (Domingo and Holland, 1994), and although data of this class are not available for viroids, similar or even higher rates can be anticipated considering that the multiplication of these pathogens is mediated by cellular RNA polymerases whose usual templates are double-stranded DNAs (Diener, 1996). Thus, genomic diversity of viroid isolates is most probably the result of the frequent generation of mutants and of the selection processes operating on them.

There are numerous reports on the sequence variability of different members of the *Pospiviroidae* family (Visvader and Symons, 1985; Koltunow and Rezaian, 1988; Lakshman and Tavantzis, 1993; Polivka *et al.,* 1996; Kofalvi *et al.,* 1997), and information of this type, although still more limited, has also become available for hammerhead viroids in recent years. Direct RNA sequencing of an ASBVd Spanish isolate revealed the same sequence as that of the original Australian isolate (Symons, 1981), but sequencing of several cDNA clones showed that the predominant (master) sequence was accompanied by other containing minor changes that were concentrated in the terminal regions (Fig. 6.1) (Pallás *et al.,* 1988). A study including three ASBVd-infected trees of different Australian origins identified 16 sequence variants between 246 and 251 nt accompanying the original sequence

(Rakowski and Symons, 1989). Again, most of the nucleotide differences were found in both terminal regions of the ASBVd molecule. Two sequence variants contained nucleotide changes in both plus and minus hammerhead structure, but they did not affect the *in vitro* self-cleavage of the corresponding RNAs. Sequence difference have also been detected in ASBVd RNAs extracted from tissues showing different symptoms (Section VII.B).

Molecular characterization of three PLMVd isolates, one severe and two latent according to their reaction on the peach indicator GF-305, showed that they are composed of a population of closely related molecular variants (Ambrós *et al.,* 1998). The determination of the primary structure of 29 different sequence variants revealed some flexibility in the PLMVd size, from 335 to 338 nt, and a large number of polymorphic positions in the viroid molecule. Despite the high heterogeneity detected in PLMVd, the variability pattern found supports the existence of three types of structural constraints limiting the genetic divergence of this viroid: the preservation of the stability of plus and minus hammerhead structures, the adoption of a branched secondary structure of minimal free energy, and the conservation of a potential pseudoknot-like element between two hairpin loops of the proposed secondary structure. Differences were found in the quasi-species that compose the isolates, in accordance with their distinct biological properties. Mechanical inoculation of GF-305 peach seedlings with representative cDNA clones from each PLMVd isolate showed that latent isolates are formed by a mixture of sequence variants, which, when bioassayed individually, induced symptomless infections. In contrast, most but not all of the GF-305 peach seedlings inoculated with cDNA clones from the severe isolate incited the symptomatic infections. In light of these results, different balances reached over time between variants endowed with different pathogenicity could explain the variable phenotype observed in natural infections caused by PLMVd severe isolates, which display a fluctuating symptomatology and frequently revert to an asymptomatic condition. On the other hand, the phenotypical stability of the PLMVd latent isolates is in agreement with the homogeneous biological behavior of the variants that compose this type of isolates (Ambrós *et al.,* 1998).

Sequence analysis of numerous cDNA clones obtained from one symptomatic and one nonsymptomatic isolate of CChMVd has also shown that they are composed of a mixture of variants following a quasi-species distribution. As already stated (Section VI.B), the pathogenicity determinant of CChMVd has been mapped in a tetraloop of its computer-predicted branched conformation (Navarro and Flores, 1997;

De la Peña *et al.*, 1999). Moreover, the sequence heterogeneity detected in variants from both CChMVd isolates strongly supports the biological significance of the proposed computer-predicted branched conformation, either because the changes are found in loops or because, when affecting a base-pair, the substitutions are compensatory (Fig. 6.4). Because covariations or compensatory mutations are considered the most robust approach for testing computer-predicted structures in RNA (Gutell *et al.*, 1994), these results show that the rod-like or quasi-rod-like secondary structure is not a universal paradigm for viroids because CChMVd, and most probably PLMVd, presents clearly branched conformations *in vivo*.

B. Tissue-Specific Variants

Observation of ASBVd-infected trees under greenhouse conditions has led to the distinction of two types of foliar symptoms appearing sequentially: first an intense chlorosis (bleached symptoms) associated with the vascular tissue and restricted to only a few leaves, and then a variegated pattern, occasionally presented by some leaves growing from shoots containing bleached leaves. In other instances new tissue growing from infected trees was symptomless (Semancik and Szychowski, 1994). The viroid was readily detected in bleached, variegated, and symptomless leaves; but the concentration was highest in the bleached portions and very low in the symptomless adjacent areas of the same leaves (Semancik and Desjardins, 1980). Discrete variants were identified as predominant in bleached tissue (249–250 nt), as well as in the variegated and symptomless leaves that were indistinguishable in size (247–248 nt). Moreover, most of the changes noted in cDNA clones from bleached tissue were concentrated in the poly(A) right terminal loop leading to its enlargement (Semancik and Szychowski, 1994). Because regardless of the inoculum source, inoculations with tissue implants from ASBVd-infected plants always induce bleached leaves as the initial foliar symptom, it has been proposed that the ASBVd variants that predominate in this type of tissue are also present in the variegated and symptomless leaves. Expression of ASB disease may then be viewed as a transition from a severe acute and self-limiting reaction in terms of ASBVd accumulation, to a persistent mild chronic form of infection accompanied by high viroid titers throughout the host (Semancik and Szychowski, 1994). Unfortunately, the difficult and unpredictable mechanical transmission of ASBVd using nucleic acid preparations has precluded testing this proposal directly by relating specific viroid variants with differential symptoms of the disease.

C. Sequence Heterogeneity in Progenies from Individual Variants

Viroids offer a unique opportunity to follow up the *de novo* generation of a "fitness landscape" from a single sequence variant, because their small size allows easy analysis of their complete genomes. As stated previously, the continuous emergence of new variants is due to the lack of proofreading of RNA polymerases. This lack and the competition between the arising variants are supposed to be the main factors determining the sequence structure of the new viroid populations.

The genomic stability of individual PLMVd sequences has been tested by inoculating peach seedlings GF-305 with their corresponding cDNA clones (Ambrós *et al.*, 1999). Four PLMVd variants, two of which induced symptomatic infections and the other two latent infections on the peach indicator, were selected as parental sequences. The structure of the populations derived from single PLMVd sequences differed according the observed phenotype, but in all cases a rapid accumulation of point mutations was detected. Indeed, determination of the primary structure of 36 progeny variants yielded 33 distinct PLMVd sequences that differed from those reported previously for this viroid (Hernández and Flores, 1992; Ambrós *et al.*, 1998; Ambrós and Flores, 1998). The funding sequence was retained exclusively in one of the four progenies analyzed and only as a minor component, illustrating the rapid evolution that may undergo the PLMVd molecule. These results are in contrast with those obtained from the analysis of progenies derived from natural variants of PSTVd, the type species of the *Pospiviroidae* family, when inoculated in the experimental host tomato (Góra-Sochacka *et al.*, 1997). Quick generation of new quasi-species from single cDNA sequences was also reported for PSTVd, but the rate of accumulation of sequence heterogeneity was notably lower than for PLMVd. It has been speculated that this could reflect involvement of different RNA polymerases with distinct mutation rates in the replication of members of each viroid family (Ambrós *et al.*, 1999), a likely possibility considering that replication of PSTVd and ASBVd occur in different cellular organella.

The high complexity found in the PLMVd populations generated *de novo* indicates that the extreme genomic variability observed in natural isolates of this viroid is probably not the consequence of repeated infections of the same plant, but rather an intrinsic property of this RNA to evolve rapidly. The polymorphism in the newly established PLMVd quasi-species seemed to be under the same structural constraints proposed to limit genetic divergence in natural isolates (Ambrós *et al.*, 1998).

VIII. Origin and Evolution of Hammerhead Viroids

A. Evolutionary Relationships with Other Viroid and Viroid-Like RNAs

Hammerhead viroids display structural and functional similarities with other small pathogenic RNAs. Within the plant world, at least two groups of RNAs have features in common with hammerhead viroids: members of the other viroid family, *Pospiviroidae,* and viroid-like satellite RNAs. The latter are small circular self-cleaving molecules whose most remarkable difference with hammerhead viroids refers to their functional dependence on a helper RNA virus. Viroid-like satellite RNAs do not share significant similarity with the RNA of their helper viruses but are encapsidated by their corresponding coat proteins. So far, nine viroid-like satellite RNAs have been identified, three of them associated with members of the nepovirus genus (sTRSV, sArMV, and sCYMV), five associated with members of the sobemovirus genus (sLTSV, sSNMoV, sVTMoV, sSCMoV, and sRYMV), and one associated with a member of the luteovirus genus (sBYDV) (Bruening, 1989; Keese and Symons, 1987; Collins *et al.,* 1998) (for abbreviations see Section III.C). Viroid-like satellite RNAs contain ribozymatic domains in one or both polarity strands. The ribozymes of those associated with sobemovirus and nepovirus genera are of the hammerhead type. However, viroid-like satellite RNAs assisted by nepoviruses can form hammerhead ribozymes in their plus polarity strands and hairpin ribozymes in their minus polarity strands. As in hammerhead viroids, the ribozymes of viroid-like satellite RNAs are most likely involved in the replication cycle that also occurs through a rolling-circle model (Bruening *et al.,* 1991).

Additionally, two other plant RNAs that bear some resemblance to hammerhead viroids have been described. A cherry small circular RNA (csc RNA1) able to form hammerhead structures in both polarity strands has been isolated from cherry trees affected by a specific malady. It shares limited sequence and structural similarity with viroids and particularly with viroid-like satellite RNAs, but whether it belongs to either of these two groups remains to be determined (Di Serio *et al.,* 1997). Another related, although nonpathogenic plant RNA forms part of a retroviroid-like element described in carnation (Darós and Flores, 1995). This RNA, called carnation small viroid-like RNA (CarSV RNA) has 275 nt and circular structure and can adopt hammerhead ribozymes in both polarity strands, but its lack of infectivity and, particularly, the existence of a homologous DNA form establish differences between this molecule and members of the

Avsunviroidae family. Within the animal kingdom, only the RNA of human hepatitis delta virus (HDV), a defective virus functionally dependent on human hepatitis B virus, is known to undergo self-cleavage, although through structures more complex than those of the hammerhead ribozymes (Rosenstein and Been, 1991; Perrota and Been, 1991). HDV RNA is a circular molecule able to fold into a rod-like conformation that contains a region termed the *viroid-like domain*, with significant similarities to viroid and viroid-like satellite RNAs (Branch *et al.*, 1989). HDV RNA also replicates through a rolling-circle model (Taylor, 1997).

Evidence supporting an evolutionary relationship between hammerhead viroids, members of the *Pospiviroidae* family, and viroid-like satellite RNAs is available. Phylogenetic analyses conducted with most of these small pathogenic RNAs are consistent with a monophyletic origin (Elena *et al.*, 1991; Hernández and Flores, 1992). In the first consensus phylogenetic tree reported, viroid-like satellite RNAs (including the viroid-like domain of HDV RNA) were clustered on one side and nonhammerhead viroids on the other side, with ASBVd, the only hammerhead viroid known at that time, located between the two clusters (Elena *et al.*, 1991). Results were similar when the phylogenetic analysis was carried out with some representatives of the nonhammerhead viroids, the viroid-like satellite RNAs and two hammerhead viroids, ASBVd and PLMVd (Hernández and Flores, 1992). The intermediate position of the two latter viroids in the resulting trees agreed with their functional properties. They share autonomous replication with nonhammerhead viroids and self-cleavage with viroid-like satellite RNAs, suggesting that they might represent an evolutionary link between these two groups of small RNAs.

B. Molecular Fossils of RNA World

Besides the obvious interest of viroids from the molecular and agricultural points of view, these small pathogenic RNAs have gained increasing relevance because of their potential evolutionary implications. Although viroids could be regarded as primitive or degenerate representatives of conventional viruses at the time they were discovered (Diener, 1971b, 1972), our knowledge since then points to a phylogenetic distance between viroids and viruses considerably higher than initially thought (Diener, 1996).

The origin of viroids has been a matter of a great speculation. Based on some sequence similarities between members of the *Pospiviroidae* family and the ends of transposable genetic elements, it was suggested

that viroids could have evolved from such DNA elements or from retroviral proviruses by deletion of internal sequences (Kiefer *et al.,* 1983). Another hypothesis proposed that viroids may represent "escaped" introns taking into account the presence in some of them of a consensus sequence believed to play a key role in the self-splicing of group I introns (Dinter-Gottlieb, 1987). However, a reexamination of such potential similarities revealed that they could be fortuitous (Diener, 1989). Moreover, none of the hammerhead viroids contain recognizable cognates of group I intron elements, which together with the different chemistry of self-cleavage in both types of molecules makes a phylogenetic relationship between viroids and introns unlikely (Diener, 1989, 1996). Another view proposes that viroids might have evolved comparatively recently from "signal" or "antenna" RNAs, two classes of molecules assumed to be involved in genetic exchange between cells (Zimmern, 1982).

In recent years, an alternative and attractive proposal about the origin of viroids and viroid-like RNAs has been advanced. It is now generally accepted that RNA preceded DNA as carrier of genetic information during evolution (Gilbert, 1986). Several factors have contributed to the acceptance of this hypothesis, especially the finding of RNAs with catalytic properties and the phenotype-genotype duality of RNA, permitting darwinian evolution to occur at the molecular level in the absence of DNA or proteins (Joyce, 1989). The postulated RNA world would have been composed of self-replicating RNAs, implying the existence of primitive RNA enzymes. Although most models for self-replicating precellular systems have been based on properties derived from the known self-splicing mechanism of the rRNA gene intron from *Tetrahymena thermophila,* the ribozyme involved in this process is relatively large and with a complex secondary and tertiary structure essential for the catalytic activity. Thus, several difficult to answer questions emerge, in particular the way in which this complex ribozyme could have evolved extracellularly, and the mechanisms for initiating and terminating replication precisely.

The catalytic structures found in viroids and viroid-like satellite RNAs have shown that ribozymes far simpler than those involved in intron splicing, but with similar capabilities, do exist. In this context it has been proposed that viroid and viroid-like satellite RNAs are molecular fossils of the primitive RNA world (Diener, 1989; Elena *et al.,* 1991). Many features of these pathogenic RNAs support that they might be relics of precellular evolution (Diener, 1989). The low fidelity copy of ancient polymerases sets a limit on the length of the molecules that could be faithfully replicated (Eigen, 1971), and viroid and viroid-

like satellite RNAs fulfill the requirement of small size. Moreover, G+C-rich sequences, such as those found in these small RNAs, would be favored because their higher stability would attenuate the effects of the error-prone nature of primitive polymerases (Eigen and Schuster, 1978). The circularity, another distinctive feature of viroids and viroid-like satellite RNAs, would ensure the complete copy of the template, excluding the need of genomic tags for specific initiation and termination sites that replication of a linear molecule requires. In addition, systems able to produce multiple copies of their genomes would likely have advantages given the high error rate presumed for prebiotic enzymes (Inoue and Orgel, 1983), and the rolling-circle transcription of a circular template leads to tandem repeats of genetic information. Last but not least, the finding of catalytic properties in hammerhead viroids and viroid-like satellite RNAs are of particular relevance in that they are remnants of a precellular RNA world (Diener, 1989).

As already mentioned, phylogenetic analyses of viroids and viroid-like satellite RNAs are consistent with the concept that these RNAs have a common origin with hammerhead viroids representing ancestral links between both types of RNAs. Whether viroids may have evolved from satellite RNAs, or vice versa, is still unclear. In any case, after the emergence of cellular organisms, these initially free-living molecules would have become intracellular parasites, with viroids acquiring dependence on their host and viroid-like satellite RNAs on the helper viruses (Diener, 1996).

IX. Concluding Remarks

The most outstanding feature of the *Avsunviroidae* members is their potential to adopt hammerhead structures in both polarity strands and to self-cleave *in vitro* accordingly. The evidence that these ribozymes also operate *in vivo* is sound. Therefore, the second step of the replication cycle (cleavage of oligomeric RNAs to the monomeric forms) is fairly well understood and certainly more than the other two steps (RNA polymerization and ligation). That the hammerhead ribozymes, a presumable relic of the postulated RNA world, have survived in a world based on DNA and proteins might indicate that they are endowed with a catalytic efficiency enabling them to avoid being replaced by the more efficient protein enzymes. This is not to exclude the possibility that hammerhead ribozymes are assisted *in vivo* by host proteins that may considerably enhance their efficiency, but the chemistry of the catalytic mechanism is almost certainly RNA-based.

In this context it is intriguing that the experimental data show that at least two of the three hammerhead viroids, ASBVd and PLMVd, replicate and accumulate in the chloroplast, in sharp contrast with PSTVd and typical *Pospiviroidae* members that replicate and accumulate in the nucleus (Section IV.C). This coincidence is unlikely to be casual. Because there is solid evidence supporting the hypothesis that chloroplasts are organelles that evolved from cyanobacteria by symbiosis (Margulis, 1993), these free-living prokaryotes might have hosted viroids, in particular the ancestors of the *Avsunviroidae* family, before they became specific to plants (Chela-Flores, 1994; Lima *et al.,* 1994). This implies that the *Avsunviroidae* members are the oldest viroids, a view that agrees with their harboring of hammerhead ribozymes and their position in the phylogenetic trees, and that also suggests that viroids might exist in present-day cyanobacteria.

Another fascinating question concerns the molecular determinants targeting ASBVd and PLMVd (and presumably CChMVd) to the chloroplast. Considering their limited sequence similarity, it is unlikely that this determinant may be a conserved motif of primary structure and rather points to a secondary structure element that would be specifically recognized by a chloroplastic factor. A parallel question regarding how *Pospiviroidae* members are imported to the nucleus still remains essentially unanswered, although some progress on the nuclear import of PSTVd in permeabilized protoplasts has been reported recently (Woo *et al.,* 1999). These latter results illustrate that viroids may be excellent model systems to study the factors governing RNA traffic through different cell compartments.

Boundaries in science are diffuse if not artificial in most cases. When the characterization of human hepatitis delta virus RNA revealed that its circular and rod-like secondary structure resembled that typical of most viroids, the possibility that this RNA could replicate through a rolling-circle mechanism with RNA self-cleavage became immediately apparent. With this enlightening hypothesis in mind, it was just a matter of time before it proved to be correct with the discovery of a new class of ribozymes (Sharmeen *et al.,* 1988; Wu *et al.,* 1989). Yet another far-reaching lesson remains to be learned. Ribozymes, one of the most promising tools in biotechnology, are particularly well represented in viroid and viroid-like RNAs in which the two structurally simplest self-cleaving domains, the hammerhead and the hairpin, have been characterized. Therefore, the study of the structure and replication of a group of small and opaque plant RNAs is having unexpected implications and applications far beyond virology and the plant kingdom.

Do viroids hold still more unveiled surprises? In members of the *Pospiviroidae* family, the cleavage reaction leading to monomeric RNA from oligomers is generally assumed to be catalyzed by an unidentified host RNase (Baumstark *et al.,* 1997). However, recent results have challenged this view by showing that transcripts of the C domain of the CCCVd, a member of the *Pospiviroidae* family, self-cleave specifically under defined (and unusual) *in vitro* conditions. The cleavage site is located in the bottom strand of the C domain within a potential hairpin structure preserved in all members of this family, suggesting that they may also self-cleave through one or more novel type of ribozyme (Liu and Symons, 1998).

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REFERENCES

Albanese, G., Giunchedi, L., La Rosa, R., and Pollini, P. C. (1992). Peach latent mosaic viroid in Italy. *Acta Hortic.* **309,** 331–338.

Allison, L. A., Simon, L. D., and Maliga, P. (1996). Deletion of *rpoB* reveals a second distinct transcription system in plastid of higher plants. *EMBO J.* **15,** 2802–2809.

Allen, R. N., and Dale J. L. (1981). Application of rapid biochemical methods for detecting avocado sunblotch disease. *Ann. Appl. Biol.* **98,** 451–461.

Allen, R. N., Palukaitis, P., and Symons, R. H. (1981). Purified avocado sunblotch viroid causes disease in avocado seedlings. *Australas. Plant Pathol.* **10,** 31–32.

Ambrós, S., and Flores, R. (1998). *In vitro* and *in vivo* self-cleavage of a viroid RNA with a mutation in the hammerhead catalytic pocket. *Nucleic Acids Res.* **26,** 1877–1883.

Ambrós, S., Desvignes, J. C., Llácer, G., and Flores, R. (1995). Peach latent mosaic and pear blister canker viroids: Detection by molecular hybridization and relationships with specific maladies affecting peach and pear trees. *Acta Hortic.* **386,** 515–519.

Ambrós, S., Hernández, C., Desvignes, J. C., and Flores, R. (1998). Genomic structure of three phenotypically different isolates of peach latent mosaic viroid: Implications of the existence of constraints limiting the heterogeneity of viroid quasi-species. *J. Virol.* **72,** 7397–7406.

Ambrós, S., Hernández, C., and Flores, R. (1999). Rapid generation of genetic heterogeneity in progenies from individual cDNA clones of peach latent mosaic viroid in its natural host. *J. Gen. Virol.* **80,** 2239–2252.

Badenes, M. L., and Llácer, G. (1998). Occurence of peach latent mosaic viroid in American peach and nectarine cultivars in Valencia, Spain. *Acta Hortic.* **472,** 565–570.

Barba, M., Cupidi, A., Loreti, S., Faggioli, F., and Martino, L. (1995). *In vitro* micrografting: a technique to eliminate peach latent mosaic viroid from peach. *Acta Hortic.* **386,** 531–535.

Bar-Joseph, M., Segev, D., Twizer, S., and Rosner, A. (1985). Detection of avocado sunblotch viroid by hybridization with synthetic oligonucleotide probes. *J. Virol. Methods* **10,** 69–73.

Baumstark, T., Schröder, A. R. W., and Riesner, D. (1997). Viroid processing: switch from cleavage to ligation is driven by a change from a tetraloop to a loop E conformation. *EMBO J.* **16,** 599–610.

Beaudry, D., Bussiere, F., Laureau, F., Lessard, C., and Perrault, J. P. (1995). The RNA of both polarities of the peach latent mosaic viroid self-cleavag *in vitro* solely by single hammerhead structures. *Nucleic Acids Res.* **23,** 745–752.

Bonfiglioli, R. G., McFadden, G. I., and Symons, R. H. (1994). *In situ* hybridization localizes avocado sunblotch viroid on chloroplast thylakoid membranes and coconut cadang cadang viroid in the nucleus. *Plant J.* **6,** 99–103.

Bonfiglioli, R. G., Webb, D. R., and Symons, R. H. (1996). Tissue and intra-cellular distribution of coconut cadang cadang viroid and citrus exocortis viroid determined by *in situ* hybridization and confocal laser scanning and transmission electron microscopy. *Plant J.* **9,** 457–465.

Boyé, R., and Desvignes, J. C. (1986). Biological techniques used for the study of new fruit virus diseases. *Acta Hortic.* **193,** 261–268.

Branch, A. D., and Dickson, E. (1980). Tomato DNA contains no detectable regions complementary to potato spindle tuber viroid as assayed by Southern hybridization. *Virology* **104,** 10–26.

Branch, A. D., and Robertson H. D. (1981). Longer-than-unit-length viroid minus strands are present in RNA from infected plants. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 6381–6385.

Branch, A. D., and Robertson, H. D. (1984). A replication cycle for viroids and other small infectious RNAs. *Science* **223,** 450–454.

Branch, A. D., Robertson, H. D., Greer, C., Gegenheimer, P., Peebles, C., and Abelson, J. (1982). Cell-free circularization of viroid progeny RNA by an RNA ligase from wheat germ. *Science* **217,** 1147–1149.

Branch, A. D., Benenfeld, B. J., and Robertson, H. D. (1988). Evidence for a single rolling circle in the replication of potato spindle tuber viroid. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 9128–9132.

Branch, A. D., Benenfeld, B. J., Baroudy, B. M., Wells, F. V., Gerin, J. L., and Robertson, H. D. (1989). An ultraviolet-sensitive RNA structural element in a viroid-like domain of the hepatitis delta virus. *Science* **243,** 649–652.

Brown, F., and Martin, S. J. (1965). A new model for virus ribonucleic acid replication. *Nature (London)* **208,** 861–863.

Bruening, G. (1989). Compilation of self-cleaving sequences from plant virus satellite RNAs and other sources. *Methods Enzymol.* **180,** 546–558.

Bruening, G., Gould, A. R., Murphy, P. J., and Symons, R. H. (1982). Oligomers of avocado sunblotch viroid are found in infected avocado leaves. *FEBS Lett.* **148,** 71–78.

Bruening, G., Feldstein, P. A., Buzayan, J. M., van Tol, H., Debear, J., Gough, G. R., Gilham, P. T., and Eckstein, F. (1991). Satellite tobacco ringspot virus RNA: Self-cleavage and ligation reactions in replication. *In* "Viroids and Satellites, Molecular Parasites in the Frontiers of Life" (K. Maramorosch, ed.), pp. 141–158. CRC Press, Boca Raton.

Bussière, F., Lehoux, J., Thompson, D. A., Skrzeczkowski, L. J., and Perreault, J. -P. (1999). Subcellular localization and rolling circle replication of peach latent mosaic viroid: Hallmarks of group A viroids. *J. Virol.* **73,** 6353–6360.

Chela-Flores, J. (1994). Are viroids molecular fossils of the RNA world?. *J. Theor. Biol.* **166,** 163–166.

Coit, J. E. (1928). Sunblotch of the avocado, a serious physiological disease. *Calif. Avocado Soc. Yearb.* 27–32.

Collins, R. F., Gellatly, D. L., Sehgal, O. P., and Abouhaidar, M. G. (1998). Self-cleaving circular RNA associated with rice yellow mottle virus is the smallest viroid-like RNA. *Virology* **241,** 269–275.

Conejero, V., and Semancik, J. S. (1977). Exocortis viroid: Alterations in the proteins of *Gynura aurantiaca* accompanying viroid infection. *Virology* **77,** 221–232.

Conejero, V., Picazo, I., and Segado, P. (1979). Citrus exocortis viroid (CEV): Protein alterations in different hosts following viroid infection. *Virology* **97,** 454–456.

Côte, F., and Perrault, J. P. (1997). Peach latent mosaic viroid is locked by a 2′,5′-phosphodiester bond produced by *in vitro* self-ligation. *J. Mol. Biol.* **273,** 533–543.

Da Graça, J. V., and Martin, M. M. (1981). Ultrastructural changes in avocado leaf tissue infected with avocado sunblotch viroid. *Phytopath. Z.* **102,** 185–194.

Da Graça, J. V., and Moon, T. E. (1983). Detection of avocado sunblotch viroid in flower buds by polyacrylamide gel electrophoresis. *Phytopath. Z.* **108,** 267–280.

Da Graça, J. V., and van Vuuren, S. P. (1980). Transmission of avocado sunblotch disease to cinnamon. *Plant Dis.* **64,** 475.

Da Graça, J. V., and van Vuuren, S. P. (1981). Use of high temperature to increase the rate of avocado sunblotch symptom development in indicator seedlings. *Plant Dis.* **65,** 46–47.

Dale, J. L., and Allen R. N. (1979). Avocado affected by sunblotch disease contains low molecular weight ribonucleic acid. *Australas. Plant Pathol.* **8,** 3–4.

Dale, J. L., Symons, R. H., and Allen, R. N. (1982). Avocado sunblotch viroid. *CMI/AAB Descrip. Plant Viruses.* No. 254.

Daròs, J. A., and Flores, R. (1995). Identification of a retroviroid-like element from plants. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 6856–6860.

Daròs, J. A., Marcos, J. F., Hernández, C., and Flores, R. (1994). Replication of avocado sunblotch viroid: Evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12813–12817.

Davies, J. W., Kaesberg, P., and Diener, T. O. (1974). Potato spindle tuber viroid. XII. An investigation of viroid RNA as a messenger for protein synthesis. *Virology* **61,** 281–286.

Davies, C., Sheldom, C. C., and Symons, R. H. (1991). Alternative hammerhead structures in the self-cleavage of avocado sunblotch viroid RNAs. *Nucleic Acids Res.* **19,** 1893–1898.

De la Peña, M., Navarro, B., and Flores, R. (1999). Mapping the molecular determinat of pathogenicity in a hammerhead viroid: A tetraloop within the *in vivo* branched RNA conformation. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 9960–9965.

Desjardins, P. R. (1987). Avocado sunblotch. *In* "The Viroids" (T. O. Diener, ed.), pp. 299–313. Plenum, New York.

Desjardins, P. R., Drake R. J., and Swiecki S. A. (1980). Infectivity studies of avocado sunblotch disease causal agent, possibly a viroid rather than a virus. *Plant Dis.* **64,** 313–315.

Desjardins, P. R., Drake, R. J. Sasaki, P. J. Atkins E. L., and Bergh B. O. (1984). Pollen transmission of avocado sunblotch viroid and the rate of the pollen recipient tree. *Phytopathology* **74,** 845.

Desvignes, J. C. (1976). The virus diseases detected in greenhouse and field by the peach seedlings GF-305 indicator. *Acta Hortic.* **67,** 315–323.

Desvignes, J. C. (1980). Different symptoms of the peach latent mosaic. *Acta Phytopath. Acad. Sci. Hung.* **15,** 183–190.

Desvignes, J. C. (1982). Resistance of some *Prunus* species to peach latent mosaic virus disease (PLMV). *Acta Hortic.* **130,** 89–91.

Desvignes, J. C. (1986). Peach latent mosaic and its relation to peach mosaic and peach yellow mosaic virus diseases. *Acta Hortic.* **193,** 51–57.

Diener, T. O. (1971a). Potato spindle tuber "virus": A plant virus with properties of a free nucleic acid. III. Subcellular location of PSTV-RNA and the question of whether virions exist in extracts or *in situ*. *Virology* **43,** 75–89.

Diener, T. O. (1971b). Potato spindle tuber "virus" IV. A replicating low molecular weight RNA. *Virology* **45,** 411–428.

Diener, T. O. (1972). Potato spindle tuber viroid VIII. Correlation of infectivity with a UV-absorbing component and thermal denaturation properties of the RNA. *Virology* **50,** 606–609.

Diener, T. O. (1989). Circular RNAs: relics of precellular evolution?. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9370–9374.

Diener, T. O. (1996). Origin and evolution of viroids and viroid-like satellite RNAs. *Virus Genes* **11,** 119–131.

Diener, T. O., and Raymer, W. B. (1967). Potato spindle tuber virus: A plant virus with properties of a free nucleic acid. *Science* **158,** 378–381.

Dimock, A. W., and Geissinger, C. M. (1969). A newly recognized disease of chrysanthemum caused by a graft-transmissible agent. *Phytopathology* **59,** 1024.

Dimock, A. W., Geissinger, C. M., and Horst, R. K. (1971). Chlorotic mottle: A newly recognized disease of chrysanthemum. *Phytopathology* **61,** 415–419.

Dinter-Gottlieb, G. (1987). Possible viroid origin: Viroids, virusoids, and group I introns. *In.* "The Viroids" (T. O. Diener, ed.), pp. 189–204. Plenum, New York.

Di Serio, F., Darós, J. A., Ragozzino, A., and Flores, R. (1997). A 451-nucleotide circular RNA from cherry with hammerhead ribozymes in its strands of both polarities. *J. Virol.* **71,** 6603–6610.

Di Serio, F., Malfitano, M., Flores, R., and Randles, J. W. (1999). Detection of PLMVd in Australia. *Australas. Plant Pathol.* **28,** 80–81.

Domingo, E., and Holland, J. J. (1994). Mutation rates and rapid evolution of RNA viruses. *In* "Evolutionary Biology of Viruses" (S. S. Morse, ed.), pp. 171–180. Raven Press, New York.

Eigen, M. (1971). Self-organization of matter and the evolution of biological macromolecules. *Naturwissenschaften* **58,** 465–523.

Eigen, M. (1993). The origin of genetic information: Virus as models. *Gene* **135,** 37–47.

Eigen, M., and Schuster, P. (1978). The hypercycle: A principle of self-organization. Part C: The realistic hypercycle. *Naturwissenschaften* **65,** 341–369.

Elena, S. F., Dopazo, J., Flores, R., Diener, T. O., and Moya, A. (1991). Phylogeny of viroids, viroid-like satellite RNAs, and the viroidlike domain of hepatitis δ virus RNA. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 5631–5634.

Epstein, L. M., and Gall, J. G. (1987). Self-cleaving transcripts of satellite DNA from the newt. *Cell* **48,** 535–543.

Faggioli, F., Loreti, S., and Barba, M. (1997). Occurrence of peach latent mosaic viroid (PLMVd) on plum in Italy. *Plant Dis.* **81,** 423.

Feldstein, P. A., Hu, Y., and Owens, R. A. (1998). Precisely full length, circularizable, complementary RNA: An infectious form of potato spindle tuber viroid. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6560–6565.

Ferbeyre, G., Smith, J. M., and Cedergren, R. (1998). *Schistosome* satellite DNA encodes active hammerhead ribozymes. *Mol. Cell. Biol.* **18,** 3880–3888.

Fernow, K. H. (1967). Tomato as a test plant for detecting mild strains of potato spindle tuber virus. *Phytopathology* **57,** 1347–1352.

Flores, R., and Llácer, G. (1988). Isolation of a viroid-like RNA associated with peach latent mosaic disease. *Acta Hortic.* **235,** 325–332.

Flores, R., and Semancik, J. S. (1982). Properties of a cell-free system for synthesis of citrus exocortis viroid. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 6285–6288.

Flores, R., Hernández, C., Desvignes, J. C., and Llácer, G. (1990). Some properties of the viroid inducing the peach latent mosaic disease. *Res. Virol.* **141,** 109–118.

Flores, R., Hernández, C., Avinent, L., Hermoso, A., Llácer, G., Juárez, J., Arregui, J. M., Navarro, L., and Desvignes, J. C. (1992). Studies on the detection, transmission and distribution of peach latent mosaic viroid in peach trees. *Acta Hortic.* **309,** 325–330.

Flores, R., Di Serio, F., and Hernández, C. (1997). Viroids: The non-coding genomes. *Semin. Virol.* **8,** 65–73.

Flores, R., Hernández, C., Llácer, G., and Desvignes, J. C. (1998). Peach latent mosaic viroid. *AAB Descrip. Plant Viruses.* No. 362.

Flores, R., Randles, J. W., Bar-Joseph, M., and Diener, T. O. (2000). Viroids. *In* "Virus Taxonomy, Seventh Report of the International Committee on Taxonomy of Viruses." (M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, D. J. McGeoch, J. Maniloff, M. A. Mayo, C. R. Pringle, and R. B. Wickner, eds.), pp. 1009–1024. Academic Press, San Diego.

Forster, A. C., Davies, C., Sheldon, C. C., Jeffries, A. C., and Symons, R. H. (1988). Self-cleaving viroid and newt RNAs may only be active as dimers. *Nature* **334,** 265–267.

Forster, A. C., and Symons, R. H. (1987). Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell* **49,** 211–220.

Gilbert, W. (1986). The RNA world. *Nature (London)* **319,** 618.

Gilbert, W., and Dressler, D. (1968). DNA replication: The rolling circle model. *Cold Spring Harbor Symp. Quant. Biol.* **33,** 473–484.

Gispert, C., Perring, T. M., and Creamer, R. (1998). Purification and characterization of peach mosaic virus. *Plant Dis.* **82,** 905–908.

Gluck, A., Endo, Y., and Wool, I. G. (1992). Ribosomal-RNA identity elements for ricin A-chain recognition and catalysis-analysis with tetraloops mutants. *J. Mol. Biol.* **226,** 411–424.

Góra-Sochacka, A., Kierzek, A., Candresse, T., and Zagórski, W. (1997). The genetic stability of potato spindle tuber viroid (PSTVd) molecular variants. *RNA* **3,** 68–74.

Grill, L. K., and Semancik, J. S. (1978). RNA sequences complementary to citrus exocortis viroid in nucleic acid preparations from infected *Gynura aurantiaca*. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 896–900.

Gross, H. J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H., and Sänger, H. L. (1978). Nucleotide sequence and secondary structure of potato spindle tuber viroid. *Nature* **273,** 203–208.

Gutell, R. R., Larsen, N., and Woese, C. R. (1994). Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev.* **58,** 10–26.

Hadas, R., Ashulin, L., and Bar-Joseph, M. (1992). Transmission of a citrus viroid to avocado by heterologous grafting. *Plant Dis.* **76,** 357–359.

Hadidi, A., Giunchedi, L., Shamloul, A. M., Poggi-Pollini, C., and Amer, M. A. (1997). Occurrence of peach latent mosaic viroid in stone fruits and its transmission with contaminated blades. *Plant Dis.* **81,** 154–158.

Hall, T. C., Wepprich, R. K., Davies, J. W., Weathers, L. G., and Semancik, J. S. (1974). Functional distinctions between the ribonucleic acids from citrus exocortis viroid and plant viruses: Cell-free translation and aminoacylation reactions. *Virology* **61,** 486–492.

Harders, J., Lukacs, N., Robert-Nicoud, M., Jovin, J. M., and Riesner, D. (1989). Imaging of viroids in nuclei from tomato leaf tissue by *in situ* hybridization and confocal laser scanning microscopy. *EMBO J.* **8,** 3941–3949.

Hari, V. (1980). Ultrastructure of potato spindle tuber viroid-infected tomato leaf tissue. *Phytopathology* **70,** 385–387.

Haseloff, J., and Symons, R. H. (1981). Chrysanthemum stunt viroid: Primary sequence and secondary structure. *Nucleic Acids Res.* **9,** 2741–2752.

Hedtke, B., Börner, T., and Weihe, A. (1997). Mitochondrial and chloroplast phage-type RNA polymerase in *Arabidopsis*. *Science* **277,** 809–811.

Hernández, C., and Flores, R. (1992). Plus and minus RNAs of peach latent mosaic viroid self-cleave *in vitro* via hammerhead structures. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 3711–3715.

Hernández, C., Daròs, J. A., Elena, S. F., Moya, A., and Flores, R. (1992). The strands of both polarities of a small circular RNA from carnation self-cleave *in vitro* through alternative double- and single-hammerhead structures. *Nucleic Acids Res.* **20,** 6323–6329.

Hertel, K. J., Pardi, A., Uhlenbeck, O. K., Koizumi, M., Ohtsuka, E., Uesugi, S., Cedergren, R., Eckstein, F, Gerlach, W. L., Hodgson, R., and Symons, R. H. (1992). Numbering system for the hammerhead. *Nucleic Acids Res.* **20,** 3252.

Heus, H. A., and Pardi, A. (1991). Structural features that give rise to the unusual stability of RNA hairpins containing GNRA loops. *Science* **253,** 191–194.

Horne, W. T. (1929). Progress in the study of certain diseases of avocado. *Phytopathology* **19,** 1144.

Horne, W. T., and Parker E. R. (1931). The avocado disease called sunblotch. *Phytopathology* **21,** 235–238.

Horne, W. T., Parker E. R., and Rounds M. B. (1941). The nature of sunblotch and its practical control. *Calif. Avocado Soc. Yearb.* 35–38.

Horst, R. K. (1975). Detection of a latent infectious agent that protects against infection by chrysanthemum chlorotic mottle viroid. *Phytopathology* **65,** 1000–1003.

Horst, R. K. (1987). Chrysanthemum chlorotic mottle. *In* "The Viroids" (T. O. Diener, ed.), pp. 291–295. Plenum, New York.

Hutchins, L. M. (1932). Peach mosaic—a new virus disease. *Science* **76,** 123.

Hutchins, C. J., Keese, P., Visvader, J. E., Rathjen, P. D., McInnes, J. L., and Symons, R. H. (1985). Comparison of multimeric plus and minus forms of viroids and virusoids. *Plant Mol. Biol.* **4,** 293–304.

Hutchins, C., Rathjen, P. D., Forster, A. C., and Symons, R. H. (1986). Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. *Nucleic Acids Res.* **14,** 3627–3640.

Inoue, T., and Orgel, L. E. (1983). A non-enzymatic RNA polymerase model. *Science* **219,** 859–862.

James, D., and Howell, W. E. (1998). Isolation and partial characterization of a filamentous virus associated to peach mosaic. *Plant Dis.* **82,** 909–913.

Joyce, G. F. (1989). RNA evolution and the origins of life. *Nature (London)* **338,** 217–224.

Kaper, J. M., Tousignant, M. E., and Steger G. (1988). Nucleotide sequence predicts circularity and self-cleavage of 300-ribonucleotide satellite of arabis mosaic virus. *Biochem. Biophys. Res. Comm.* **154,** 318–325.

Kapoor, S., Suzuki, J. Y., and Sugiura, M. (1997). Identification and functional significance of a new class of non-consensus-type plastid promoters. *Plant J.* **11,** 327–337.

Kawamoto, S. O., Horst, R. K., and Wong, S. M. (1985). Solubility of chrysanthemum chlorotic mottle viroid in LiCl solutions. *Acta Hortic.* **164,** 333–340.

Keese, P., and Symons, R. H. (1985). Domains in viroids: Evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 4582–4586.

Keese, P., and Symons, R. H. (1987). The structure of viroids and virusoids. *In* "Viroids and Viroid-like Pathogens" (J. S. Semancik, ed.), pp. 1–47. CRC Press, Boca Raton.

Kiberstis, P. A., Haseloff, J., and Zimmern, D. (1985). 2′ Phosphomonoester, 3′–5′ phosphodiester bond at a unique site in a circular viral RNA. *EMBO J.* **4,** 817–822.

Kiefer, M. C., Owens, R. A., and Diener, T. O. (1983). Structural similarities between viroids and transposable elements. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 6234–6238.

Kishi, K., Takanashi, K., and Abiko, K. (1973). New virus diseases of peach, yellow mosaic, oil blotch and star mosaic. *Bull. Hort. Res. Sta. Japan, Ser. A.* **12,** 197–208.

Kofalvi, S. A., Marcos, J. F., Cañizares, M. C., Pallás, V., and Candresse, T. (1997). Hop stunt viroid (HSVd) sequence variants from *Prunus* species: Evidence for recombination between HSVd isolates. *J. Gen. Virol.* **78,** 3177–3186.

Koltunow, A. M., and Rezaian, M. A. (1988). Grapevine yellow speckle viroid: Structural features of a new viroid group. *Nucleic Acids Res.* **16,** 849–864.

Konarska, M., Filipowicz, W., Domdey, H., and Gross, H. J. (1981). Formation of a 2′-phosphomonoester, 3′, 5′-phosphodiester linkage by a novel RNA ligase in wheat germ. *Nature* **293,** 112–116.

Lafontaine, D., Beaudry, D., Marquis, P., and Perrault, J. P. (1995). Intra- and inter-molecular non enzymatic ligations occur within transcripts derived from the peach latent mosaic viroid. *Virology* **212,** 705–709.

Lai, M. C. C., and Cavanagh, D. (1997). The molecular biology of coronaviruses *Adv. Virus Res.* **48,** 1–100.

Lakshman, D. K., and Tavantzis, S. M. (1993). Primary and secondary structure of a 360-nucleotide isolate of potato spindle tuber viroid. *Arch. Virol.* **128,** 319–331.

Lerbs-Mache, S. (1993). The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: Single-subunit enzyme or catalytic core of multimeric enzyme complex? *Proc. Natl. Acad. Sci. U.S.A.* **90,** 5509–5513.

Liere, K., and Maliga, P. (1999). *In vitro* characterization of the tobacco *rpoB* promoter reveals a core sequence motif conserved between phage-type plastid and plant mitochondrial promoters. *EMBO J.* **18,** 249–257.

Lima, M. I., Fonseca, M. E. N., Flores, R., and Kitajima, E. W. (1994). Detection of avocado sunblotch viroid in chloroplasts of avocado leaves by *in situ* hybridization. *Arch. Virol.* **138,** 385–390.

Liu, Y. -H., and Symons, R. H. (1998). Specific RNA self-cleavage in coconut cadang cadang viroid: Potential for a role in rolling circle replication. *RNA* **4,** 418–429.

Lomonossoff, G. P. (1995). Pathogen-derived resistance of plant viruses. *Annu. Rev. Phytopathol.* **33,** 323–343.

López-Herrera, C., Pliego, F., and Flores, R. (1987). Detection of avocado sunblotch viroid (ASBV) in Spain by double polyacrylamide gel electrophoresis. *J. Phytopathol.* **119,** 184–189.

Loreti, S., Faggioli, F., and Barba, M. (1995). A rapid extraction method to detect peach latent mosaic viroid by molecular hybridization. *Acta Hortic.* **386,** 560–564.

Marcos, J. F., and Flores, R. (1992). Characterization of RNAs specific to avocado sunblotch viroid synthesized *in vitro* by a cell-free system from infected avocado leaves. *Virology* **186,** 481–488.

Marcos, J. F., and Flores, R. (1993). The 5′ end generated in the *in vitro* self-cleavage reaction of avocado sunblotch viroid RNAs is present in naturally occurring linear viroid molecules. *J. Gen. Virol.* **74,** 907–910.

Margulis, L. (1993). "Symbiosis in Cell Evolution." 2nd ed. W. H. Freeman and Co., New York.

Matthews, R. E. F. (1991). "Plant Virology." 3rd ed. Academic Press, New York.

Mathews, D. E., and Durbin, R. D. (1990). Tagetitoxin inhibits RNA synthesis directed by RNA polymerases from chloroplasts and *Escherichia coli*. *J. Biol. Chem.* **265,** 493–498.

Miller, W. A., Hercus, T., Waterhouse, P. M., and Gerlach. W. L. (1991). A satellite RNA of barley yellow dwarf virus contains a novel hammerhead structure in self-cleavage domain. *Virology* **183,** 711–720.

Mohamed, N. A., and Thomas W. (1980). Viroid-like properties of an RNA species associated with the sunblotch disease of avocados. *J. Gen. Virol.* **46,** 157–167.

Monsion, M., Bachelier, J. C., Candresse, T., Desvignes, J. C., Macquaire, G., and Dunez, J. (1988). Investigations on the infectious agent responsible for peach latent mosaic disease. *Acta Hortic.* **235,** 247–255.

Mühlbach, H. P., and Sänger, H. L. (1979). Viroid replication is inhibited by α-amanitin. *Nature* **278,** 185–188.

Navarro, B., and Flores, R. (1997). Chrysanthemum chlorotic mottle viroid: Unusual structural properties of a subgroup of viroids with hammerhead ribozymes. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11262–11267.

Navarro, J. A., Daròs, J. A., and Flores, R. (1999). Complexes containing both polarity strands of avocado sunblotch viroid: Identification in chloroplasts and characterization. *Virology* **253,** 77–85.

Navarro, J. A., and Flores, R. (2000). Characterization of the initiation sites of both polarity strands of a viroid RNA reveals a motif conserved in sequence and structure. *EMBO J.* **19,** 2662–2670.

Navarro, J. A., Vera, A., and Flores, R. (2000). A chloroplastic RNA polymerase resistant to tagetitoxin is involved in replication of avocado sunblotch viroid. *Virology* **268,** 218–225.

Niblett, C. L., Dickson, E., Fernow, K. H., Horst, R. K., and Zaitlin, M. (1978). Cross-protection among four viroids. *Virology* **91,** 198–203.

Owens, R. A., and Diener, T. O. (1982). RNA intermediates in potato spindle tuber viroid replication. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 113–117.

Pallás, V., García-Luque, I., Domingo, E., and Flores, R. (1988). Sequence variability in avocado sunblotch viroid (ASBV). *Nucleic Acids Res.* **16,** 9864.

Palukaitis, P., Hatta, T. Alexander D. M., and Symons R. H. (1979). Characterization of a viroid associated with avocado sunblotch disease. *Virology* **99,** 145–151.

Palukaitis, P., Rakowski, A. G., Alexander D. M., and Symons R. H. (1981). Rapid indexing of the sunblotch disease of avocados using a complementary DNA probe to avocado sunblotch viroid. *Ann. Appl. Biol.* **98,** 439–449.

Perrota, A. T., and Been, M. D. (1991). A pseudoknot-like structure required for efficient self-cleavage of hepatitis δ virus RNA. *Nature* **350,** 434–436.

Pine, T. S. (1976). Peach blotch, peach calico, peach mosaic. *USDA Agric. Handb.* **437,** 56–70.

Pley, H., Flaherty, K. M., and McKay, D. B. (1994a). Three-dimensional structure of a hammerhead ribozyme. *Nature* **372,** 68–74.

Pley, H., Flaherty, K. M., and McKay, D. B. (1994b). Model for an RNA tertiary interaction from the structure of an intermolecular complex between a GAAA tetraloop and an RNA helix. *Nature* **372,** 111–113.

Polivka, H., Staub, U., and Gross, H. J. (1996). Variation of viroid profiles in individual grapevine plants: Novel grapevine yellow specke viroid 1 mutants show alteration of hairpin I. *J. Gen. Virol.* **77,** 155–161.

Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., and Bruening, G. (1986). Autolytic processing of dimeric plant virus satellite RNA. *Science* **231,** 1577–1580.

Querci, M., Owens, R. A., Vargas, C., and Salazar, L. F. (1995). Detection of potato spindle tuber viroid in avocado growing in Peru. *Plant Dis.* **79,** 196–202.

Rakowski, A. G., and Symons, R. H. (1989). Comparative sequence studies of variants of avocado sunblotch viroid. *Virology* **173,** 352–356.

Riesner, D., Henco, K., Rokohl, U., Klootz, G., Kleinschmidt, Domedey, H., Jank, P., Gross, H. J., and Sänger, H. L. (1979). Structure and structure formation of viroids. *J. Mol. Biol.* **133,** 85–115.

Rohde, W., and Sänger, H. L. (1981). Detection of complementary RNA intermediates of viroid replication by Northern blot hybridization. *Biosci. Rep.* **1,** 327–336.

Romaine, C. P., and Horst, R. K. (1975). Suggested viroid etiology for chrysanthemum chlorotic mottle disease. *Virology* **64,** 86–95.

Rosenstein, S. P., and Been, M. D. (1991). Evidence that genomic and antigenomic RNA self-cleaving elements from hepatitis δ virus have similar secondary structures. *Nucleic Acids Res.* **19,** 5409–5416.

Rosner, A., Spiegel, S., Alper, M., and Bar-Joseph, M. (1983). Detection of avocado sunblotch viroid (ASBV) by dot-spot self-hybridization with a ^{32}P-labelled ASBV-RNA. *Plant Mol. Biol.* **2,** 15–18.

Sänger, H. L. (1988). Viroids and viroid diseases. *Acta Hortic.* **234,** 79–87.

Sänger, H. L., Klotz, G., Riesner, D., Gross, H. J., and Kleinschmidt, A. (1976). Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 3852–3856.

Schindler, I. M., and Mühlbach, H. P. (1992). Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: A reevaluation. *Plant Sci.* **84,** 221–229.

Schnell, R. J., Kuhn, D. N., Ronning, C. M., and Harkins, D. (1997). Application of RT-PCR for indexing avocado sunblotch viroid. *Plant Dis.* **81,** 1023–1026.

Schnölzer, M., Haas, B., Ramm, K., Hofmann, H., and Sänger, H. L. (1985). Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). *EMBO J.* **4,** 2181–2190.

Semancik, J. S., and Desjardins, P. R. (1980). Multiple small RNA species and the viroid hypothesis for the sunblotch disease of avocado. *Virology* **104,** 117–121.

Semancik, J. S., and Szychowski. J. A. (1994). Avocado sunblotch disease: A persistent viroid infection in which variants are associated with differential symptoms. *J. Gen. Virol.* **75,** 1543–1549.

Semancik, J. S., and Weathers, L. G. (1972a). Exocortis virus: An infectious free-nucleic acid plant virus with unusual properties. *Virology* **47,** 456–466.

Semancik, J. S., and Weathers, L. G. (1972b). Exocortis disease: Evidence for a new species of "infectious" low molecular weight RNA in plants. *Nature New Biol.* **237,** 242–244.

Semancik, J. S., Conejero, V., and Gerhart, J. (1977). Citrus exocortis viroid: Survey of protein synthesis in *Xenopus laevis* oocytes following addition of viroid RNA. *Virology* **80,** 218–221.

Shamloul, A. M., Minafra, A., Hadidi, A., Waterworth, H. E., Giunchedi, L., and Allam, E. K. (1995). Peach latent mosaic viroid: Nucleotide sequence of an Italian isolate, sensitive detection using RT-PCR and geographic distribution. *Acta Hortic.* **386,** 522–530.

Sharmeen, L., Kuo, M. Y. P., Dinter-Gottlieb, G., and Taylor, J. (1988). Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *J. Virol.* **62,** 2674–2679.

Skrezeczkowski, L. J., Howell, W. E., and Mink, G. I. (1996). Occurrence of peach latent mosaic viroid in commercial peach and nectarine cultivars in the *US. Plant Dis.* **80,** 823.

Sogo, J. M., Koller, T., and Diener, T. O. (1973). Potato spindle tuber viroid. X. Visualization and size determination by electron microscopy. *Virology* **55,** 70–80.

Spiesmacher, E., Mühlbach, H. P., Schnölzer, M., Haas, B., and Sänger, H. L. (1983). Oligomeric forms of potato spindle tuber viroid (PSTV) and of its complementary RNA are present in nuclei isolated from viroid-infected potato cells. *Biosci. Rep.* **3,** 767–774.

Stern, D. B., Higgs, D. C., and Yang, J. (1997). Transcription and translation in chloroplasts. *Trends Plant Sci.* **2,** 308–315.

Sugiura, M. (1992). The chloroplast genome. *Plant Mol. Biol.* **19,** 149–168.

Symons, R. H. (1981). Avocado sunblotch viroid: Primary sequence and proposed secondary structure. *Nucleic Acids Res.* **9,** 6527–6537.

Tabler, M., Tzortzakaki, S., and Tsagris, M. (1992). Processing of linear longer-than-unit-length potato spindle tuber viroid RNAs into infectious monomeric circular molecules by a G-specific endoribonuclease. *Virology* **190,** 746–753.

Tanaka, K., Oikawa, K., Ohta, N., Kuroiwa, H., Kuroiwa, T., and Takahashi, H. (1996). Nuclear encoding of a chloroplast RNA polymerase sigma subunit in a red alga. *Science* **272,** 1932–1935.

Tanaka, K., Tozawa, Y., Mochizuki, N., Shinozaki, K., Nagatani, A., Wakasa, K., and Takahashi, H. (1997). Characterization of three cDNA species encoding plastid RNA polymerase sigma factors in *Arabidopsis thaliana:* Evidence for the sigma heterogeneity in higher plant plastid. *FEBS Lett.* **413,** 309–313.

Taylor, J. M. (1997). Organization and expression of the hepatitis delta virus genome. *Semin. Virol.* **8,** 59–64.

Thomas, W., and Mohamed N. A. (1979). Avocado sunblotch — a viroid disease? *Australas. Plant Pathol.* **8,** 1–3.

Tsagris, M., Tabler, M., Mühlbach, H. P., and Sänger, H. L. (1987). Linear oligomeric potato spindle tuber viroid (PSTV) RNAs are accurately processed *in vitro* to the monomeric circular viroid proper when incubated with a nuclear extract from healthy potato cells. *EMBO J.* **6,** 2173–2183.

Uhlenbeck, O. C. (1990). Tetraloops and RNA folding. *Nature* **346,** 613–614.

Utermohlen, J. G., Drake, R. J. Desjardins P. R., and Semancik J. S. (1981). The transmission of sunblotch disease with a purified RNA species. *Phytopathology* **71,** 909.

Vera, A., and Sugiura, M. (1995). Chloroplast rRNA transcription from structurally different tandem promoters: An additional novel-type promoter. *Curr. Genet.* **27,** 280–284.

Vera, A., Hirose, T., and Sugiura, M. (1996). A ribosomal protein gene *(rp132)* from tobacco chloroplast DNA is transcribed from alternative promoters in non-photosynthetic plastids: Similar promoter region organization in plastid housekeeping genes. *Mol. Gen. Genet.* **251,** 518–525.

Visvader, J. E., and Symons, R. H. (1985). Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. *Nucleic Acids Res.* **13,** 2907–2920.

Visvader, J. E., and Symons, R. H. (1986). Replication of *in vitro* constructed viroid mutants: Location of the pathogenicity-modulating domain in citrus exocortis viroid. *EMBO J.* **13,** 2051–2055.

Wallace, J. M. (1958). The sunblotch disease of avocados. *J. Rio Grande Val. Hortic. Soc.* **12,** 67–74.

Wallace, J. M., and Drake R. J. (1962). A high rate of seed transmission of avocado sunblotch virus from symptomless trees and the origin of such trees. *Phytopathology* **52,** 237–241.

Whitsell, R. (1952). Sunblotch disease of avocado. *Calif. Avocado Soc. Yearb.* **37,** 217–240.

Woese, C. R., Winker, S., and Gutell, R. R. (1990). Architecture of ribosomal RNA: Constraints on the sequence of tetraloops. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 8467–8471.

Woo, Y. -M., Itaya, A., Owens, R. A., Tang, L., Hammons, R. W., Chou, H. -C., Lai, M. M. C., and Ding, B. (1999). Characterization of nuclear import of potato spindle tuber viroid RNA in permeabilized protoplasts. *Plant J.* **17,** 627–635.

Wu, H. -N., Lin, Y. -J., Lin, F. -P., Makino, S., Chang, M. -F., and Lai, M. M. C. (1989). Human hepatitis δ virus RNA subfragments contain an autocleavage activity. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1831–1835.

Zaitlin, M., Niblet, C. L., Dickson, E., and Goldberg, R. B. (1980). Tomato DNA contains no detectable regions complementary to potato spindle tuber viroid as assayed by solution and filter hybridization. *Virology* **104,** 1–9.

Zentmeyer, G. A. (1959). Avocado diseases in Latin America. *Plant Dis. Rep.* **43,** 1229–1230.

Zhang, Y., and Epstein, L. M. (1996). Cloning and characterization of extended hammerheads from a diverse set of caudate amphibians. *Gene* **172,** 183–190.

Zimmern, D. (1982). Do viroids and RNA viruses derive from a system that exchanges genetic information between eukaryotic cells? *Trends Biochem. Sci.* **7,** 205–207.

Zuker, M. (1989). On finding all suboptimal foldings of an RNA molecule. *Science* **244,** 48–52.