Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3711–3715, May 1992
Biochemistry

Plus and minus RNAs of peach latent mosaic viroid self-cleave *in vitro* via hammerhead structures

(viroids/viroid-like satellite RNAs/self-cleavage)

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Communicated by Theodor O. Diener, December 13, 1991

ABSTRACT **Peach latent mosaic viroid (PLMVd), the causal agent of peach latent mosaic disease, has been sequenced and found to be a circular RNA molecule of 337 nucleotide residues, which adopts a branched conformation when it is folded in the model of lowest free energy. PLMVd exhibits limited homologies with other viroids and some satellite RNAs, but it does not have any of the central conserved sequences characteristic of the subgroups of typical viroids. However, a segment of approximately one-third of the PLMVd sequence has the elements required to form in the RNAs of both polarities the hammerhead structures proposed to act in the *in vitro* self-cleavage of avocado sunblotch viroid (ASBVd) and some satellite RNAs. Plus and minus partial- and full-length RNA transcripts of PLMVd containing the hammerhead structures displayed self-cleavage during transcription and after purification as predicted by these structures. These data are consistent with the high stability of the PLMVd hammerhead structures, more similar to the corresponding structures of some satellite RNAs than to those of ASBVd, and indicate that the self-cleavage reactions of PLMVd are most probably mediated by single hammerhead structures. Our results support the inclusion of PLMVd in a viroid subgroup represented by ASBVd, whose members are characterized by their ability to self-cleave *in vitro*, and probably *in vivo*, through hammerhead structures. A consensus phylogenetic tree has been obtained suggesting that PLMVd, together with ASBVd, may represent an evolutionary link between viroids and viroid-like satellite RNAs.**

Analysis of the 15 known sequences of different viroids (small plant pathogenic single-stranded circular RNAs) has revealed that they can be classified into two groups, one formed by avocado sunblotch viroid (ASBVd) and one formed by all other typical viroids. These groups, in turn, can be further divided into either two or four subgroups depending on the criteria adopted (1, 2). The very special position of ASBVd in this scheme is accompanied by other peculiar features, prominent among which is the ability of ASBVd dimeric transcripts of both polarities to self-cleave *in vitro* to unit length strands (3). Since this cleavage, proposed to occur through a hammerhead structure, is shared by some satellite RNAs, particularly by those with a circular viroid-like structure (virusoids), but not by typical viroids (4), the possibility has been raised that ASBVd might be a viroid-like satellite RNA, especially because avocado trees often are infected by what appear to be one or more seed-transmissible viruses that could potentially act as helper viruses (5). Although a recent phylogenetic analysis indicates that ASBVd may represent an evolutionary link between typical viroids and viroid-like satellite RNAs (2), as it had been advanced previously on the basis of different criteria (6), the description of additional viroids containing hammerhead structures and consequently exhibiting self-cleavage *in vitro* would help to establish more firmly that this property is not exceptional among viroids. Here we report that peach latent mosaic viroid (PLMVd), the causal agent of PLM disease (7), contains self-cleavage domains that can adopt hammerhead structures in the RNAs of both polarities.†

MATERIALS AND METHODS

Viroid Source. PLMVd was purified as reported (7) from fruits (diameter, 2–4 cm) of the peach cultivar Armking S5615, which is infected by a typical isolate of this viroid as indicated by field symptomatology and cross-protection bioassay in the greenhouse (8).

Cloning and Sequencing of PLMVd cDNA. PLMVd fragments were generated by limited digestion of the circular RNA with RNases T1 and U2, and their sequences were determined by partial enzymic hydrolysis and PAGE (9).

First-strand cDNA was synthesized on circular PLMVd as described (10), using reverse transcriptase and either the complementary oligodeoxyribonucleotide primer 1 (5′-GCGTCCCTACCTGGATCACACCCCCC-3′) or primer 2 (5′-CTATCGGGAACTGCAGTGCTCCGAATAGGGC-ACC-3′), whose sequences were derived from RNA and DNA sequencing, respectively. Second-strand DNA was synthesized by the method of Gubler and Hoffman (11), and the resulting double-stranded cDNA was cloned in the *Sma* I site of pUC18.

Viroid cDNA inserts were sequenced by the dideoxynucleotide chain-termination method (12) using T7 DNA polymerase and in some cases *Taq* DNA polymerase with 7-deaza-dGTP.

Analysis of the *in Vitro* Self-Cleavage of PLMVd RNAs. A cDNA clone containing the full-length sequence of PLMVd plus an additional repeat of 16 residues was subcloned in plasmids pSPT18 and pSPT19 (Boehringer Mannheim), and transcripts of plus and minus polarities, respectively, were obtained with T7 RNA polymerase. A PLMVd cDNA fragment of 207 residues between the *Pst* I and *Sma* I sites was also subcloned in the same plasmids, and transcripts of both polarities were obtained in the same way. Radioactive and nonradioactive transcription reactions were carried out and the products were analyzed as described (13).

Self-cleavage of the purified transcription products was performed by incubating them in 50 mM Tris·HCl, pH 8/5 mM $MgCl_2$/0.5 mM EDTA (self-cleavage buffer) at 40°C for 1 h (13). Unless otherwise stated, the samples were previously heated in 1 mM EDTA (pH 6) at 100°C for 1 min and

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Abbreviations: PLMVd, peach latent mosaic viroid; ASBVd, avocado sunblotch viroid; vLTSV, lucerne transient streak virusoid.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M83545).

snap cooled on ice. In some experiments, the heat treatment was in 1 mM EDTA (pH 6) containing 25%, 50%, or 75% formamide. In other experiments, the snap cooling was in methanol/dry ice. Products from the reaction mixtures were examined by PAGE (in some cases the gels contained 40% formamide in addition to 7 M urea to improve the resolution) and identified by autoradiography.

Sequence Comparisons. Alignment of viroid and satellite RNA sequences and obtainment of the consensus phylogenetic tree were as indicated (2). The secondary structures of lowest free energy were determined by using the FOLD program (Genetics Computer Group package) based on that of Zuker and Stiegler (14).

RESULTS

Nucleotide Sequence of PLMVd. Two overlapping and apparently full-length cDNA clones of PLMVd, one obtained with primer 1 (26-mer) (Fig. 1, positions 193–218) and the other obtained with primer 2 (34-mer) (positions 91–124) were selected and sequenced in both directions. The second cDNA clone made it possible to correct the sequence corresponding to the 5′ end of primer 1. Fig. 1 (*Inset*) shows the primary structure of PLMVd, which is a circular RNA of 337 residues consisting of 90 G (26.7%), 87 C (25.8%), 80 A (23.7%), and 80 U (23.7%) residues. Therefore, the PLMVd G+C content (52.5%) is lower than that of the other viroids (56–60%) with the exception of ASBVd (38%) (1).

The sequence of PLMVd was compared with those of typical viroid subgroup members (2): potato spindle tuber viroid, apple scar skin viroid, coconut cadang-cadang viroid, hop stunt viroid, and the recently described *Coleus blumei* viroid 1 (15). Other sequences included in the comparative analysis were those of ASBVd, lucerne transient streak virusoid (vLTSV), solanum nodiflorum mottle virusoid, and the linear satellite RNA of tobacco ringspot virus. Although PLMVd shares sequence homologies with all the aforementioned RNAs (see below), these homologies are limited and do not include the highly conserved sequences characteristic of the central regions of the members of the viroid subgroups. However, plus and minus PLMVd RNAs do have the conserved sequences of the hammerhead structures present in the self-cleaving RNAs of ASBVd and in some satellite RNAs (see below).

Proposed Secondary Structure for PLMVd. The search for the PLMVd secondary structure of lowest free energy led to an unexpected conformation with two branching points from which five and three stems emerge (Fig. 1), whereas the characteristic rod-like structures were found for all typical viroids with the exception of hop latent viroid, for which a conformation with two bifurcations slightly more stable than the rod-like one proposed was obtained (16). The application of the same approach to the case of ASBVd also led to a rod-like conformation but one with a short bifurcation at one of its ends, in agreement with previous results (17). The value of the $\Delta G/N$ parameter (difference in free energy per nucleotide residue during the formation of the structure in the ground state from the completely coiled polynucleotide strand) for the branched conformation of PLMVd (1.58 kJ/mol) was within the range of those corresponding to the rod-like conformations of apple scar skin viroid and other members of this subgroup (apscaviroids) (1.45–1.59 kJ/mol); higher and lower $\Delta G/N$ values were obtained for those of the potato spindle tuber viroid subgroup (pospiviroids) (1.62–1.74 kJ/mol) and ASBVd (1.12 kJ/mol). In the proposed secondary structure for PLMVd, paired nucleotide residues represent the 71.2% of the total, including 52.5% G+C, 40.8% A+U, and 6.6% G+U.

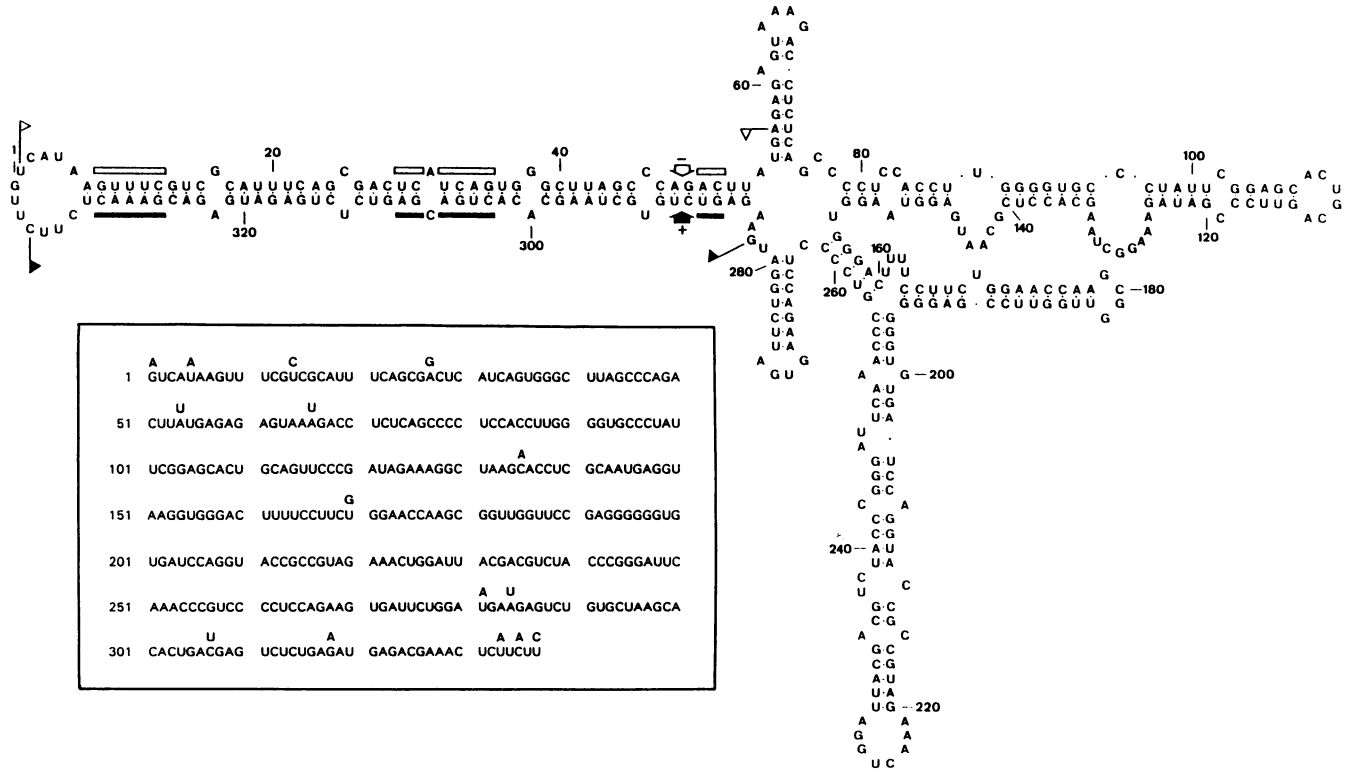

FIG. 1. Proposed secondary structure of lowest free energy for PLMVd. Plus and minus self-cleavage domains are delimited by flags, the 13 conserved residues present in all hammerhead structures are indicated by bars, and the predicted self-cleavage sites are shown by arrows. Solid and open symbols refer to plus and minus polarities, respectively. (*Inset*) Primary structure of PLMVd with the sequence heterogeneity resulting from base substitutions indicated above the reference sequence. *Pst* I and *Sma* I sites are located between positions 109–114 and 241–246, respectively.

Hammerhead Structures in PLMVd RNAs. Fig. 2 shows that PLMVd RNAs of plus and minus polarities have the 13 conserved residues together with the other elements characteristic of the hammerhead structures responsible for the *in vitro* self-cleaving reactions of ASBVd, of a group of satellite RNAs, and of the transcript of the newt satellite 2 DNA (4, 18).

Both hammerhead structures of PLMVd, included in a segment representing approximately one-third of the viroid sequence, have very stable stems III and short loop-outs at the ends of stems I and II (Fig. 2), resembling in these two aspects the hammerhead structures of virusoids and satellite RNA of tobacco ringspot virus more than those of ASBVd. A cytidylate residue precedes the predicted self-cleavage sites of the PLMVd hammerhead structures, as occurs in most of the other known hammerhead structures. The residue between the conserved sequences CUGA and GA is a C and a U in the plus and minus PLMVd hammerhead structures, respectively, conforming to the known hammerhead structures where the residue found in this position is C, U, or A.

It is interesting to note that the base substitutions found in the second PLMVd clone in the region of the hammerhead structures either do not affect the stability of stems II and III or are located in loops (Fig. 2). PLMVd plus and minus hammerhead structures share extensive sequence homologies (Fig. 2).

Self-Cleavage of PLMVd RNAs. The self-cleavage of PLMVd RNAs was investigated by means of RNA transcripts of both polarities synthesized *in vitro* from linearized clones containing PLMVd cDNAs of partial and full length (Fig. 3*A*). Both plus and minus PLMVd RNAs self-cleaved extensively during transcription (Fig. 3*B*, lanes 2, 4, 6, and 8), generating fragments of lengths consistent with the sites of self-cleavage predicted from previous studies with other self-cleaving RNAs having hammerhead structures (3, 4, 18, 20, 21). The exact positions of the phosphodiester bonds cleaved were determined by enzymic sequencing of the 5′ ends of the 3′ self-cleavage fragments from both partial-length transcripts and were found to coincide with the predicted self-cleavage sites (data not shown), which are separated by 96 nucleotide residues.

When the complete plus and minus RNA transcripts from full-length clones were purified from preparative gels, denatured by heating, and incubated under standard self-cleavage conditions, both RNAs (referred to as FC+ and FC−), but especially FC−, showed self-cleavage (Fig. 3*B*, lanes 3 and 5). However, in similar experiments with complete plus and minus RNA transcripts from partial-length clones, none of the RNAs (referred to as PC+ and PC−) self-cleaved (lanes 7 and 9). Assuming that the treatment (heat denaturation and snap cooling on ice) preceding the incubation with the self-cleavage buffer was not sufficient to form in PC+ and PC− the conformations active in self-cleavage that are present during transcription and that PC+ contained instead the inactive conformation present in the native structure (Fig. 1), and PC− contained a similar one, we first modified the treatment by performing the snap cooling on dry ice/methanol. Under these conditions, self-cleavage was detected with PC− (lane 10) as well as with PC+ (data not shown). As a second modification of the treatment, heat denaturation was carried out in the presence of 25%, 50%, or 75% formamide and was followed by snap cooling on ice. Under this new set of conditions, an increase in self-cleavage with increasing formamide concentration was detected with PC− (lanes 11–13) and PC+ (data not shown), and a more extensive self-cleavage than in the absence of formamide was observed with FC+ and FC− (data not shown).

Place of PLMVd Among Viroids and Viroid-like Satellite RNAs. The phylogenetic tree relating PLMVd to members of

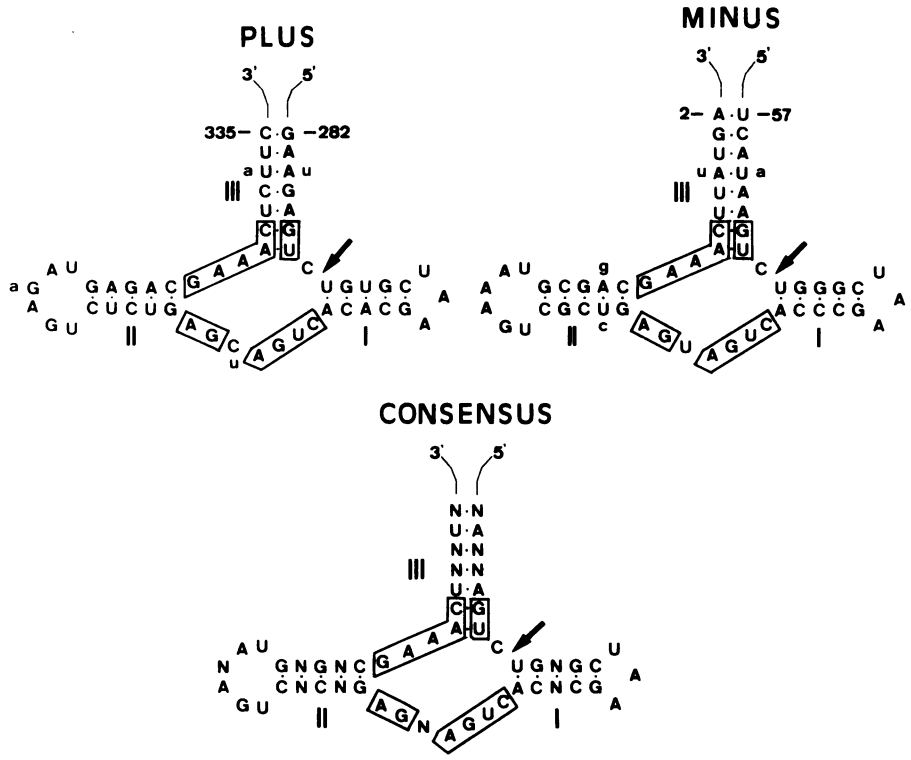

FIG. 2. Hammerhead structures of plus and minus PLMVd RNAs with arrows indicating the predicted self-cleavage sites. Residues boxed by solid lines are the conserved sequences present in all hammerhead structures in similar positions, and lowercase letters indicate base substitutions. The same numbers are used in the minus as in the plus polarity. The hammerhead structure consensus between the plus and minus ones of PLMVd is also presented, where N indicates nonconserved residues. The terminal base pair of stem III of the hammerhead structure of plus PLMVd RNA does not exist in the sequence derived from the second PLMVd clone, where the C at position 335 has been substituted by an A.

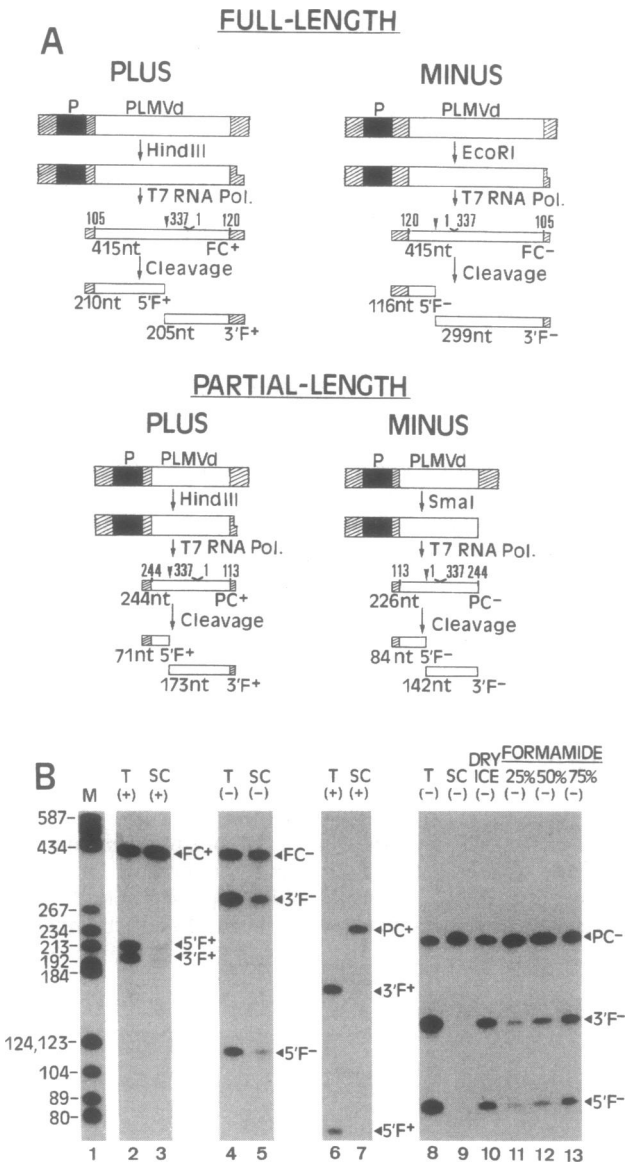

FIG. 3. *In vitro* synthesis of RNAs containing sequences of PLMVd and self-cleavage reactions of purified RNAs. (*A*) Diagrams of plus and minus DNA templates (the full-length template was in fact a greater-than-unit-length 353-residue cDNA clone of PLMVd) and of the T7 RNA polymerase products generated by transcription of templates linearized with appropriate restriction enzymes. Hatched boxes, vector sequences; solid boxes, T7 promoter; open boxes, PLMVd sequences. Following the nomenclature proposed in ref. 19, the transcription products are C+ and C− (the complete transcripts, with a preceding F or P to indicate full or partial length, respectively), and 5′F+, 3′F+, 5′F−, and 3′F− (the cleavage fragments). The sites of self-cleavage are indicated by arrowheads. Nucleotide (nt) residue numbers in PLMVd RNAs are given above the products and their expected size is below. (*B*) Analysis of the T7 RNA polymerase transcription (T) reactions and of self-cleavage (SC) reactions of purified products by PAGE and autoradiography. Lane 1, radioactive DNA markers (prepared by digesting pBR322 with *Hae* III and end-labeling) with their sizes in residues on the left. Lanes 2 and 4, transcription products + and − from full-length clones. Lanes 3 and 5, self-cleavage of purified FC+ and FC− after heating at 100°C for 1 min in 1 mM EDTA (pH 6), snap cooling on ice for 5 min, and incubation at 40°C for 1 h in the self-cleavage buffer. Lanes 6 and 8, transcription products + and − from partial-length clones. Lanes 7 and 9, self-cleavage of purified PC+ and PC− after the same treatment as in lanes 3 and 5. Lanes 10–13, same as lane 9 but with the following modifications: snap cooling on dry ice/methanol (lane 10), and heating at 100°C for 1 min in 1 mM EDTA (pH 6) containing 25% (lane 11), 50% (lane 12), and 75% (lane 13) formamide (after addition of the self-cleavage buffer the initial formamide concentrations were reduced to one-half).

the subgroups of typical viroids, ASBVd and some representatives of the viroid-like satellite RNAs, is presented in Fig. 4. It can be concluded that the monophyletic origin proposed for viroids and viroid-like satellite RNAs (2) can be extended to PLMVd (as well as to *Coleus blumei* viroid 1, which was not included in the previous study). PLMVd appears separate from typical viroids and close to ASBVd and particularly to vLTSV; the latter two RNAs have, like PLMVd, hammerhead structures in the strands of both polarities.

DISCUSSION

A notable feature of PLMVd is its ability to form the hammerhead structure that has been described in some satellite RNAs (4, 18, 20) but so far only in one viroid, ASBVd (3). PLMVd can, like ASBVd, assume hammerhead structures in the RNAs of both polarities, although with the latter double-hammerhead structures have been proposed to operate in the self-cleavage reactions (especially in that of the plus polarity), since the ASBVd single-hammerhead structures are unstable (19, 22). Conversely, the hammerhead structures of PLMVd are stable, and RNAs containing only subsets of PLMVd sequences of either polarity self-cleaved efficiently during transcription, indicating that the corresponding reactions most probably occur through an intramolecular mechanism. The hammerhead structures of plus and minus PLMVd are more closely related to each other (Fig. 2) than to any of the other known hammerhead structures, a situation that has also been observed between the hammerhead structures of vLTSV (23). Moreover, the 13 conserved residues of the plus and minus PLMVd hammerheads and their corresponding self-cleavage sites (separated by 96 nucleotide residues) occur in opposite positions in the proposed conformations of plus (Fig. 1) and minus PLMVd RNAs (data not shown), as also happens in vLTSV (23), although the self-cleavage sites of vLTSV are separated by only 6 nucleotide residues and part of the self-cleavage domains are overlapping. The conformations of lowest free energy of neither PLMVd RNA contain the hammerhead structures and, consequently, are not expected to self-cleave; these conforma-

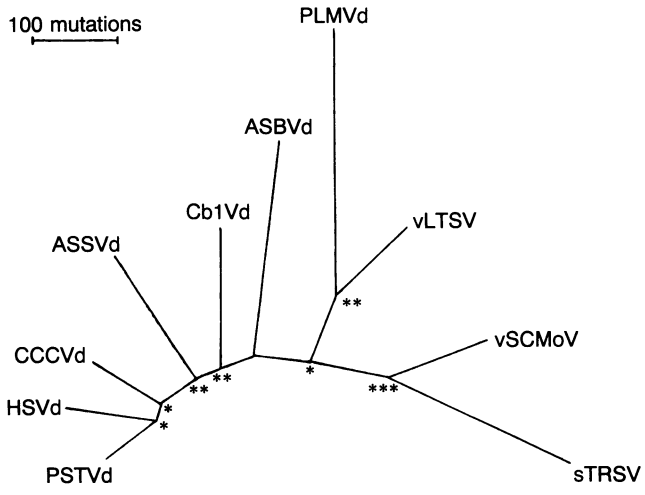

FIG. 4. Consensus phylogenetic tree (based on 100 replicates) obtained for the sequences of some representative viroids and satellite RNAs. ***, Group monophyletic in all of the replicates; **, monophyletic in >95%; *, monophyletic in >70%; the ASBVd branch appeared in the indicated position in 59% of the replicates. PSTVd, potato spindle tuber viroid; ASSVd, apple scar skin viroid; CCCVd, coconut cadang-cadang viroid; HSVd, hop stunt viroid; Cb1Vd, *Coleus blumei* viroid 1; sTRSV, satellite RNA of tobacco ringspot virus; vSCMoV, subterranean clover mottle virusoid.

tions probably exist in the purified complete transcripts as well as in the circular infectious viroid (preventing their self-cleavage). Alternatively, the active conformations may form during transcription and promote self-cleavage before RNA synthesis has been completed and the most stable inactive conformations can be formed. It is also interesting to note that there are homologies between the nucleotide residues forming stems I and II of the hammerhead structures of plus and minus PLMVd, particularly of those close to the interior single-stranded loop, and the corresponding residues of the hammerhead structures of ASBVd (plus), subterranean clover mottle virusoid (plus), and of vLTSV (plus and minus) (4, 21, 23).

A second remarkable feature of PLMVd is that the secondary structure model of lowest free energy (Fig. 1) presents two branching points, not conforming, therefore, to the rod-like structures characteristic of most typical viroids. The branched structure of PLMVd nevertheless has a stability within the range of those corresponding to the rod-like conformations of typical viroids, as deduced from the values obtained for the $\Delta G/N$ parameter. This type of branched structure has been reported in some viroids and virusoids (17, 21, 24), although in the case of PLMVd the number of branches is higher and they are longer. The physiological significance of the proposed secondary structure is not known, although the segment containing the sequences involved in the hammerhead structures is very stable (a similar stable segment can also be formed in the minus polarity) and, as indicated above, it probably protects the circular viroid molecules from self-cleavage.

Data have been presented previously demonstrating that the causal agent of PLM disease is a viroid, since purified preparations of it induced characteristic symptoms of this malady in GF-305 peach seedlings (*Prunus persica* L. Batsch) (7). The possibility that PLMVd could rather be a viroid-like satellite RNA can be discarded, since this same experimental host has been used extensively as an indicator for detection of different virus and virus-like diseases of fruit trees (8), and there is no evidence that it could be originally infected in a symptomless way by a potential helper virus, which also should be transmitted very efficiently by seed. Moreover, PLMVd, like ASBVd, does not contain the sequence GAUUUU, conserved in all virusoids in a similar position on their proposed secondary structures, which, as has been suggested (21), may play a role in replication of these viroid-like satellite RNAs.

The very limited host range of PLMVd, which is a peach-specific disease (25), is reminiscent of a parallel situation in ASBVd, which can infect only avocado (*Persea americana* Mill.) and other species of the Lauraceae (26). PLM disease is probably the same disease known as peach mosaic in the United States and as peach yellow mosaic in Japan; the term "latent" in the name of the disease as described in France refers to the observation that most of the infections do not induce symptoms in leaves, with the first pathological alterations (delay in foliation, flowering, and ripening; fruit deformations and cracked sutures; bud necrosis; and rapid aging of trees) becoming visible 2 years after planting (25). Symptoms caused by ASBVd in leaves are also sporadic, and some infections are symptomless or latent (26).

Sequencing of PLMVd has shown its partial homology with other viroids as well as with some satellite RNAs, an observation consistent with the proposed monophyletic origin for all these RNAs (2). The consensus phylogenetic tree obtained (Fig. 4) suggests a relationship between PLMVd and vLTSV, although the length of the PLMVd branch is indicative of a long independent evolution of this sequence, and the biological properties of PLMVd are those of a viroid. Therefore, PLMVd may represent an even closer evolutionary link between viroids and viroid-like satellite RNAs than ASBVd.

PLMVd is the second member of a subgroup of viroids characterized by having hammerhead structures responsible for self-cleavage *in vitro*, which probably also play a role in the processing of oligomeric precursors *in vivo*. ASBVd is the type member of this subgroup for which we propose the name avsunviroids following established criteria (2).

We want to express our deep appreciation to J. C. Desvignes (Centre Technique Interprofessionel des Fruits et Légumes, Lanxade, France) for providing us with the PLMVd-infected tissue and for encouragement and support. The assistance of S. F. Elena and A. Moya (Servei de Bioinformàtica, Universitat de València) with the phylogenetic analysis and of M. Climent (Instituto de Agroquímica y Tecnología de Alimentos) with the processing of the tissue is acknowledged, as well as the comments of G. Llácer, V. Pallás, J. Marcos, J. A. Darós, J. Dopazo, and A. Navarro. Fig. 3*A* was kindly drawn by C. Alvarez and M. Flores. This work was partially supported by a grant from the Dirección General de Investigación Científica y Técnica of Spain (PB87-0346) and by an Integrated Action between France and Spain (164-1990). C.H. is recipient of a predoctoral fellowship from the Generalitat Valenciana.

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