Virology **271,** 37–45 (2000)
doi:10.1006/viro.2000.0288, available online at http://www.idealibrary.com on IDEAL®

Sequencing of Peach Latent Mosaic Viroid Variants from Nine North American Peach Cultivars Shows that This RNA Folds into a Complex Secondary Structure

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Received December 27, 1999; accepted March 6, 2000

We sequenced 34 new peach latent mosaic viroid (PLMVd) variants isolated from nine different peach cultivars. This study provides the widest view of PLMVd diversity reported to date and includes the original characterization of North American variants, which cannot be differentiated from European sequences. PLMVd appears as a species in which each isolate is a complex mixture of RNAs. Analysis of base-pair covariations supports the hypothesis that PLMVd folds into a complex branched structure with the potential of including three new pseudoknots. The resulting "globular-like" structure is in contrast to the rod-like one adopted by most other viroids.

INTRODUCTION

Viroids, which are small (~300 nucleotides), single-stranded, circular RNAs that infect higher plants and cause significant losses in agriculture, are the smallest nucleic acid-based pathogens known (Symons, 1997). They replicate through a rolling circle mechanism involving only RNA intermediates and do not encode any proteins. Consequently, the distinct biological properties of viroids, including the identification of their natural hosts, depends strictly on their RNA sequences and structures. Peach latent mosaic viroid (PLMVd), the causal agent of peach latent mosaic disease, is a group A viroid and thus possesses self-cleaving hammerhead motifs essential for its replication (Hernandez and Flores, 1992). This viroid is generally latent in peach trees for 5–7 years before symptoms begin to appear. The most conspicuous symptoms under field conditions are a delay in foliation, flowering, and ripening, as well as fruit deformation, bud necrosis, and rapid aging of the tree. PLMVd is widely distributed (~55%) in peach germ plasm from Europe, Asia, and both North and South America (Flores *et al.,* 1990; Hadidi *et al.,* 1996). Furthermore, PLMVd, or a closely related viroid, is occasionally detected in apple, cherry, pear, plum, and apricot germ plasm from countries in Europe, Asia, and the Middle East (Hadidi *et al.,* 1996; El-Dougdoug, 1998); however, only a 337-nt sequence of PLMVd isolated from cherry has been reported to date (Hadidi *et al.,* 1996). In contrast, several PLMVd sequences were established from peach tree cultivars originating from European countries (Ambros *et al.,* 1998; Hernandez and Flores, 1992; Shamloul *et al.,* 1995). The first two variants were from one French cultivar, whereas the third came from an Italian one. Together, these variants showed that mutations are scattered throughout the RNA molecules (Hernandez and Flores, 1992; Shamloul *et al.,* 1995). An analysis of 29 sequence variants derived from one severe and two latent European peach cultivars showed that PLMVd is a quasi-species (Ambros *et al.,* 1998). In this work, phylogenetic analysis resulted in the division of PLMVd molecules into three major groups. Recently, the rapid generation of genetic heterogeneity in progeny from individual cDNA clones of 3 of these 29 variants demonstrated that the evolution rate exhibited by PLMVd is considerably higher than that reported for other viroid species, such as potato spindle tuber viroid (Ambros *et al.,* 1999).

To learn more about PLMVd sequence heterogeneity, we embarked on a sequencing project to determine the breadth of PLMVd sequence variability between populations of RNA extracted from various peach cultivars. We then used this information to determine the *in vivo* secondary structure of PLMVd by sequence covariations and see how it compared with that based on nuclease mapping experiments in solution (Bussière *et al.,* 2000). An inherent goal of this work was to determine PLMVd sequence variants from a broad range of North American peach cultivars for which no sequence data were available before this work.

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0042-6822/00 $35.00

RESULTS

Sequencing of North American PLMVd

PLMVd RNA was extracted from the leaves of nine different peach trees belonging to nine different cultivars grown on the west coasts of Canada (Sidney, British Columbia) and the United States (Prosser, WA) (see Table 1). Each peach tree was naturally infected by PLMVd, so a broad range of sequence heterogeneity should be expected. The PLMVd RNAs were RT–PCR amplified using a pair of primers that hybridize to the PLMVd P3 stem (positions 97–134 of PLMVd isolated from the Armking cultivar; Ar2, Table 1) because this region shows little sequence variation. Furthermore, because the sequences reported by Ambros *et al.* (1998) were obtained using primers that hybridized to another region, the two sets of data should give a clear picture of the sequence variability of the entire PLMVd genome. The resulting DNA fragments were cloned, and four PLMVd clones from each of eight different peach cultivars, and two from the Agua cultivar, were sequenced in both directions. The four clones isolated from the Suncrest cultivar were identical, as were those isolated from the Golden Health cultivar. Therefore, 28 sequences are reported in the GenBank Nucleotide Sequence Database under accession nos. AF170496 to AF170523. With the exception of one clone from the Harrow Beauty cultivar (i.e., HB4) that is 342 nt in size, all other PLMVd variants ranged in size from 336 to 339 nt, with no correlation between size and cultivar source being observed (see Table 1, Fig. 1).

Before undertaking the analysis of this new collection of nucleotide sequences, different controls were performed to establish the background error rate of the method used (data not shown). More specifically, we attempted to determine the sequence heterogeneity introduced during the RT–PCR amplification. We analyzed the fidelity of the PCR amplification, which was performed using *Taq* DNA polymerase with the number of thermocycles limited to 30 so as to limit the number of PCR artifacts. First, we performed the PCR amplification using the linearized plasmid with the PLMVd insert cloned from the peach Suncrest cultivar (i.e., Su2; see Table 1) as template. The Suncrest insert was used because the four original clones were identical. After gel-purification, the PCR product was cloned and sequenced. The new sequences revealed four clones identical to the original. Second, a dimeric PLMVd insert corresponding to the isolate from the Armking cultivar (i.e., Ar2; pPD1 plasmid, see Materials and Methods) was used as template. Using the same protocol as above, three clones identical to the original sequence were obtained. A fourth clone possessed two mutations: the substitution of an adenosine for an uridine at position 105 and an insertion of a guanosine between positions 253 and 254. Therefore, the PCR amplification appeared to have good fidelity, with only two mutations in eight clones being detected.

Next, we questioned whether the RT could have generated a bias in our data. Although the avian myeloblastosis virus (AMV) reverse transcriptase is an error-prone polymerase, it was used in this work because all other reverse transcriptases tested were inefficient in synthesizing full-length cDNA using the highly structured PLMVd RNA as template. PLMVd RNA strands were synthesized by *in vitro* transcription using plasmid pPD1 and were purified on 5% polyacrylamide gels. Greater-than-unit-length linear PLMVd transcripts were used as template to establish the fidelity of the procedure. RT–PCR amplification, coupled with cloning and sequencing of four clones, was performed. Although the *in vitro* transcription might introduce mutations into the RT–PCR amplification template, two clones were found to be identical to the plasmid sequence. Two other clones were found to contain a single mutation each: one with the adenosine at position 61 mutated to a cytosine and the other with deletion of the adenosine at position 282. Regardless of whether any potential bias is introduced by the *in vitro* transcription, the RT–PCR amplification and further steps of the procedure appear to have good fidelity. In these control experiments, the mutations were detected at different positions, indicating that they do not result from a systematic bias in the method. In fact, the controls revealed that the introduction of sequence heterogeneity is limited during the procedure, that it is at least an order of magnitude less than the heterogeneity observed in the collection of new PLMVd variants. Thus, it seems that most of the sequence heterogeneity observed resulted from the evolution of PLMVd in the infected leaves.

Sequence analysis

To facilitate sequence analysis, all PLMVd variants (i.e., including those reported previously; Ambros *et al.,* 1998; Hernandez and Flores, 1992; Shamloul *et al.,* 1995), with the exception of those obtained from cDNA clones (Ambros *et al.,* 1999), were aligned using the CLUSTAL W program (Thomson *et al.,* 1994) (Fig. 1). For purposes of the alignment, the 5′ end was arbitrarily fixed between the P2 and P3 stems because they are well conserved and contiguous (i.e., without a single-stranded region between them; see later). Some variants from the Armking, Redgold, Hardired, and Siberian C cultivars were found to be identical, indicating that a sequence is not exclusive to a cultivar. No stretch of sequence differentiates PLMVd variants isolated from European and North American cultivars. The observed sequence variability is restricted to a relatively small number of positions, with 220 positions being highly conserved in the 61 different sequences analyzed. Among the positions that showed variability, about half had only one other base. This lim-

TABLE 1

PLMVd Sequences Known

Cultivar[a]	Variant[b]	Length (nt)	Reference	Accession no.[c]
Armking	Ar1	337	Hernandez and Flores, 1992	332747
Armking	Ar2	338	Hernandez and Flores, 1992	332747
Suncrest	Su1	338	Shamloul *et al.,* 1995	
Bing[d]	Bi1	337	Itadidi *et al.,* 1996	
GF-305	gds2	337	Ambros *et al.,* 1998	3445334
GF-305	gds21	337	Ambros *et al.,* 1998	3445335
GF-305	gds15	338	Ambros *et al.,* 1998	3445336
GF-305	gds23	338	Ambros *et al.,* 1998	3445337
GF-305	gds18	338	Ambros *et al.,* 1998	3445338
GF-305	gds1	338	Ambros *et al.,* 1998	3445339
GF-305	gds3	338	Ambros *et al.,* 1998	3445348
GF-305	gds19	338	Ambros *et al.,* 1998	3445349
GF-305	gds13	337	Ambros *et al.,* 1998	3445350
GF-305	gds6	337	Ambros *et al.,* 1998	3445351
GF-305	gds16	336	Ambros *et al.,* 1998	3445352
GF-305	esc8	337	Ambros *et al.,* 1998	3445353
GF-305	esc16	337	Ambros *et al.,* 1998	3445354
GF-305	esc5	337	Ambros *et al.,* 1998	3445355
GF-305	esc12	337	Ambros *et al.,* 1998	3445356
GF-305	esc10	336	Ambros *et al.,* 1998	3445357
GF-305	esc14	336	Ambros *et al.,* 1998	3445358
GF-305	1s4b	336	Ambros *et al.,* 1998	3445359
GF-305	1s16b	337	Ambros *et al.,* 1998	3445360
GF-305	1s17b	337	Ambros *et al.,* 1998	3445361
GF-305	1s1	337	Ambros *et al.,* 1998	3445362
GF-305	1s18b	337	Ambros *et al.,* 1998	3445363
GF-305	1s11	337	Ambros *et al.,* 1998	3445364
GF-305	1s8	338	Ambros *et al.,* 1998	3445365
GF-305	1s19b	335	Ambros *et al.,* 1998	3445366
GF-305	1s5b	335	Ambros *et al.,* 1998	3445367
GF-305	1s11b	336	Ambros *et al.,* 1998	3445378
GF-305	1s6b	338	Ambros *et al.,* 1998	3445379
GF-305	1s14b	337	Ambros *et al.,* 1998	3445380
Redgold	Rg11	338	Present work	AF170512
Redgold	Rg15	338	Present work	AF170513
Redgold	Rg16	338	Present work	AF170514
Redgold	Rg20	338	Present work	AF170515
Agua	Ag5	338	Present work	AF170516
Agua	Ag11	338	Present work	AF170517
Hardired	Hd1	338	Present work	AF170500
Hardired	Hd6	337	Present work	AF170501
Hardired	Hd7	338	Present work	AF170502
Hardired	Hd8	336	Present work	AF170503
Siberian C	SC21	339	Present work	AF170498
Siberian C	SC22	339	Present work	AF170499
Siberian C	SC24	338	Present work	AF170496
Siberian C	SC29	338	Present work	AF170497
Redhaven	Rh11	339	Present work	AF170504
Redhaven	Rh12	339	Present work	AF170505
Redhaven	Rh13	339	Present work	AF170506
Redhaven	Rh15	339	Present work	AF170507
Harrow Beauty	HB2	339	Present work	AF170508
Harrow Beauty	HB3	339	Present work	AF170509
Harrow Beauty	HB4	342	Present work	AF170510
Harrow Beauty	HB5	339	Present work	AF170511
Suncrest[e]	Su2	337	Present work	AF170518
Golden Health[e]	GH1	337	Present work	AF170519
Tylor	Ty1	337	Present work	AF170520
Tylor	Ty2	337	Present work	AF170521
Tylor	Ty3	337	Present work	AF170522
Tylor	Ty4	337	Present work	AF170523

[a] Peach tree cultivars.
[b] PLMVd isolates.
[c] Accession number in Genbank database.
[d] Bing cultivar is the cherry tree.
[e] We sequenced four identical clones of this variant.

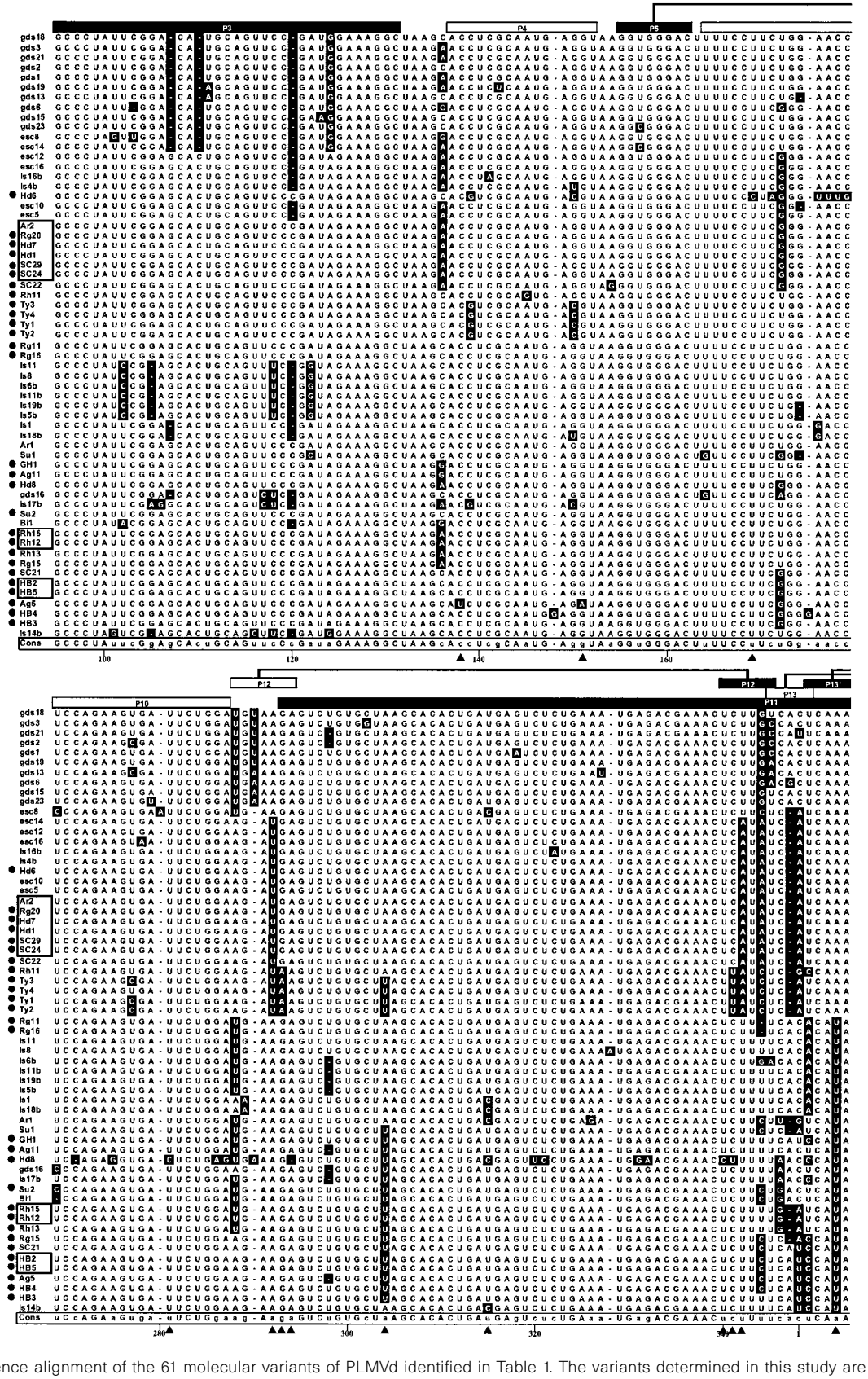

FIG. 1. Sequence alignment of the 61 molecular variants of PLMVd identified in Table 1. The variants determined in this study are indicated by a dot in the left margin, whereas variants that are perfectly identical are boxed. Nucleotide variations are indicated with black boxes. The consensus sequence is shown at the bottom. Uppercase letters indicate nucleotides found in all PLMVd variants, whereas lowercase letters indicate nucleotides found in most sequences. Filled triangles under the consensus sequence indicate covariations. Bars at the top show the secondary structure helices in which a given position is included.

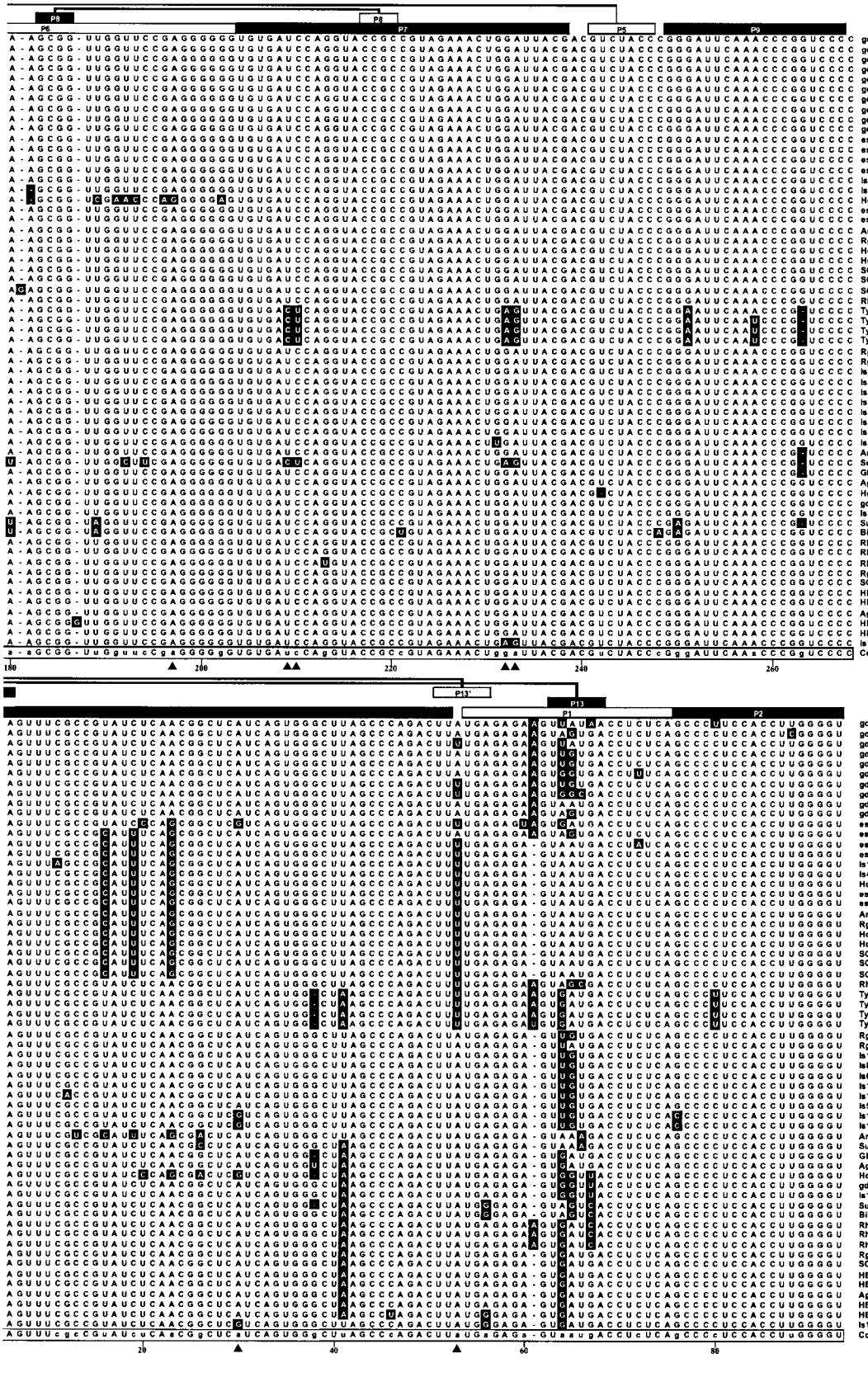

FIG. 1—*Continued*

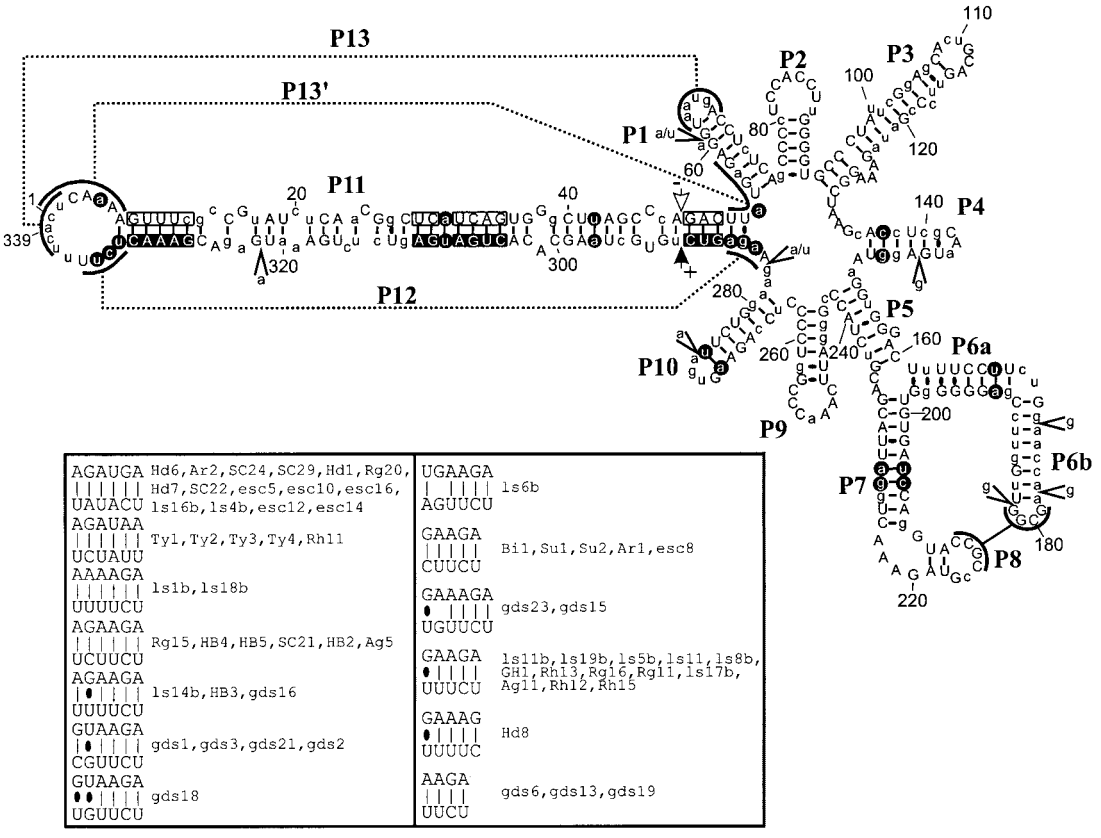

FIG. 2. Predicted secondary structure of PLMVd using base-pair covariation data. The helix numbering has been arbitrarily fixed as if the circular RNA was synthesized from position 1 and follows their order of appearance. GU wobble base-pairs are indicated with black ovals. Pseudoknots for which no biochemical evidence exists are illustrated with dotted lines. Base-pairs showing covariation are circled. Insertions observed in only one PLMVd sequence variant are illustrated. The P11 stem includes the hammerhead motifs whose highly conserved sequences are boxed. Arrows show the hammerhead cleavage sites of both the plus (+) and minus (−) polarities. Inset, base-pair composition of the P12 pseudoknots for all variants.

ited variability strongly suggests that constraints exist to limit the heterogeneity of PLMVd. We also generated a phylogenetic tree, as described previously (Ambros *et al.,* 1998) (data not shown). Inclusion of the new sequences clearly shows that primary structure is not sufficient to unequivocally determine the group to which a variant belongs. Even so, it seems that each isolate is composed of a complex mixture of RNA species.

The hammerhead sequences of both polarities were folded into their active secondary structures (data not shown). The three helices (stems I, II, and III) can be formed in each variant, and covariation of the base-paired nucleotides is observed for all stems, supporting their existence and suggesting selective pressure in favor of self-cleavage activity. The various mutations occurring in the hammerhead domains have been previously studied (Ambros *et al.,* 1998). We did not observe any new mutations in the region that may be of interest for further characterization.

Nucleotide covariations and secondary structure of PLMVd

The secondary structures of lowest free energy were predicted for several PLMVd variants using the MFOLD program (Zuker, 1989). Although the main core, which included several stems, was always present in the resulting structures, the occurrence of a significant amount of structural diversity was observed between the variants (data not shown; see Fig. 2 for a stable secondary structure). Specifically, stems P1, P2, P10, and P11 were present in each predicted structure, whereas the sequence forming stems P3 to P9 was predicted to fold in various ways. Surprisingly, most of the observed sequence variations were found in stems P1, P2, P10, and P11, whereas the region forming stems P3 to P9 shows less variation. To take advantage of the large number of available PLMVd sequences, we ran the aligned sequence file (Fig. 1) through a covariation program (i.e., the Bioedit package; Brown, 1991). Using the consensus sequence derived from the alignment, the most stable secondary structure was predicted using MFOLD and readjusted based on the base-pair covariation data. To facilitate the description of this secondary structure (Fig. 2), we numbered the helices as if this circular RNA had been synthesized from position 1, which was arbitrary fixed, and followed the order of appearance of the nucleotides forming each stem. If two helices have the potential to stack but are separated by an internal loop,

the same number was given to both stems, and they are then differentiated by a letter (i.e., the P6a and P6b stems). This numbering of PLMVd secondary structure was also used in a report of PLMVd nuclease mapping (Bussière *et al.,* 2000).

Base-pair covariations support the presence of all stems except P5 and P8, which are highly conserved. However, "quasiperfect" base-pair covariation (i.e., no detrimental mismatches) was limited to only a few positions (circled in Fig. 2). One of these base-pairs (i.e., positions A_{270}–U_{275}) formed the extremity of the P10 stem that possesses the conserved loop sequence GUGA. For the Hd8 variant, this base-pair is G_{270}-C_{275}, producing a GNRA tetraloop that is proposed to be ultrastable (Varini, 1995). However, covariation analysis did not unambiguously establish any nucleotide triplet, which would have been useful in establishing how PLMVd folds in three dimensions.

This structure obtained by *in vivo* sequence variability analysis is similar to that derived *in vitro* by nuclease mapping and oligonucleotide probing using a variant sequence isolated from the Armking cultivar (Bussière *et al.,* 2000). In fact, the differences are minor and localized, suggesting that *in vitro* and *in vivo* PLMVd structures are almost identical. For example, nuclease mapping data suggest that the P6a stem is formed by base-pairing between $_{162}$UUUCCUUC$_{169}$ and $_{191}$GAGGGGGG$_{198}$, where U_{161} is a bulge separating the P5 and P6a stems, and U_{170} is a bulge separating the P6a and P6b stems (Bussière *et al.,* 2000). In contrast, the secondary structure based on covariation shows that stem P6a is formed by base-pairing between $_{161}$UUUUCCUU$_{168}$ and $_{191}$GAGGGGGG$_{198}$ (Fig. 2). As a consequence, no bulge is present between stems P5 and P6a, whereas $_{169}$CU$_{170}$ forms an internal loop between stems P6a and P6b.

The proposed secondary structure includes several potential pseudoknots. The P8 stem discovered during nuclease mapping, and supported by oligonucleotide probing (Bussière *et al.,* 2000), has a sequence that is highly conserved in all PLMVd variants, most likely because the four ordered GC base-pairs are required for formation of a helix in this stretched region. Strong sequence covariation supported the presence of another pseudoknot between $_{284}$AAGA$_{287}$ and $_{232}$UCUU$_{235}$ (i.e., P12; see Fig. 2, inset). In some sequence variants, the length of this stem may be extended up to 6 bp. This pseudoknot corresponds to a portion of stem III of the plus polarity hammerhead structure. The presence of pseudoknot P13, formed by nucleotides of the left-hand and P1 loops, specifically between nucleotides $_{337}$UCACU$_{2}$ and $_{64}$AAUGA$_{68}$ (the underlined nucleotides are base-paired), had been suggested previously (Ambros *et al.,* 1998). The presence of this putative pseudoknot was supported by compensatory mutations in the two single-stranded regions (see Fig. 1 and Ambros *et al.,* 1998). Unlike the P12 pseudoknot, base-pair covariation analysis performed here only partially supports the presence of the P13 pseudoknot. However, the adoption of the P12 pseudoknot would require the closing of the left-hand loop and the single-stranded region between P10 and P11 (i.e., to roll the P11 stem), thereby placing the left-hand loop in close proximity to the P1 loop and possibly favoring formation of the P13 pseudoknot. Alternatively, it is possible that if stem III of the plus polarity hammerhead motif forms a pseudoknot (i.e., P12), then a P13′ pseudoknot may be formed by the analogous stem III of the minus polarity hammerhead motif. More specifically, a P13′ pseudoknot would be formed by nucleotides $_{1}$CUCAAAA$_{7}$ and $_{52}$UUAUGAG$_{58}$ (the underlined nucleotides are base-paired); however, this requires unfolding the bottom of the P1 stem.

DISCUSSION

We report here 34 new PLMVd variants isolated from nine different North American peach cultivars. This study gives the widest view of PLMVd diversity reported to date and is the first characterization of North American isolates, which cannot be differentiated from European ones. PLMVd appears as a species for which each isolate is composed of a complex mixture of RNA species; however, the sequence variability appears to be restricted to a relatively small fraction of the RNA genome, suggesting that constraints limit the heterogeneity of PLMVd. The variable positions are scattered all around the RNA molecules, but the majority are concentrated in the region that includes the hammerhead self-cleaving motifs (i.e., stems P1, P2, P10, and P11). In contrast, the region forming stems P3 to P9 shows less variation. Therefore, it appears reasonable to suggest that PLMVd is composed of two domains: (1) the left domain, which includes the hammerhead sequences and has a variable sequence and a conserved secondary structure, and (2) the right domain, which has a relatively conserved sequence but a variable structure. Most likely, these domains serve different functions in the PLMVd life cycle and consequently are under different selective pressures. Perhaps PLMVd arose from the linkage of two different RNA molecules (i.e., the left and right domains), as is proposed for the human hepatitis delta virus (Brazas and Ganem, 1996).

Recently, the secondary structure of PLMVd was characterized in solution by nuclease mapping experiments coupled to binding shift assays using oligonucleotides (Bussière *et al.,* 2000). To verify whether this *in vitro* structure is comparable to the *in vivo* one, analyses of base-pair covariations and predictions of secondary structure were performed (Fig. 2.). Briefly, the *in vitro* and *in vivo* structures are in good agreement and correlate with the refinement of the computer-predicted structure (Ambros *et al.,* 1998). In fact, with the exception of the pseudoknots, the differences are minor and localized.

PLMVd appears to fold into a complex branched structure with the potential of including three pseudoknots (P8, P12, and P13′); however, the existence of the previously proposed pseudoknot P13 (Ambros *et al.,* 1998) is not supported by our covariation analysis. The P8 pseudoknot appears to be part of this secondary structure, whereas the P12 and P13′ pseudoknots are only putative stems awaiting biochemical support. All three pseudoknots (P8, P12, and P13′) have the potential to positively contribute to the stabilization of the structure by increasing the net number of base-pairs. Moreover, it is possible that *in vivo* PLMVd adopts various alternative structures that coexist with that determined by nuclease mapping and oligonucleotide binding shift assays and that these alternative structures are more complex and include several pseudoknots. This possibility is increased by the fact that *in vitro* analyses have not been able to resolve potential alternative secondary structures such as the hammerhead motifs (Bussière *et al.,* 2000). It is also possible that host conditions, which may include the interaction with proteins, allow formation of the P12 and P13′ pseudoknots and thereby the folding of the RNA molecule into a compact structure. Furthermore, preliminary electron microscopy of PLMVd under native conditions supports the hypothesis that a significant portion of PLMVd molecules adopt a "globular-like" structure (P. Magny, D. Beaudry, and J. P. Perreault, unpublished data).

This structure is in contrast to the rodlike one proposed for nearly all other viroids and may be responsible for the insolubility of PLMVd in 2 M lithium chloride, a solution in which a number of group B viroids (e.g., potato stunt transient viroid, PSTVd; see Symons, 1997), as well as avocado sunblotch viroid (i.e., another group A viroid), that fold into rodlike secondary structures are soluble (Navarro and Flores, 1997). Chrysanthemum chlorotic mottle viroid (CChMVd) was also reported to be insoluble in 2 M lithium chloride (Navarro and Flores, 1997). If this biophysical property is due to a PLMVd "globular-like" structure, CChMVd should also have the potential to form such a structure. The most stable secondary structure predicted for CChMVd (Navarro and Flores, 1997) reveals many similarities to that of PLMVd. The left domain includes the hammerhead sequences, whereas the right domain is organized as a center from which several hairpins emerge (Fig. 3). In the right domain, it has been proposed that a pseudoknot analogous to P8 in PLMVd may be formed (Bussière *et al.,* 2000). No evidence for the presence of a pseudoknot between the left-hand loop and the first hairpin around the center of the right domain, equivalent to pseudoknot P13 in PLMVd, was discovered. However, pseudoknots corresponding to hammerhead stem III of both polarities may be formed and would produce a closed structure (Fig. 3). These pseudoknots are the equivalent of the PLMVd P12 and P13′ stems and give CChMVd the potential to fold into a compact form that includes several pseudoknots in a manner similar to PLMVd. These two viroids appear to share both a structure and a replication mechanism (Bussière *et al.,*1999) and are therefore proposed to belong to the same viroid subgroup.

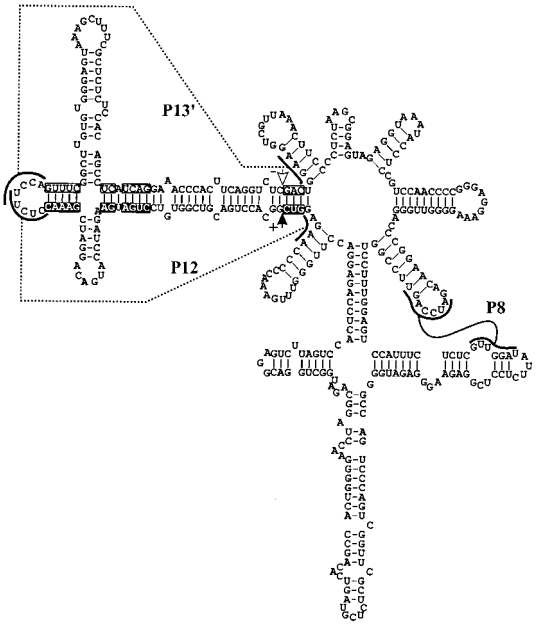

FIG. 3. Potential secondary structure of CChMVd. Pseudoknots are identified with dotted lines and are numbered as described for the PLMVd secondary structure (see Fig. 2). This secondary structure is based on one reported previously (Navarro and Flores, 1997).

MATERIALS AND METHODS

RNA isolation and amplification

Leaves of nine different peach cultivars (natural infected by PLMVd) grown on the west coasts of Canada (Sidney, British Columbia) and the United States (Prosser, WA) were used as source of PLMVd (see Table 1). RNA was prepared from the leaves (150 mg) using the RNeasy Plant mini kit (Qiagen, Studio City, CA) as recommended by the manufacturer. RNA samples were quantified by UV spectroscopy, and their quality was verified by 1% agarose gel electrophoresis. First-strand cDNA of PLMVd RNA of plus polarity was synthesized using avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and primer D97-113 (5′-TGCAGTGCCCGAATAGG-′3). Double-stranded cDNA was produced by PCR amplification of the RT reaction mixture (using 1/10th of the total volume) with primers D97-113 and D114-134. To limit the number of PCR artifacts, freshly purified *Taq* DNA polymerase was produced as previously described (Engelke *et al.,* 1990), and the number of thermocycles (1 min at 94°C, 1 min at 57°C, 1 min at 72°C) was limited to 30. The RT–PCR products were fractionated on 1% agarose gels.

To access the fidelity of the procedures used, different

controls were performed. For the PCR amplification, two different templates (5 ng) were used: (1) the *Eco*RI-linearized plasmid with the insert corresponding to the PLMVd Suncrest variant (i.e., Su2, see Table 1) produced during this work, and (2) the *Eco*RI-linearized plasmid pPD1 (Beaudry *et al.,* 1995). Briefly, the latter construction possesses two tandemly repeated PLMVd sequences (Ar2 variant, see Table 1) cloned into the *Pst*I restriction site of pBluescript II KS. To control the whole RT–PCR amplification, transcripts were produced by *in vitro* transcription using plasmid pPD1 as template according to the procedure reported previously (Beaudry *et al.,* 1995). The insert is flanked by the T3 and T7 promoters, which can be used for the production of plus and minus polarity transcripts, respectively. After gel-purification of the RNA transcripts, 1 ng of the longer fragment (761 nt, a dimer) was used to perform RT–PCR amplification as described above.

Cloning and sequencing

The gel bands containing full-size PLMVd cDNA were isolated, and the DNA fragments were extracted and cloned using the property of *Taq* DNA polymerase of adding an adenosine to the 3′ ends of the PCR products. Consequently, the PCR-amplified fragments are easily ligated in a "sticky end" fashion to a linearized pCRII vector, which possesses an extra thymidine residue at each 5′ end, as recommended by the manufacturer (TA cloning kit; InVitrogen, San Diego, CA). Four PLMVd clones from eight different peach cultivars and two from the Agua cultivar were sequenced in both directions by the dideoxyribonucleotide chain termination method using the T7 sequencing kit (Pharmacia Biotech, Piscataway, NJ) (see Table 1). The sequences are reported in the GenBank Nucleotide Sequence Database under accession nos. AF170496 to AF170523.

ACKNOWLEDGMENTS

This work was sponsored by a grant from Natural Sciences and Engineering Research Council (NSERC) of Canada to J.P.P. J.P.P. is a Medical Research Council (MRC) scholar.

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