Genomic Structure of Three Phenotypically Different Isolates of Peach Latent Mosaic Viroid: Implications of the Existence of Constraints Limiting the Heterogeneity of Viroid Quasispecies

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Viroids, the smallest agents of diseases in plants (11), are appropriate experimental systems for the study the genetic diversity and population dynamics of pathogenic RNAs. Previous sequence comparisons among viroids led to a model of structural and functional domains (29) that is valid for most of the known members of the group, which form a family characterized by having a central conserved region (15, 32). However, three other members, avocado sunblotch viroid (ASBVd) (27), peach latent mosaic viroid (PLMVd) (23) and the recently characterized chrysanthemum chlorotic mottle viroid (CChMVd) (38), are included in a second family because they lack a CCR. Furthermore, strands of each polarity have the ability to undergo self-cleavage via hammerhead ribozymes. Hammerhead viroids are also characterized by restricted host ranges: they replicate only in their natural hosts or closely related species.

Although there are numerous reports on the analysis of structure-function relationships in different members of the first family of viroids, information on viroids with hammerhead ribozymes is much more limited. Studies focused on ASBVd have revealed sequence heterogeneity affecting mainly the left- and right-hand terminal regions of the rod-like structure proposed for this RNA (39, 44, 49). Different ASBVd sequence variants have been associated with distinct symptoms (49), but a clear assignment of a defined phenotype to a given variant is not feasible in the ASBVd-avocado system because successful infections with nucleic acid preparations are difficult to achieve and, moreover, a long assay period (1 year or more) is usually required to observe the symptoms (44). Conversely, successful mechanical inoculation of PLMVd on the peach indicator GF-305 has been reported (19), and the time elapsed between inoculation and the onset of symptoms is relatively short (8 to 12 weeks), making this system very attractive for the study of different aspects of the interaction between a viroid with hammerhead ribozymes and its natural host.

PLMVd, a 337-nucleotide (nt) circular RNA adopting a branched conformation of minimum free energy content (23), is the causal agent of peach latent mosaic disease (19). This disease was initially identified in France (8, 9), and its most conspicuous symptoms under field conditions are a delay in foliation, flowering, and ripening, fruit deformation, bud necrosis, and rapid aging of the trees; only very rarely is a yellow mosaic or blotch observed on the infected leaves. In the greenhouse, PLMVd natural isolates are divided into severe or latent strains depending on whether they induce leaf symptoms on seedlings of the peach indicator GF-305 (8, 9). PLMVd, like many other viroids (15, 25, 43, 54), probably propagates in its host as a population of similar but not identical molecular strains existing as quasi-species (14). Different PLMVd sequence variants on GF-305 peach seedlings have shown that the biological properties of the PLMVd isolates may be correlated with both the complexity of their viroid populations and the presence of specific sequence variants. In this work, PLMVd isolates with different phenotypic effects were chosen for a study of the sequence polymorphism in the viroid RNA. We have obtained data on the distribution of the variability along the PLMVd molecule and on the structure of the viroid populations which form the isolates. In addition, a biological assay has been used to evaluate the infectivity and pathogenicity of individual PLMVd sequence variants on its natural host in an effort to obtain some insight into the structure-function relationships in this viroid.
MATERIALS AND METHODS

Viroid sources. PLMVd was purified as reported previously (19) from leaves of GF-305 peach seedlings infected either by the severe isolate (D168), inducing blotch symptoms in this indicator, or by either of two latent isolates LS35 and Esc76906, which elicit symptomless infections but protect the plants from challenge inoculations with the severe isolate (8).

cDNA synthesis. First-strand cDNA was synthesized on purified circular PLMVd RNA by using avian myeloblastosis virus reverse transcriptase (RT) and primer RF-43, 5′-d(TCTGTACCAAGCCCTGGAAACCACCGCT)-3′, as reported previously (23). For synthesis of second-strand cDNA, an aliquot (1/20) of the RT reaction mixture was PCR amplified with primer RF-43 and RF-44, 5′-d(TGTGATCCAGTACCGCTGAGAAC)-3′, and 2.5 U of cloned Pfu DNA polymerase. Primers RF-43 and RF-44 are complementary and identical to positions 206 to 179 and 199 to 225, respectively, of the PLMVd reference sequence (23), and they overlap a Sau3A restriction site located in a domain of the molecule where very low variability was observed in preliminary experiments in which a set of PLMVd clones was prepared by the same RT-PCR approach but with a pair of primers overlapping a PstI site and covering positions 91 to 135 of the PLMVd reference sequence. The amplification reaction was carried out with the buffer suggested by the producer (Stratagene) for maximal fidelity: 20 mM Tris-HCl (pH 8.8)–2 mM MgSO4–10 mM KCl–10 mM NH4SO4–0.1% Triton X-100–100 μg of nuclelease-free bovine serum albumin per ml–400 μM each deoxynucleoside triphosphate. The PCR cycling profile consisted of a hot start of 94°C for 2 min and 72°C for 3 min (with the enzyme added at this stage) followed by 30 cycles of 94°C for 40 s, 60°C for 30 s, and 72°C for 2 min, with a final extension step at 72°C for 10 min (7).

Cloning and sequencing. PCR products were separated by polyacrylamide gel electrophoresis, and after ethidium bromide staining the cDNAs of the expected size were eluted, digested with Sau3A, and cloned into plasmid pBSII KS+ (Stratagene) linearized with BamHI. In some cases, PCR products were cloned directly into pBSII KS+ linearized with Smal. Viroid cDNA sequences were determined in both directions by chain-terminating inhibitors (46) and T7 DNA polymerase. In some experiments, single-stranded DNA templates were used to avoid gel compressions and an additional extension step with terminal transferase was included to eliminate nonspecific stops.

RNA self-cleave. Recombinant plasmids containing full-length cDNA inserts of different PLMVd variants were used to study self-cleave during in vitro transcription reactions carried out at 37°C for 1 h as indicated (23). The lengths of the 9 and 3 vector tails depended on the structure of the recombinant plasmid. Inserts from gds23, ls16b, and esc10 clones had the same plasmid orientation, with the first being cloned in the BamHI site and the other two being cloned in the Smal site. EcoRI- and XbaI-linearized plasmids were used to synthesize plus- and minus-polarity transcripts, respectively. Inserts from gds6 and gds2 clones had the opposite plasmid orientation with respect to the three previous ones and were cloned in the BamHI site. Plus-polarity transcripts were obtained with XbaI-linearized plasmids. The primary transcripts and their self-cleave products were separated in 5% polyacrylamide gels containing TBE/1× plus 8 M urea and 40% formamide, which were stained with ethidium bromide or, when radioactive, scanned and quantified with a bioimage analyzer (Fuji BAS Plus 8 M urea and 40% formamide, which were stained with ethidium bromide cleavage products were separated in 5% polyacrylamide gels containing TBEX1)

RESULTS

Uneven distribution of the high sequence variability found in PLMVd. A number of complete cDNA clones was obtained for each of the three PLMVd isolates in this study. Eleven cDNA clones derived from the severe isolate D168 were chosen at random, and their sequences revealed that they corresponded to different molecular variants. These variants, given the prefix gd, differed in both their length (336 to 338 nt) and primary structure. Ten clones derived from the latent isolate Esc76906 were also sequenced, but in this case only six different molecular variants of 336 or 337 nt were found; they were designated by the prefix ls. Regarding the latent isolate LS35, 13 clones were analyzed which yielded 12 molecular variants with sizes ranging from 335 to 338 nt; they were given the prefix esc. Figure 1 shows the primary structure of the 29 PLMVd RNAs characterized in this study aligned with respect to the PLMVd reference sequence (23). Of a total of 342 nucleotide positions in the alignment (Fig. 1), 47 (13.7%) were polymorphic for isolate D168, 32 (9.3%) were polymorphic for isolate Esc76906, and 55 (16%) were polymorphic for isolate LS35. The number of polymorphic positions in the PLMVd molecule, considering all the sequence variants analyzed, was 75 (21.9%).

Phylogenetic analysis of the 29 sequence variants characterized here by the distance matrix method led to an unrooted tree (Fig. 2) which supports their clustering into three major groups. A similar tree topology was obtained by using the Wagner parsimony criterion (17) (data not shown). Group I is composed of all gd variants except gds16, group II is made up of all esc variants plus two variants from isolate LS35 (ls16b and esc6), and group III is composed of all ls variants from isolate LS35 plus gds16 from isolate D168. Phylogenetic groups of PLMVd variants can be distinguished by informative changes (Fig. 1). Sequences from group I are characterized by five of these changes (Fig. 1), although only the insertion at position 285 is unique to group I members; the G at position 339 is present in ls6b of group III, and the three other point mutations are also present in esc8 and esc14 of group II. Similarly, all members of group II bear three specific point mutations whereas four other changes are detected in the sequences of this group, with the exception of esc8. Finally, PLMVd sequences of group III have a U at position 5 instead of the A present in members of the other two groups. The presence of a C or A at position 2 and a U at position 339 is also characteristic of group III members, with the exceptions of gds16 and ls6b, respectively.

FIG. 1. Sequence alignment of 29 molecular variants of PLMVd derived from three different isolates. The reference sequence of PLMVd is included for comparative purposes and is shown at the top with two corrections, a deletion of one of the three Cs at positions 117 to 119 and a duplication of the G at position 257, with its nucleotide substitutions indicated. Dots denote residues identical to the reference sequence, and dots indicate residues identical to the nucleotide sequence of the group. Other nucleotide substitutions present in most sequences of groups II and III are in green and light blue, respectively. Primers used for RT-PCR amplification cover positions 178 to 225. Residues involved in a potential pseudoknot-like element are boxed.
Figure 3 shows the secondary structure of lowest free energy predicted for the PLMVd reference sequence by applying a version of the MFOLD program updated from that used previously (23). With this same program, branched conformations very similar to that proposed for the reference sequence were obtained for all the newly characterized PLMVd RNAs (data not shown). However, some sequence variants showed local modifications in their most stable foldings, and in this respect the conformations predicted for all gds variants, most ls variants (except ls16b and ls4b), and esc8 exhibited a cruciform structure in the hammerhead arm (Fig. 3, inset). Approximately one-third of the molecule, delimited by positions 180 and 270, is relatively invariant (Fig. 1 and 3). Most of the changes found between different sequence variants were located in a region which includes both hammerhead structures and, particularly, in the single-stranded regions designated loops A and B as well as in the PstI arm (Fig. 3), showing the existence of specific domains in the PLMVd molecule capable of tolerating different sequence alternatives. The number of residues forming loops A and B was variable: loop A, composed of 12 nt in the PLMVd reference sequence, is reduced to 3 nt in some variants, whereas loop B, composed of 4 nt in the reference sequence, is enlarged to 10 nt in some variants (data not shown). The hammerhead arm, in either the rod-like or cruciform alternatives, as well as the hairpin ending with loop B, was formed in the optimal and the three closest suboptimal secondary structures of all PLMVd variants, supporting the proposed conformation for this region of the PLMVd molecule.

Conserved stability of hammerhead structures in PLMVd variants. As indicated above, a considerable number of changes in the PLMVd variants were found in positions involved in both self-cleavage domains. This was an unexpected finding in view of the key functional role they are assumed to play (see below). However, with the exception of one case (see below), mutations observed within the sequences forming the plus- and minus-polarity hammerhead structures do not affect the nucleotides which are strictly conserved among the other known natural hammerhead structures (4, 12, 23, 24, 27, 38, 52). Interestingly, most of the variations found in these domains are located in loops or do not affect the stability of the stems because of double compensatory mutations, and some other changes should have only minor effects (Fig. 4). However, regarding the plus-polarity hammerhead structure, it is noticeable that several variants lack the U located 3’ to the cleavage site (Fig. 4A and C), positions 1.1 of the hammerhead structure and 293 in the alignment, respectively (Fig. 1). Furthermore, gds1 has the peculiarity that the nucleotides preceding and following the conserved sequences GAAA and GA, respectively, do not form a canonical base pair as a consequence of the G-to-A substitution (Fig. 4A), affecting positions 10.1 of the hammerhead structure and 313 of the alignment. A similar situation has been reported previously for the plus-polarity hammerhead structure of the satellite RNA of barley yellow dwarf virus (sBYDv) (36) and for the minus-polarity hammerhead structure of the carnation small viroid-like RNA (24).

With regard to the minus-polarity hammerhead structure, it is worth noting the presence of a G-to-U mutation in the conserved GAAA motif in ls16b (Fig. 4B). In addition, 7 of the 10 sequence variants forming group I (all except gds6, gds13, and gds21), together with esc14 of group II (Fig. 4A and B), have a disruption of the base pair formed by nt 15,5 and 16,5 of the hammerhead structure because of the occurrence of uncompensated mutations.

Analysis of the self-cleavage efficiencies of the PLMVd RNAs during in vitro transcription showed that, as expected, no detectable self-cleavage was observed for the minus-polarity RNA of ls16b (Fig. 5), in agreement with previous results demonstrating that any change in residue G12 of the hammerhead ribozyme destroys its catalytic activity (45). Other mutations mentioned above affecting PLMVd hammerhead structures, such as those found in gds1 and gds23, gave rise to minor or no reduction of self-cleavage, but the U1.1 deletion detected in the plus-polarity hammerhead structure of several variants, such as gds2, strongly reduced self-cleavage (Fig. 5).

A postulated pseudoknot-like element for PLMVd on the basis of covariation. Many nucleotide changes have been found in the single-stranded regions designated loops A and B on the most stable secondary structure proposed for PLMVd (Fig. 3). In spite of this variability, conservation of a potential base-pairing interaction between the two hairpin loops has been observed (Fig. 6), suggesting the existence of a pseudoknot-like element (41) that could also be regarded as an intramolecular “kissing” interaction (35). For some sequence variants, we have considered loops slightly larger than those predicted in the most stable secondary structures in order to indicate the most likely interactions. It should be mentioned that a similar interaction can be formed in the minus PLMVd strand, but for simplicity we present only that corresponding to the plus strand.

The complementarities found between loops A and B in PLMVd sequences of group I would allow the formation of a stem composed of two sets of 3 or 4 bp separated by a mismatch of 1 or 2 bases. Alternative, more stable base pairings between loops A and B are possible for gds15, gds23, and gds21 (Fig. 6A).

The number of base pairs contained in the pseudoknot-like structure is 4 bp in the sequence variants belonging to group II, with the exception of esc8, which may establish a longer interaction of 6 bp between loops A and B (Fig. 6B). On the other hand, variants from group III may form a pseudoknot-like element between loops A and B very similar to those found for PLMVd sequences of group II, although 5 bp is involved for all sequences except ls6b, for which 4 bp is involved (Fig. 6C).
Comparison, for example of ls17b and ls14b shows that the 5-bp interaction is preserved as a result of two concurrent co-variations.

Since covariations or compensatory mutations are regarded as the most powerful method of predicting elements of higher-order structure in RNA (22), including pseudoknots (42), our data suggest that a motif of such a kind may exist in PLMVd RNA.

Differential infectivity and pathogenicity of PLMVd cDNAs.

To set up an appropriate system to evaluate the infectivity of individual PLMVd sequences, a preliminary bioassay was carried out by inoculating a series of PLMVd cDNAs and their corresponding transcripts to GF-305 peach seedlings. The PLMVd cDNA clone used in this initial experiment corresponded to the second sequence variant reported previously (23), which is identical to esc5. Dot-blot hybridization showed that the dimeric RNA, the monomeric cDNA with cohesive ends, and the plasmid containing the dimeric cDNA were the most infectious molecules (Table 1). Because of their quicker preparation and easier handling, the last two types of inocula were chosen for subsequent experiments. It should be also noted that no symptoms were observed in the infected plants, indicating that this particular PLMVd sequence variant, obtained from the latent isolate S5615 (23), was not pathogenic.

Dot-blot analysis of plants inoculated mechanically with PLMVd cDNAs from the severe isolate D168 showed that with the exception of gds2, all were infectious, although differences in the infectivity levels were observed (Table 2). It is difficult at this stage to explain why some sequence variants are more efficient at establishing infections than others, but at least for gds2 and gds16, a role for the U1.1 deletion of their plus-polarity hammerhead structures may be presumed, since this mutation, as mentioned above, has a strong effect on the in vitro self-cleavage. Similarly, it is likely that the low infectivity of gds1 could come at least in part from the G-to-A change in residue 10.1 of the plus-polarity hammerhead structure which disrupts the first base pair of helix II (Fig. 4A), because this variant displayed a reduced self-cleavage during in vitro transcription (Fig. 5). All gds sequences were pathogenic, although the type and intensity of the induced symptoms were variable: symptoms were observed in all infected plants upon inoculation with gds1, gds3, gds6, gds13, gds16, and gds18, whereas the phenotypes incited by gds15, gds19, and gds23 were variable, with some plants developing symptoms and others remaining symptomless in spite of being infected. Interestingly, the symptoms induced by the pathogenic clones very closely resembled those induced by the original isolate D168, with the single exception of gds1, which produced a severe chlorotic-necrotic reaction (Table 2).

Bioassays with cDNAs derived from the two latent PLMVd isolates revealed that of the three different sequence variants tested from isolate Esc76906, esc8 showed low infectivity, as opposed to esc10 and esc14 (Table 2). On the other hand, variants ls1, ls8, ls11, and ls14b from isolate LS35 showed high infectivity levels; the three other variants, ls5b, ls6b, and ls17b, were not infectious, although they contain the U1.1 deletion of...
FIG. 4. Hammerhead structures of the plus and minus strands of PLMVd variants. Sequence heterogeneity has been indicated on the self-cleaving domains of the PLMVd reference sequence (23). The nucleotide variations found between variants belonging to groups I (A), II (B), and III (C), as defined in Fig. 1, are shown. Nucleotide substitutions are indicated within circles, insertions are indicated within squares, and deletions are indicated by triangles pointing out in each case the PLMVd sequence variants affected by the corresponding mutation. Arrows indicate the predicted self-cleavage sites. The 13 conserved nucleotides present in all natural hammerhead structures (with the exception of the plus strand of sBYDV RNA [36] and the minus strand of a viroid-like RNA found recently in cherry [12], in which only 11 nt are conserved), are boxed. The same numbering is used for the plus and minus polarities and corresponds to that of the alignment shown in Fig. 1. Asterisks denote nucleotide changes present in all sequence variants of the corresponding group. (Inset) Scheme of PLMVd hammerhead structures with the proposed numbering system (26).
loss of essential functions of the viroid RNA. So far, the only identified structure-function relationship in viroids endowed with self-cleavage through hammerhead structures is the presumed involvement of these ribozyme domains in the autolytic processing of the RNA strands, a key step in the rolling-circle mechanism proposed for the replication of these pathogens (2, 3, 6; for reviews, see references 20 and 52). The results reported here, showing that changes observed in most PLMVd variants in the sequences which form both hammerhead structures do not affect their stabilities, extend the results observed for the two first PLMVd variants characterized (23) and strongly indicate that PLMVd ribozymes are operative in vivo, as they also appear to occur with ASBVd and CChMVd ribozymes (6, 38).

The case of the U1.1 deletion found in the plus-polarity hammerhead structure of gds2 and several PLMVd variants strongly reducing self-cleavage (Fig. 5) deserves a comment. We suspect that this mutation may have been introduced during reverse transcription, because it was detected only in the plus-polarity hammerhead structure. In this respect, it is interesting that deletions in residue 17 of the plus-polarity hammerhead structure, which precedes the site of self-cleavage, have been found in cDNA clones of the RNAs of satellite of lucerne transient streak virus (51) and sBYDV (36), although in the latter case it could not be determined whether the deletion affects position 17 or 11 because both are A residues. It has been suggested that this type of deletion may have been introduced by the RT because of the presence of a 2′-phosphate at the nucleotide preceding the self-cleavage/ligation site, as previously shown for the encapsidated satellite RNA of Solanum nodiflorum mottle virus (30). Taking into account these results, the deletion of nucleotide U1.1 following the self-cleavage site, detected here in some PLMVd cDNA clones, could reflect the presence of similar atypical bond at the ligation site of the plus-strand linear monomeric RNA, at least in a fraction of the viroid population, which would lead to anomalies in the functioning of the RT. Another possibility for such an atypical bond is the 2′,5′-phosphodiester linkage formed in the in vitro self-ligation of PLMVd (5), if this result has any significance in vivo.

On the other hand, we do not have any clue about the selective advantages that a branched secondary structure may confer on PLMVd, but this conformation is different from the rod-like or quasi-rod-like structures proposed for most other viroids. Since a similar branched conformation has been obtained only for CChMVd and there is evidence that PLMVd and CChMVd have unique in vitro conformations because

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**DISCUSSION**

An analysis of the genomic structures of three PLMVd isolates with different biological properties has been performed. Isolates were maintained in the greenhouse by graft inoculation on the peach indicator GF-305, preventing any bias in the viroid diversity as a consequence of selection processes for certain variants which may take place in species other than the natural host (48). Moreover, a thermostable DNA polymerase endowed with proofreading activity has been used to minimize the introduction of artificial changes during PCR amplification. The characterization of sequence variants from the severe isolate D168 and from the latent isolates LS35 and Esc76906 has revealed a large number of polymorphic positions in the viroid RNA. The length of the sequence variants ranged from 335 to 338 nt, indicating that strict conservation of size is not a essential feature of PLMVd, in accordance with what has been found for other viroids (25, 31, 44, 54). However, on the basis of the variability pattern found, at least three types of structural constraints limiting the genetic divergence of PLMVd sequences can be distinguished: (i) formation of stable hammerhead structures in both polarity strands, (ii) conservation of a similar branched secondary structure of minimal free energy for the different sequence variants, and (iii) preservation of a potential pseudoknot-like element between loops A and B of the secondary structure proposed for PLMVd.

It is very likely that these structural constraints prevent the

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**TABLE 1. Infectivity of PLMVd cDNAs and transcripts**

<table>
<thead>
<tr>
<th>Type of inoculum</th>
<th>Infectivity</th>
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<tbody>
<tr>
<td>Monomeric cDNA (cohesive ends)</td>
<td>12/15</td>
</tr>
<tr>
<td>Monomeric cDNA (blunt ends)</td>
<td>7/15</td>
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<tr>
<td>Monomeric cDNA with a short repeat</td>
<td>0/15</td>
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<tr>
<td>Dimeric cDNA plus short vector tails</td>
<td>6/15</td>
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<tr>
<td>Plasmid with a dimeric cDNA</td>
<td>10/15</td>
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<tr>
<td>Monomeric plus RNA</td>
<td>2/15</td>
</tr>
<tr>
<td>Monomeric plus RNA with a short repeat</td>
<td>0/15</td>
</tr>
<tr>
<td>Dimeric plus RNA</td>
<td>14/15</td>
</tr>
</tbody>
</table>

* The PLMVd cDNA clone used is the second sequence variant reported previously (20).
* Monomer starting at position 105 plus a short repeat of 18 nt (positions 106 to 123).
* With short tails from the vector.
* Number infected/total number.
they are insoluble in 2 M LiCl, as opposed to typical viroids, which remain soluble (38), this trait might reflect common functional features exclusive to these two viroids. Finally, the functional relevance of pseudoknots has been demonstrated or postulated for a number of different RNAs (42). However, to our knowledge, this is the first case in which covariation evidence suggests the existence of a pseudoknot-like element in a viroid. Although the significance of this motif in PLMVd replication and/or pathogenesis needs further investigation, it should be noted that pseudoknotted structures have been found to be crucial for the function of some viroid-like molecules such as sBYDV RNA (37) and the hepatitis delta virus RNA (28, 40).

The viroid content of tissues infected with the three isolates analyzed here were similar (data not shown), indicating that the observed phenotypes do not correlate with the accumulation levels of PLMVd. However, differences at the sequence level have been found between the viroid populations which comprise the isolates, showing that they consist of a pool of closely related molecules. Most of the sequence variants obtained from each isolate can be clustered on the basis of informative changes, and therefore three major groups can be defined. Different alternatives for the putative pseudoknot-like interaction are found for the three groups, indicating that distinctions between their members may not be restricted to the primary structure.

It is worth noting that isolates D168 and LS35 contain sequences belonging to more than one phylogenetic group. Isolate heterogeneity may be the consequence of repeated natural infections of the same individual tree, although it is very likely that most of the observed variability results from the accumulation of mutants emerging de novo during the viroid replication due to the error-prone nature of RNA polymerases (13). The strength and direction of selection processes will ultimately determine the rate at which substitutions spread throughout the viroid populations. The different constraints operating on each nucleotide position are expected to lead to an irregular distribution of the variability, and in fact, this is the observed situation (Fig. 1). Moreover, we have apparently detected in the present work the majority of the polymorphic positions in PLMVd, since most of the changes, with respect to the reference sequence, of the only variant characterized from an Italian PLMVd isolate (50) are represented in our sequence spectrum and the same occurs with PLMVd variants from other PLMVd sources which are currently being characterized in our laboratory (data not shown).

**FIG. 6.** Potential pseudoknot-like element in the PLMVd molecule. Shown are the proposed long-range interactions between nucleotides of loops A and B (Fig. 2) of the predicted secondary structures of variants belonging to groups I (A), II (B), and III (C), as defined in Fig. 1. The type interaction of each group is given in the left panel, with continuous and broken lines indicating the existence of a base pair in all and some cases, respectively. Free energy values (at 25°C) for the proposed interactions are shown in parentheses.
fore, the PLMVd severe isolate is formed by a mixture of symptomatic or asymptomatic infections in different plants. There is no clear onset of symptoms, but three cDNAs incited either symptomatic or asymptomatic infections in different plants. By contrast, the biological effects produced by the cDNAs of latent isolates were always symptomatic. This has led to interesting data in this respect. Infections induced by specific PLMVd cDNAs in an effort to obtain further insight into the molecular basis of PLMVd pathogenesis.

The separate inoculation of specific PLMVd cDNAs independently by each of them, these studies rely on the availability of sequences whose biological properties are precisely known. The separate inoculation of specific PLMVd cDNAs has led to interesting data in this respect. Infections induced by cDNA clones from the latent isolates were always symptomless. By contrast, the biological effects produced by the cDNAs from the severe isolate were variable: most of them induced the onset of symptoms, but three cDNAs incited either symptomatic or asymptomatic infections in different plants. Therefore, the PLMVd severe isolate is formed by a mixture of sequence variants with different pathogenicity, as is also the case for viroids lacking hammerhead ribozymes (21, 54). The results of the bioassays also provide a molecular framework for explaining the pattern usually observed in PLMVd infections, which are phenotypically stable when caused by latent isolates but exhibit fluctuations in symptoms when caused by severe isolates. These fluctuations may be the result of different balances reached in the course of the infection between variants with different pathogenicity coexisting in the severe isolates. It is tempting to speculate that the frequent reversions to an asymptomatic condition observed upon propagation of PLMVd severe isolates reflect a tendency of the pathogen to evolve to lower virulence, as predicted by the hypothesis that considers highly severe parasites to be poorly adapted to their hosts (for a review, see reference 33). In this context, PLMVd latent isolates may have a possible evolutionary advantage because they do not provoke severe debilitation of the infected plants, thus preserving the reservoirs for viroid replication and transmission. Nevertheless, evolution of pathogens toward being harmless to the plant is only one of the possible evolutionary trajectories, because selective pressures experienced by many animal and human pathogens seem to favor increasing virulence or convergence to some intermediate level (16, 34).

We cannot at this stage assign the pathogenic effect of PLMVd variants to a defined structural motif. Sequence comparison between gds23 and esc14, the closest characterized variants inciting different host responses, suggests that at least 12 nucleotide changes are required to restore pathogenicity to variants that give rise to asymptomatic infections. Site-directed mutagenesis of PLMVd cDNAs should help to clarify this point, although a complex situation might emerge, with determinants for various aspects of the symptoms being located in different regions of the viroid molecule, as has been reported for other viroids without hammerhead structures (47, 55).

Finally, it is very likely that the host response observed upon inoculation with individual PLMVd sequence variants will be induced by the quasispecies generated de novo from each particular variant. Under controlled conditions such as those used in our bioassays, the sequences derived from specific cDNAs must differ from each other in a reproducible way, because we have observed the same phenotypic effect for a given PLMVd variant in independent experiments. However, for gds15, gds19, and gds23, the situation is more complex, and they can give rise to quasispecies with different phenotypic effects, as inferred from the diverse symptoms observed in the infected plants. We are currently analyzing the progenies originating from different PLMVd cDNAs in an effort to obtain further insight into the molecular basis of PLMVd pathogenesis.

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