Rapid generation of genetic heterogeneity in progenies from individual cDNA clones of peach latent mosaic viroid in its natural host

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Viroids, small single-stranded circular RNAs endowed with autonomous replication, are unique systems to conduct evolutionary studies of complete RNA genomes. The primary structure of 36 progeny variants of peach latent mosaic viroid (PLMVd), evolved from inoculations of the peach indicator GF-305 with four individual PLMVd cDNAs differing in their pathogenicity, has been determined. Most progeny variants had unique sequences, revealing that the extremely heterogeneous character of PLMVd natural isolates most probably results from the intrinsic ability of this RNA to accumulate changes, rather than from repeated inoculations of the same individual trees under field conditions. The structure of the populations derived from single PLMVd sequences differed according to the observed phenotype. Variant gds6 induced a reproducible symptomatic infection and gave rise to a more uniform progeny that preserves some parental features, whereas variant gds15, which induced a variable phenotype, showed a more complex behaviour, generating two distinct progenies in symptomatic and asymptomatic individual plants. Progenies derived from variants esc10 and ls11, which incited latent infections, followed a similar evolutionary pattern, leading to a population structure consisting of two main groups of variants, one of which was formed by variants closely related to the parental sequence. The evolution rate exhibited by PLMVd, considerably higher than that reported for potato spindle tuber viroid, may contribute to the fluctuating symptomatology of the severe PLMVd natural isolates. However, the polymorphism observed in PLMVd progenies does preserve some structural and functional elements previously proposed for this viroid, supporting the fact that they act as constraints limiting the genetic divergence of PLMVd quasispecies generated de novo.

Introduction

Viroids are the smallest replicons able to infect higher plants and to incite in most of their hosts specific diseases (Diener, 1991; Flores et al., 1997, 1998). They consist of a circular single-stranded RNA that does not code for proteins and which ranges in size from 246 nt to 399 nt. Sequence variability has been reported in different viroid populations (Visvader & Symons, 1985; Keese et al., 1988; Koltunow & Rezaian, 1988; Lakshman & Tavantzis, 1993; Góra et al., 1994; Kofalvi et al., 1997), and shows the high plasticity of these minimal genetic systems (Diener, 1996). The heterogeneity may result from the de novo emergence of new variants as an effect of the error-prone nature of RNA polymerases (Domingo & Holland, 1994), or from repeated infections of the same individual, a feasible situation in woody hosts such as citrus and other fruit trees with a long productive life.

There are numerous reports on the population dynamics resulting from infections with in vitro-generated sequences of potato spindle tuber viroid (PSTVd) containing different artificially introduced mutations. These studies have revealed a reversion to wild-type, stable maintenance of the introduced mutations, and the appearance of new spontaneous changes (Hammond & Owens, 1987; Owens et al., 1991; Qu et al., 0001-6308 © 1999 SGM

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The data reported in this paper are in the EMBL nucleotide sequence database and assigned the accession nos AI241818–AI241850.
which the isolates were obtained, or of the unusual ability of PLMVd to evolve rapidly. To fathom this question, an analysis of the populations generated de novo by inoculating several individual PLMVd cDNA clones is presented here. Moreover, the quasispecies derived from PLMVd variants inducing symptomatic and asymptomatic infections have been compared in order to find out if there are any significant differences in the evolutionary dynamics between both types of founding sequences.

**Methods**

- **Viroid inocula and infectivity bioassay.** Four representative PLMVd variants differing in their biological properties when inoculated on the peach indicator GF-305 were chosen to study the sequence spectrum of their progenies. Two of the variants, gds6 and gds15 from the D168 severe isolate, belong to group I of PLMVd sequences, whereas gds6 incited a reproducible severe infection, gds15 incited either symptomatic or asymptomatic infections in different plants. The other two variants, esc10 from the Esc76906 latent isolate and ls11 from the LS35 latent isolate, belong to group II and III of PLMVd sequences, respectively, and both induce symptomless stable infections (Ambrós et al., 1998).

  Young GF-305 peach seedlings were inoculated by slashing the stems with cDNA monomeric inserts having cohesive ends (0.25 μg per plant) or plasmids with dimeric inserts (5 μg per plant) obtained as indicated (Ambrós et al., 1998). GF-305 plants were kept under greenhouse conditions for 2–3 months, the period required by pathogenic PLMVd sequences to induce leaf symptoms. Detection of PLMVd-infected plants was performed by dot-blot hybridization using radioactive probes of PLMVd cRNA as reported previously (Ambrós et al., 1995).

- **RT–PCR amplification, cloning and sequencing of PLMVd variants.** PLMVd RNA circular forms, purified by two electrophoresis steps in non-denaturing and denaturing polyacrylamide gels, were reverse-transcribed with avian myeloblastosis virus reverse transcriptase and primer RF-43, 5′ d(TGTGATCCAGGTACCGCCGTAGAAACT) 3′, complementary to positions 208–178 of the PLMVd reference sequence (Hernández & Flores, 1992). First-strand cDNA was precipitated with ethanol and dissolved in 10 μl water. Aliquots of 1–2 μ1 of the cDNA solution were PCR-amplified using primers RF-43 and RF-44, 5′ d(TGTGATCCAGGTACCGCCGTAGAAACT) 3′, identical to positions 199–225 of the PLMVd reference sequence (Hernández & Flores, 1992), and 2 U Taq or Pfu DNA polymerase. Primers RF-43 and RF-44 overlap a Sau3A restriction site located in a domain of the molecule with low sequence variability (Ambrós et al., 1998). PCR amplifications were performed in 50 μl using the buffers recommended by the suppliers for maximal fidelity (Boehringer Mannheim and Stratagene, respectively). The PCR cycling profile was as reported previously (Ambrós et al., 1998) and following separation by PAGE, the PCR products of the expected size were eluted and cloned into the Smal-linearized pBSII KS+ plasmid (Stratagene) when amplified with Pfu DNA polymerase, or into the linearized and thymidylated pT7Blue plasmid (Novagen) when amplified with Taq DNA polymerase. Inserts were sequenced in both directions with chain-terminating inhibitors (Sanger et al., 1977). The cDNA clones obtained from each progeny were designated with the prefix and number of the parental variant (gds6, gds15, esc10 or 1993; Wassenegger et al., 1994). However, only quite recently an analysis of the populations evolving from inoculations with naturally occurring variants of PSTVd has shown the genetic stability of some parental sequences which were recovered in the progenies upon one to six plant passages and in some cases were predominant (Góra-Sochacka et al., 1997). The considerable stability of PSTVd variants could be due to strong structural constraints limiting variability, and in this respect it has been shown that the conservation of a rod-like secondary structure and the formation of a stable hairpin II are both indispensable for PSTVd infectivity and maintenance in vivo (Loss et al., 1991; Owens et al., 1991; Lakshman & Tavantzis, 1992; Qu et al., 1993; Wassenegger et al., 1994; Hu et al., 1997).

All the aforementioned studies have been carried out with viroids belonging to the Pospiviroidae family (Flores et al., 1998), characterized by having a central conserved region (CCR) within a central domain (Keese & Symons, 1985), and most of the analyses were performed using experimental hosts, for example tomato in the case of PSTVd. However, the available data on the variability of the components of the second viroid family, Avsunviroidae, formed by avocado sunblotch viroid (ASBVd) (Hutchins et al., 1986), peach latent mosaic viroid (PLMVd) (Hernández & Flores, 1992) and chrysanthenum chlorotic mottle viroid (CChMVd) (Navarro & Flores, 1997), are much more limited. The members of this family lack a CCR but are endowed with the ability to self-cleave through hammerhead ribozymes, with PLMVd and CChMVd being more closely related to each other and forming the pelamoviroid genus (Navarro & Flores, 1997; Flores et al., 1998). Natural isolates of ASBVd (Pallás et al., 1988; Rakowski & Symons, 1989; Semancik & Szczowski, 1994), PLMVd (Hernández & Flores, 1992; Ambrós & Flores, 1998; Ambrós et al., 1998) and CChMVd (Navarro & Flores, 1997), are heterogeneous populations that also fit the quasi-species model (Eigen, 1993), but nothing is known about their stability over time or with successive passages in their natural hosts.

PLMVd is the causative agent of peach latent mosaic disease (Desvignes, 1976; Flores et al., 1990). We have previously shown the existence of high sequence variability in the populations of three natural isolates of PLMVd of different pathogenicity and we suggested that the polymorphism is distributed unevenly along the molecule as a consequence of at least three structural constraints: the preservation of active hammerhead structures, an overall branched conformation of the RNA, and a potential pseudoknot-like interaction between two loops (Ambrós et al., 1998). Analysis of the variability pattern of 29 PLMVd sequences led to their classification into three major groups, each characterized by a series of informative changes and a particular type of pseudoknot-like interaction. One question raised by this work is whether the observed PLMVd genomic divergence is a consequence of repeated inoculations of the same individual field trees from
In vitro self-cleavage reactions. Recombinant plasmids with full-length cDNAs inserts of some esc10 progeny variants, including the parental sequence, were selected for the study of their self-cleavage activities during in vitro transcription as reported (Ambros & Flores, 1998). The lengths of the 5′ and 3′ vector tails depended on the structure of the recombinant plasmids. Inserts of variants esc10-P and esc10-eb had the same plasmid orientation and were cloned into the SmaI site of PBsII KS+. The EcoRI–XbaI fragment of the recombinant pT7Blue plasmid containing the full-length cDNA insert of variant esc10-1 was subcloned into the pBSII KS+ and had also the same orientation as the two previous ones. Minus polarity transcripts of these constructs were obtained with T3 RNA polymerase from XbaI-linearized plasmids as reported (Ambros & Flores, 1998). The insert of esc10-4 variant was cloned into pT7Blue and had the opposite orientation. The corresponding minus polarity transcript was obtained with T7 RNA polymerase from the EcoRI-linearized plasmid. Transcriptions products were separated in 5% polyacrylamide gels containing 1×TBE plus 8 M urea and 40% formamide, which were stained with ethidium bromide or, when radioactive, scanned and quantified with a bioimage analyser (Fuji BAS 1500).

Sequence analysis. Multiple alignments of the PLMVd parental sequences and their progeny variants were obtained with the CLUSTAL W program, version 1.5 (Thomson et al., 1994) with minor adjustments introduced manually to optimize them. Nucleotide diversity values and their corresponding variances were determined as in Nei (1987) using the DnaSP program, version 1.00 (Rozas & Rozas, 1995). Genetic distances were estimated according to the model of Jukes & Cantor (1969) and the phylogenetic tree was constructed by the Neighbour-Joining method (Saitou & Nei, 1987) using the MEGA program, version 1.01 (Sudhir et al., 1993). The bootstrap test, based on 1000 replicates, was used to determine the statistical value of the nodes and the final trees were rooted using the corresponding parental variants. The most stable secondary structures were obtained with the circular version of the MFOLD program (Zuker, 1989) from the GCG package (Genetics Computer Group, Madison, WI, USA). Free energy values for the stems of the proposed pseudoknot-like interaction were calculated using Turner tables (http://www.ibc.wustl.edu/~zuker/rna/energy/index.shtml).

Results

Genomic variability in the progeny from a typical PLMVd severe variant

Each of the eight cDNA clones obtained from the progeny of variant gds6 corresponded to a new PLMVd sequence variant. From a total of 339 positions in the alignment, 19 were polymorphic (Figs 1 and 2). These were not randomly distributed because 17 known to be polymorphic in a previous analysis of the variability of the PLMVd molecule (Ambros et al., 1998). The progeny sequences differed from the parental variant, not found in the progeny, in four to ten nucleotide changes (genetic distances for pairwise comparisons from 0.0090 to 0.0273), whereas the most distant variants gds6-1 and gds6-22 were separated by 12 changes (genetic distance 0.0335) (Fig. 1). The nucleotide diversity value for this progeny was 0.02045 ± 0.00001 (± variance). Phylogenetic analysis did not provide a tree topology with a significant value to exclude a star topology for the evolution of the progeny of gds6 variant.

The five informative positions (61, 109, 122, 283 and 336; Fig. 1) characteristic of group I PLMVd sequences, to which variant gds6 belongs, were preserved in a significant fraction of the progeny sequences, gds6-3, -12, -7, -1b and -1. In the three remaining variants, gds6-8, -22 and -11, only the informative position 336 showed the change G → U (Fig. 1), which implies a transition from an informative position characteristic of group I to another of group III.

Changes observed in ribozyme domains of the progeny of gds6 variant did not affect either the conserved residues present in all known natural hammerhead structures (Bruening, 1989; Flores et al., 1997; Navarro & Flores, 1997; Symons, 1997) or their thermodynamic stabilities because they were found in loops or in distal base pairs of the stems (Fig. 3). However, as previously found for other PLMVd variants (Ambros et al., 1998), a deletion of the U located 3′ to the self-cleavage site of the plus hammerhead structure was observed in variant gds6-8 (Fig. 3). This change strongly reduced self-cleavage during in vitro transcription and it is most probably an artefact introduced by the reverse transcriptase (Ambros et al., 1998).

Some base changes found within the gds6 progeny variants would affect the pseudoknot-like element that the parental sequence gds6 may potentially form (Ambros et al., 1998). Although a detailed description of the base-pairings involved in this element is not shown, it is remarkable that the mutations found in some progeny variants, as those present in gds6-1b, would lead to stronger interactions (Fig. 4A).

Analysis of the progenies from a PLMVd variant inducing two different phenotypes

When bioassayed on the peach indicator GF-305 the gds15 variant, which differs in 10 nucleotides from the gds6 variant, was phenotypically unstable: it induced the typical PLMVd symptoms of D168 isolate in some plants whereas in others the infection was symptomless (Ambros et al., 1998). To obtain an insight into this differential pathogenicity, several cDNA clones of the progenies from a symptomatic and a non-symptomatic plant were characterized.

As observed for the gds6 variant, the 12 cDNA clones characterized from the progeny of the gds15 parental sequence (five and seven from the symptomatic and non-symptomatic plant, respectively) were new PLMVd variants. The number of polymorphic positions taking into account the two populations derived from the gds15 variant, 34 out of a total of 341 in the alignment (Fig. 5), almost doubled that of the progeny of the gds6 variant, although both gds6 and gds15 progenies were similarly heterogeneous (nucleotide diversity 0.02045 ± 0.00001 vs 0.01855 ± 0.0001, tfl = 1.32, P = 0.1017). As in the case of the progeny of the gds6 variant, phylogenetic analysis did not exclude a star topology for the
Fig. 1. Sequence alignment of the progeny variants derived from the gds6 parental sequence. Dots indicate residues identical to the parental sequence (gds6-P), shown on the top, and dashes denote gaps. Regions involved in forming hammerhead structures are flanked by flags, the conserved nucleotides present in most natural hammerhead structures are indicated by bars and self-cleavage sites are shown by arrows; solid and open symbols refer to plus and minus polarities, respectively. Informative changes dividing PLMVd variants into groups I and III are shown on light-grey and black backgrounds, respectively. Other specific changes present in most sequences of group II are denoted with regular lowercase letters. Residues from loops A and B involved in a potential pseudoknot-like interaction are boxed. Primers used for RT–PCR amplification cover positions 178–225. Asterisks denote new polymorphic positions in the PLMVd molecule.

The evolution of the progenies of the gds15 variant. Considering the two populations derived from the gds15 variant separately, the number of polymorphic positions was significantly higher in the progeny from the symptomatic plant than in that from the non-symptomatic plant (nucleotide diversity 0.02182 ± 0.00004 vs 0.01322 ± 0.00001, t₀ = 2.8, P = 0.0094), but the parental sequence was not recovered in either. In the symptomatic plant population one of the variants, gds15-4s, had 20 changes with respect to the parental sequence (genetic distance 0.0435) and the variants of this progeny had four to 20 changes (genetic distances from 0.0030 to 0.0435) (Fig. 5). By contrast, the number of changes between the parental and progeny sequences varied from four to ten in the asymptomatic plant population (genetic distances from 0.0120 to 0.0212) in which the most closely related variants, gds15-6as and -7as, differed only in one position (genetic distance 0.0030) (Fig. 5).

Four or five informative positions of the parental sequence were preserved in most gds15-derived variants from the symptomatic plant, a situation similar to that found in the progeny of gds6 variant (Fig. 5); only the gds15-4s variant had lost all of them and had acquired the three informative changes defining group III sequences (positions 2, 5 and 338 in Fig. 5). In contrast to the progeny from the symptomatic plant inoculated with gds15 variant, none of the characterized cDNA clones from the asymptomatic plant inoculated with gds15 variant retained the five group I informative changes (Fig. 5, positions 61, 110, 123, 284 and 338). Although the small number of cDNA clones analysed does not permit one to draw firm conclusions, the distinctive attribute of this population appeared to be the gradual loss of these informative positions reflected in the existence of variants conserving only four, three or two positions.

As for the population derived from variant gds6, the most remarkable change affecting the ribozyme domains of progenies from the gds15 variant was the deletion in three of the cDNAs of the residue located 3’ to the cleavage site of the plus hammerhead structure (Fig. 3). On the other hand, a potential
Rapid evolution of a hammerhead viroid

Fig. 2. Proposed secondary structure of PLMVd (Ambrós et al., 1998) showing the distribution of polymorphic positions for each progeny along the reference sequence (Hernández & Flores, 1992). Nucleotide changes, either substitutions or indels, as well as new variable sites along the molecule and the number of affected sequences, are indicated. Ribozyme domains involved in the hammerhead structure formation are flanked by flags, conserved residues present in most natural hammerhead structures are indicated by bars and self-cleavage sites are marked by arrows with solid and open symbols referring to plus and minus polarities, respectively. Numbering of the reference sequence is marked every twenty residues. Inset, alternative cruciform conformation of the hammerhead arm region predicted for most progeny variants. Changes observed in this region are indicated by encircled letters.
Fig. 3. Hammerhead structures of variants from four PLMd progenies. Nucleotide changes found between variants belonging to different progenies are indicated on the self-cleavage domains of the corresponding parental sequences. Nucleotide substitutions, insertions or deletions are indicated within circles, within squares and by triangles respectively, pointing out in each case the variants affected by the corresponding change. The 13 conserved residues present in most natural hammerhead structures are boxed. The same numberings are used for the plus and minus polarities and correspond to those of the alignments shown in Figs 1, 5, 6 and 8.
Rapid evolution of a hammerhead viroid

Fig. 4. Potential pseudoknot-like element in PLMVd RNA. (A)–(C) Interactions proposed for some parental and progeny variants belonging to groups I, II and III, respectively. The names of the parental sequences are in a black background and those of progeny variants in a grey background. Consensus interactions of each group according to Ambros et al. (1998) are shown on the left, with continuous and broken lines indicating the existence of a base pair in all and some cases, respectively. (D) A new alternative interaction found in one progeny variant. Free energy values (at 25 °C) for the proposed interactions are shown in parentheses following the name of the variant. Numberings correspond to those of the alignments shown in Figs 1, 5, 6 and 8.

A pseudoknot-like interaction may be formed in all progeny variants from the gds15 parental sequence with approximately half of them maintaining the group I-type interaction as the parental sequence (Ambros et al., 1998), and the rest adopting an interaction characteristic of group III (data not shown).

Multiple compensatory changes and a novel substitution in the hammerhead structures of the progeny from a PLMVd latent variant

Characterization of nine cDNAs clones from the progeny of esc10 variant showed a nucleotide diversity significantly higher than that found in populations derived from gds6 (0.03493 ± 0.00002 vs 0.02045 ± 0.00001, t16 = 7.78, P < 10^-6) and gds15 variants (0.03493 ± 0.00002 vs 0.01855 ± 0.00001, t16 = 9.41, P < 10^-7). Of a total of 341 positions in the alignment of the progeny from esc10 variant, 36 were polymorphic including six new ones, with three variants, esc10-5, -5b and -12, forming a phylogenetic cluster separated from the parental sequence by 19–22 changes (genetic distances from 0.0338 to 0.0433) (Fig. 6). With the exception of the esc10-5b cDNA clone, identical to the gds21 variant characterized previously (Ambros et al., 1998), the other eight cDNA clones characterized from the esc10 variant were new PLMVd sequences.

One of the most interesting attributes of the progeny from esc10 variant was a shift in the pattern of informative changes that lead to two major subpopulations (Fig. 6). Although the group II parental sequence was not retained in the progeny, variants from the first subpopulation were closer to it because they preserved two or three of the informative positions exclusive to group II (1, 24 and 341) as well as a set of point changes specific for most of the group II members (Fig. 6).
Moreover, the same pseudoknot-like interaction proposed for the parental variant or a similar one resulting from a new covariation in the third base pair (compare for example esc10-2 and esc10-1 variants in Fig. 4B) could be formed in the progeny variants. The second subpopulation in the progeny from the esc10 parental sequence, formed by variants esc10-5, -5b and -12, had the five informative positions defining group I (Fig. 6) together with the potential to form a pseudoknot-like interaction of this group (data not shown). Variant esc10-6b represents an intermediate sequence between the two subpopulations (Fig. 6), conserving the three informative positions and most of the specific point changes of group II sequences, but having also two informative positions of group I. The most stable pseudoknot-like interaction for variant esc10-6b contains five base pairs and represents a new alternative of this potential element of tertiary structure (Fig. 4D).
Some unusual ribozyme mutants were detected in the progeny from the esc10 variant (Fig. 3). In the minus hammerhead structure of the esc10-1 variant the substitution $G12 \rightarrow A$ in the GAAAC sequence conserved in all natural hammerhead ribozymes (Bruening, 1989; Di Serio et al., 1997; Flores et al., 1997; Navarro & Flores, 1997; Symons, 1997) was
The minus hammerhead structure of a third variant, esc10-6b, presented two concurrent point substitutions disrupting the fourth and fifth base pairs of helix II and III, respectively, that might in part explain the low self-cleavage activity of this RNA (Fig. 7). Regarding the plus polarity hammerhead structure, esc10-2 and -5b variants showed the same deletion of the residue located 3′ to the self-cleavage site observed previously (Fig. 3). It is interesting to note that in variants esc10-5, -5b and -12, covariations restored the fifth base pair of helix II and helix III of the minus and plus hammerhead structures, respectively (Fig. 3).

**Recovery of the parental sequence in the progeny from a latent variant**

The progeny from the ls11 variant was unique in having the parental sequence preserved (variant ls11-13) and one variant (ls11-4) represented by two cDNAs with the same sequence. The nucleotide diversity of this progeny, although significantly lower than that from the esc10 variant (0.02800 ± 0.00002 vs 0.03493 ± 0.00002, \(t_{14} = 3.08, P = 0.0041\)), was higher that those derived from gds6 (0.02800 ± 0.00002 vs 0.02045 ± 0.00001, \(t_{18} = 3.72, P = 0.0013\)) and gds15 variants (0.02800 ± 0.00002 vs 0.01855 ± 0.00001, \(t_{17} = 4.92, P = 0.0001\)). Of a total of 339 positions in the alignment of the progeny from the ls11 variant, 22 were polymorphic, three of them new, and included informative changes characteristic of group III, to which the parental sequence belongs (positions 2, 5 and 336), and group I (positions 61, 109, 122, 283 and 336) (Figs 2 and 8).

Variants ls11-12 and -3 were closely related to the parental sequence, retaining three and two of the group III informative changes, respectively, as well as the same type of pseudoknot-like interaction (Fig. 4D). Conversely, variants ls11-4 and ls11-6 form a phylogenetic cluster separated from the parental sequence. They have a much closer relationship to group I sequences as reflected by the presence of four or five of the informative changes defining this group (Fig. 8), and the potential to form a similar pseudoknot-like-element (data not shown). The remaining sequence of this progeny, ls11-2b, appears in the phylogenetic trees as an intermediate variant between group III and group I sequences (Fig. 8), a situation similar to that proposed for variant esc10-6b from the other latent progeny.

Variants from the progeny of the ls11 variant did not present relevant changes affecting the stabilities of their hammerhead structures except the deletion U1.1 present in the plus hammerhead structure of the ls11-12 variant detected previously in other PLMVd sequences (Fig. 3).

**Discussion**

The evolution of PLMVd on its natural host has been approached by inoculating single infectious cDNAs on the peach indicator GF-305. Molecular characterization of the
progenies derived from four PLMVd variants differing in their pathogenicity, revealed the rapid accumulation of sequence heterogeneity 2–3 months after infection, the time required by the pathogenic variants to induce the onset of symptoms. The wide separation in the sequence space between the parental and some of the progeny variants, as well as the high polymorphism of the latter, showed the very dynamic nature of the viroid populations. From a total of 36 cDNAs characterized, 33 differed from each other and from the 31 sequence variants reported previously (Hernández & Flores, 1992; Ambrós et al., 1998; Ambrós & Flores, 1998) and, in fact, 18 new polymorphic positions in the PLMVd molecule were observed. These results indicate that the genetic variability found in natural isolates of this viroid (Ambrós et al., 1998) is probably not the consequence of repeated infections of the same plant but rather an intrinsic property of this RNA to evolve rapidly.

The distribution of the polymorphism along the molecule showed a predominant localization in loops A and B and in the PstI arm (Fig. 2), the same three regions where an important fraction of the informative changes of PLMVd has been previously found (Ambrós et al., 1998). One main trend of PLMVd evolution appears to be the gradual accumulation of point changes, as supported by the cases of the progenies of esc10 and ls11 variants, with most, but not all, of the polymorphic positions representing independent hotspots whose combination greatly enlarges the number of new sequences. However, some point changes were not independent since most of those found within the hammerhead structures were compensatory and did not affect their stabil-
quences must follow defined routes in the sequence space. The PLMVd quasispecies evolving from the different parental parents is necessary to consider the quasispecies as a whole. However, higher genetic stability (Góra-Sochacka et al., 1994) as compared to PSTVd, a system with considerable systematics, in agreement with the functional role presumed for these self-cleaving domains (Hernández & Flores, 1992; Ambros et al., 1998). Moreover, all the new PLMVd variants characterized maintain the global branched secondary structure (data not shown) predicted for this viroid (Ambros et al., 1998). In addition, covariations consistent with the potential pseudo-knot-like interaction between loops A and B proposed previously (Ambros et al., 1998) were observed in different progenies.

The rapid evolutionary pattern shown by PLMVd fits the quasispecies model proposed for different RNA replicons, including viruses (Domingo et al., 1985; Domingo & Holland, 1994), satellite RNAs (Kurath & Palukaitis, 1989; Aranda et al., 1993; Sheldon & Symons, 1993) and other viroids (Keese et al., 1988; Elena et al., 1991). Within this latter group of subviral pathogens, only one systematic analysis of the progenies evolving from single natural PSTVd variants inoculated in the experimental host tomato has been reported recently (Góra-Sochacka et al., 1997). This study revealed the rapid generation of new quasispecies of PSTVd, but the maximal number of nucleotide changes accumulated with respect to the parental variants and between the progeny sequences, three in both cases, is considerably lower than that found for PLMVd. Therefore, the relative homogeneity of PSTVd progenies in which the parental sequence prevails as a main component (Góra-Sochacka et al., 1997), represents a case of population equilibrium (Domingo et al., 1985; Domingo & Holland, 1994), as opposed to the PLMVd situation in which the parental sequence was retained as a minor component in only one of the four progenies studied here. It may be speculated that the different rate in accumulating sequence heterogeneity observed in PSTVd and PLMVd may reflect the involvement of distinct RNA polymerases in the replication of the two viroids. ASBVd, the type species of the Avsunviroidae family, to which PLMVd belongs, accumulates in the chloroplast (Bonfiglioli et al., 1994; Lima et al., 1994), whereas PSTVd and other members of the Pospiviroidae family accumulate in the nucleus (Harders et al., 1989; Bonfiglioli et al., 1996). If the subcellular replication and accumulation sites coincide and represent a distinctive feature within each viroid family, this would imply that the RNA polymerases involved in the replication of PSTVd and PLMVd would not be the same and, consequently, this could lead to different mutation rates. Moreover, PLMVd seems to be endowed with a particularly high flexibility to accommodate an extensive number of polymorphic positions, suggesting that different selective constraints operate on this viroid as compared to PSTVd, a system with considerable higher genetic stability (Góra-Sochacka et al., 1997).

The extreme variability found in PLMVd quasispecies impedes the establishment of a correlation between the observed phenotype and any individual genotype, making it necessary to consider the quasispecies as a whole. However, the PLMVd quasispecies evolving from the different parental sequences must follow defined routes in the sequence space because, in most cases, the same phenotypic effect was observed for a given PLMVd variant in independent experiments (Ambros et al., 1998). In this context, the reproducible symptomatic infections incited by the gds60 variant might be explained, at least in part, by the establishment of a quasispecies with a relatively low intrapopulation heterogeneity and with essentially no changes in the pattern of group I informative positions; the evolution of the progeny from gds18 variant, also inducing a symptomatic infection, provided similar results (data not shown). Although the pathways leading to symptomatic or asymptomatic infections in plants inoculated with the gds15 variant are unknown, the population structure of the two progenies derived from that variant has provided interesting data. Despite the higher heterogeneity found in the symptomatic plant, all variants except gds15-4s maintained most of the group I informative positions characteristic of variants giving rise to symptomatic infections, whereas a gradual loss of the group I informative positions was observed in the progeny from the asymptomatic plant inoculated with the gds15 variant.

Progenies from PLMVd parental sequences esc10 and ls11 leading to asymptomatic infections appeared to follow a similar evolutionary trend regardless of the founding variant. In both populations a major fraction of variants preserving the parental characteristics were observed, together with others containing informative positions of the distant group I. Interestingly, group I variants appearing in these progenies converged into a similar region of the sequence space as illustrated by the case of ls11-4 and esc10-5 variants that only differ in one position. However, transitions from group II to group III sequences, or vice versa, were never detected.

A characteristic of PLMVd infections is the unstable symptomatology observed under field conditions, particularly when caused by severe isolates. The rapid generation of genetic diversity observed upon propagation of individual PLMVd genomes in its natural host suggests that repeated fluctuations in the sequence spectrum, due to progressive accumulation of point changes, may determine to a large extent the variable phenotype observed in natural PLMVd infections.

We thank J. C. Desvignes for supplying material and bioassays, A. Ahuir and N. Grasseau for technical assistance, Drs S. F. Elena and V. Pallás for critical reading of the manuscript and suggestions, and D. Donnellan for English revision. R.F. was supported by grant PB95-0139 from the Dirección General de Investigación Científica y Técnica of Spain and by contract AIRCT93-1567 from the European Commission. S.A. was a recipient of a predoctoral fellowship from the Generalidad Valenciana and C.H. was a recipient of contract ERBFMBICT950143 from the European Commission.

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Received 15 March 1999; Accepted 30 April 1999