Towards dissecting the structural determinant of *Peach latent mosaic viroid* inducing mosaic symptoms

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Abstract

Most isolates of *Peach latent mosaic viroid* (PLMVd) do not incite foliar symptoms, but a few number of isolates cause peach mosaic (PM) or peach calico (PC), an extreme albino phenotype. The PC determinant has been previously mapped at an insertion of 12-13 nt folding into a hairpin capped by a U-rich loop but the PM determinant, which is not associated with a specific insertion, remains unidentified and could reside in one or more domains of the branched conformation proposed for PLMVd. To tackle this problem we have selected for further dissection one variant (GDS6), recovered from a typical PM isolate (GDS), which is very infectious and elicits consistently a characteristic PM. We have initially focused on G337, a position that appears associated with PM in multiple alignments that include GDS6 and other PM-inducing and latent variants. To determine the role of G337 in infectivity and symptoms, GF-305 peach seedlings were inoculated with *in vitro* transcripts of recombinant plasmids containing dimeric tandem inserts of PLMVd-cDNAs with all possible changes at this position introduced by site-directed mutagenesis. Deletion of G337 abolished infectivity, while substitutions by A, C or U incited, in most inoculated plants, PM symptoms. Cloning and sequencing showed that the A substitution at position 337 was preserved in the progeny or reverted to G, while C or U substitutions at this position were not stable and reverted to A or G in the progenies. Extending this approach to additional nucleotides of loop A, or of other PLMVd domains, may provide hints in identifying the determinant of PM.

Keywords: Viroids, Pathogenesis, Peach disease

Introduction

Peach latent mosaic viroid (PLMVd) (Hernández and Flores, 1992; Flores et al., 2006), is the type species of the genus *Pelamoviroid* within the family *Avsunviroidae* (Flores et al., 2005). Members of this family do not have a central conserved region, but are able to form hammerhead ribozymes in both polarity strands that mediate self-cleavage of the replicative intermediates generated through a symmetric rolling-circle mechanism (Flores et al., 2000). There is direct evidence indicating that replication and accumulation occurs in the chloroplast in two members of this family, *Avocado sunblotch viroid* (ASBVd) (Bonfiglioli et al., 1994; Lima et al., 1994; Navarro et al., 1999) and PLMVd (Bussière et al., 1999), a property that is presumably shared by the other members of the family *Avsunviroidae*.

PLMVd infection of peach cultivars grown under field conditions, and of the peach indicator GF-305 grown in greenhouse, may induce a wide variety of leaf symptoms and, accordingly, isolates are classified into three types: i) latent, the most frequent as reflected in the name of the disease (Desvignes, 1976; Desvignes 1980; Desvignes 1986), ii) peach mosaic (PM) or peach blotch of variable severity, and iii) peach calico (PC), an extreme chlorosis that completely covers the leaf area. Previous results have shown that the structural determinant of PC maps at an insertion of 12-13 nt folding into a hairpin capped by a U-rich loop (Malfitano et al., 2003; Rodio et al., 2006; Rodio et al., 2007). However, the molecular determinant of PM remains unidentified because, not being associated with a specific insertion, it could reside in one or more domains of the branched PLMVd conformation. Moreover, molecular characterization of latent and PM-inducing PLMVd isolates has revealed that they are formed by complex populations of variants (Ambrós et al., 1998), and bioassays on GF-305 peach seedlings of some individual variants have shown that the biological properties of PLMVd isolates depend on the complexity of their populations and on the presence of specific variants (Ambrós et al., 1999). Therefore, mapping the structural determinant of PLMVd inducing PM may be considerably more complicated than in the case of PC. Here, we report our first attempts in this mapping.

Material and methods

Infectivity bioassays: Inoculations were performed by slashing the stems of GF-305 peach seedlings, which were kept in a greenhouse and periodically examined for symptom expression 2-3 months postinoculation (Ambrós et al., 1999). Infection of GF-305 peach seedlings was additionally tested by dot-blot hybridization of nucleic acid preparations with a radioactive full-length PLMVd-cRNA probe, and by RT-PCR with a pair of adjacent primers (RF-43 and RF-44) of sense and antisense polarity derived from a region with low variability (Ambrós et al., 1998).

Inocula: The recombinant plasmid pGDS6, containing a head-to-tail dimeric full-length cDNA insert of the GDS6 variant (Ambrós et al., 1999), served as starting material for generating by site-directed mutagenesis (Byrappa et al., 1995) plasmids pGDS6Δ337, pGDS6A337, pGDS6C337 and pGDS6U337, wherein the G337 nucleotide was deleted or substituted in both cDNA copies by A, C or T, respectively. *In vitro* transcripts from these plasmids, synthesized as reported previously (Hernández and Flores, 1992), were used as inocula.

Progeny characterization and sequence analysis: RT-PCR products were fractionated by electrophoresis in polyacrylamide gels (5 %) and, after ethidium bromide staining, the PLMVd-cDNAs of the expected size were eluted and cloned into a plasmid vector. The sequence of the corresponding inserts were automatically sequenced.

Results and discussion

Our working hypothesis is that the molecular determinant for PC may map at the same loop (loop A) at which the 12-13 nt insertion characterictic of PC has been reported before (Malfitano et al., 2003). As an experimental system to start testing this hypothesis we have selected the PLMVd variant GDS6, recovered from a typical PM isolate (GDS), because it is very infectious and incites consistently a characteristic PM (Ambrós et al., 1998; Ambrós et al., 1999). Multiple alignments of previously characterized PLMVd variants inducing latent and PM phenotypes, including GDS6 (Ambrós et al., 1998; Ambrós et al., 1999), suggest that nucleotide G337 of variant GDS6, located in the loop A (Figure 1), is associated with PM.

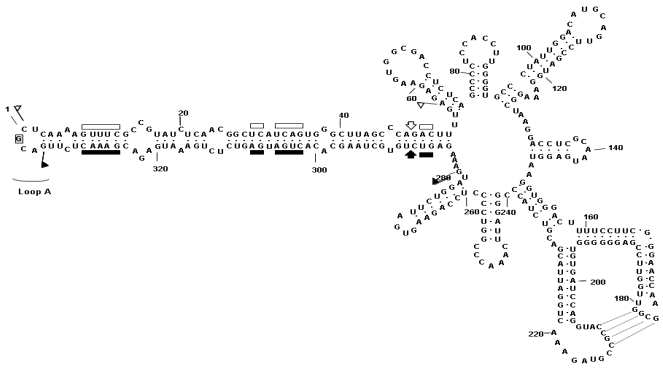

Fig. 1 Proposed secondary structure of PLMVd variant GDS6 (Ambrós et al., 1998). Sequences involved in forming the hammerhead structures are flanked by flags, conserved nucleotides present in most natural hammerhead structures are indicated by bars, and self-cleavage sites are marked by arrows; solid and open symbols refer to plus and minus polarities, respectively. Nucleotides involved in a pseudoknot between positions 176 to 179 and 209 to 212 (Bussière et al., 2000) are denoted by broken lines. The G337 residue within the loop A is boxed and with a grey background.

To examine this possibility we generated the recombinant plasmids pGDS6Δ337, pGDS6A337, pGDS6C337 and pGDS6U337, containing head-to-tail dimeric full-length cDNAs in which the G337 nucleotide was deleted or substituted by A, C or T, respectively (Table 1). The infectivity and pathogenicity of the resulting GDS6-mutated variants was tested by slash inoculation of the corresponding *in vitro* transcripts into blocks of eight GF-305 seedlings. Symptom observation and dot-blot hybridization and RT-PCR analyses revealed that deletion of G337 abolished infectivity of the GDS6Δ337 transcript, while substitutions by A, C or U had moderate effects, with most plants showing clear PM symptoms although less severe than the GDS6 wild-type control. Cloning and sequencing of the PLMVd progeny accumulating in seedlings inoculated with variant GDS6A337 showed that it was composed by

variants characterized by having a G or an A residue at position 337, which are the nucleotides found at this same position in all the natural variants characterized previously from the GDS isolate (Ambrós et al., 1998; Ambrós et al., 1999) (Table 1). In contrast, the progenies resulting from variants GDS6C337 and GDS6U337 did not preserve the artificially-introduced C337 and U337 nucleotides, respectively (Table 1).

Tab. 1 Nucleotide at position 337 in the progeny of the mutated variants with respect to the parental GDS6 variant (with a G at this position).

Variant	Sequenced clones	A337	G337	C337	U337
GDS6*	8	2	6	0	0
GDS6Δ337	NI	-	-	-	-
GDS6U337	9	3	6	0	0
GDS6A337	20	10	10	0	0
GDS6C337	10	4	6	0	0

*Data from Ambrós et al. (1999); NI: not infectious variant

Instead, in both cases they were substituted by G or A as in the progeny resulting from variant GDS6A337. Additionally, other point mutations with respect to the parental inoculated transcripts were detected in two positions (but not in another two) of the loop A in the characterized progeny variants (Figure 2). However, these substitutions have been already reported in the progeny of the natural GDS6 variant (Ambrós et al., 1999), and no specific correlation with induction of PM could be established in the present study. As expected, a relatively high sequence variability distributed throughout the viroid genome at positions similar to those previously reported for the progeny of the natural GDS6 variant (Ambrós et al., 1999), was also noticed (data not shown).

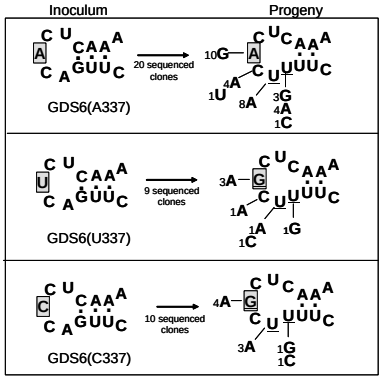

Fig. 2 Schematic representation of the loop A and the adjacent stem of the GDS6-mutated infectious variants (left) and of their respective progeny variants (right). The nucleotide at the position 337 is boxed and with a grey background. The number of variants sequenced in the progeny of each infectious variant is indicated below the arrows. The most abundant point mutations in progeny variants are underlined, and the number of clones in the progeny in which additional mutations were found, as well as their location, are also indicated.

Our results show that a deletion at position 337 of variant GDS6 abolish infectivity, most likely because a nucleotide at this position is critical for a minimal replication threshold that might facilitate, due the high mutation rate observed in some chloroplastic viroids (Gago et al., 2009), incorporation of a 1-nt insertion restoring the original size of loop A. In line with this view, the three substitutions at the same position would allow replication above the threshold and ultimately lead to selection of a purine. Because of the natural reversion of substitutions at position 337, it is not possible to establish a direct correlation between PM and the G337 residue in the GDS6 variant. However, further experiments extending this approach to additional nucleotides of loop A, or of other PLMVd domains, may provide hints to identify the molecular determinant of PM.

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