GENOMIC STRUCTURE OF NEW TUNISIAN PEACH LATENT MOSAIC VIROID VARIANTS

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SUMMARY

Peach latent mosaic viroid (PLMVd) is a single-stranded circular RNA that do not code for proteins and ranges in size from 335 to 351 nucleotides. It mainly infects peach. In this study, the sequence of 20 complete cDNA clones derived from seven PLMVd isolates detected in five Tunisian peach cultivars was analysed in 3 steps: primary structure, phylogeny and secondary structure. The analysis of the primary structure revealed that all the 20 cDNA clones sequences corresponded to different variants. They ranged in size from 336 to 341 nt. Sequence alignment of our variants with reference sequences revealed 81 polymorphic positions. Among them, 15 were never described in the literature so far. The variable positions are scattered all around the RNA molecules, but the majority of them were concentrated in the region corresponding to nucleotides 1 to 70 and 170 to 346 in the alignment. Sequence homologies between variants of the same isolate or variants of different isolates ranged from 96% to 100%. This confirms that a PLMVd isolate is composed by a complex mixture of closely related molecules. Moreover, some variants isolated from different cultivars were found to be similar, indicating that a sequence is not exclusive to a cultivar.
Phylogenetic analysis of our sequences allowed their clustering into two groups: group I (16 variants) and group II (4 variants) that differed by 18 polymorphic positions. Further phylogenetic analysis and sequence alignment of our sequences and the reference sequences were done. It revealed that our sequences were similar to the reference sequence Hd8 in the regions delimited by nucleotides 1 to 69 (region 1) and 268 to 343 (region 5) and to the reference sequence Hd6 in the region between nucleotides 150 and 200 (region 3). The other regions corresponding to nucleotides 70 to 149 (region 2) and 201 to 267 (region 4) were similar for all the sequences. These observations revealed that our Tunisian PLMVd variants correspond to a new population never reported in the literature. Analysis of the secondary structure confirmed that all PLMVd Tunisian variants presented a branched secondary structure and revealed a new potential pseudoknot-like interaction between two loops.

Key words: PLMVd, molecular characterization, sequence analysis, peach, viroids, Tunisia

INTRODUCTION

Viroids are the smallest pathogen able to infect and to cause severe disease in higher plants. They are small single stranded circular RNA, that do not code for any proteins and that range in size from 246 to 401 nucleotides (nt) (Tabler and Tsagris, 2004). They rely on interaction with host component for their replication, spread and pathogenicity. Peach latent mosaic viroid (PLMVd) is the causal agent of peach latent mosaic disease. It replicates autonomously through a rolling circle mechanism involving self-cleavage

hammerhead motif (Hernandez and Flores, 1992). The PLMVd infection can be either latent or not. The symptom expression corresponds to mosaic symptoms or large white patches covering most of the leaf bade, ageing of peach trees and irregularly shaped, flattened, and colorless fruits (Desvignes, 1986; Flores *et al.* 1990; Llacer, 1998; Malfitano *et al.*, 2003). PLMVd can infect several plant species including peach (*Prunus persicae*) from Europe, Asia, North Africa and both North and South America (Flores *et al.*, 1990; Hadidi *et al.*, 1997; Fekih Hassen *et al.*, 2004) but also apricot (*Prunus armeniaca*), plum (*Prunus domestica*), sweet cherry (*Prunus avium*), cultivated pear (*Pyrus communis*), wild pear (*Pyrus amygdaliformis*), mume (*Prunus mume*) and almond (*Prunus amygdalus*) from Europe, North America and Tunisia (Hadidi *et al.*, 1997; Faggioli *et al.*, 1997; Giunchedi *et al.*, 1998; Kyriakopoulou *et al.*, 2001; Fekih Hassen *et al.*, 2004; 2005). Up to now, several PLMVd sequences have been characterized from peach trees cultivars originating from Europeans countries and North America. The first characterized PLMVd sequences (Ar1) was obtained in 1992 (Hernandez and Flores, 1992). Since ever, other molecular variants, isolated from severe or latent isolates have been isolated and sequenced (Shamloul *et al.*, 1995; Ambros *et al.*, 1998; Pelchat *et al.*, 2000; Malfitano *et al.*, 2003; Rodio *et al.*, 2006). Interestingly, PLMVd exhibited high sequence polymorphism in comparison with other viroids such as Potato spindle tuber viroid (PSTVd) (Ambros *et al.*, 1999).

The main objective of this work was to study the sequence polymorphism of 20 new Tunisian PLMVd variants obtained from 5 different peach cultivars in order to obtain data on the distribution of the sequence variability along the PLMVd molecule and on the secondary structure.

MATERIAL AND METHODS

Viroid sources

Total RNA was extracted as previously reported (Fekih Hassen *et al.*, 2006) from PLMVd infected leaves of 7 peach trees belonging to 5 different cultivars grown in the North and the centre of Tunisia. Characteristics of PLMVd isolates are illustrated in Table 1.

Table 1. Characteristics of PLMVd variants

Isolate	Variant	Province (Region)	Cultivar	Rootstock origin	Scion origin
377	377.1-377.2-377.3-377.4	Bizerte (North)	Red Top	Tunisia	USA
210	210.2-210.3-210.4	Ben Arous (North)	Carnival	Spain	USA
151	151.1-151.2-151.3-151.4	Manouba (North)	Alberta	Spain	USA
143	143.1-143.4	Ben Arous (North)	*	Spain	USA
240	240.4	Nabeul (North)	Royal Glory	Spain	USA
162	162.1-162.2-162.4	Ben Arous (North)	Early May Crest	Spain	USA
287	287.1-287.2-287.3-287.4	Kairouane (Centre)	Early May Crest	Spain	USA

* Missing data

RT-PCR amplification, cloning and sequencing

RT-PCR was carried out on RNA preparations with the help of the One tube RT-PCR Titan kit (Roche Diagnostics, Penzberg, Germany) according to the protocol described by Fekih Hassen *et al.* (2006). The amplified fragments were then purified and cloned with the TA cloning Kit (Invitrogen, Groningen, The Netherlands). Two to four clones were sequenced in both directions following the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

Multiple sequence alignments were carried out using the Clustal W program (Thompson *et al.*, 1994). Phylogenetic analyses were performed with the DNAMAN software, version 5.2.2 (Lynnon Biosoft, Quebec, Canada). Secondary structures of lowest free energy for PLMVd were generated with the MFOLD program (Zuker, 1989) of the sequence analysis package of the University of Wisconsin (Genetics Computer Group) (Devereux *et al.*, 1984).

RESULTS AND DISCUSSION

Analysis of the primary structure and the phylogeny of Tunisian PLMVd variants

Twenty complete cDNA clones were obtained from 7 PLMVd isolates. They corresponded to different molecular variants ranging in size from 336 to 341 nt. This observation suggested that conservation of the sequence length is not required for PLMVd as it has been reported for other viroids (Herold *et al.*, 1992, Kofalvi *et al.*, 1997). Multiple sequence alignments of the Tunisian variants with all the published PLMVd variants revealed 81 polymorphic positions. Among them, 15 were never described in the literature. These mutations are distributed around the PLMVd molecule, but the majority were located in the regions delimited by positions 1 to 70 and 170 to 346 (data not shown). The sequence homologies between variants of the same isolate were 96% to 100%. These same percentages were observed between variants of different isolates showing that PLMVd fits the quasi-species model described by Eigen (1993) and proposed for other PLMVd variants (Ambros *et al.*, 1999), for some viruses (Domingo *et al.*, 1985, Domingo and Holland, 1994) and satellite RNA (Kurath and Palukaitis, 1989).
Analyses of the phylogeny and the primary structures of the 20 Tunisian PLMVd variants allowed their clustering into 2 sub-groups presenting 92% homologies between each other and distinguished by 18 informative positions. Group I was composed of 16 PLMVd variants and group II was made up of 4 variants (Figure 1).

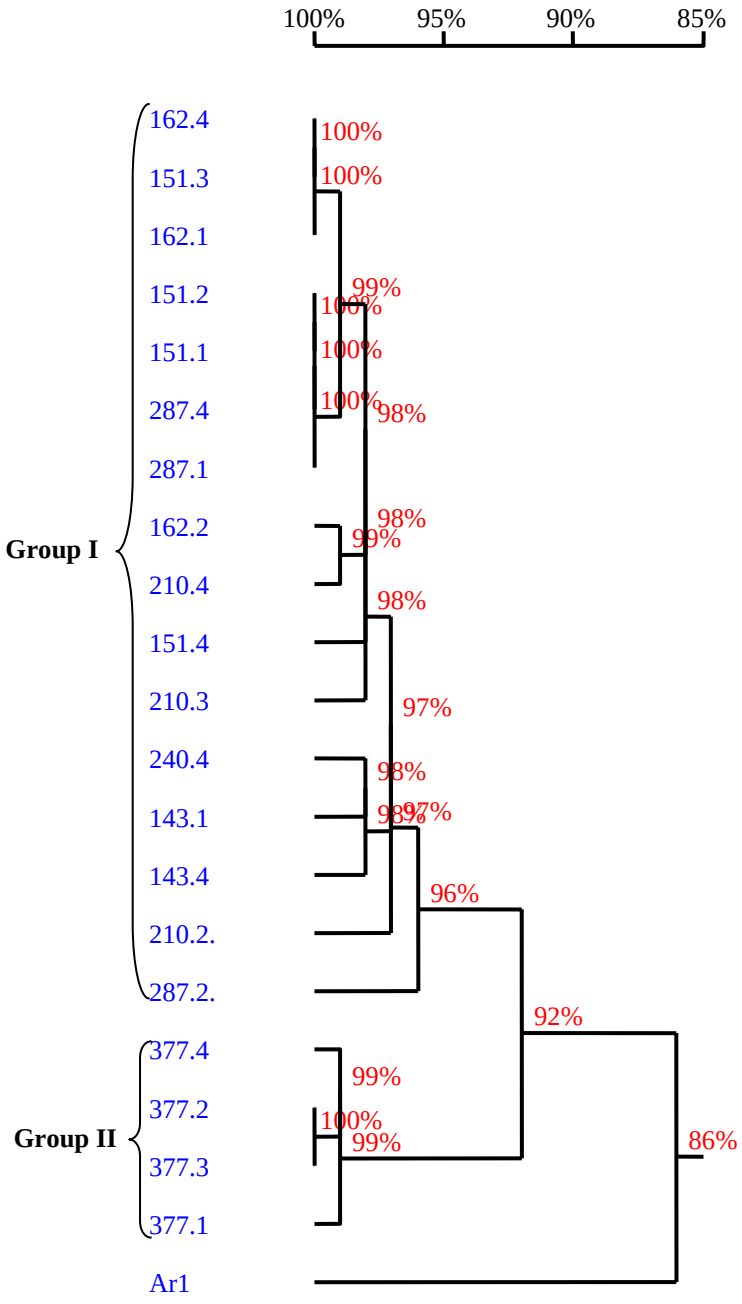

Figure 1. Homology tree between the Tunisian PLMVd variants in comparison with the reference sequence Ar1 (Hernandez and Flores, 1992). Subdivisions in groups are indicated by bracket

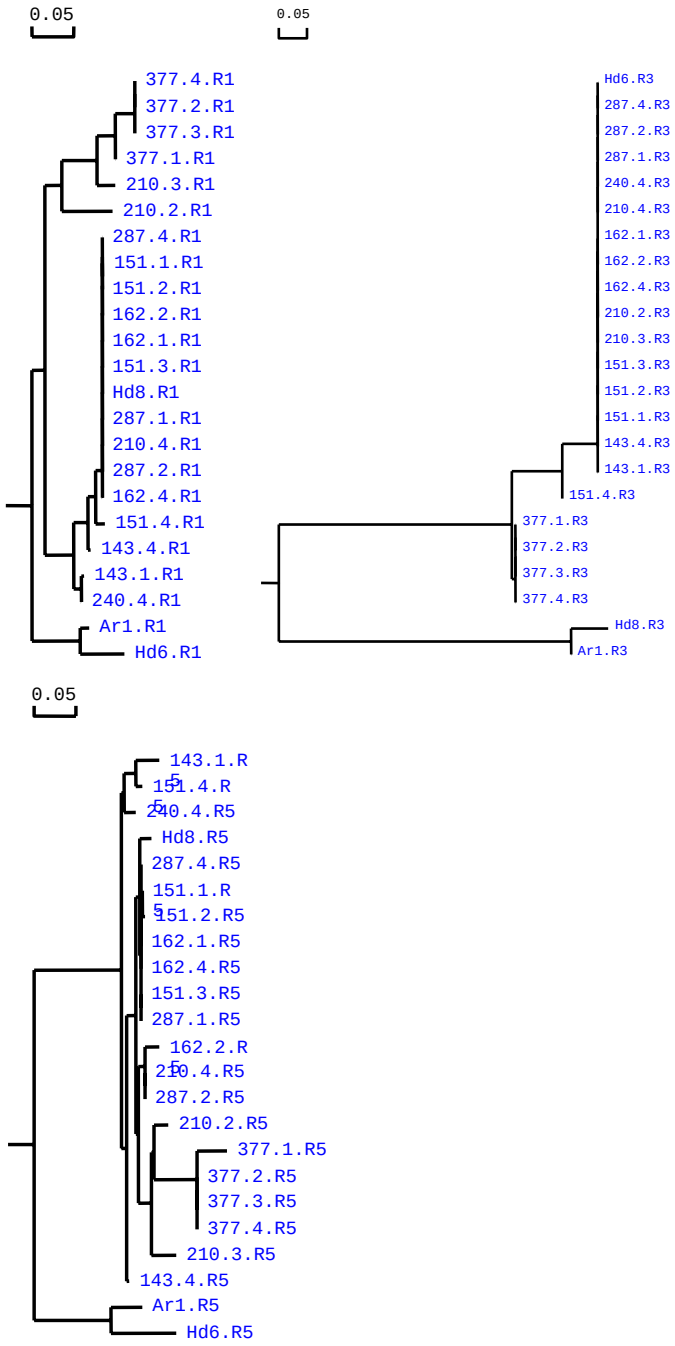

Figure 2. Phylogenetic trees of the Tunisian PLMVd variants with the reference sequences Ar1, Hd6 and Hd8 for the regions R1 (a), R3 (b) and R5 (c) which are delimited by nucleotides 1-69, 150-200 and 268-343, respectively

The sequence comparison of the Tunisian PLMVd variants with all the PLMVd variants previously described revealed 82% to 85% sequence homologies between each other. These values were lower than the arbitrary level of 90% separating variants from species. So, a multiple sequence alignment of the 20 Tunisian PLMVd variants with all the published PLMVd variants was done in order to determine if the Tunisian PLMVd variants were variants of the same species or of different species. Results showed that the PLMVd molecule of the Tunisian variants could be divided into 5 regions. The regions R1 (1 to 69) and R5 (268 to 343) were similar to the corresponding regions of the variant Hd8 (GeneBank accession number AF170503). However, the region R3 (150 to 200) was similar to the corresponding region of the PLMVd variant Hd6 (GeneBank accession number AF170501). The regions R2 (70-149) and R4 (201 to 267) were common to all the variants. These results were confirmed by phylogenetic analyses of the Tunisian variants with the reference sequences Ar1 (GeneBank accession number M83545), Hd6 and Hd8 for each of the 5 regions. Figures 2-a, 2-b and 2-c showed the phylogenetic results for the regions R1, R3 and R5, respectively. This study suggests that the Tunisian PLMVd variants may emerge from recombinations between previously coexisting Hd6 and Hd8 variants. So, Tunisian PLMVd sequences were new variants rather than species in spite of the low sequences homologies (82% to 85%) with the previously published PLMVd variants. Such events have previously been described for Hop stunt viroid (HSVd) isolates clustered into five groups including three major groups (plum-type, hop-type and citrus-type) and two minor groups that could be considered as the results of recombination events between members of the plum- and citrus- types or between members of the plum- and hope-types (Amari *et al.*, 2001). Furthermore, a previous work revealed evidence for exchange of RNA fragments between structural domains of different viroid species (Stasys *et al.*, 1995). They described a Citrus viroid III-a (CVdIII-a) isolate which contained an Apple scar skin viroid (ASSVd) derived upper CCR and a Pear blister canker viroid (PBCVd) derived lower CCR. An other case of recombination event was described for Australian grapevine viroid (AGVd) showing a genome divided into 12 portions belonging to four different viroids which were Grapevine yellow speckle 1 (GYSVd-1), ASSVd, Citrus exocortis viroid (CEVd) and PSTVd (Mc Innes and Symons, 1991).

Secondary structure analyses

The secondary structures of lowest free energy were predicted for all the Tunisian PLMVd variants using the MFOLD program (Zuker, 1989). This analysis showed the conservation of a highly branched secondary structure despite the nucleotide sequence polymorphism. The ribozyme domains folded into active hammerhead structures of plus and minus polarities (Figure 3). Indeed, mutations affecting these regions were located in the loops or, when located in the stems, covariations of base-pair nucleotides or compensatory mutations were observed. These data suggested the existence of a selective pressure in favour of self cleavage activity. It is worth noting that all the Tunisian PLMVd variants showed a potential pseudoknot like interaction between loops 11 and 1 which was strongly supported by base pair covariations (Figure 3). A similar interaction can be formed in the minus PLMVd

strand. Other interaction types between these two loops have already been suggested (Ambros *et al.*, 1998; 1999). However, *in vitro* assays have not been able to show evidence for such interaction (Pelchat *et al.*, 2000). This could be explained by the fact that *in vivo* conditions may involve host factors such as proteins or nucleic acids which may stabilize the pseudoknot like interactions.

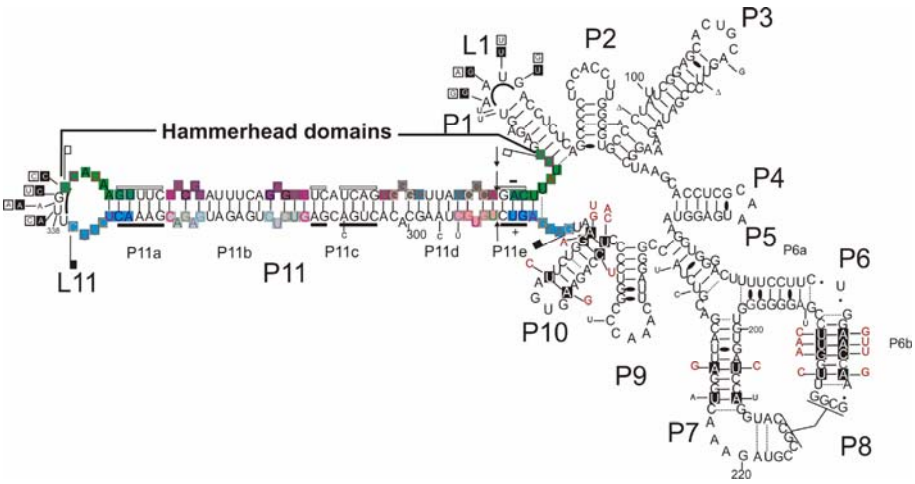

Figure 3. Predicted secondary structure of the PLMVd reference sequence (Hernandez and Flores, 1992). Fifteen new mutations are indicated along the PLMVd molecule. Black backgrounds correspond to nucleotide covariation. Regions involved in forming plus and minus polarity of hammerhead structures are flanked by flags. The conserved nucleotides present in most natural hammerhead structures are indicated by bars. The hammerhead cleavage sites for the plus and minus polarities are shown by arrows. Solid and open symbols refer to the plus and minus polarities, respectively. Stems I, II and III of the hammerhead structures of the plus polarity are in pink, grey and blue backgrounds. Stems I, II and III of the minus polarity hammerhead are in brown, purple and green backgrounds. Two examples of nucleotide covariation involved in a potential pseudoknot like interaction between loops 11 and 1 are indicated in black and white backgrounds, respectively.

In conclusion, despite the dynamic population of PLMVd illustrated in this work by the sequence polymorphism and the recombination hypothesis there are structural constrains limiting the sequence heterogeneity in this viroid as the conservation of a branched secondary structure, the preservation of the stability of the hammerhead ribozymes and potential pseudoknot like intra-molecular interactions in the PLMVd molecule (Ambros *et al.*, 1998; Bussière *et al.*, 2000; Pelchat *et al.*, 2000).
The ongoing experiments will be focused on the study of the structure-pathogenicity relationship of the new PLMVd population of variants by bioassays on GF-305 peach seedling.

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