Identification and characterization of *Peach latent mosaic viroid* and *Hop stunt viroid* in different peach cultivars showing dapple fruit, fruit yellow mosaic and cracked suture symptoms

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Abstract

From the early 1990s, a fruit peach syndrome characterized mainly by small discoloured spots (dapple fruit) and/or yellow areas on the skin (yellow mosaic), cracked suture and deformations was identified in most commercial orchards in the Emilia Romagna region (Northern Italy). In the past, *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) have been detected in trees with symptomatic fruits. In order to ascertain the presence and spread of these two viroids, symptomatic fruit samples were collected from five different cultivars: 'Royal Glory', 'Crimson Lady', 'Grenat', 'Diamond Princess' and 'Laura'. Dapple fruit symptoms affected all cultivars, 'Grenat' samples also showed evident yellow mosaic and fruit deformation, and 'Royal Glory' severe cracked sutures. The results showed a large diffusion of the two viroids, mainly in mixed infections. Anvaluation of the role the viroids could play in symptom expression has been complicated by the high number of samples infected by both viroids (60%). Nonetheless, PLMVd was confirmed to be strictly associated with the yellow mosaic, cracked suture and fruit deformation symptoms. The aetiological origin of the dapple fruit disease, however, seems to be more complicated, since in the 'Diamond Princess', only PLMVd has been found to be associated with the symptoms, whereas in all other cultivars, the presence of HSVd could have influenced the symptom expression. Moreover, the molecular characterization of some PLMVd isolates does not show any correlation between nucleotide sequence and symptoms although new PLMVd variants were identified.

Keywords: peach fruit symptoms, PLMVd, HSVd, mixed infection

Introduction

Viroids are pathogens of food, industrial and ornamental plants. Despite their small genome (single strand RNA of 240-400 nucleotides), they can affect a lot of plants causing severe damage. In fruit tree cultivation, the economic impact of viroids could be very important, since fruit quality is the aspect which is mostly affected (Daros et al., 2006). More specifically, two viroids were found on peach cultivars: *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd). PLMVd is the causal agent of Peach latent mosaic disease (Flores et al., 1990). The most severe symptoms of PLMVd on the peach fruit are deformation, discolored spot, the presence of cracked sutures and flattened stones. Symptoms induced on foliage are rare. In a few cases a particular albino pattern (peach calico) that covered most of the leaf area was observed. This last symptom was associated to a specific PLMVd variant, characterized by an insertion of 12-13 nucleotides (nts) causing the white coloration (Rodio et al., 2007). PLMVd is a member of the family of *Avsunviroidae* and consists of 335-338 nts for most strains and 347-351 nts for the calico strain (Flores et al., 2003) and an Egyptian strain that does not show any symptoms on the foliage (Hassen et al., 2007).

Dapple fruit symptoms on peach were also associated with the presence of HSVd (Sano et al., 1989; Zhou et al., 2006). HSVd belongs to the genus of *Hostuviroid* within the *Pospiviridae* family and consists of a circular single-strand molecule of RNA with a size that ranges between 294-303 nt.

From the early 1990s, a fruit peach syndrome characterized mainly by discoloured spots (dapple fruit) and/or yellow mosaic, cracked suture and deformations occurred in most commercial orchards in the Emilia Romagna region (Northern Italy) (Albanese et al., 1992). In order to ascertain the presence and the spread of the two viroids in the symptomatic trees, fruit samples have been collected from five peach cultivars: 'Royal Glory', 'Crimson Lady', 'Grenat', 'Diamond Princess' and 'Laura' and molecularly analyzed. Dapple fruit symptoms affected all cultivars (Figure 1), whereas 'Grenat' samples also showed evident yellow mosaic and fruit deformation (Figure 2) and 'Royal Glory' severely cracked sutures.

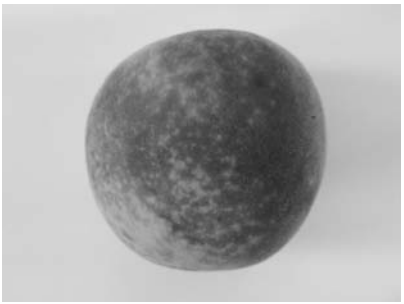

Fig. 1 Fruit of 'Diamond Princess' showing typical dapple fruit symptom

Fig. 2 **'**Grenat' fruits showing dapple fruit, yellow mosaic and fruit deformation symptoms

Materials and methods

Source of material: Symptomatic peach fruits of different cultivars originating from several orchards located in the Emilia Romagna region (Northern Italy) were used as source of materials (Table 1). More specifically, symptomatic fruits have been collected from an average of 20 trees per cultivars. PLMVd-infected, HSVd-infected and healthy GF 305 were used as positive and negative controls.

Tab. 1 List of peach cultivars collected, fruit symptoms observed, viroids detected, sequence analysis and accession numbers of the sequences. The underlined accession numbers refer to isolates showing the new mutations. DF= dapple fruit; CS= cracked suture; YM= yellow mosaic; Def= deformations.

				PLMVd sequence analysis		
Peach cultivar	**Symptoms**	**PLMVd**	**HSVd**	**Length**	**New mutations**	**Accession numbers**
Royal Glory	DF, CS	100%	90%	338-339	G280A	GQ872131- GQ872132- GQ872133
Crimson Lady	DF	70%	100%	339	-	GQ872128- GQ872129- GQ872130
Grenat	DF, YM, Def	90%	30%	337-339	-	GQ872125- GQ872126- GQ872127
Diamond Princess	DF	100%	0%	337-339	C165A	GQ872122-GQ872123- GQ872124
Laura	DF	100%	100%	350	12 nt insertion	GQ872134- GQ872135- GQ872136

RNA target preparation and viroids detection: For the detection of HSVd and PLMVd, total nucleic acids (TNA) were extracted from fruit skin according to the protocol established by Faggioli et al, (2001). TNA was finally eluted in 100 µL of DEPC water and analyzed following a two step/one tube RT-PCR protocol using specific primer pairs (Loreti et al., 1999; Astruc et al., 1996). All amplified products were analyzed using electrophoresis in a 1.5% agarose gel and stained with ethidium bromide.

Cloning and sequence analysis: At least three PLMVd positive samples per cultivar were selected for the sequence analysis. Amplified products were purified and cloned into pGEM®-T easy vector (Promega, Madison, WI, USA). Obtained sequences from the recombinant plasmids were multiple aligned using the Clustal W program and compared with the PLMVd isolates retrieved from the GeneBank database. Secondary structures were predicted using the mFold program (Zucker, 1989).

Results and discussion

The results showed a large diffusion of the two viroids in the assayed samples, mainly in mixed infections; more specifically PLMVd was found in 100% of 'Royal Glory', 'Diamond Princess' and 'Laura' samples; in 90% of 'Grenat' samples and in 70% of 'Crimson Lady' samples; HSVd affected 100% of 'Crimson Lady' and 'Laura' samples, 90% of 'Royal Glory' samples and 30% of 'Grenat' samples, whereas it was not found in any 'Diamond Princess' sample (Table 1). The evaluation of the role that the viroids could play in symptoms expression has been complicated by the high number of samples infected by both viroids (60%). Nevertheless, as already reported, PLMVd was confirmed to be strictly associated with yellow mosaic, cracked suture and fruit deformation symptoms. The aetiological origin of dapple fruit disease, however, seems to be less clear, since in 'Diamond Princess' only PLMVd has been found to be associated with the symptoms, whereas in all other cultivars the presence of HSVd could have influenced the symptom expression.

The molecular characterization of the PLMVd isolates does not show any correlation between nucleotide sequence and symptoms, although new variants were identified. In fact, the sequence alignment of our PLMVd isolates with the previously characterized ones revealed the presence of 15 new PLMVd variants (Figure 3): The molecular analysis of the PLMVd clones obtained from the cultivars 'Royal Glory', 'Diamond Princess', 'Grenat' and 'Crimson Lady' did not show any peculiarity with reference to specific symptoms (Figure 3), although variants isolated from the 'Royal Glory' and 'Diamond Princess' showed two mutations that had never been previously described (Table 1). More specifically, the clone RG2 showed a nucleotide change from G to A at position 280 and the clone DP3 showed a nucleotide change from C to A at position 165. However, all the novel variants were clustered in Group III (data not shown). More interesting was the sequencing of clones of the PLMVd isolate detected in the cultivar 'Laura', that showed an insertion of 12 nucleotides in the hammerhead region (Figure 3) Isolates with a similar insertion were previously reported by Hassen et al. (2007), but a comparison of the nucleotide sequences with the 'Laura' isolate highlight these differences. In order to confirm the results, amplification products, obtained with a proof reading *Taq* polymerase, were cloned and twenty clones sequenced. Eighteen out of twenty of the obtained sequences confirmed the presence of the insertion. Clustal W analysis showed some differences between the 18 analysed clones, that are summarized in Table 2. A Blast analysis was performed to understand the origin of this insertion and showed that the most common 12 nt sequence (UUUCGGAAGAAA) has a 100% homology with 12 nt of a previously published PLMVd sequence. The 12 nt are homologous from 113 to 124 nt of the stem 3 of some peach calico sequences published by Rodio et al., 2007. This evidence supports the hypothesis that the insertion could originate from a recombination event between two PLMVd isolates.

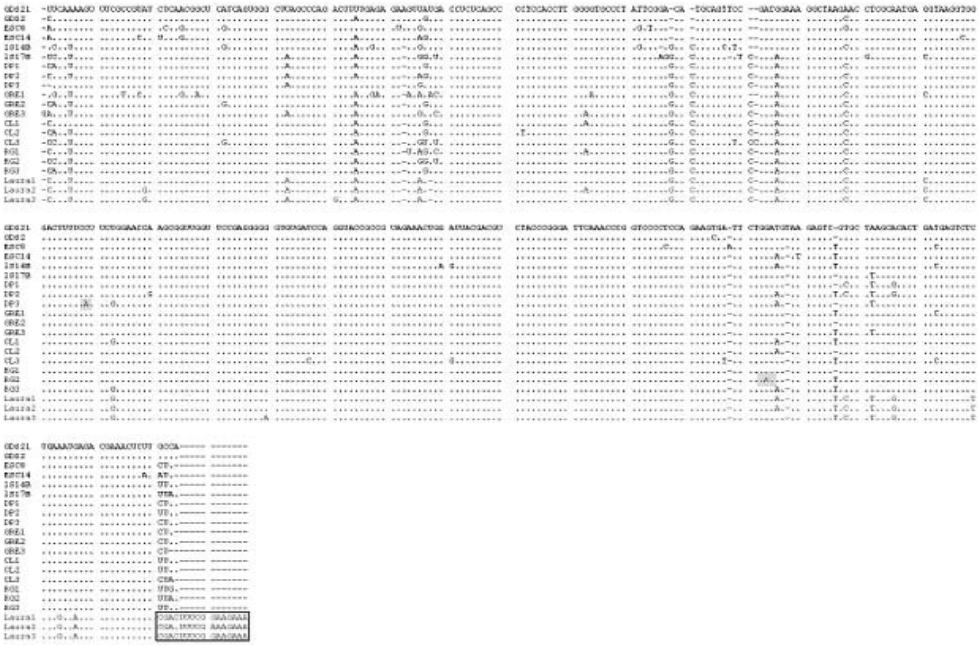

Fig. 3 Sequence alignment of PLMVd clones obtained from peach cultivars showing different symptoms. The reference sequences of group I, II and III are reported in black, the sequence of three clones of cvs Diamond Princess, Grenat, Crimson Lady, and Royal Glory are reported in blue and the PLMVd sequences from cv Laura with the 12 nt insertion are reported in red (highlighted by the red box). In the grey boxes show the two new mutations observed in clones DP3 and RG2

Tab. 2 Variants of the 12 nt insertion among the 18 clones of PLMVd 'Laura' isolate. The changes are underlined

Sequence	Number of clones with the same sequence insertion	Accession numbers
UUUCGGAAGAAA	9/18 (50%)	CQ872134- CQ872135- CQ872140- CQ872143- CQ872146- CQ872148- CQ872149- CQ872150- CQ872151
UUUCGAAAGAAA	5/18 (28%)	CQ872136- CQ872138- CQ872141- CQ872144- CQ872147
UUUCGUAAGAAA	1/18 (5.5%)	CQ872139
UUCCGGAAGAAA	1/18 (5.5%)	CQ872142
UUUCAAAAGAAA	1/18 (5.5%)	CQ872145
UUUCGUUUGGAA	1/18 (5.5%)	CQ872137

The other 'Laura' variants, reported in table 2, seem to be only polymorphisms belonging to the first insertion. In fact, according to the predicted secondary structure analysis, the mutations occurred only in the loop and not in the stem, as happens also for the Egyptian isolates (Figure 4), underlying the importance of the stem-loop secondary structure in the hammerhead region.

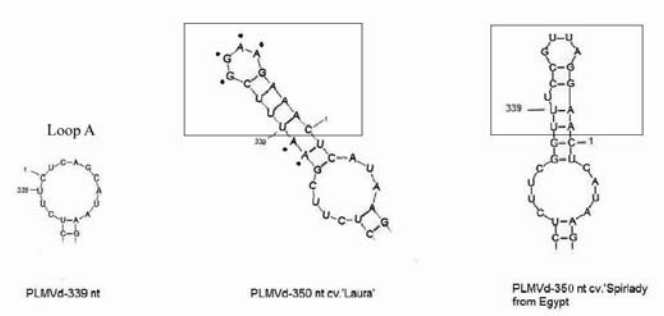

Fig. 4 Predicted secondary structures of the lowest free energy of the loop A of: a typical PLMVd isolate (339 nt), the 'Laura' isolate (350 nt) and the Egyptian isolate cv Spirlady (350 nt). The nucleotides of the characteristic insertion are highlighted in the boxes.

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