Arch Virol (2012) 157:343–347
DOI 10.1007/s00705-011-1156-z

BRIEF REPORT

New Iranian and Australian peach latent mosaic viroid variants and evidence for rapid sequence evolution

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Received: 8 August 2011 / Accepted: 19 October 2011 / Published online: 11 November 2011

Abstract Peach latent mosaic viroid isolates from peach and plum in Iran have been compared with an Australian isolate from nectarine. Thirteen sequence variants 336-338 nt in size were obtained. All variants clustered phylogenetically with variants reported from several hosts and countries. A total nucleic acid extract, a slightly longer than full-length RT-PCR amplicon, and a recombinant plasmid clone from the Australian isolate were all infectious to, and symptomatic in, mechanically inoculated peach seedlings. The infectious clone generated two progeny viroid molecules, which each showed 10 different mutations compared with the parent clone inoculated 30 days previously.

Viroids are infectious, small, naked, single-stranded, circular, non-coding RNAs that are dependent on host plant factors for their replication [13]. They replicate either in the nucleus (*Pospiviroidae*) or chloroplast (*Avsunviroidae*) [4]. The family *Avsunviroidae* includes the species *Avocado sunblotch viroid* (ASBVd), *Chrysanthemum chlorotic mottle viroid* (CChMVd), *Peach latent mosaic viroid* (PLMVd) and *Eggplant latent viroid* (ELVd), whose members contain a hammerhead structure in strands of both polarities and lack a central conserved region [4]. They replicate by a symmetric rolling-circle mechanism in which the hammerhead structure is responsible for processing by self-cleavage [13]. PLMVd is a circular RNA comprising 335-351 nt, which adopts a highly branched secondary structure containing one or more pseudoknots [13, 17].

PLMVd was first reported in France during graft indexing of peach germplasm imported from the US and Japan on the peach indicator "GF-305" [5], and its hosts include peach, sweet cherry, plum, apricot, and wild and cultivated pear [6, 7, 9, 10, 19, 23]. Symptoms observed in peach include mosaic, blotching, chlorosis, vein banding, or calico symptoms on leaves, bud necrosis, delayed shoot development, dieback of branches, small circular discoloured areas on fruit, open habit, and rapid aging of trees [12]. PLMVd is transmitted by grafting and budding but not through seed [6] and is transmissible mechanically and by aphids [11, 15].

PLMVd was detected in samples collected from economically important cultivars of peach and plum in 2009 in Khorasan Razavi, Iran, and compared with an Australian nectarine-derived isolate of the viroid [7]. We describe here the sequences of 13 new variants of PLMVd, demonstrate the infectivity and pathogenicity of a slightly longer than full-length amplicon generated from an Australian isolate, and also of a recombinant vector containing the clone, and provide further evidence that PLMVd rapidly generates sequence heterogeneity.

Leaves of peach and plum were collected from Iran and Australia (Table 1). Iranian samples were transported as freeze-dried leaves to Australia. The Australian PLMVd isolate was maintained in a greenhouse at the Waite Campus (University of Adelaide, Australia) on two peach

Electronic supplementary material The online version of this article (doi:10.1007/s00705-011-1156-z) contains supplementary material, which is available to authorized users.

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Table 1 Source of samples, locality, symptoms and size of PLMVd RT-PCR amplicons

Sample number	Host	Cultivar	Region	Symptoms[a, b]	Size of PLMVd amplicon (nt)
s1	Peach	Sorkhosepide	Chenaran, Iran	Calico	337*
s2	Peach	Sorkhosepide	Quchan, Iran	M, C	338
s3	Peach	Sorkhosepide	Chenaran, Iran	M, C	336
s4	Peach	Sorkhosepide	Mashhad, Iran	M, C	337
s5	Peach	Sorkhosepide	Chenaran, Iran	Calico	337*
s6	Peach	Sorkhosepide	Mashhad, Iran	M, C	338
s7	Peach	Sorkhosepide	Chenaran, Iran	Calico	337*
s8	Peach	Sorkhosepide	Neyshabur, Iran	M, C	337
s9	Plum	Shiro	Chenaran, Iran	Y	338
s10	Nectarine	Maygrand	Riverland, Australia	R	338
s11	Nectarine	Maygrand	Riverland, Australia	R	337
s12	Peach	Nemaguard	Adelaide, Australia	Mo, C	337
s13	Peach	Nemaguard	Adelaide, Australia	Mo, C	338

[a] M, mosaic; C, chlorosis; Mo, mottling; R, leaf rosetting and open habit; Y, yellowing

[b] Symptoms on Iranian isolates were seen in the orchard and therefore may be caused by factors other than viroid infection

* In the single clones obtained from s1, s5 and s7, with calico symptoms, we did not detect the nt 349-350 sequence described as calico-specific by Malfitano et al. [22]

seedlings patch-bark grafted with the nectarine, cv Maygrand, isolate in 1998 [7].

Nucleic acids were prepared either by a silica capture method [14] using freeze-dried leaves or by a modified TRIzol® method (Gibco-BRL) using fresh leaves [3]. The TRIzol® method was modified as follows: fresh leaves were ground in liquid nitrogen, and immediately, 10 vol (w/v) of a mixture of 38% TE-saturated phenol, 1.2 M guanidine thiocyanate, 5% glycerol, and 0.1 M sodium acetate was added, shaken for 20 s and incubated at room temperature for 10 min. One-fifth volume of chloroform-isoamyl alcohol (24:1 v/v) was added to the extract, vortexed vigorously for 20 s and incubated at room temperature for 5 min. Nucleic acids were precipitated by adding 0.5 volumes of isopropanol in the presence of 0.8 M sodium citrate and 1.2 M sodium chloride. We have found that these modifications of the RNA precipitation can help to remove proteoglycan- and polysaccharide-contaminating compounds from the isolated RNA. First-strand cDNA was synthesized using specific reverse primers, either RF-43 or C1 [1, 21; and Suppl. Table S1] with Transcriptor Reverse Transcriptase (Roche Diagnostics) according to the manufacturer's directions. When PCR reactions were carried out using primers RF-43 and RF-44, the PCR cycling profile had an initial heating step of 94° C for 2 min, followed by 32 cycles of 94° C for 30 s, 58° C for 30 s, 72°C for 2 min, with a final extension step at 72° C for 10 min. When using primers C1 and C2, the annealing temperature was reduced to 55° C and the final extension was reduced by 3 minutes. Primer sequences are shown in Suppl. Table S1.

Amplicons primed with RF-43 and RF-44 comprised a unit-length PLMVd sequence plus a 9-nucleotide overhang (Suppl. Table S1 and Figure S2). They were purified using a QIAquick gel extraction kit (QIAGEN, Australia) and cloned into pGEM-T Easy Vector according to the manufacturer's directions (Promega, Australia). Extraction of plasmid DNA was carried out using a HiYield mini kit (Real Biotech Corporation, Taiwan). Sequencing was performed on recombinant plasmids using universal M13 forward and reverse primers at the Australian Genome Research Facility (Adelaide, Australia). Cloned fragments were sequenced in both directions. Sequence data were compiled with BioEdit v7.0 [16]. Secondary RNA structure of the lowest free energy state was generated using Mfold software [24]. Multiple sequence alignments were done with the Clustal W algorithm [20]. Phylogenetic trees were generated using MEGA 4.0 [18] with bootstrapping from 1000 replicates.

The s10 Australian isolate (Table 1) was inoculated by high-pressure injection (two concurrent injections of 100 µl) combined with bark slashing of the stem (8-10 times with an inoculum-contaminated scalpel blade: see Suppl. Figure S1) to nine dormant peach seedlings cv. Nemaguard per inoculum. The peach seedlings were one year old with a height of ~20 cm. The three inocula prepared in nuclease-free water were recombinant plasmid with the longer than full-length 347-nt PLMVd insert (2 µg per plant), the 347-nt amplicon alone (0.2 µg per plant), and total nucleic acids extracted from peach infected with the viroid (6 µg per plant). Positive controls were

patch-bark graft inoculated (5 seedlings), and negative controls were untreated seedlings. Plants were maintained in a growth chamber at 30°C with a 14:10 h day:night cycle.

A number of peach and plum cultivars from four regions of north-eastern Iran tested positive for PLMVd (Table 1). One hundred Sorkhosepide peach and 10 Shiro plum trees were assayed for PLMVd by RT-PCR. For peach from the Chenaran region, 7 out of 10 samples were positive, and four amplicons (from s1, s3, s5, s7) were cloned. In the Quchan region, 10 out of 35 samples were positive, and one amplicon (from s2) was cloned. In the Mashhad region, 5 out of 20 were positive, and two amplicons (from s4, s6) were cloned. In the Neyshabour region, 10 out of 25 were positive, and one amplicon (from s8) was cloned. For plum from the Chenaran region, 5 out of 10 were positive and one amplicon (from s9) was cloned. Positives were also obtained from Elberta peach (2 out of 10) in Mashhad but were not cloned. The two Australian clones originated from each of the two peach seedlings patch-bark grafted from Maygrand nectarine (s10, 11). The samples s12 and s13 were two of the nine peach seedlings inoculated with recombinant plasmid from s10. One clone was obtained and sequenced from s12, and one from s13.

The viroid sequences varied between 336 and 338 nucleotides in size (Table 1). They had 94-99 % similarity to reported PLMVd sequences. Variable sites comprising substitutions, insertions, and deletions were unevenly distributed across different regions of the PLMVd molecule, and some areas showed very low or no variability at all. In agreement with other workers, we found no variation in regions previously defined as conserved [1, 8, 17], such as the hammerhead region, shown by solid and dotted boxes for plus and minus polarities, respectively, in Suppl. Figure S2. The kissing loop region of all our PLMVd molecules, that is, from nucleotide 160 to 200, showed low sequence variability (Fig. 2 and Suppl. Figure S2).

Phylogenetic analysis based on full-length PLMVd RNA showed that the Iranian isolates were more variable than the Australian isolates, which grouped together (Fig. 1). The sequences from the two Australian isolates (s10 and s11), the sequences of the two Australian progeny clones (from s12 and s13), one Iranian isolate (from s2) and one Spanish isolate (AJ005313) grouped together with 96-97 % similarity.

All three inocula were infectious to peach seedlings. When the recombinant plasmid was used as inoculum, the viroid was detected by RT-PCR three weeks after inoculation, and symptoms started to appear at four weeks. With total nucleic acid inoculum, initial symptoms appeared at 5 weeks, and at 6 weeks after the amplicon inoculations. The rates of successful transmission were 7, 8 and 5 for the recombinant plasmid, total nucleic acid and PCR amplicon

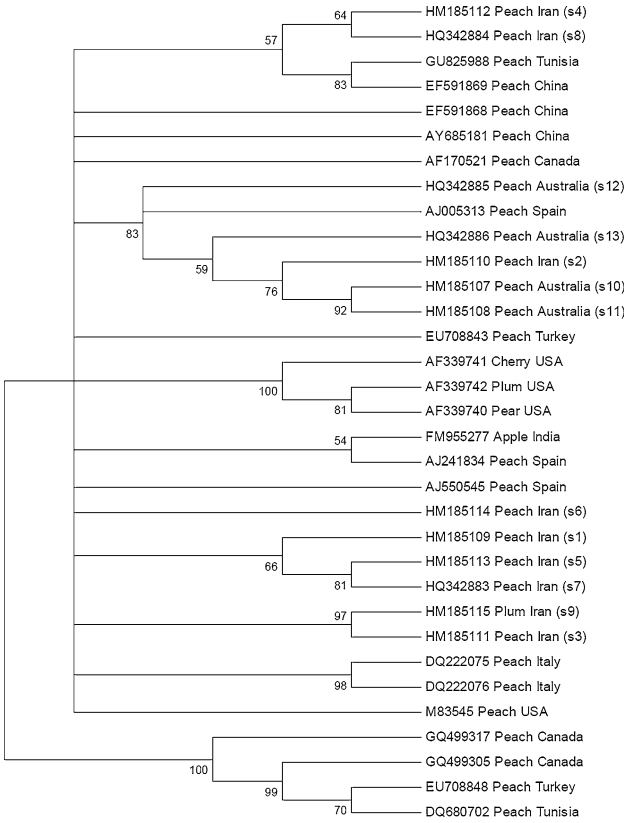

Fig. 1 Phylogenetic tree showing relationships between sequences of Iranian, Australian and other selected PLMVd isolates from different hosts and countries (with GenBank accession number, host, country and the sample number used for this study). The tree is a condensed version (<50%) based on a multiple alignment using Clustal W and the neighbour-joining method within the MEGA 4.0 program. Groupings are shown after bootstrapping with 1000 replicates. s1 – s13 are described in Table 1 and Suppl. Figure S2

inocula, respectively (n = 9). Following patch bark grafting, 4 out of 5 plants tested positive after 6 weeks. Mottling and chlorosis of leaves (especially around the mid vein) was prominent in infected plants. While the symptoms were clear under the conditions of 30°C with a 14-h day cycle, they disappeared after the seedlings were transferred to a glasshouse maintained at a temperature below 25°C. The symptoms in this study were expressed earlier than reported for previous work, and it is possible that the placing of the inoculated seedlings, which were at a late stage of dormancy, into a 30°C environment, which immediately broke dormancy, could have led to the appearance of symptoms on newly emergent leaves as early as 4 weeks post-inoculation. Earlier symptom emergence may also have been due to the use of Nemaguard instead of GF-305 peach seedlings.

The sequences of the progeny isolates (from s12 and s13; see above) from s10 showed that there had been a high rate of parent-progeny mutation. Figure 2 maps the sites of mutation on the secondary structural diagram of the parent

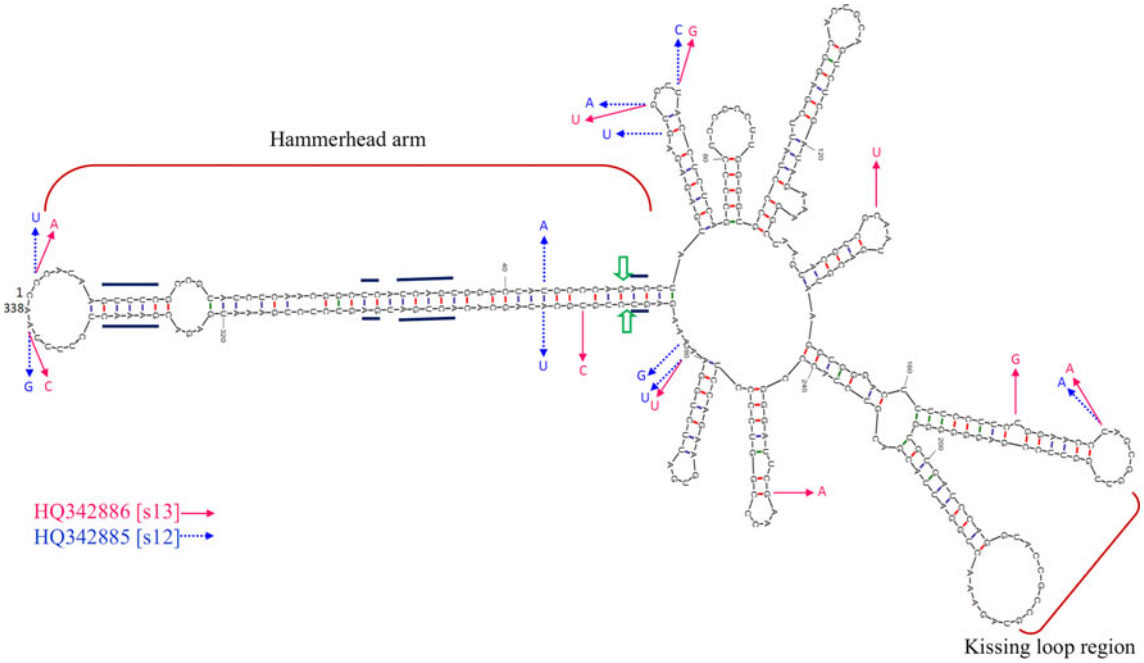

Fig. 2 Nucleotide sequence and secondary structure of the Australian PLMVd clone HM185107, generated by Mfold. The mutation sites of two progeny of this isolate, HQ342885 [s12] and HQ342886 [s13], are shown with dotted and solid arrows, respectively. The conserved nucleotides in the hammerhead region are indicated by dark bars. Open arrows show the self-cleavage sites

clone. In total, 20 different mutations were detected in this single passage. While this confirms previous evidence for genetic instability of PLMVd [2], our observation that the mutations occurred within 30 days of inoculation is an indication of a rapid rate of mutation. It also confirms that the processing sites are conserved, thus supporting the conclusion that they probably have an essential role in viroid replication [2].

Isolates of PLMVd from peach and plum in Iran have been studied here at the molecular level and compared with Australian isolates originating from a single inoculum obtained from nectarine. The sequence variation observed among these isolates (Suppl. Figure S2 and Fig. 1) agrees with the view that PLMVd can be considered a quasispecies with a closely related population of sequence variants, which may or may not be biologically/functionally different. A hallmark of PLMVd is that the nucleotide changes that define variants generally occur in certain highly variable regions, and these are not obviously associated with molecular functions that lead to phenotypic differences.

The high sequence variability among PLMVd isolates may reflect the high mutation rate of members of the family *Avsunviroidae* generally. The mutation rate in this family is ~10-fold higher than the mutation rate in the family *Pospiviroidae* [8]. According to Ambros et al. [2], sequence variability in different isolates of PLMVd is not the result of repeated inoculations. Rather, it is related to the intrinsic property of this viroid to evolve rapidly. Sequence analysis of the progeny isolates from the Australian isolate s10 confirms that there is a high rate of genomic variability generated from parent to progeny. The progeny differed from the single parent in genome size (Table 1) and nucleotide substitutions (Fig. 2). Our results, with 20 substitutions in only one passage, confirm those of others that the PLMVd RNA molecule is highly prone to mutation [2]. This paper shows that the generation of genomic variability is more rapid than previously described, as the period from inoculation to isolation of progeny was around 4 weeks.

Populations of PLMVd sequences in various hosts have a tendency for geographic clustering. The variation among isolates within Australia is not known, as the nectarine isolate used here is the only one to have been sequenced. However, there is marked variation among the Iranian isolates collected from the same peach cultivar in different regions of Iran. In regions such as Iran, there are endemic PLMVd host species, and plants have been traded with other countries for hundreds of years, providing significant scope for evolution. This is reflected in Fig. 1, which shows that the Iranian isolates are distributed among 3 of the 5 groups in the phylogenetic tree.

Acknowledgments This study was supported by The University of Adelaide, Australia, and Ferdowsi University of Mashhad, Iran. We thank Dr. Richard Glatz for his comments, and Dr. Kieren Arthur for her assistance.

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