Identification and Molecular Characterisation of *Peach Latent Mosaic Viroid* Isolate Infecting Peach in Egypt

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Abstract

In Egypt, a survey covering five commercial peach orchards of the new reclaimed lands, El Khatatba region in Al Minufiya governorate, was carried out.

During the last two years, 73 peach trees (cv. Florida grafted on Nemagard rootstock) were visually inspected and sampled. No symptom characteristic of PLMVd infection was observed. All samples were tested for the presence of PLMVd using molecular hybridization (Dot-blot) and reverse transcription-polymerase chain reaction (RT-PCR).

Only one of the 73 peach trees resulted positive for PLMVd as evidenced by Dot-blot and RT-PCR. Following RT-PCR amplification cloning and nucleotide sequence, the Egyptian PLMVd isolate showed an identity of 86 to 95% when compared with other PLMVd sequences in Genbank.

INTRODUCTION

Viroids are the smallest know agents of infectious disease; they are circular molecules, a few hundred nucleotides long, with a high degree of secondary structure, they do not code for any polypeptides and replicate independently of any associated plant virus, therefore, viroids must rely on pre-existing host enzymes for their replication and elicit their pathogenic effects (Owens, 2000).

Peach latent mosaic viroid (PLMVd), belongs to genus *Pelamoviroid* within the family *Avsunviroidae* and consists of a 337–339 nucleotides circular RNA folds into a complex branched secondary structure of minimum free energy content (Hernandez and Flores, 1992; Bussiere et al., 2000).

Presence of PLMVd has been proved in the North and South America, China, Japan, Mediterranean basin, Europe and Australia (Flores et al., 2003). The interchange of propagative material of infected peach and nectarine cultivars has most probably been the major factor of PLMVd spread, particularly considering that this viroid does not incite conspicuous leaf symptoms (Hadidi et al., 1997). The viroid is graft and possibly aphid transmitted (Desvignes, 1999).

PLMVd is considered a dangerous pathogen of peach and a quarantine pathogen by European Plant Protection Organization (EPPO/CABI, 1992).

Previous studies, carried out by Torres et al. (2004) in commercial orchards of Egypt, did not evidence the presence of PLMVd in peach trees; only HSVd was detected.

In this work, we report, for the first time, the occurrence of PLMVd in commercial peach orchards in Egypt; in addition, we determined PLMVd sequence of the unique PLMVd isolate found during our survey.

MATERIALS AND METHODS

Plant Materials

During the last two years, 73 peach trees (cv. Florida grafted on Nemagard rootstock) were visually inspected and sampled. Samples were collected from five commercial peach orchards of the new reclaimed lands, El Khatatba region in Al Minufiya governorate (North West Egypt).

Nucleic Acid Extraction and Dot Blot Hybridization

Leaf tissue (0.2 g) was powdered with liquid nitrogen and homogenized with 900 µl of 0.2M Tris–HCl pH 8.2, 17.5 µl of 5M NaCl, 8 µl of 10% Triton X-100 and 2 µl of 2-mercaptoethanol. After centrifugation at 9,000 g for 20 min, the pellet was discarded and the supernatant was mixed with 500 µl of water-saturated phenol pH 7.0, 100 µl of 5% sodium dodecyl sulfate (SDS) and 100 µl of 0.1mM (EDTA) pH 7.0. The nucleic acids present in the aqueous phase, were recovered by ethanol precipitation and then re-suspended in 500 µl of distilled sterile water (Faggioli et al., 2001). Aliquots (5 µl) were spotted on nylon membrane; hybridized over night at 70ºC with full length digoxigenin labeled PLMVd riboprobe and detected as recommended by supplier (Roche, DIG Luminescent Detection Kit for Nucleic Acid).

RT-PCR Amplification and cDNA Cloning

The reverse primer (5'-AACTGCAGTGCTCCGAATAGGGCAC-3'), complementary to bases 131 to 166 and the forward primers (5'-CCCGATAGAAAGGCTAAGCACCTCG-3') homologous to bases 167 to 202 of the PLMVd reference sequence (Hernandez and Flores, 1992), previously reported for amplification of the PLMVd (Loreti et al., 1999), were used.

Amplification was carried out using the Qiagen® One step RT-PCR kit (Qiagen). The PCR cycling profiles were as reported previously (Loreti et al., 1999). The amplified product was gel purified using QIAquick PCR purification Kit (Qiagen), cloned into pGEM-T Easy Vector and transformed into competent *E. coli* (Promega pGEM-T Easy Kit). Transformants were screened and selected using standard protocol (Sambrook et al., 1989). Cloned viroid cDNAs were sequenced on a fluorescent DNA sequencer CEQ2000XL, employing the dye terminator sequencing kit according to the manufacturer's instructions (Beckman Coulter). Multiple alignments of nucleotide sequences of PLMVd were obtained using the default options of Clustal X 1.8, a Windows interface for the Clustal W multiple sequence alignment program (Thomson et al., 1994). The most stable secondary structures were obtained with the circular version of the MFOLD program (Zuker, 2003).

RESULTS AND DISCUSSIONS

Dot-blot hybridization with a PLMVd-specific riboprobe, revealed the presence of PLMVd in only one of 73 peach trees (Fig. 1). The infected tree showed no symptom characteristic of PLMVd infection.

Analysis by RT-PCR with a pair of PLMVd-specific and adjacent primers led to amplify a DNA fragment of expected size from the leaf tissue of the infected peach tree. The fragment was of the same size of the amplified PLMVd positive control. No amplification was obtained with extracts from uninfected peach leaf tissues (data not shown).

Nucleotide sequence analysis of cloned PCR products showed that the two full length cDNA clones derived from the PLMVd-Egyptian isolate denoted as Eg18 and Eg19, (GenBank Accession Nos. DQ839564 and DQ839565), had a sequence of 339 and 338 nucleotides length, respectively.

The deletion of variant Eg19 located at 3' of the self-cleavage site of the plus polarity hammerhead structure (Fig. 2, position 292) has been reported previously for other PLMVd variants and interpreted as an artifact of reverse transcription (Ambros et

al., 1998, 1999; Malfitano et al., 2003; Rodio et al., 2006). Comparisons between the two sequences variants obtained from PLMVd-Egyptian isolate and the reference PLMVd variant (PL-ref) (Hernandez and Flores, 1992) showed that 20 out of total 339 nucleotide positions (5.89%) were polymorphic (Table 1). These changes were mainly located in the upper and lower strands of the hammerhead arm (P11 stem) (Fig. 2). The observed mutations did not affect significantly either the branched secondary structure of minimal free energy (Fig. 2) or the hammerhead structures in both variants (E18 and E19), confirming a selective pressure in favor of self-cleavage activity (Bussière et al., 2000; Pelchat et al., 2000).

To our knowledge, this is the first report of the presence of PLMVd in Egypt.

Considering the economic impact of PLMVd, it is advisable to develop certification program for peach in Egypt to prevent further spread of the pathogen.

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Tables

Table 1. Polymorphic positions observed in two sequence variants derived from PLMVd-Egyptian isolate with respect to the reference sequence (PL-ref) (Hernandez and Flores, 1992). Nucleotide insertion and deletion are indicated by (+) and (-), respectively.

Residue position	Helix number	Changes in Egyptian sequence variants	
		Eg18	Eg19
1	P11	G → A	
5	P11	U → A	
14	P11	U → C	
17	P11	C → U	
20	P11	U → C	
24	P11	G → A	
27	P11	A → G	
54	P11	A → U	
57	P11	A → G	
62	P1	G → A	
67	P1	G → C	G → U
162	P6a	U → G	
174	P6b	A → G	
187	P6b	U → C	
258	P9	G (+)	
283	P10	U (+)	C (+)
290	P11		U → (-)
307	P11	C → U	
336	P11		U → C
337	P11	U → C	

Figures

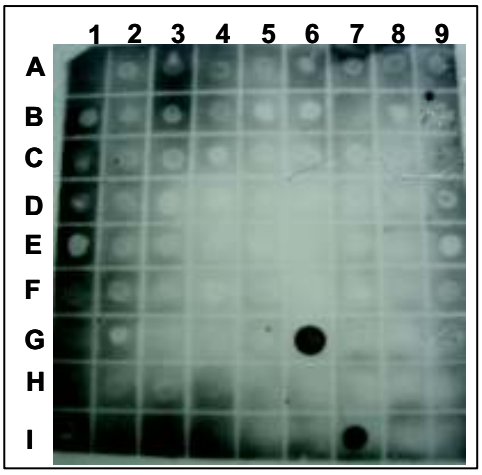

Fig. 1. Dot blot analysis with a PLMVd-specific digoxigenin-labeled riboprobe of TNA extracted from 73 peach samples. 6I and 7I, healthy and PLMVd infected controls, respectively.

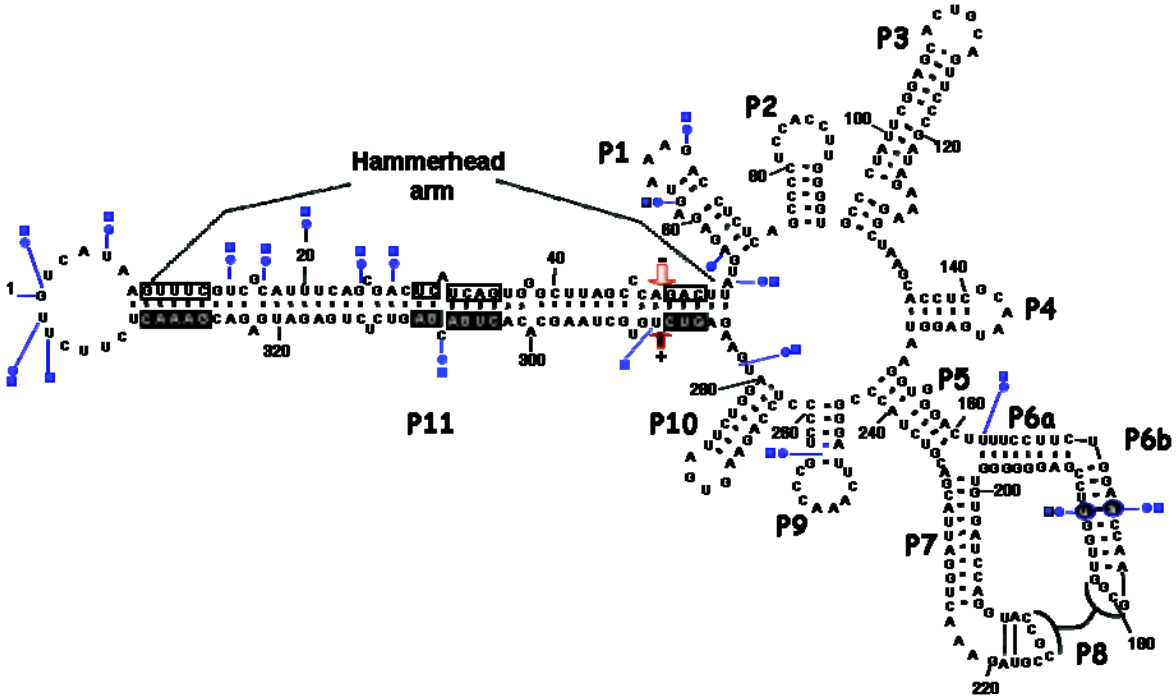

Fig. 2. Secondary structure proposed for PLMVd (Ambros et al., 1998) showing the distribution of polymorphic positions for each sequence variant along the reference sequence (Hernandez and Flores, 1992). Nucleotide changes, either substitutions or indels of the Egyptian PLMVd sequence variants (Eg18 and Eg19) are denoted by black circles and squares, respectively. Conserved residues present in hammerhead structures are boxed and self-cleavage sites are marked by black and white arrows. Base pairs showing compensatory mutations are denoted with lowercase letters and circled. The helices are numbered as described by Bussiere et al. (2000).