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Improvement of the reverse transcription loop mediated isothermal amplification (RT-LAMP) method for the detection of *Peach latent mosaic viroid* (PLMVd)

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Abstract

Peach latent mosaic viroid (PLMVd) is the most known peach viroid. Among the diagnostic techniques used for its detection, the most recent described being the reverse transcription loop-mediated isothermal amplification method (RT-LAMP). Several modifications were done on the basic protocol proposed by Boubourakas et al. (2009), additional experiments were preformed in order to further evaluate the method. Namely, the reaction time was further reduced and traces of leaf tissue, taken by a sterile toothpick, instead of tRNA were used as the intial material. Moreover, the AMV reverse transcriptase proved to be more effective than Thermoscript, while restriction enzyme analysis was performed on the RT-LAMP products in order to confirm that products had the respective sequences of the selected target. Finally, the extremely high efficiency and sensitivity of RT-LAMP proved to be sufficient for the detection of PLMVd in hosts other than peach.

Keywords: PLMVd, RT-LAMP, peach, reverse transcriptases

Introduction

Peach latent mosaic viroid (PLMVd), a member of the family *Avsunviroidae* of the genus *Pelamoviroid* (Navarro and Flores, 1997), is the causal agent of an economically important disease of peach, responsible for reduction of fruit quality, tree vigor, and increased susceptibility to biotic and abiotic stresses. The term latent in the name of PLMVd refers to the observation that the vast majority of natural infections of peach occur without leaf symptoms and the prolonged time required for the onset of symptoms. Therefore, a sensitive, accessible, reliable, cost effective and fast diagnostic method that could contribute to the restriction of viroid spread and the production of healthy and of high quality propagation material is needed. Reverse Transcription-Loop Mediated Isothermal Amplification (RT-LAMP) seems to be a good candidate method for this purpose, since it combines the following characteristics, such as: 1) amplification of nucleic acids under isothermal conditions in the range of 65 ºC, 2) high specificity, 3) high amplification efficiency, 4) easy detection of amplified target DNA. LAMP is a novel nucleic acid amplification method, relative simple, characterized by the use of a DNA polymerase with strand displacement activity and a set of four different primers designed specifically to recognize 6 distinct regions on the target sequence (Nagamine et al., 2002; Mori et al., 2001; Notomi et al., 2000). Boubourakas et al. (2009) developed, for the first time, an RT-LAMP protocol for the detecion of PLMVd. According to the findings of this study, the combination of the OLD1 primer set with the degenerate loop primer, under 62.5 ºC and 0.8 M betaine concentration, led to the detection of PLMVd within almost 30 min, with a detection limit of 10^{-5}.

In the present study we introduced some modifications in RT-LAMP in order to make it simpler and the improved method was used for the detection of PLMVd in several host species in addition to peach.

Materials and methods

<u>Plant material</u>: In the present study, the Greek PLMVd isolate 52, coming from naturally infected peach trees cv. SpringCrest exhibiting the characteristic symptom of fruit cracked sutures, and the Italian isolates P51 and P39 (calico isolate) were used as source of PLMVd material. Leaf and fruit samples of apricot, peach, plum, cultivated and wild pear and quince were also collected from North-Eastern Peloponnesus, Greece. Leaves of healthy peach GF305 were used as negative controls. Also, Italian isolates of *Apple scar skin viroid* (ASSVd), *Pear blister cancer viroid* (PBCVd), *Hop stunt viroid* (HSVd) and *Potato spindle tuber viroid* (PSTVd) were used for specificity evaluation of the method. All the Italian isolates and the healthy seedling material came from the collection of the CRA-Centro di Ricerca per la Pathologia Vegetale in Rome.

RT-LAMP method: The amplification reaction was preformed at 62.5 ºC for 1 h followed by 2 min at 80 ºC using the OLD1 primer set in combination with the degenerate loop set, as described by Boubourakas et al. (2009). The parameters examined during this study were: 1) the type of the template used: total RNA using the protocol described by Rott and Jelkmann (2001) or traces of leaf tissue taken by a sterilized toothpick, and 2) the types of reverse transcriptase: AMV (Promega, Madison, USA) or Thermoscript (Invitrogen, Ltd, Paisley, England, UK). All components were assembled and reactions were preformed either in a Real Time Turbimeter LA200 (Teramecs Co. Ltd, Kyoto, Japan) which measures the turbidity of each reaction mixture in real time or in a PTC-200 DNA Engine Cycler (MJ Research, Waltham, Massachusetts, USA). The amplified products were also visualized with agarose gel electrophoresis (2 %). In addition, the amplified products were digested with *RsaI* (Promega, Madison, USA) that cleaves at the site GT⇓AC within the primer B2c and the digestion products were analysed by electrophoresis. The Loopamp Fluorescent Detection Reagent (Eiken Chemical Co. Ltd., Tochigi, Japan) was added in the reaction mixture to obtain a direct visual observation of the reaction tube under UV light.

Specificity of RT-LAMP: In order to determine the specificity of the method, other viroids such as ASSVd, PBCVd, HSVd and PSTVd were subjected to RT-LAMP using the PLMVd OLD1 and degenerate loop primer sets; and all reactions were analyzed in parallel.

Results and discussion

This RT-LAMP protocol was able to specifically detect various PLMVd peach isolates from Greece and Italy, including an Italian calico isolate. No signal was generated when template from other viroids, ASSVd, HSVd, PBCVd and PSTVd or when extracts from healthy peach plants were subjected to the RT-LAMP assay using the PLMVd specific primer sets (Figure 1). The above results indicate that the amplified products were PLMVd-specific and not the result of cross-contaminations.

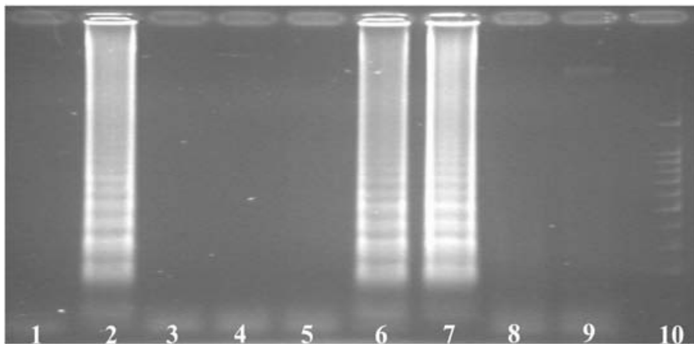

Fig. 1 Gel electrophoresis of RT-LAMP products from various PLMVd isolates and other viroids. Lane 1: water, Lane 2: PLMVd 52 (Greece), lane 3: PBCVd, lane 4: ASSVd, lane 5: HSVd, lane 6: PLMVd P51 (Italy), lane 7: PLMVd P39 calico (Italy), lane 8: PSTVd, lane 9: healthy peach control, lane 10: molecular weight marker (100 bp, New England Biolabs, Hertfordshire, England, UK).

It was shown previously that total RNA extracted using the Rott and Jelkmann (2001) protocol provides a reliable template for RT-LAMP on PLMVd (Boubourakas et al., 2009). In the present study it was proven that traces of leaf tissue, taken by a sterilized toothpick, can be used as template (Figure 2). However, the intensity of the signal obtained was lower than that of using tRNA as template. The fact that the method could be employed using traces of plant tissue results in: a) further reduction of the reaction time, and b) allowing the use of RT-LAMP in the field when the internal fluorescent dye is used (Figure 3).

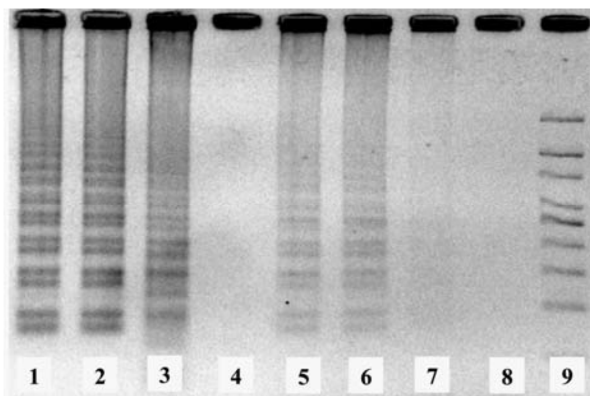

Fig. 2 Gel electrophoresis of RT-LAMP products using AMV (lanes 1-4) or Thermoscript (lanes 5-8); using as template tRNA (lanes 1-2 and 5-6) or traces of leaf tissue taken by a sterilized toothpick (lanes 3 and 7). Lanes 4 and 8: healthy peach control, lane 9: molecular weight marker (100 bp, New England Biolabs, Hertfordshire, England, UK).

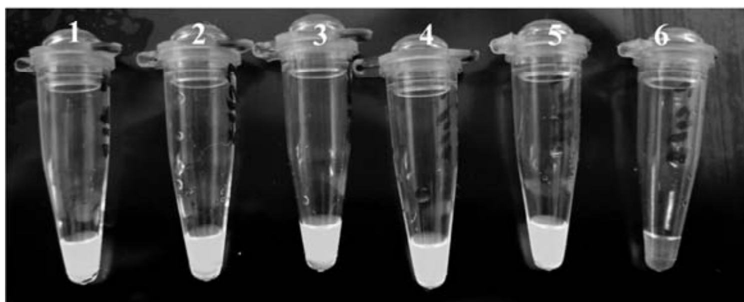

Fig. 3 Detection of PLMVd by RT-LAMP using fluorescent internal dye, on peach (tube 1, 2), on pear (tube 3), on wild pear (tube 4) and on quince (tube 5). Healthy control (tube 6).

When the reverse transcriptases AMV and Thermoscript were compared in RT-LAMP, a less intense, however positive, signal was observed when Thermoscript was used, when either tRNA or traces of plant tissue were used as templates (Figure 2); thus Thermoscript is considered to be less efficient in RT-LAMP.

In order to confirm that the RT-LAMP products have the corresponding sequences of the selected target, a portion of the amplified products is subjected to restriction enzyme analysis (Kubota et al., 2008). Based on the sequence of the expected amplified products and using the NEBcutter V2.0 software (New England BioLabs, Hertfordshire, England, UK, http://tools.neb.com/NEBcutter2/), the *RsaI* restriction enzyme was found to cut the RT-LAMP products in four fragments, namely 131 bp, 171 bp, 198 bp και 225 bp (Figure 4).

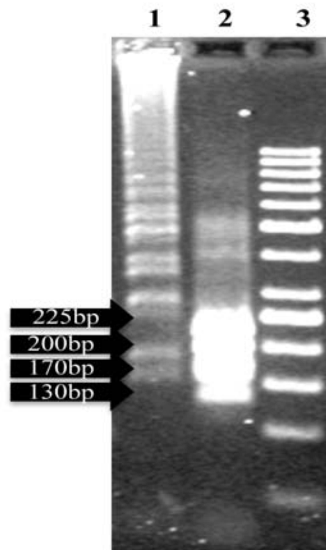

Fig. 4 Restriction enzyme digestion of RT-LAMP products. RT-LAMP products (lane 1) were digested by *RsaI* (lane 2). Lane 3: molecular weight marker (50 bp, New England Biolabs, Hertfordshire, England, UK).

Detection of PLMVd in stone fruit hosts, other than peach, such as plum, or pome fruits is difficult, presumably due to viroid concentration in infected tissue at relative low titers (Flores et al., 1992). Since RT-LAMP has an extremely high efficiency and sensitivity, it proved to be sufficient for the detection of PLMVd in hosts such as plum (2/2), apricot (1/2), pear(1/2), wild pear (2/2) and quince (1/1) (Figure 5).

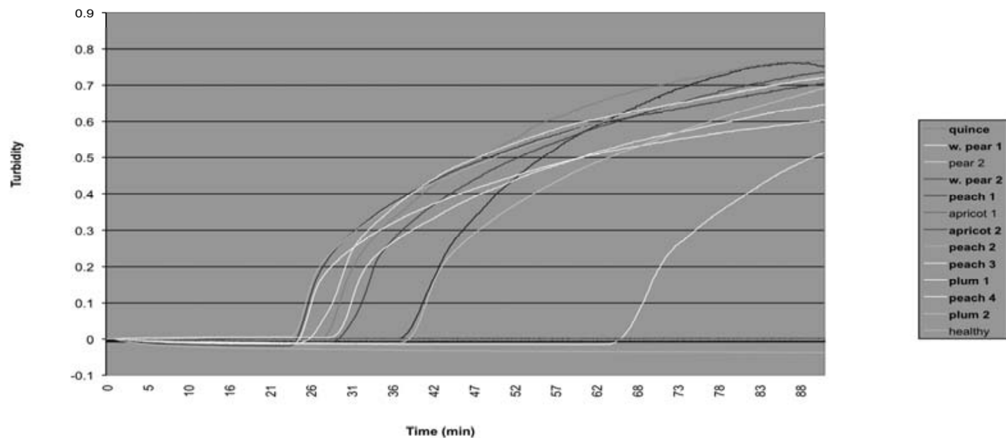

Fig. 5 Real time detection of PLMVd by RT-LAMP assay in several hosts such as apricot, peach, pear, plum, quince and wild pear.

With the results of the present work, we think that the study for the use of RT-LAMP in PLMVd detection has got closed to its end. RT-LAMP method is easy, relatively cheap, fast, extremely sensitive, highly specific, reliable, with its improved version to have the possiblility to be performed in the field.

Acknowledgments

We would like to thank Dr Andreas Voloudakis for creetically reviewing the text.

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