Development of one-tube real-time RT-qPCR for the universal detection and quantification of *Plum pox virus* (PPV)

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ABSTRACT

In this study a one-tube real-time RT-qPCR assay was developed using the TaqMan chemistry for the universal detection and quantification of PPV, one of the most important pathogens affecting stone fruit trees. In order to design appropriate primers and probe, nucleotide sequences from different PPV isolates originating from all known strains were recovered from the databases. Various genomic regions were screened and finally primers were selected from a conserved region of the 3′- terminal part of the CP gene amplifying a 146 bp DNA fragment while the probe was designed to bind within the amplicon. Ten-fold serial dilutions of *in vitro* synthesized RNA transcripts were applied for the construction of standard curve. The amplification efficiency of the assay was 93.8% and the linear range of quantification was from 40 up to 4×10^8 RNA copies. The real time RT-PCR was successfully tested with a collection of genetically diverse isolates with different geographical origin belonging to seven PPV strains. The present method is proposed as a useful tool for various basic or applied research studies of PPV as well as for routine testing of plant material during phytosanitary control or in certification schemes of *Prunus* species.

Plum pox virus (PPV) is the causal agent of sharka, which is characterized as the most important viral disease of Prunus species, due to its significant financial and agronomic impact in afflicted areas (Cambra et al., 2006a). The virus displays remarkable genetic variability, having so far nine characterized strains, namely M, D, EA, C, Rec, T, W, CR and An (James et al., 2013; Palmisano et al., 2012). The strains are mainly differentiated based on their molecular traits and only in a few cases (*e.g.* PPV-C, -CR) these differences are clearly depicted on the biological properties of the virus. PPV-D, –M and -Rec are the major strains of the virus while the rest exhibit a geographically limited distribution (Garcia et al., 2014). PPV is easily transmitted non-persistently in short distances by various aphid-vectors (Cambra et al., 2006b). Long distance trade of infected propagative material enables disease spread in previously unaffected regions (Barba et al., 2015) and even the introduction of new viral isolates into pre-existing PPV populations (James et al., 2003). Consequently, PPV has been designated as a quarantine pest by a number of phytosanitary agencies (CABI, 2016; EPPO, 2016), while regulations governing *Prunus* plant material trade were implemented on both national and international levels

(Cambra et al., 2006a; Mavrodieva et al., 2013).

Reliable detection methods are a key component of successful certification schemes of PPV-free stone fruit tree material, aiding in both halting pathogen spread and minimizing any potential economic and agricultural disease impact (Barba et al., 2015; Rimbaud et al., 2015). Owing to its scientific and regulatory significance, several variations of PCR have been adapted for PPV detection (Olmos et al., 2006; Kim et al., 2008; Sochor et al., 2012). Additionally, two official international protocols were established for the detection and characterization of PPV strains (EPPO, 2004; IPPC-FAO, 2012). The latter protocol includes three real time PCR methods for universal PPV detection: two TaqMan methods, which were evaluated using isolates from D, M (Olmos et al., 2005) and D, M, C, EA strains (Schneider et al., 2004), respectively and a SYBR green I method, evaluated using D, M, C, EA strains (Varga and James, 2006). The purpose of our study was to develop a highly-sensitive, rapid and specific single-tube real-time RT-PCR assay for the universal detection and quantification of all known PPV strains.

PPV isolates from various geographic regions belonging to M, D, Rec, T, C, CR, EA strains and uncharacterized ones were kindly

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Table 1

PPV isolates used in this study for the development and validation of the real time RT-PCR assay.

Isolate code	PPV strain	Origin	Provided by
Rc 1	Unknown	Greece	Dr C. Varveri (BPI, Greece)
Rc 2	Unknown		
Rc 7	Unknown		
Rc8	PPV-T or M	Slovakia	Dr M. Glasa (SAS, Slovakia)
Rc9	PPV-Rec		
Rc10	PPV-Rec		
Rc11	Unknown		
Rc12	Unknown		
Rc13	PPV-M		
Rc14	PPV-D		
Iso4	PPV-C		
Iso5	PPV-CR		
Iso6	PPV-CR		
Rc24	Unknown	Spain	Dr A. Olmos (IVIA, Valencia)
Rc26	PPV-D		
Rc27	PPV-D		
Rc28	PPV-M		
Rc29	PPV-EA		
Iso1	PPV-Rec		
Iso2	PPV-Rec		
Iso3	PPV-T		
Rc19	Unknown	Cyprus	Dr L. Papayiannis (ARI, Cyprus)
Rc20	PPV-M		
Rc21	Unknown		
Sam 12	Unknown	Greece	Laboratory of Plant Pathology, School of
Sam 13	Unknown		Agriculture, Aristotle University of
Sam 14	Unknown		Thessaloniki, Greece
Sam 15	Unknown		
Sam 16	Unknown		
Sam 17	Unknown		
Sam 18	Unknown		
–	PPV-T	Turkey	Dr Kadriye Caglayan (MKU, Turkey)

provided by several researchers or originated from the collection of the Laboratory of Plant Pathology (School of Agriculture, Aristotle University of Thessaloniki, Greece) (Table 1) and were used for the validation of the developed assay.

Total ribonucleic acids of plant material were extracted from 200 mg of leaf tissue, using a modification of the RNA extraction method A described by Pappi et al. (2015). Briefly, plant tissue was homogenized with 2000 µl of Lysis buffer 1. The lysate was incubated at 70 °C for 10 min and 500 µl were transferred to a new microcentrifuge tube. The rest of the procedure for extracting the ribonucleic acids was essentially as described by Pappi et al. (2015). The total concentration and purity of the extracted RNA was estimated after determining the absorbance at 260 and 280 nm spectrophotometrically, using Biophotometer (Eppedorf, AG, Hamburg, Germany).

Primers and TaqMan probe design was based on highly conserved regions of the PPV genome, after aligning homologous nucleotide sequences from 64 different PPV isolates deposited in the EMBL-EBI and NCBI databases. More specifically, conserved regions of the capsid protein (CP) gene that were also previously used by Schneider et al., 2004 were selected. The designed primers PPV-F (5′-CACAAGTGGAR-TATCCAATAAAGCCATTG-3′) and PPV-R (5′-CTGAATTCCATACCTTG GCATGTATGC-3′) amplify a 146 bp fragment. The Integrated DNA Technologies (IDT) OligoAnalyzer software Version 3.1, (https://eu. idtdna.com/analyzer/Applications/OligoAnalyzer) was used for *Tm* analysis, as well as for verification of the absence of possible hairpins and secondary structures. PPV TaqMan probe (5′-CACATTTCAGTAAC-GTBGCTGAAGCG- ZNA4-3′) was labeled with hexachloro-6-carboxy-fluo- rescine (HEX) at the 5′-end and with ZNA-4-Black Hole Dark Quencher 2 (BHQ-2) at the 3′-end (Metabion International AG, Martinsread, Germany).

For the determination of the absolute number of gene copies detected, PPV RNA transcripts were synthesized *in vitro* and serial dilutions were used to generate standard curves in the RT-qPCR assays. In particular, for the development of the PPV detection system, a 852 bp CP fragment of an isolate kindly donated by Dr. C. Varveri (BPI, Athens, Greece) (Isolate Rc 2, Table 1) was PCR amplified using degenerate primers (PPV Ex F: 5′-TTYACKCCAGCAACAAC-3′, PPV Ex R: 5′−CCA CTA CAC TCC CCT CAC-3′) (suppl. Table 1) and was further cloned into the pCR®II-TOPO® vector (Invitrogen-Life Technologies, Groningen, The Netherlands). NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany) was used for the purification of the recombinant plasmid, which then was linearized with the restriction enzyme XhoI (New England Biolabs, Ipswich, USA) and gel purified with the NucleoTrap® extraction II kit (Macherey-Nagel, Düren, Germany). Positive single strand RNA was transcribed using SP6 polymerase (New England Biolabs, Ipswich, USA), after incubation with 1 µg of linearized plasmid DNA, at 40 °C for 2 h. The RNA transcripts were subsequently treated with the enzyme DNase I (RNase-free) (New England Biolabs, Ipswich, USA) and the residual RNA was finally purified according to the RNA extraction method C as described by Pappi et al. (2015). PPV RNA transcripts were quantified spectrophotometrically (NanoPhotometer® P-Class, P 330 (Implen, München, Germany) and ten-fold serial dilutions (10^8 down to 10 transcripts/µl) were prepared in siliconized tubes, with DEPC-treated water containing 50 ng/µl of RNA carrier (Qiagen, Hilden, Germany) and stored at −80 °C. The dilutions of these RNA standards were used to determine the amplification efficiency, the dynamic range of quantification and the detection limit of the RT-qPCR assay.

The one-tube real-time RT-qPCR was performed in a final volume of 25 µl and reaction mixture consisted of buffer F-517 (Optimized Detergent-free DyNAzyme™ EXT Buffer; 50 mM Tris−HCl, 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$)(Thermo Scientific, Vantaa, Finland), 0.2 mM of each dNTP, 4 mM DTT, 1 U of Superscript™ III RNaseH⁻ Reverse Transcriptase, 1.5 mM additional $MgSO_4$ (Invitrogen-Life Technologies, Groningen, The Netherlands) and 3 U of HotStartTaq DNA polymerase (Qiagen, Hilden, Germany).

The concentrations of primers and TaqMan probe (0.2 up to 1 µM) were tested using 40 up to 4×10^8 synthetic PPV-RNA transcripts as templates and the combination of 0.8 µM of the PPV-F primer, 0.4 µM of the PPV-R primer and 0.3 µM of the TaqMan probe were the optimal. Thermal cycling conditions were 50 °C for 30 min, followed by 95 °C for 15 min and 50 cycles in 2 steps: a) 30 s at 95 °C and b) 60 s at 60 °C. Fluorescence levels were measured at the end of each cycle and the analysis of fluorescence data was conducted using the MxPro-Mx3005 P software (Version 4.00; Agilent Technologies, California, USA).

The developed RT-qPCR was applied in samples collected during 2012–2014 from apricot trees exhibiting typical PPV symptoms originating from the Prefecture of Kavala (Eastern Macedonia) as well as on randomly collected field samples from peach, cherry, and plum from the Prefecture of Imathia (Table 2). RNA extraction was performed as described above.

The RT-qPCR amplification efficiency of the optimized assay was 93.8%, revealing optimal amplification conditions for the tested pathogen. The linear range of quantification was from 40 to 4×10^8 PPV RNA transcripts. The equation of the standard curve obtained was y = -3.481·log(nr of copies) + 45.59, and the correlation coefficient 0.999 (Fig. 1). Different thermal conditions of the RT-qPCR along with various primers and TaqMan probes concentrations were tested in order to achieve the optimum detection system for the pathogen.

Table 2

Detection of PPV in different plant samples collected from the Prefectures of Imathia and Kavala.

Plant Species	Number of samples tested	Origin	PPV positive
Apricot	23	Kavala	23
Peach	90	Imathia	38
Cherry	36		0
Plum	2		0
Total number of samples	151		61

Number of PPV copies	Mean Ct±SD
4×10^8	15,85±0,13
4×10^7	19,07±0,32
4×10^6	22,43±0,17
4×10^5	26,22±0,01
4×10^4	29,44±0,06
4×10^3	32,90±0,35
4×10^2	36,45±0,32
4×10^1	40,27±0,59

E=93.8% RSq=0.999
y= -3.481 · log (nr of copies) +45.59
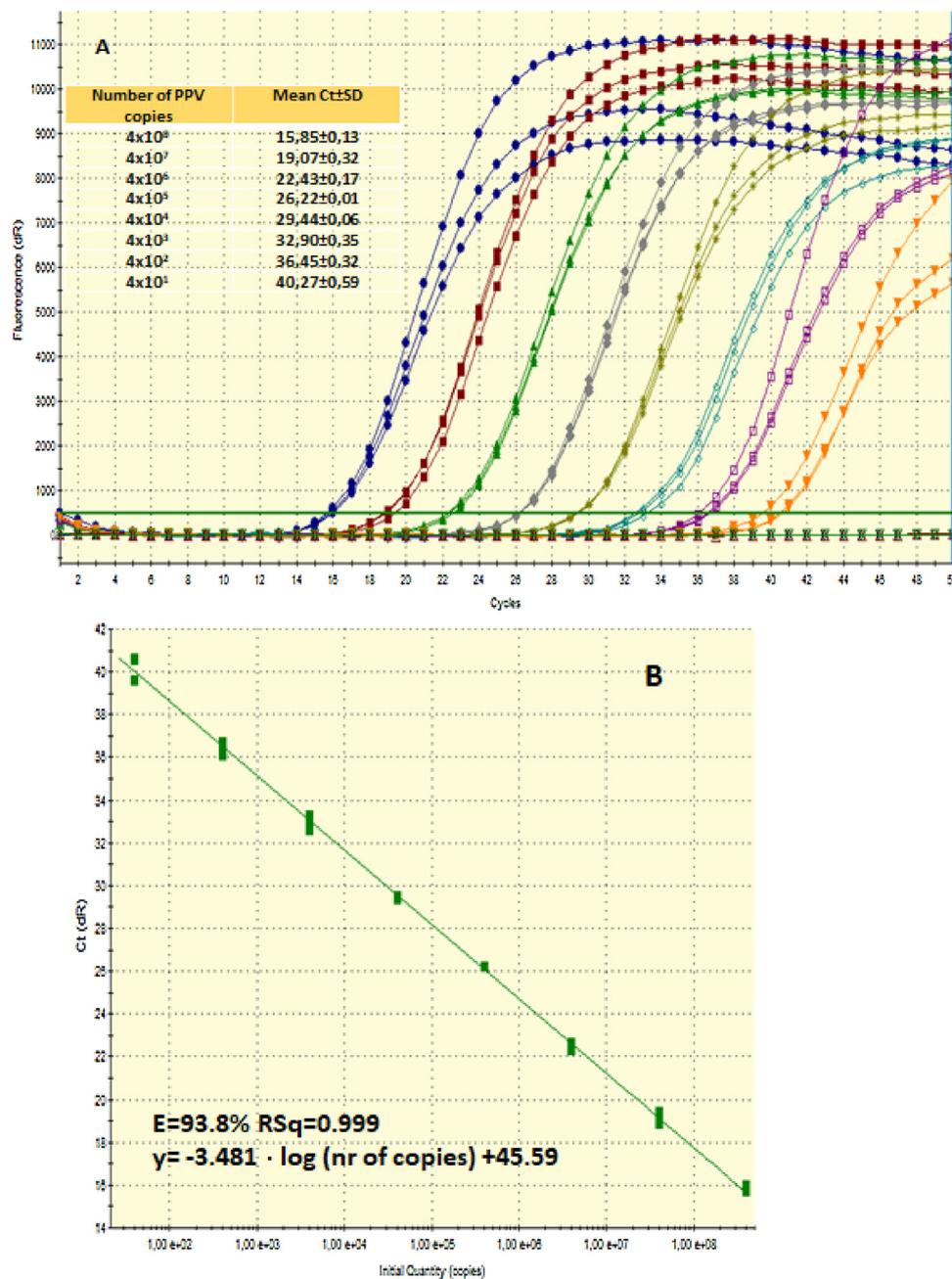
Fig. 1. Amplification plots of PPV-RNA detection in the RT-qPCR assay. (A) HEX fluorescent signals generated from a dilution series of PPV-RNA transcripts. From the right to the left, the curves represent RNA ten-fold serial dilutions, from 40 to 4×10^8 copies per reaction, performed in three replicates. Mean Ct values and standard deviation (SD) among replicates are shown. (B) Corresponding standard curve. The equation of the standard curve obtained was y = -3.481·log(nr of copies) + 45.59, and the correlation coefficient 0.999. Reactions of the no-template controls tested showed no Ct values.

The method successfully amplified all 32 isolates from the seven different PPV strains tested (Table 1) thus exhibiting a broad detection range. Even though isolates from the W and An strains could not be acquired in order to be tested, *in silico* analysis of the designed primers and probe indicated that these strains are also detectable with the developed method. No fluorescence signal was obtained using total RNA from fruit tree samples hosting other *Prunus*-infecting viruses such as prune dwarf virus, prunus necrotic ringspot virus and apple chlorotic leaf spot virus nor from PPV-free *Prunus* samples (data not shown).

The application of the developed RT-qPCR in a small number of samples collected from symptomatic apricot trees confirmed the presence of PPV. Moreover testing of randomly collected field samples from the Prefecture of Imathia revealed a significant presence of PPV in peach (42.2%). The virus was not detected in the few samples from

cherry and plum trees tested (Table 2).

Given the high economic significance of PPV, the existence of reliable techniques for its sensitive and fast diagnosis is of paramount importance. It is well known that the molecular assays surpass serological ones in the case of fruit tree virus diagnostics and our own observations for PPV confirm this aspect (data not shown). Several RT-PCR and real time RT-PCR assays have been developed so far (Kim et al., 2008; Olmos et al., 2005, 2006; Schneider et al., 2004; Varga and James, 2006; Sochor et al., 2012), with most of them exhibiting a broad detection range such as the universal detection of five PPV strains by the method of Kim et al. (2008). However, the constant identification of new genetic variants that might slip currently available methods call for a continuous update and the development of new polyvalent PPV diagnostics. In this respect the herein developed RT-qPCR assay exhibits

broad detection range covering all known strains of PPV and at the same time it is highly sensitive detecting low numbers of viral molecules. Moreover, the use of the Taqman probe in this scheme will allow future development of multiplex real time PCR assays for the simultaneous detection of important virus pathogens infecting cultivated *Prunus* species. Therefore the developed RT-qPCR assay represents a useful tool that could be applied for epidemiological studies of PPV, screening of plant genotypes for virus resistance, plant-virus interactions studies, phytosanitary control as well as for routine testing of plant material in certification schemes of *Prunus* species.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.10.006.

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