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Specific detection and quantification of Plum pox virus by real-time fluorescent reverse transcription-PCR

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Abstract

Plum pox virus (PPV), a destructive and economically devastating pathogen of *Prunus* species, was recently discovered in Pennsylvania and Canada. Current containment efforts involve eradication of infected trees based on ELISA surveys, which are laborious and less sensitive than PCR-based techniques. A real-time, fluorescent, reverse transcription-polymerase chain reaction (RT-PCR) assay was developed for the detection of PPV in the Smart Cycler (Cepheid). The methods developed are reproducible, specific to PPV, and sensitive enough to consistently detect PPV transcripts at the 10–20 fg level. The assay is more sensitive than either ELISA or traditional PCR followed by visualization with ethidium–bromide. PPV was detected from multiple hosts and from multiple *Prunus* tissues (leaf, stem, bud, and root). A dilution series using an in vitro synthesized transcript containing the target sequence as a standard demonstrated that the assay was effective for quantitation of viral template. The real-time PCR assay is a valuable tool for PPV detection and liter quantification in field or laboratory settings.

Keywords: Plum pox virus; Real-time PCR; Virus detection

1. Introduction

Plum pox virus (PPV) is responsible for the disease Sharka, considered the most economically destructive virus disease of cultivated *Prunus* (Lopez-Moya et al., 2000; Nemeth, 1986; Dunez and Sutic, 1988; Roy and Smith, 1992). Originally described as an abnormality of plums in Bulgaria in 1915, PPV was shown to be of viral origin in 1934 (Christoff, 1934). PPV has been reported in numerous economically important *Prunus* hosts, including peach, plum, apricot, nectarine, almond, and sweet and sour cherries. In Europe PPV also infects a number of naturally occurring woody and herbaceous hosts. Multiple strains of PPV have been identified, including the most common D strain, which causes chlorotic blotches and lines on fruit and chlorotic vein clearing and ringspots on leaves, and the M strain, which generally causes more severe symptoms (Damsteegt et al., 1997). PPV spread steadily throughout Europe from its origin, eventually reaching the Middle-East, northern Africa, India, Chile, US, and Canada. Although eradication efforts have succeeded in rare instances in Europe, the overall course of the disease in Europe indicates that PPV moves and evolves effectively (Gottwald et al., 1995; Levy et al., 2000a,b; Lopez-Moya et al., 2000). PPV causes severe losses to the *Prunus* industry wherever it occurs, with losses easily reaching hundreds of millions US dollars. It spreads rapidly within orchards, where it can infect 50–100% of the trees within 100 m of an infected tree within 10 years (Jordovic, 1968).

PPV is a member of the genus *Potyvirus* in the family Potyviridae. The genome consists of a 9.7 kb, positive sense, ssRNA molecule (Fig. 1A), expressed as a 350 kDA polyprotein precursor that is proteolytically processed by self-encoded proteases into nine smaller functional proteins (Revers et al., 1999; Reichman et al., 1992). PPV has been detected using a variety of biological, serological, and molecular methods (Desvignes, 1976; Varveri et al., 1987; Wetzel et al., 1991; Asensio et al., 1994; Levy and Hadidi, 1994; Damsteegt et al., 1997). Several polyclonal and monoclonal antibodies have been raised against all

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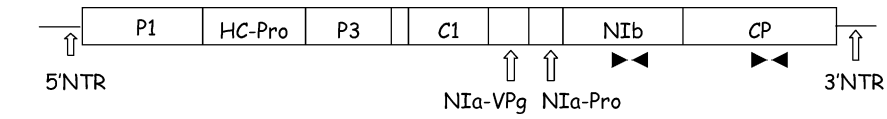


(A)

NIb Target (152 nt)

```
  FWD primer .         .         .         .         .         .
1- CTTGAATGGGACAGATCAAATGAgccaatccatcgattggaggcaatttgtgcatcaatggtggaagcgt -70
   gaacttaccctgtctagtttagtcggttaggtagctaaccTCCGTTAAACACGTAGTTACCACCTTCgca
                                                    Probe
           .         .         .         .         .         .
71- ggggttataaggagctgctgagggagatccgaaaatttacagttgggttcttgaacaagcaccatacaa -140
    ccccaatattcctcgacgactccctctaggcttttaaaatgtcaacccaagaacttgttCGTGGTATGTT
           .
141- tgctctttcaaa -152
     ACGAGAAAGTTT
     REV primer
```

CP Target (131 nt)

```
  FWD primer .         .         .         .         .         .
1- CCAATAAAGCCATTGTTGGATCatgcgaaacccacttttagacaaattatggcacatttcagtaacgtgg -70
   ggttatttcggtaacaacctagtacgctttgggtgaaaatctgtttaatACCGTGTAAAGTCATTGCACC
                                                          Probe
           .         .         .         .         .         .
71- ctgaagcgtatattgaaaaacgaaattatgaaaaagcatacatgccaaggtatggaattca -131
    GACTTCgcatataacttttgctttaatacttttcgtaTGTACGGTTCCATACCTTAAGT
                                          REV primer
```

(B)

Fig. 1. (A) Genomic map of Plum pox virus. The PPV genome is composed of positive sense ssRNA and contains one long ORF that, upon translation, yields a large polyprotein precursor. Viral and host proteases cleave the polyprotein into nine smaller proteins. Approximate locations of PCR primers for amplification of target regions are shown as arrows. (B) Sequences of target regions. Primer and probe sequences are shown in bold capitalized letters.

strains of PPV (Asensio et al., 1994; Boscia et al., 1997; Cambra et al., 1994; Candresse et al., 1994; Kerlan and Dunez, 1979; Olmos et al., 1997) and epitope maps have been prepared for specific monoclonals (Candresse et al., 1998). Enzyme-linked immunosorbent assay (ELISA) remains the most common survey detection tool. However, strain variability, low virus titers, and uneven distribution of the virus in infected plants lead to detection inconsistencies (Nemeth, 1986). Viral titers can fluctuate greatly with host species, the age of the tree and the time of year (Polak, 1998). In addition, cross-reactivity with other potyviruses has been a problem with polyclonal antisera (Lopez-Moya et al., 2000).

The polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) are widely used as diagnostic tools in virus research (Mackay et al., 2002). PCR and RT-PCR are particularly effective in the detection of viral pathogens because of the high sensitivity and reduced labor involved in both the production of and use of diagnostic protocols. Not surprisingly, numerous PCR formats have been adapted for the detection of PPV. PPV has been successfully detected using traditional PCR (Wetzel et al., 1991; Levy and Hadidi, 1994; Candresse et al., 1995; Faggoli et al., 1998), immuno-capture PCR (Wetzel et al., 1992), silicacapture PCR (Mallinoski, 1997) and print-capture PCR (Olmos et al., 1996). Despite the fact that all of these techniques were quite successful in detecting low titers of the virus, none of these protocols were quantitative in nature. Additionally, most surveys continue to use ELISA detection protocols even though PCR detection methods are considered to be more sensitive (Kolber et al., 1997). Real-time PCR is an adaptation of the traditional PCR protocol that allows for the rapid detection of target-specific amplicons without post-PCR electrophoresis. The hybridization step of real-time PCR, which uses a defined probe sequence to target specific amplicons, eliminates the need for confirmation by Southern blot. In addition, real-time PCR allows for the accurate quantification of target when used with a standard curve. Real-time PCR is gaining wider acceptance for its increased sensitivity, increased speed and reduced risk of carry over contamination (Mackay et al., 2002), and it has been adapted for use with other plant viruses (Schoen et al., 1996; Eun et al., 2000; Mumford et al., 2000; Roberts et al., 2000; Boonham et al., 2002; Korimbocus et al., 2002).

PPV was identified in Pennsylvania in 1999, and subsequently in Canada in 2000, resulting in immediate eradication efforts, largely based on the results of extensive surveys using ELISA detection methods. Difficulties with sensitivity combined with the time and labor involved make surveys based on ELISA less than desirable. In addition, a quantitative method for studying the varied distribution and titer of PPV throughout the host and growing season is highly desirable. The high sensitivity, high specificity and high throughput potential associated with real-time PCR suggest that this protocol may be a viable alternative. Primer and probe sequences were selected for conserved regions of the PPV genome, and tested in a Smart Cycler (Cepheid). The assay proved to be highly sensitive and very specific to PPV. In addition, the real-time format allows for very accurate quantitation of the target RNA.

2. Materials and methods

2.1. Virus isolates

The four recognized PPV strains (D, M, C and El Amar) were obtained from M. Ravelonandro (INRA) and grafted into woody bioindicators *Prunus persica* (peach) cv GF305 and *Prunus tomentosa* (Nanking cherry) as described previously (Damsteegt et al., 1997). Pennsylvania isolates (PPV-D) were harvested from 14 different sites in the four quarantined counties (Adams, Cumberland, York and Franklin), either as budwood from infected trees or as young infected trees. Infected materials were bagged and transported under USDA Animal and Plant Health Inspection Service and Maryland Department of Agriculture permits (no. 46471) to the BSL-3 biocontainment facility at Fort Detrick, MD, USA. Budwood was grafted onto GF305 and *P. tomentosa*. Buds were allowed to grow out and symptoms were observed in scion leaves and new bioindicator growth. Confirmation of Pennsylvania strain type was done by ELISA with strain specific PPV monoclonal antibodies according to manufacturer's protocol and strain specific PCR as previously described (Damsteegt et al., 1997; Levy et al., 2000a,b). Presence or absence of additional *Prunus* viruses were determined by ELISA utilizing antibodies specific for Prunus necrotic ringspot and Prune dwarf viruses, Apple chlorotic leaf virus and Tomato ringspot virus. Other potyviruses (Potato virus Y virus, Clover yellow vein virus, and an uncharacterized Datura potyvirus (D437)) as well as other *Prunus* viruses (Myrobalan latent ringspot virus, Peach rosette mosaic virus, Prunus necrotic ringspot virus, Apple chlorotic leaf spot virus and Tomato ringspot nepovirus) were maintained in plants at Fort Detrick.

2.2. Nucleic acid extraction

Total RNA was extracted from 50 mg fresh or frozen (in RNALater, Ambion, Austin, TX, USA) leaf (from all hosts) or flower (from peaches) material using RNAqueous small scale phenol-free total RNA isolation kit (Ambion) following the protocol provided by the manufacturer. Cell disruption was accomplished by using a disposable pestle attached to an electric drill, homogenization took place in a 1.5 ml microcentrifuge tube. Following purification the RNA was eluted in 25–50 μl of manufacturer-provided elution buffer. Total RNA concentrations were estimated using spectrophotometer. The quality of the total RNA preps was periodically assessed using gel electrophoresis. Total RNA was extracted from peach buds, green stems, woody stems and roots by finely chopping the plant material with a razor blade followed by RNAqueous extraction according to manufacturer protocol.

2.3. Oligonucleotide primers

Primer and probe sequences were selected by determining suitable conserved regions in the PPV genome using multiple sequence alignments. All current PPV sequences available in Genbank, including the D, M, C and EA strains, were aligned using the program Pileup from the Genetics Computer Group (GCG) sequence analysis software package (version 9.0, Genetics Computer Group, Madison, WI, USA) (Devereaux et al., 1984). Target regions were selected from conserved regions in the 3′ end of the NIB gene (nucleotides 8280–8431) and the middle of the CP gene (nucleotides 9198–9329) (Fig. 1B). Nucleotide positions correspond to the reported sequence for PPV D, Genbank accession number X16415 (Teycheney et al., 1989). Probes (synthesized by Applied Biosystems Inc., Foster City, CA, USA) were labeled at the 5′ end with the fluorescent reporter dye 6-carboxy-flourescin (FAM), and labeled at the 3′ end with the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA).

2.4. Preparation of RNA transcripts for standard curves

A recombinant plasmid carrying the cloned 3′ terminal 1400 nucleotides of a European PPV-D strain including the 131 nucleotide target sequence (courtesy of Dr. Laurene Levy, USDA-APHIS) was linearized with *Bam*HI (Promega, Madison, WI, USA) and purified using the MinElute PCR purification kit (Qiagen, Valencia, CA, USA). RNA transcripts were generated using MAXIscript in vitro transcription kit's (Ambion, Austin TX, USA) T7 enzyme mix at 37 °C for 1 h. Plasmid DNA was digested twice with RNase-free DNase I at 37 °C for 15 min. The transcript was isolated by two ammonium acetate and ethanol precipitations. RNA concentration was determined through gel quantitation, using 0.8% agarose gel with 0.5× TBE, 75 volts for 2 h and visualized with ethidium bromide. The transcripts were diluted serially in 2 μl aliquots (300 pg/μl) of total RNA extracted from healthy peach variety GF305.

2.5. RT-PCR assay

The Superscript One-Step RT-PCR with Platinum Taq kit (Invitrogen) was used throughout as 25 μl reactions in a Smart Cycler (Cepheid). The RT-PCR mixture contained 1× Reaction Mix (0.2 mM each of dATP, dTTP, dCTP, and dGTP, 1.2 mM $MgSO_4$) additional 4.8 mM $MgSO_4$, 200 nM forward and reverse primers, 100 nM FAM-TAMRA probe, and 0.5 μl RT/Platinum Taq Mix per reaction. Thermal cycling conditions were 52 °C for 15 min for reverse transcription, 95 °C for 5 min for Platinum Taq activation, and 60 cycles of 95 °C for 15 s and 60 °C for 30 s for PCR. Between 300 pg and 2 μg of total RNA or DNA template was used per assay. Fluorescence from FAM reporter was detected at 505–537 nm wavelength. The cycle threshold (C_t) values for each reaction were calculated automatically by the Smart Cycler (Cepheid) detection software by determining the point in time (PCR cycle number) at which the reporter fluorescence exceeded 10 times the computer determined standard deviation for background. The size of the PCR product was checked periodically using electrophoresis.

A standard curve for quantitation was generated using four independent assays, where C_t values were plotted from 10-fold serial dilutions of the PPV RNA transcript. The C_t values were calculated by the Smart Cycler software. Calculations for each dilution set included, standard deviation and average C_t. Regression analysis was completed on the average C_t values.

3. Results

3.1. Design of primers and probes

Sequence and immunological data identified the Pennsylvania isolates of PPV as belonging to the D strain group (Levy and Damsteegt, personal communication). Primers and probe sequences for real-time PCR assays were designed by first aligning the sequences of several PPV strains, including several known D strain isolates, the M strain, the EA strain and the C strain. Two sets of primers and probes were selected based on conserved regions in the alignments. The first region was a 152 nucleotide (nt) segment in the 3′ region of the NIB gene, the second region was a 131 nt segment in the middle of the coat protein (CP). In addition to conserved regions of the PPV genome, probes and primers were also selected following suggested protocol to contain

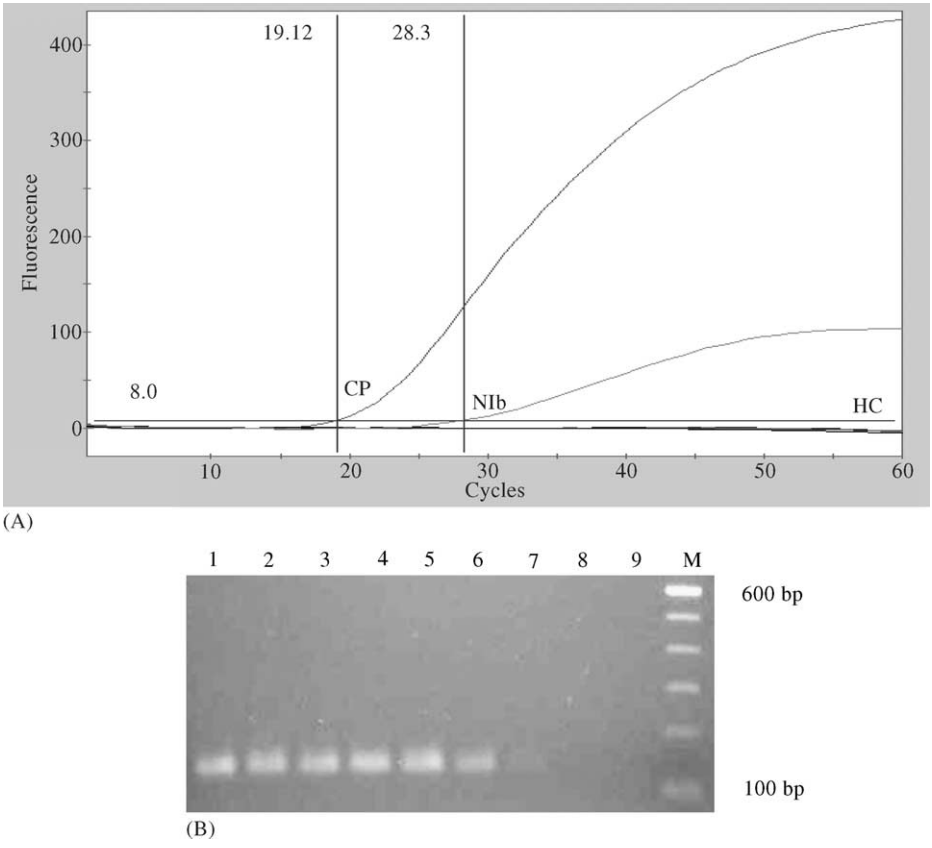


Fig. 2. (A) Real-time PCR assay comparing CP and Nib primer/probe sets. Using the same PPV-D template the CP primer/probe set (CP) produces stronger signal and lower C_t values than the Nib primer probe set (Nib). Healthy control for both primer/probe sets show no signal (HC). (B) Gel electrophoresis analysis of PCR product generated by CP primers. Lanes 1–7 show the correctly sized 132 bp product. Lane 8 is a negative control using total RNA from healthy peaches, lane 9 is an RT-PCR reaction with no RNA template.

Table 1
Real-time PCR assay specificity

Virus and strain	Host	Results (+/−)
Plum pox virus—D (PPV-D)	*Prunus persica*	+
Plum pox virus—El Amar (PPV-EA)	*Prunus persica*	+
Plum pox virus—Cherry (PPV-C)	*Prunus tomentosa*	+
Plum pox virus—M (PPV-M)	*Prunus persica*	+
Plum pox virus—PA (PPV-PA)	*Prunus persica*	+
Apple chlorotic leafspot virus (ACLV)	*Prunus persica*	−
Tomato ringspot virus (TomRSV)	*Prunus persica*	−
Myrobalan latent ringspot virus (MLRV)	*Nicotiana tabacum*	−
Peach rosette mosaic virus (PRMV)	*Prunus persica*	−
Prunus necrotic ringspot (PNRV)	*Prunus persica*	−
Potato virus Y (PVY)	*Nicotiana tabacum*	−
Datura potyvirus—D437[a]	*Nicotiana tabacum*	−
Clover yellow vein virus (CYVV)	*Nicotiana tabacum*	−
Plum pox virus—PA	*Melilotus officinalis*	+
Plum pox virus—PA	*Nicotiana benthamiana*	+
Plum pox virus—PA	*Nicotiana edwardsonii*	+
Plum pox virus—PA	*Nicotiana tabacum*	+
Plum pox virus—PA	*Prunus americana*	+
Plum pox virus—PA	*Nicotiana occidentalis* 37-B	+
Plum pox virus—PA	*Prunus serotina*	+
Plum pox virus—PA	*Prunus tomentosa*	+
Plum pox virus—PA	*Prunus persica*	+
Plum pox virus—PA	*Prunus persica* flower	+
Plum pox virus—PA	*Prunus persica* fruit	+
Plum pox virus—PA	*Prunus persica* green stem	+
Plum pox virus—PA	*Prunus persica* leaf	+
Plum pox virus—PA	*Prunus persica* leafbud	+
Plum pox virus—PA	*Prunus persica* root	+
Plum pox virus—PA	*Prunus persica* woody stem	+

Positive results are those where reaction fluorescence exceeds 10 times the computer-determined standard deviation for the background.
[a] D-437 is a previously undescribed potyvirus found in *Datura* (V. Damsteegt, personal communication).

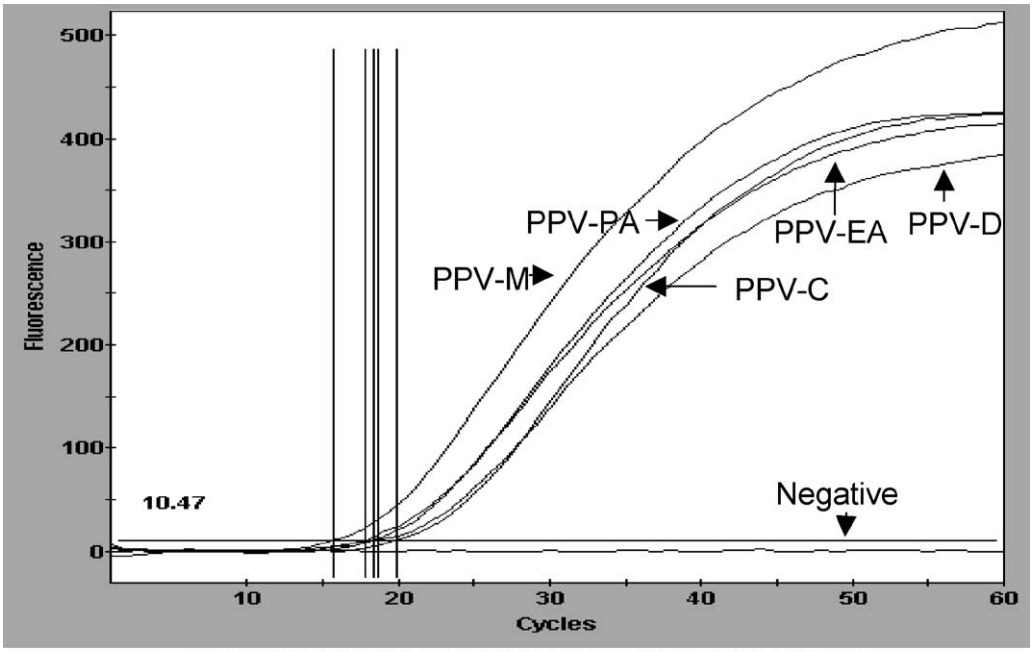
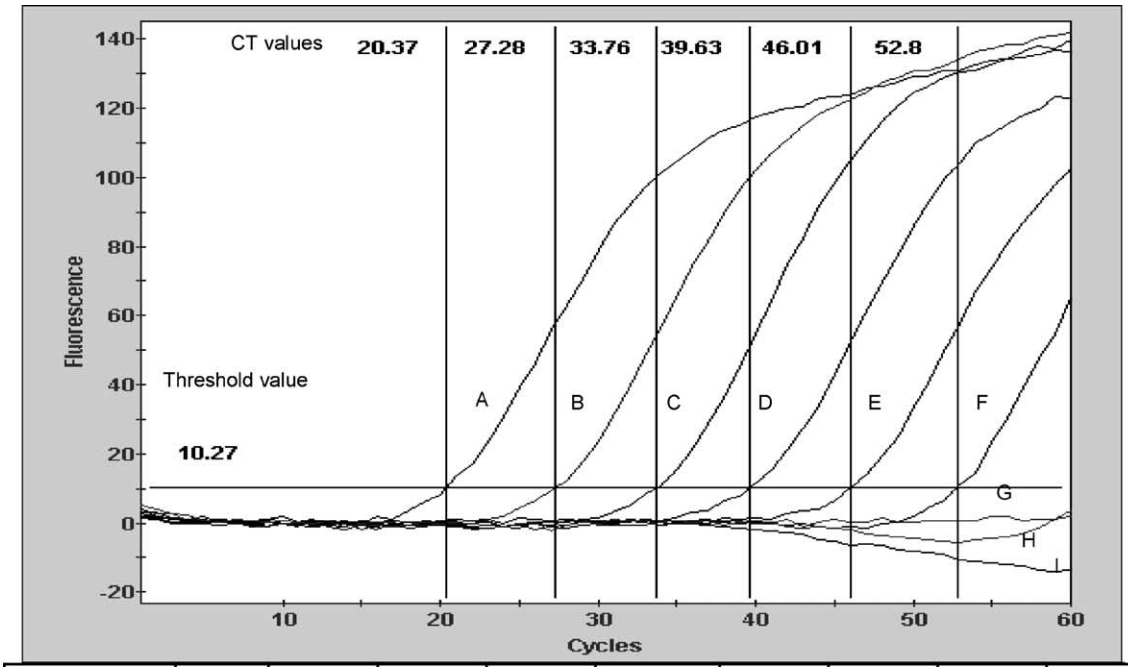


Fig. 3. Detection of PPV strains with real-time PPV assay. All PPV strains were isolated from peach leaves, negative control is a healthy peach. PPV-C, D, EA and M are previously described strains maintained at Fort Detrick. PPV-PA is an isolate of PPV obtained from Pennsylvania.

30–80% G–C content with more cytosine residues than guanines, melting temperatures approximately 65 °C, with no runs of identical nucleotides. Both sets of primers and probes were first tested using a DNA template containing the target regions. A correctly sized DNA fragment which specifically annealed to the synthesized probe in real-time PCR assays was synthesized with both sets of primers (data not shown).

3.2. Assay specificity

Following confirmation of effective amplification using a DNA template the real-time PCR assay was combined with reverse-transcription and tested on total RNA from healthy control and PPV infected peach trees and Colmo peas. No signal was detected using total RNA from healthy control



Sample	A	B	C	D	E	F	G	H	I
Transcript Conc.	2 ng	200 pg	20 pg	2 pg	200 fg	20 fg	2 fg	Healthy RNA	No temp.
Ct value	20.37	27.28	33.76	39.63	46.01	52.80	-	-	-

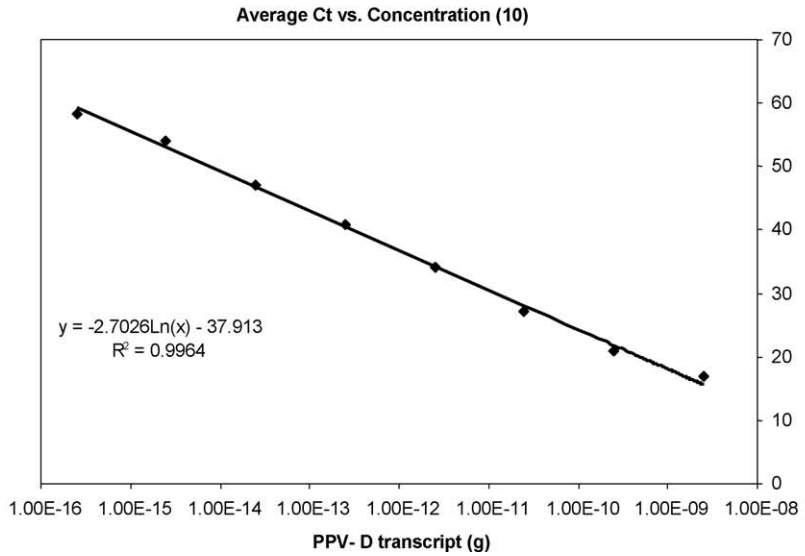


Fig. 4. Use of the PPV real-time PCR for quantitation of viral RNAs. The Smart Cycler output from a typical assay using serial dilutions of PPV transcript as template. Shown above is the regression analysis of the results of four independent serial dilution assays.

plants. Both sets of primers and probes successfully detected virus in both hosts efficiently. However, for identical samples the CP primer probe set consistently generated better curves with lower C_t values than the NIB primer/probe set (Fig. 2A). Thus, the CP primer/probe set was chosen for continued use in subsequent experiments. The PCR product generated by the CP primers was determined to be the expected size by electrophoresis (Fig. 2B). Using the CP primer/probe set the RT-PCR assay successfully detected all Pennsylvania isolates tested as well as the four known strains of PPV (Table 1, Fig. 3). The C_t values for PPV-C were consistently slightly higher than C_t values for PPV-D (European or Pennsylvanian), PPV-EA or M strains (Fig. 3 an data not shown), but it was not determined if this was related to assay efficiency or viral titers. The assay did not detect any other potyviruses tested, nor did it detect other common viral pathogens from *Prunus* (Table 1). In particular, the assay was able to distinguish PPV from an uncharacterized *Datura* potyvirus D-437. Antisera to D-437 cross reacts with PPV (V. Damsteegt, personal communication).

3.3. Use in multiple hosts and tissues

The assay was tested on a limited PPV host range (Table 1). The virus has been successfully detected from numerous *Prunus* hosts including peach, plum, apricot, Nanking cherry and *Prunus americana*. In addition, the assay detected PPV in a number of herbaceous hosts including peas, *Nicotiana benthamiana*, *Nicotiana occidentalis* 37-B, *Nicotiana edwardsonii*, and *Melilotus officinalis*. No signal was detected in healthy control plants of any of these species. The assay detected PPV in most types of *Prunus* tissue, including buds, leaves, flowers, fruit, roots and young stems (less than one year old growth). However, in limited tests the assay was not successful in detecting virus in older woody stems (data not shown).

3.4. Assay sensitivity and quantitation

In order to determine the lower detection limits and the quantitative ability of the assay in vitro, transcripts were synthesized from a 1.4 kb PPV clone that contained the target region of the CP. The assay consistently detects femtogram (fg) levels and sometimes as little as 500 ag of an in vitro synthesized PPV transcript in a healthy plant sap background (Fig. 4A). The assay detects virus in combined or batched samples, where tissue from one infected leaf is included with tissue from four healthy leaves, indicating samples could be batched (data not shown). The assay was repeated four times to assess variability. Regression analysis of the four independent standard curves demonstrates that the assay is highly repeatable (Fig. 4B), indicating the assay can be used with a standard curve to accurately estimate viral titer.

4. Discussion

We have designed primers and probes for a PPV specific real-time PCR assay. The assay is highly sensitive and very specific to PPV. The primers and probes detect all strains of PPV without amplifying product from other potyviruses, other *Prunus* viruses or RNA from healthy control plants of any species tested. The assay was slightly more efficient at detecting the D and M strains of PPV, but this is to be expected due to single base mismatches between the selected CP primer and probe sequences and the corresponding sequences from PPV C and EA. The assay was consistently capable of detecting fg levels of a PPV transcript diluted in total RNA extractions from healthy plants. The assay was useful in detecting PPV from a wide range of both woody and herbaceous hosts. In addition, the assay was useful in detecting PPV in a number of different woody tissue types, including leaves, flowers, buds, roots and young stems. In a limited number of tests the assay has not been successful in detecting PPV in older woody stems, but this is more likely a flaw in the RNA extraction procedure than in the assay itself. The assay should be sensitive enough to detect minute levels of virus in previously difficult to detect backgrounds, such as dormant trees.

PCR and RT-PCR are commonly used as sensitive diagnostic tools for detection of viruses. The sensitivity of PCR detection methods can be orders of magnitude better than RNA detection by northern analysis or protein detection by ELISA. The current ELISA systems used for surveying in the US are labor intensive and subject to misinterpretation. The PPV ELISA system is not as sensitive as established PCR detection protocols (Kolber et al., 1997; Lopez-Moya et al., 2000). The real-time PCR assay described here is more sensitive than other PCR systems described for PPV, and it does not require visualization of the product by electrophoresis or additional silica or PPV antibody procedures. The cost per sample is higher for real-time PCR than for ELISA, but the real-time PCR has the added advantages of speed, accuracy, sensitivity and reproducibility. In addition, the cost per sample can be reduced significantly. Because the real-time PCR assay is so sensitive, samples can be effectively batched without losing accuracy. Also, the procedure described here makes use of a commercially available total RNA extraction kit, but the assay was equally successful amplifying specific product from standard RNA extraction procedures (data not shown).

In addition to potential uses for diagnostics, the real-time PCR protocol described serves as a helpful tool for quantifying levels of viral RNA. Quantitation using standard PCR is difficult, often involving multiple primer sets and competition between templates. Previous PPV specific RT-PCR procedures have been limited to use as a qualitative detection method. Repeated experiments using in vitro transcripts as template demonstrate that a reliable standard curve can be established to estimate RNA concentrations in experimental samples. This makes the assay a powerful research tool for

further studies. The combined sensitivity and quantitative nature of the assay allows for detailed studies of virus distribution in planta, both temporally and spatially. The high sensitivity may allow for detection and comparison of relative viral levels in aphid vectors and non-vectors as well.

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