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Detection and differentiation of Plum pox virus using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing

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Abstract

A real-time multiplex PCR procedure with melting curve analysis, using the green fluorescence dye SYBR Green I, was developed for rapid and reliable identification of Plum pox virus (PPV) isolates of strains D and M. Members of the different strains were identified by their distinctive melting temperatures (T_ms); 84.3–84.43 °C for D isolates, and 85.34–86.11 °C for M isolates. The associated amplicon sizes were 114 and 380 bp, respectively. The procedure was used for detection and identification of PPV in both herbaceous and woody hosts. The T_m for members of a particular strain was very similar, with a host effect that did not hinder strain identification. Universal primers included in the study detected all isolates of PPV tested, amplifying a 74 bp fragment. The T_m of this fragment varied from 80.12 to 81.52 °C and may have supplementary value for PPV identification. SYBR Green-based detection was compared to detection using a hybridization LUX fluorogenic primer. Better resolution of the melting peaks was observed with SYBR Green I, than with the LUX primers, hence strain identification with SYBR Green I was more reliable. This is a simple approach to PPV strain identification with the relatively inexpensive dye SYBR Green I, and eliminates any need for electrophoretic analysis of amplicons or RFLP patterns using ethidium bromide.

Keywords: Plum pox virus; Strain typing; Real-time PCR; Multiplex; Melting curve; SmartCycler

1. Introduction

Plum pox virus (PPV) is a member of the genus *Potyvirus*, and the virus is considered the most serious pathogen affecting stone fruits; apricots, cherry, nectarine, peaches, and plums (Németh, 1986; Nemchinov et al., 1998). The symptoms of plum pox disease or sharka include fruit abnormality, loss of taste, and fruits that drop prematurely (Németh, 1986). These symptoms may cause losses as high as 80–100% of a crop (Németh, 1986) and consequently it is a disease of economic and quarantine significance in every country where stone fruits are grown.

There are four widely recognized strains of PPV; D, M, C, and El Amar (Candresse et al., 1994, Nemchinov et al., 1998; Olmos et al., 1997; Szemes et al., 2001). Recently, an unusual isolate of PPV (W3174) was detected that may represent a fifth strain of PPV (James et al., 2003). PPV strains differ in the severity of associated symptoms, efficiency of aphid transmission, host range, and geographic distribution. To achieve successful eradication or effective management of the associated disease, strain typing of the virus is required. Several methods for strain typing PPV have been described; enzyme-linked immunosorbent assay (ELISA) with strain-specific monoclonal antibodies (Myrta et al., 2000; Olmos et al., 1997), RT-PCR with restriction fragment length polymorphism analysis (Glasa et al., 2002; Hammond et al., 1998; Wetzel et al., 1991), heminested PCR with strain-specific primers (Olmos et al., 1997), PCR-ELISA (Olmos

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et al., 1997; Poggi Pollini et al., 1997), and integrated RT-PCR/nested PCR (Szemes et al., 2001).

The techniques described above are valuable for strain typing PPV, but are complex and time consuming. Real-time PCR with melting curve analysis has been described as a simple, rapid, and reliable technique for the detection and identification of certain pathogenic bacteria (Shrestha et al., 2003), hemoflagellates (Nicolas et al., 2002), and RNA viruses (Beuret, 2004; Mouillesseaux et al., 2003; Richards et al., 2004). The objective of this study was the development of a simple and reliable method for simultaneous detection of PPV, and identification of the two common strains of PPV, using real-time multiplex PCR with melting curve analysis. Universal primers for PPV, as well as D-specific, and M-specific primers were combined with a pair of primers targeting the endogenous plant gene NADH dehydrogenase subunit 5 (Menzel et al., 2002). Melting curve analysis was used for PPV detection, specific identification of isolates of PPV strains D and M, and differentiation from PPV C, EA, and W3174. Melting curves generated by real-time PCR using SYBR Green I and LUX primers (Nazarenko et al., 2002) were compared. A protocol utilizing SYBR Green I in real-time multiplex PCR with melting curve analysis was found to be simple and reliable for the detection of PPV, and for the identification of D and M types.

2. Materials and methods

2.1. Virus source

PPV D was obtained from W. Jelkmann, Germany; PPV M, type member M strain, was obtained from F. Dosba, France; PPV C (a sweet cherry isolate), and PPV EA (El Amar) were obtained as freeze dried tissue samples from A. Myrta, Italy. These isolates were maintained in the herbaceous host *Nicotiana benthamiana*. Also, the type member (Marcus) of M strain was maintained at the Sidney Laboratory in *Prunus persica* (peach), along with another M isolate Q1949. PPV 2630 is a Canadian type D isolate maintained in peach (*P. persica*) and *N. benthamiana*. PPV Fantasia, and Vulcan are D type isolates (James and Upton, 2001) of PPV detected in Canada, and tested in the original hosts, nectarine and clingstone peach, respectively. The virus isolate W3174 is a Canadian isolate of PPV, detected in plum (*P. domestica*) and mechanically sap-transmitted to *N. benthamiana*. This isolate does not appear to belong to any of the four recognized strains of PPV (James et al., 2003).

2.2. Primer design

Preliminary evaluation of published PPV-specific oligonucleotide primers indicated that there were no available sets suitable for the particular objectives of this study. PPV coat protein nucleotide sequences, from GenBank database and in house sequences, were aligned and used to identify oligonucleotide primer sequences that may be used for universal detection of all PPV isolates (PPV-U), D-specific (PPV-FD), M-specific (PPV-FM), and a suitable reverse primer PPV-RR. The sequences of the various primers, primer locations, and product sizes are given in Tables 1 and 2. Additional primers used in this study include the reverse primer P1 described by Wetzel et al. (1991), and the Nad5-F and Nad5-R primers described by Menzel et al. (2002). Menzel et al. (2002) designed the Nad5-F primer so that it spans two exon regions making it mRNA-specific. LUX fluorogenic primers (Nazarenko et al., 2002) were designed using Invitrogen's LUX™ designer software, and synthesized by Invitrogen Corp. (Burlington, Ontario). The linked fluorophore was FAM (6-carboxy-fluorescein).

2.3. Isolation of total RNA and synthesis of cDNA

Total RNA was extracted as described by James et al. (2003). Approximately 4 μg of total RNA in 5 μl

Table 1
Oligonucleotide primers used in the multiplex polymerase chain reactions

Primer	Fluorophore	Sequence (5′–3′)[a]	Size	Target[b]	Position
PPV-P1[c]	–	ACCGAGACCACTACACTCCC	20	PPV	9560–9579[d]
PPV-U	–	TGAAGGCAGCAGCATTGAGA	20	PPV	9424–9443[d]
PPV-FD	–	TCAACGACACCCGTACGGGC	20	PPV-D	9384–9403[d]
PPV-FM	–	GGTGCATCGAAAACGGAACG	20	PPV-M	9118–9137[d]
Nad5-F	–	GATGCTTCTTGGGGCTTCTTGTT	23	Plant	Re: Menzel et al. (2002)
PPV-RR	–	CTCTTCTTGTGTTCCGACGTTTC	23	PPV	9475–9497[d]
Nad5-R	–	CTCCAGTCACCAACATTGGCATAA	24	Plant	Re: Menzel et al. (2002)
PPVRR-LUX	FAM	gaaacgCTCTTCTTGTGTTCCGACGT**5**TC	29	PPV	9475–9497[d]
Nad5F-LUX	FAM	gaacaagTGCTTCTTGGGGCTTCTTG**5**TC	29	Plant	213–231[e]

[a] The **5** denotes the position of the T that is FAM labeled for the LUX primers.
[b] Primers were designed to be specific for all PPV strains (primers PPV-U, and PPV-R or PPVR-LUX), D-specific (PPV-FD), M-specific (PPV-FM), or plant-specific (primers Nad5-F, Nad5-R, and Nad5F-LUX).
[c] PPV-P1 primer (Wetzel et al., 1991) used for cDNA synthesis.
[d] Relative position on Plum pox virus accessions D (X16415) and M (M92280).
[e] Relative position of the Nad5F-LUX primer on the Nad5 gene, accession number D37958. The Nad5 forward primers are mRNA-specific as they overlap two exons (Menzel et al., 2002).

Table 2
Expected amplicon size with each primer pair used in multiplex polymerase chain reactions

Forward primer	Reverse primer	Target	Expected size (bp)[a]
PPV-U	PPV-RR or PPVRR-LUX	All strains	74 (80)
PPV-FD	PPV-RR or PPVRR-LUX	D strain	114 (120)
PPV-FM	PPV-RR or PPVRR-LUX	M strain	380 (386)
Nad5-F	Nad5-R	Plant Nad5 gene	181
Nad5F-LUX	Nad5-R	Plant Nad5 gene	186[b]

[a] The number in parentheses represents the LUX primer amplicon with six additional nucleotides that were used to create the hairpin structure (Nazarenko et al., 2002).

[b] The Nad5F-LUX primer amplicon with seven additional nucleotides that were used to create the hairpin structure (Nazarenko et al., 2002), and in the design two bases were deleted at the 5′ end of the original primer.

diethylpyrocarbonate-treated (depc) H_2O was combined with 2 μl each of the antisense primers PPV-P1 (10 μM) and Nad5-R (5 μM or 10 μM stocks for herbaceous or woody, respectively), incubated at 72 °C for 5 min, then immediately placed on ice. The RT reaction was carried out in a 20 μl volume by adding 11 μl of RT mix consisting of 4 μl 5X first strand buffer (Invitrogen), 2 μl 0.1 M DTT, 1 μl 10 mM dNTP Mix, 0.5 μl RNaseOUT™ (40 U/μl, Invitrogen), 1 μl SUPERSCRIPT™ II (Invitrogen), and 2.5 μl depc H_2O. RT was carried out at 42 °C for 60 min, followed by 5 min at 99 °C, in Stratagene's (La Jolla, California) RoboCycler® temperature cycler. In evaluating the technology for simultaneous detection and identification of mixed strains, equal volumes of total RNA extracted from 100 mg of singly-infected herbaceous plant tissue were mixed in vitro prior to cDNA synthesis. In assessing reliability for detecting mixed infections, total RNA from herbaceous tissue infected with strains D and M were adjusted to the following ratios: 1:1, 1:2, 1:3, 1:4 and vice versa.

2.4. Optimization of conditions for multiplex PCR

A number of parameters were evaluated for multiplex PCR optimization as described by Arezi et al. (2003); Henegariu et al. (1997); and Lekanne Deprez et al. (2002). These included conditions such as various hot-start DNA polymerase enzymes; Platinum® Taq DNA polymerase high fidelity and Platinum *Pfx* DNA polymerase from Invitrogen, and iTaq™ DNA polymerase from BioRad (Mississauga, Ontario). Also evaluated were PCR buffers such as Karsai buffer (Karsai et al., 2002), 10× high fidelity buffer (Invitrogen), 10× *Pfx* amplification buffer (Invitrogen), and 10× iTaq™ PCR buffer (BioRad); primer concentrations in both the RT step (312.5–1250 nM, final concentration), and PCR step (50–400 nM final concentration); cDNA concentration in the PCR reaction (dilutions of 1:3, 1:4, 1:5, and 1:6 in sterile double distilled H_2O); $MgCl_2$ versus $MgSO_4$; $MgCl_2$ concentrations (2–8.5 nM, final concentration); SYBR Green I concentration (1:25,000–1:125,000); and two step PCR (denaturation at 95 °C for 15 s to 3 min, with annealing extension at 55–60 °C for 10–60 s) versus three step PCR (denaturation at 95 °C for 15 s to 3 min, annealing at 55–60 °C for 20–30 s, and extension at 72 °C for 20–30 s).

2.5. SYBR Green I real-time multiplex PCR

Optimum conditions identified included the final primer concentrations (nM) for the SYBR Green I multiplex reactions as follows; PPV-U at 400, PPV-FM at 350, PPV-FD at 150, Nad5-F and Nad5-R at 100, and PPV-RR at 200. The PCR mastermix included these primers and 2.5 μl 10× Karsai buffer (Karsai et al., 2002), 0.5 μl 10 mM dNTP, 1 μl 50 mM $MgCl_2$, 0.1 μl Platinum® Taq DNA polymerase high fidelity (Invitrogen), 3 μl 1:5000 SYBR Green I (Sigma, Oakville, Catalogue #S-9430)—final concentration 1:42,000 (diluted in TE, pH 7.5 as per Karsai et al., 2002), and sterile PCR-grade MilliPore® water to a final volume of 24 μl. Finally, 1 μl of diluted cDNA (1:4 or 1:5 dilution of cDNA generated from herbaceous or woody samples, respectively) was added to this mixture.

2.6. Multiplex real-time PCR with LUX primers

Optimum conditions identified included the final primer concentrations (nM) for the LUX multiplex reactions as follows; PPV-U at 100, PPV-FM at 400, PPV-FD at 150, Nad5-F-LUX and Nad5-R at 200, and PPVRR-LUX at 200. The PCR mastermix included these primers and 2.5 μl of 10× high fidelity PCR buffer (Invitrogen), 0.5 μl of 10 mM dNTP's, 1 μl of 50 mM $MgSO_4$, 0.1 μl of Platinum® Taq DNA polymerase high fidelity (Invitrogen), and sterile PCR-grade MilliPore® water to a final volume of 24 μl. One microliter of diluted cDNA (1:4 or 1:5 dilution of cDNA generated from herbaceous or woody samples, respectively) was added to this mixture.

2.7. Real-time PCR cycling and agarose visualization

Real-time PCR was performed using a SmartCycler® II Thermal Cycler (Cepheid, Sunnyvale, CA). For routine analysis with SYBR Green I and LUX multiplex reaction, PCR cycling consisted of two steps that included; 2 min incubation at 95 °C followed by threshold-dependent cycling for 15 s at 95 °C, and 60 s at 60 °C, where cycling advanced to melt stage once total fluorescence passed threshold (manual setting at 30), plus extra nine cycles. This was identified as optimal in preliminary studies. Fluorescence readings (channel 1 for

both SYBR Green I and FAM signals) were taken during the anneal/extension step (60 °C incubation). Melting was performed from 60 to 95 °C at 0.1 °C/s with a smooth curve setting averaging 1 point. Melting peaks were visualized by plotting the 1st derivative against the melting temperature. The melting temperature (T_m) was defined as the peak of the curve, and if the highest point was a plateau, then the midpoint was identified as the T_m. For electrophoretic analysis, PCR products (10 μl) were separated on a 2% agarose (Bio-Rad) gel in TBE buffer, at 80 V for 60 min, with ethidium bromide staining.

3. Results

3.1. cDNA concentration and optimization of real-time RT-PCR

Variability of the viral titre within the plant tissue led to varying cDNA concentrations of targets after reverse transcription. Primer P1 proved to be an effective primer for generating cDNA for all PPV isolates from all hosts. Good PCR amplification was obtained using cDNA concentrations of 1:3, 1:4, 1:5 and 1:6 dilutions for herbaceous and woody tissue samples. The concentrations 1:4 and 1:5 were selected for routine analysis of cDNA generated from total RNA extracted from infected herbaceous and woody plants, respectively. Digestion of the cDNA with RNase was not necessary prior to amplification as the oligonucleotide primers were virus-specific, and no cross reactions were observed. Platinum® Taq DNA polymerase high fidelity was effective for both SYBR Green I real-time PCR and LUX primer PCR. Interestingly, the buffering requirements differed, with the Karsai buffer being more efficient in SYBR Green real-time PCR, compared to the high fidelity buffer for LUX primer real-time PCR. Also, the two-step PCR cycling regime was found to be robust and just as effective as the three-step PCR regime for both SYBR Green I real-time PCR and LUX primer PCR.

3.2. Real-time multiplex PCR using SYBR Green I and melting curve analyses

The oligonucleotide primers amplified the expected targets in real-time PCR analysis using the SmartCycler®. The universal forward primer amplified a 74 bp fragment and detected all PPV isolates tested, in herbaceous or woody hosts (Fig. 1, lanes 2–7 and 9–11). The D-specific forward primer reacted with D isolates only (Fig. 1, lanes 2, 6, 9, and 10), and the M-specific forward primer with M (Fig. 1, lane 3). The D-specific band in lane 9 of Fig. 1 is weak but clearly visible on the gel and original photograph, which may represent low virus titre. The primer PPV-RR was used as the reverse primer in each of the above reactions. The Nad5 fragment was not observed in all lanes of the gel (Fig. 1). The cycle threshold (C_t), the cycles necessary to detect a signal, was greater for the Nad5 target, indicating more efficient amplification of PPV targets in the earlier cycles. The reactions were stopped just as the Nad5 amplicon was entering the log phase, consequently enough product did not accumulate to allow visualization on the ethidium bromide stained gel.

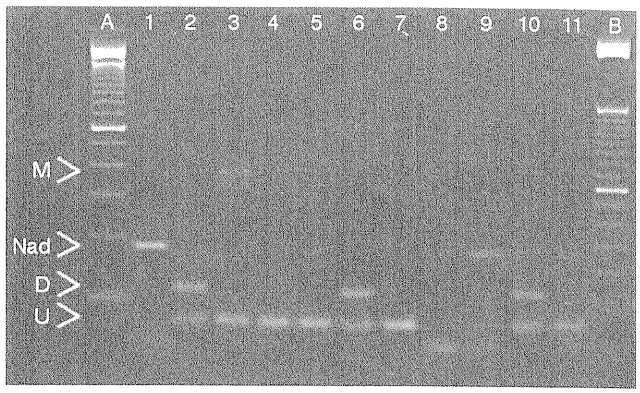

Fig. 1. Gel separation (2% agarose in TBE) of real-time multiplex PCR products. Lane A, 100 bp DNA ladder; lane 1, *N. benthamiana* healthy; lane 2, PPV D in *N. benthamiana*; lane 3, PPV M in *N. benthamiana*; lane 4, PPV C in *N. benthamiana*; lane 5, PPV EA in *N. benthamiana*; lane 6, PPV 2630 in *N. benthamiana*; lane 7, PPV W3174 in *N. benthamiana*; lane 8, healthy GF305 peach; lane 9, PPV Fantasia in nectarine (*P. persica*); lane 10, PPV Vulcan peach (*P. persica*); lane 11, PPV W3174 from *P. domestica* (plum); and lane B, 50 bp DNA ladder. M, Nad, D, and U indicate the position of the M-specific, Nad-specific, D-specific, and PPV universal fragments, respectively.

Melting curve analysis of the amplicons associated with the various isolates of PPV allowed accurate identification of D or M isolates, and differentiation from isolates belonging to other strains or groups. The T_m for isolates of the two strains varied, facilitating specific identification of isolates of strains D and M. Isolates of strain D (114 bp fragment) had a T_m of 84.3–84.43 °C, depending on the host (Figs. 2, 4 and 5). The melting curves for PPV Vulcan in peach and PPV Fantasia in nectarine were identical. Only PPV Vulcan is shown for clarity (Fig. 5). The strain M isolate (Marcus isolate in *N. benthamiana)* with a 380 bp amplified fragment, had a T_m of 86.11 °C (Fig. 2). The M-specific primer combination pro-

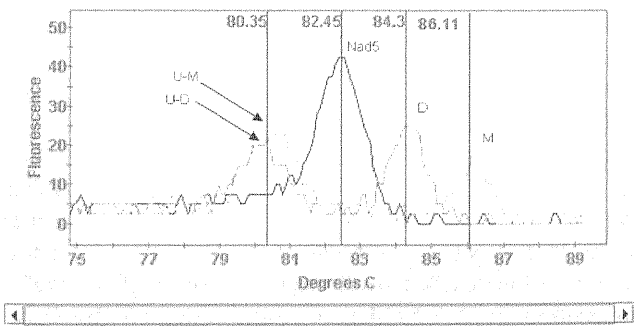

Fig. 2. Multiplex melting curves using SYBR Green I for the detection of Nad5, PPV D, and PPV M fragments amplified with specific primers, showing strain identification in infected *N. benthamiana*. The T_ms associated with the fragments amplified simultaneously using the universal PPV primer, PPV-U, also are shown (U-M for PPV M, and U-D for PPV D).

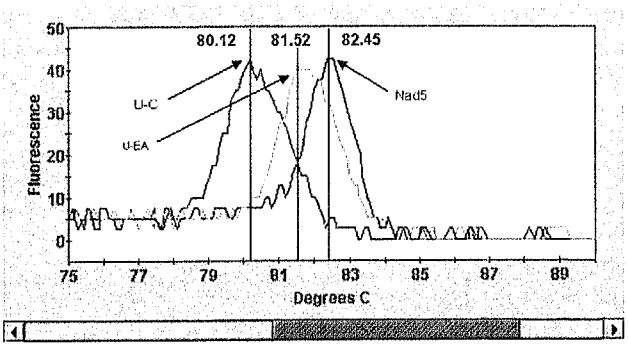

Fig. 3. Multiplex melting curves using SYBR Green I for the detection of Nad5, PPV C, and PPV EA amplicons showing PPV identification and the absence of nonspecific reactions with the D- and M-specific primers, in infected *N. benthamiana*. T_ms associated with the fragments amplified simultaneously using the universal PPV primer, PPV-U, are shown (U-C for PPV C, and U-EA for PPV EA).

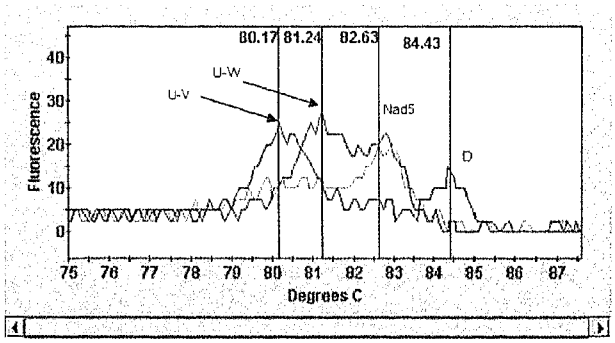

Fig. 5. Multiplex melting curves using SYBR Green I for the detection of Nad5, PPV Vulcan, and PPV W3174 amplicons showing differentiation of a D isolate from PPV-W3174 isolate, in *Prunus*. T_ms associated with the fragments amplified simultaneously using the universal PPV primer, PPV-U, for isolates Vulcan and W3174 are shown (U-V for PPV Vulcan, and U-W for PPV W3174, respectively).

duced 380 bp fragments with M isolates (Marcus and Q1949 isolates) in *P. persica* T_ms of approximately 85.34 °C were observed (results not shown), which is distinct from members of strain D. No cross reactions with the D- and M-specific primers were seen in melting curve analysis of PPV isolates PPV-C, PPV-EA and PPV-W3174 (Figs. 3–5). The universal primers amplified products 74 bp in size (Fig. 1) with T_ms varying from 80.08 to 81.52 °C (Figs. 2–5). They detected all PPV isolates tested, thereby confirming PPV detection when D or M are present (Figs. 2, 4, and 5), or indicating that the virus is a PPV isolate, other than of D or M (Fig. 3). The T_m of the C, EA, and W3174 associated 74 bp fragments produced with the universal forward primer were 80.12, 81.52, and 81.29 °C, respectively (Figs. 3 and 4), and may allow further discrimination of PPV isolates. Amplification of the Nad5 endogenous target (T_m of 82.45–82.63 °C) was included as a control for assessing extraction of amplifiable total RNA, a control for false negatives (Figs. 1–5).

When total RNA from D-infected and M-infected *N. benthamiana* were mixed, simultaneous identification and differentiation of both strains was possible as shown in Fig. 2. In this study total RNA ratios of 1:1 to 1:4 were assessed for both strains and in all cases detection and differentiation were possible.

3.3. Real-time multiplex PCR using LUX primers and melting curve analyses

Simultaneous amplification by real-time PCR of the universal PPV fragment (74 bp), the Nad5 fragment (187 bp), and the D-specific (114 bp) or the M-specific fragment (380 bp), was achieved (Fig. 6). Analysis using the LUX primers resulted in a reduction of the T_m of the D-specific and M-specific amplicons. The D-specific fragment had a T_m ranging from 82.32 °C (woody) to 82.45 °C (herbaceous) (Figs. 6 and 7, respectively). The M-specific amplicon had a T_m of approximately 82.97 °C (Fig. 6). This reduced range resulted in the peak of PPV D overlapping the peak associated with PPV M (Fig. 6). A reduction of the T_m of the Nad5 fragment was also observed; 80.18–80.52 °C for LUX

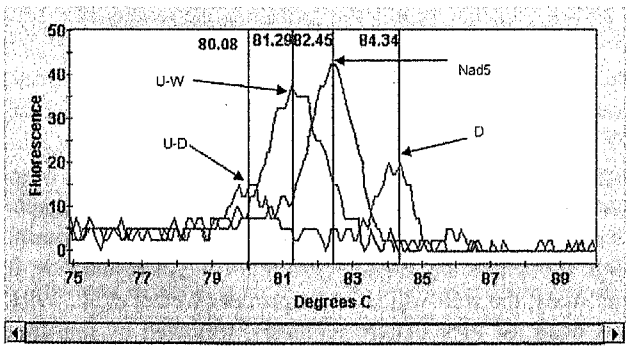

Fig. 4. Multiplex melting curves using SYBR Green I for the detection of Nad5, PPV 2630, and PPV W3174 amplicons, showing differentiation of a D isolate from PPV W3174 isolate, in *N. benthamiana*. T_ms associated with the fragments amplified simultaneously using the universal PPV primer, PPV-U, for isolates 2630 and W3174 are shown (U-D for PPV 2630, and U-W for PPV W3174, respectively).

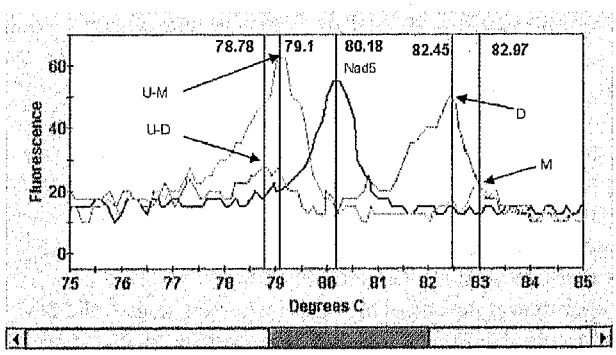

Fig. 6. Multiplex melting curves using LUX fluorogenic primers for the detection of Nad5, PPV D, and PPV M amplicons, showing overlap of the D and M melting peaks. T_ms associated with the fragments amplified simultaneously using the universal PPV primer, PPV-U, for isolates of D and M are shown (U-D for PPV D, and U-M for PPV M, respectively).

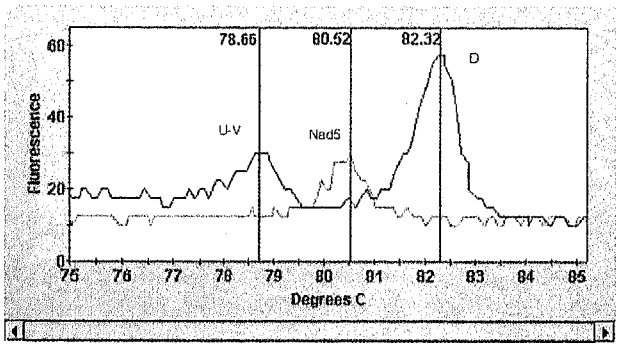

Fig. 7. Multiplex melting curves using LUX fluorogenic primers for the detection of Nad5 and PPV Vulcan amplicons, from *P. persica* (peach). Also, the T_m associated with the fragment amplified simultaneously using the universal PPV primer PPV-U is shown, D indicates PPV Vulcan.

primer analysis (Figs. 6 and 7), compared to 82.45–82.63 °C for the SYBR Green I analysis (Figs. 2–4). In general, the range of T_ms associated with fragments amplified using the LUX primers tended to be narrower, hence less resolution and clarity in interpreting the results.

4. Discussion

Plum pox virus strains D and M are the two most commonly detected groups of the virus, and they differ in their pathogenicity and aggressiveness (Roy and Smith, 1994). Consequently there is a need for strain typing isolates of PPV in any disease management or eradication program. Many of the techniques described previously for strain typing PPV consist of several steps, for example; RT-PCR, gel purification of the PCR products, digestion of the purified PCR products with restriction endonucleases, and subsequent analysis by gel electrophoresis for RFLP pattern analysis (Glasa et al., 2002; Hammond et al., 1998; Wetzel et al., 1991). Real-time PCR with melting curve analysis has been described as a relatively simple and reliable method for simultaneous detection and differentiation of *Mycobacterium tuberculosis* from nontuberculous mycobacterial isolates (Shrestha et al., 2003), differentiation of *Leishmania* species (Nicolas et al., 2002), Noroviruses (Beuret, 2004; Richards et al., 2004), and Taura syndrome virus from Yellow head virus that infect shrimp (Mouillesseaux et al., 2003). This approach to pathogen identification is based on fluorescence melting curve analysis of PCR products, with the T_m being a function of the GC/AT ratio, fragment length, and sequence (Ririe et al., 1997; Nicolas et al., 2002). Since analysis is real-time and can be carried out during the PCR reaction, rather than at the end of the reaction, testing tends to be faster than conventional PCR. Also, the need for analysis by gel electrophoresis using toxic ethidium bromide is eliminated (Ririe et al., 1997).

Mouillesseaux et al. (2003) indicated that fragment length influences T_m values with longer fragments having a higher T_m than shorter fragments. That principle was used in this study, in real-time multiplex PCR with melting curve analysis, to develop a test for rapid and accurate identification of PPV D and PPV M isolates. The D-specific amplicon is 114 bp in size, with a T_m of 84.3–84.43 °C, whereas the M-specific fragment is 380 bp in size, with a T_m of 85.34–86.11 °C, producing distinct melting peaks. The strain-specific T_m was affected by the host species but this did not affect reliable identification of D and M isolates. T_m values are known to be affected by factors such as the concentration of DMSO, magnesium chloride, template DNA, and rate of temperature transition (Giglio et al., 2003; Ririe et al., 1997). The host related response observed in this study may represent a matrix effect since this pattern was observed with identical isolates in herbaceous versus woody hosts. Also, different isolates of the same strain may have slightly different sequences of the amplified fragments, which may contribute to T_m variations. The T_m value is known to be affected by the GC content (Ririe et al., 1997). Universal primers for PPV were included in the reaction, as well as control primers targeting the Nad5 gene. This approach provides; (1) confirmation of PPV detection with strain typing, (2) detection and potential differentiation of PPV isolates other than members of strain D or M, and (3) confirmation that amplifiable total RNA was extracted thereby reducing the possibility of false negative results. Reverse transcription with real-time PCR is more sensitive than ELISA and conventional RT-PCR for PPV detection (Schneider et al., 2004). Another advantage of this approach is that it may facilitate rapid identification of mixed infections.

SYBR Green I is a non-specific fluorescence dye with a high affinity for double-stranded DNA (Witter et al., 1997). The LUX (light upon extension) primer is a self-quenching hairpin primer linked to a fluorophore that fluoresces after hybridization with a specific and complementary target (Nazarenko et al., 2002). In this study, better resolution was obtained for melting curve analysis after real-time multiplex PCR with SYBR Green I, compared to analysis using LUX fluorogenic primers. This agrees with previous reports reviewed by Wilhelm and Pingoud (2003) who described SYBR Green I as producing more precise results or results of similar precision compared to fluorogenic probes. This is encouraging since SYBR Green I is relatively inexpensive and easy to use (Giglio et al., 2003). Another benefit of using SYBR Green I compared to probe-based systems is that false negatives from probes not recognizing their target due to sequence heterogeneity is avoided (Read et al., 2001). Lekanne Deprez et al. (2002) found that the choice of cDNA priming oligo influences the sensitivity of real-time PCR analysis. In this study, the well-validated P1 primer (Wetzel et al., 1991) was used for PPV cDNA synthesis, followed by real-time PCR using primers upstream of the P1 site. This contributed to the development of a very reliable diagnostic test. It is interesting to note that for woody host samples, a higher dilution of cDNA (1:5 compared 1:4 for herbaceous host samples) gave better melting curves. This may indicate the presence of inhibitors in woody host derived cDNA samples.

In conclusion, a relatively simple and reliable real-time multiplex PCR with melting curve analysis procedure was developed for the detection of PPV and identification of isolates of PPV D and PPV M. The T_m associated with isolates of PPV strain D were consistently lower and distinct from the T_m associated with strain M, facilitating easy identification and differentiation of the two strains. If isolates of groups other than PPV D or M are present, this approach has the capacity to confirm the presence of PPV, while at the same time distinguishing them from isolates of PPV D or M. Use of a two-step PCR procedure (denaturation, with annealing and extension combined) further reduced the complexity and duration of the test, and increased the robustness of the procedure. The method described in this study should provide a useful tool for strain typing PPV, and it is possible that similar techniques may be developed for strain identification of other fruit tree viruses. Fruit trees are often infected with multiple viruses (James and Mukerji, 1993; James and Jelkmann, 1998), and real-time multiplex PCR with melting curve analysis may be a simple approach for simultaneous detection of several viruses.

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