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Use of reverse transcription loop-mediated isothermal amplification for the detection of *Plum pox virus*

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Abstract

A one step, accelerated reverse transcription loop-mediated isothermal amplification (RT-LAMP) procedure was developed for the detection of *Plum pox virus* (PPV). The six primers required for accelerated RT-LAMP were designed using a conserved region in the C-terminus of the coat protein coding region of PPV. RT-LAMP was used to detect isolates of five strains of PPV including the strains D, M, EA, C, and W. The virus was detected reliably in both infected herbaceous and woody hosts. RT-LAMP was compared to real-time RT-PCR with SYBR Green I and melting curve analysis, using serial dilutions of total RNA extracts. Similar sensitivities were observed, except that real-time RT-PCR was more consistent at lower template concentrations. The purity of the FIP and BIP primers affected the efficiency of the reaction, and incubation time and template concentration affected the ladder-like pattern observed after agarose gel electrophoresis. Although PPV could be detected after 30 min of incubation at 63 °C, a longer incubation time was required for lower concentrations of the target. RT-LAMP is a very sensitive, low cost diagnostic tool that should be of value in more accurate determination of the distribution of PPV. This should assist in preventing further spread of this devastating virus.

Keywords: *Plum pox virus*; Reverse transcription loop-mediated isothermal amplification; Sensitive; Low cost detection; Primer purity

1. Introduction

Sharka or plum pox is a severe disease of stone fruits (Nemeth, 1986). The disease was first observed around 1917 in Bulgaria on plums (Atanasoff, 1932). The causal agent is *Plum pox virus* (PPV) and its known natural host range of stone fruit species has since expanded to include apricot, peach (Nemeth, 1986), nectarine (Thompson et al., 2001), and cherry (Topchiiska, 1992; Kalashyan et al., 1994). Since 1917 the virus has spread to many countries (Nemeth, 1994), and recent reports describe finding the virus in the USA (Levy et al., 2000), Canada (Thompson et al., 2001), Kazakhstan (Spiegel et al., 2004), China (Navratil et al., 2005), and Argentina (Marini, 2005; Dal Zotto et al., 2006). This means therefore that even over 85 years after its initial detection there is an increase in the spread and/or distribution of PPV. This increase in distribution is being observed despite molecular characterization of the virus (Lain et al., 1989), and the development of serological (Dunez, 1977; Cambra et al., 1994), and molecular diagnostic tools (Varveri et al., 1987; Wetzel et al., 1991, 1992) for PPV detection.

It is possible that in some countries the virus may have been present and undiagnosed for several years. Commonly used diagnostic tools such as ELISA may not be sensitive enough to detect low inoculum levels (Candresse et al., 1994), and more sensitive nucleic acid-based techniques such as conventional RT-PCR and real-time RT-PCR are too expensive for use in routine screening (Martin et al., 2000; James et al., 2006). Sensitive diagnostic techniques that are easy to implement, and of relatively low cost, may contribute to solving this problem.

Loop-mediated isothermal amplification (LAMP) is a diagnostic technique that is sensitive and of relatively low cost. LAMP was developed for high specificity DNA amplification. It is efficient, rapid, and amplification occurs under isothermal conditions (Notomi et al., 2000). The technique utilizes a DNA polymerase with strand displacement activity, and a set of four specially designed primers that recognize six distinct sequences on the target (Notomi et al., 2000). The appeal of this technology is that no expensive thermocycling equipment is required. A simple heat block or water bath able to maintain a temperature of 60–65 °C is all that is required for a LAMP

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assay. Positive reactions can be identified by agarose gel electrophoresis (Notomi et al., 2000), however, DNA or cDNA is generated in such abundance that results may be confirmed by simple observation with the naked eye (Pham et al., 2005; Dukes et al., 2006). The LAMP reaction has since been enhanced by the addition of loop primers (Nagamine et al., 2002). This modification reduces reaction time and has the potential to increase sensitivity. Though developed originally for the amplification of DNA targets, LAMP has been adapted for the detection of viral RNA targets by a process called reverse transcription (RT)-LAMP (Fukuta et al., 2003; Parida et al., 2004; Pham et al., 2005; Dukes et al., 2006).

The PPV genome consists of positive sense single-stranded RNA of approximately 10 kilobases (Lain et al., 1989). In this study a RT-LAMP technique was developed for the detection of PPV. PPV-specific loop primers were designed to achieve improved performance, and RT-LAMP with loop primers was compared to real-time RT-PCR to determine the sensitivity and reliability of RT-LAMP for PPV detection.

2. Materials and methods

2.1. Virus isolates

PPV Fantasia, PPV Vulcan (James and Upton, 2001), and PPV 2630 are D type isolates of PPV detected in Canada. These isolates were tested in the original hosts, nectarine, clingstone peach, and Redhaven peach, respectively. Also, PPV 2630 was mechanically sap-transmitted from peach (*Prunus persica* var. Redhaven) to *Nicotiana benthamiana.* The virus isolate W3174 is a Canadian isolate of PPV, detected in plum (*P. domestica*) and mechanically sap-transmitted to *N. benthamiana*. This isolate represents a new strain of PPV, strain W (James and Varga, 2005). PPV D was obtained from W. Jelkmann, Germany; PPV M, type member Marcus strain, was obtained from F. Dosba, France; and 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 *N. benthamiana*.

2.2. Isolation of total RNA

Total RNA was extracted as described by James et al. (2003). Briefly, for extractions from *N. benthamiana* or woody tissue, 100 mg fresh tissue or 10 mg freeze dried tissue was used as starting material. Total RNA was eluted from the Qiagen RNEasy® columns with 35 μL of PCR-grade MilliQ H_2O. Approximately 1 μL (800 ng) of total RNA was diluted in 9 μL MilliQ H_2O and 5 μL of this dilution was used for each LAMP reaction. Extractions were stored long term at −80 °C.

2.3. RT-LAMP primer design and primer synthesis considerations

A conserved region in the C-terminus of the coat protein coding region was selected for primer design. The Canadian PPV isolate Fantasia (PPV-Fan) (accession AY912056.1) was used as the reference sequence. LAMP primers were designed within nucleotide positions 9153–9350 of PPV-Fan (Fig. 1) using PrimerExplorer V3 software, from Eiken Chemical Co. Ltd., Japan (http://primerexplorer.jp/elamp3.0.0/index.html) with default settings. Two sets of primers were designed initially. However, based on some initial experiments to assess range of detection, one set of primers was selected for further evaluation and use for broad spectrum PPV detection. Primer details are listed in Table 1. The relative locations of primers across various PPV strains including isolates of D, M, EA, C, and W are detailed in a multiple alignment shown in Fig. 1.

Primer quality appears to be an important factor for successful LAMP amplification. Also, batch to batch variation in synthesis can affect reactivity. During the course of the optimization process two batches of the same primers were used. The second batch of primers did not exhibit the same reactivity as the first batch. As these initial primers were only desalted, the purity and quality of the primers, especially the FIP and BIP (48- and 46-mer, respectively) were of concern. New primers of greater purity were obtained from two suppliers: Sigma–Genosys, Canada (Oakville, Ont.) and Integrated DNA Technologies (IDT, Coralville, IA). A full set of primers were obtained from Sigma–Genosys with the FIP and BIP primers Reverse-Phase HPLC purified, while the remaining primers were purified using their Reverse-Phase Cartridge method. A second set of FIP and BIP primers only were obtained from IDT. These were purified using their Rapid HPLC method. Primers were re-suspended in TE pH 8.0 and stored at −20 °C prior to use. Working primer stocks were used a maximum of 2 weeks.

2.4. Optimization of RT-LAMP amplification

Many parameters were examined during the optimization of the RT-LAMP amplification. These include: (1) amount of total RNA (neat versus serial dilutions of template in RNase-free water), (2) type and amount of reverse transcriptase (SuperScript™ III, Thermo-X™, or Cloned AMV (Invitrogen Burlington, Ont.)), (3) a one temperature combined RT-LAMP reaction or a two step procedure (an RT step then a LAMP step) with gradient temperature evaluations, depending on optimal temperatures for cDNA synthesis (40–61 °C) and *Bst* DNA polymerase activity, (4) temperature gradient (59–65.3 °C) to identify optimal temperature for *Bst* DNA polymerase activity in LAMP amplification of PPV, (5) addition or exclusion of RNaseOUT™ (Invitrogen), (6) $MgSO_4$ concentration (2–10 mM), (7) time of LAMP incubation (30, 40, 60, 80, 100, 120, 240, 360 min), (8) dNTP concentration (1.4–3.0 mM), and (9) addition of loop primers.

2.5. RT-LAMP amplification set-up and reaction conditions

Components and concentrations of the RT-LAMP mastermix are detailed in Table 2. Assembly of all components were performed in a LABCONCO Purifier® Filtered PCR Enclosure (Kansas City, MO) using dedicated PCR pipettors and gamma-irradiated filter tips. Isothermal amplifications were performed using both the Mastercycler® personal and gradient thermal

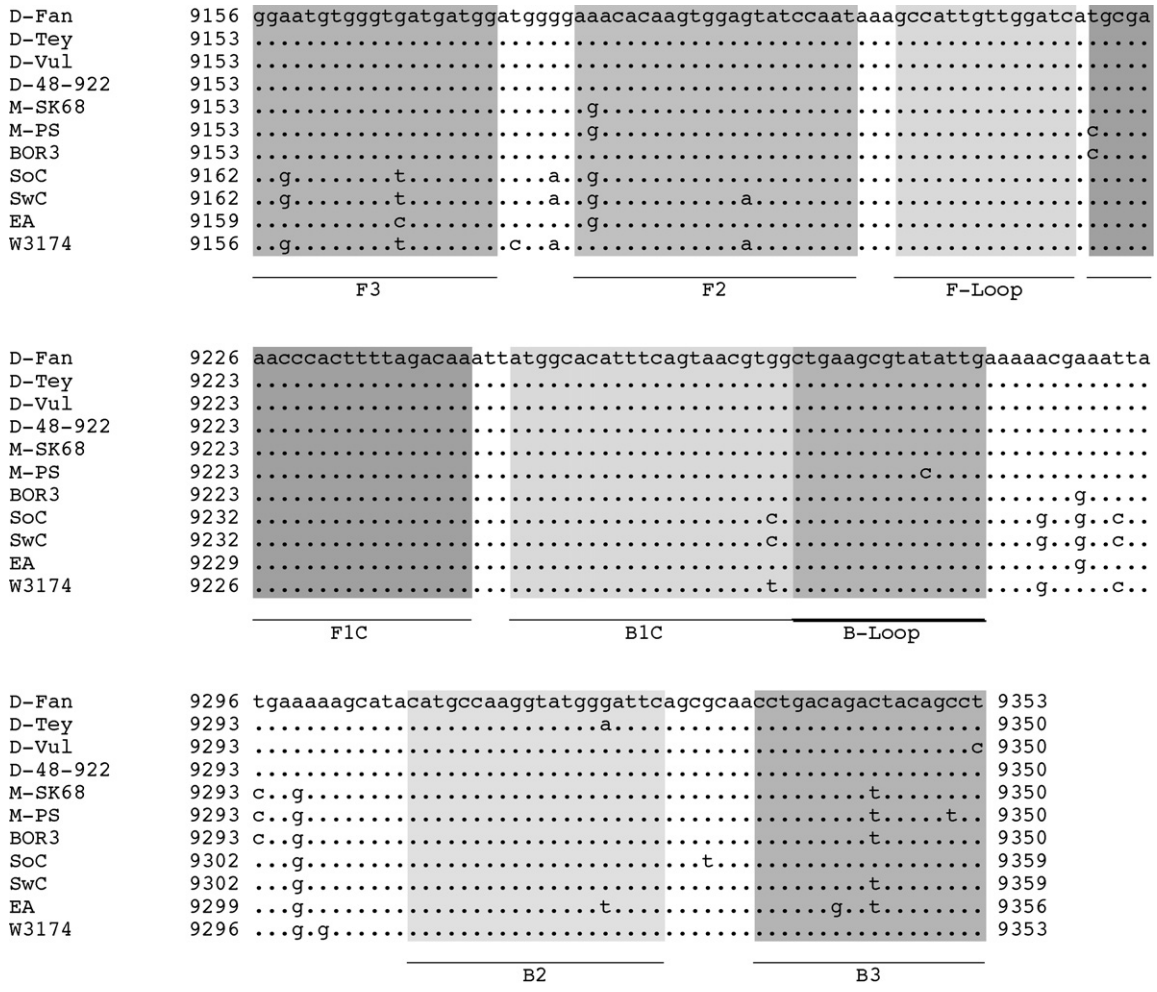

Fig. 1. Alignment of the C-terminus of the coat protein coding region of selected *Plum pox virus* isolates of the strains D, M, EA, C, Rec, and W, showing the location of the primers used for RT-LAMP amplification. The accession number of isolates used in the alignment are as follows: D isolates Fan (AY912056.1), Tey (X16415.1), Vul (AY912057.1), 48-922 (AY912058.1); M isolates SK68 (M92280.1), PS (AJ243957.1); Rec isolate BOR3 (AY028309.2); C isolates SoC (AY184478.1), SwC (Y09851.2); EA isolate EA (AM157175.1); and strain W isolate W3174 (AY912055).

cyclers from Eppendorf (Hamburg, Germany). Two incubation regimes were compared: (1) a one temperature incubation (63 °C for 1 h), and (2) a two temperature incubation (50 °C for 10 min followed with 1 h at 63°). The block was preheated in each regime and both regimes were followed by a 10 min incubation at 80 °C and a final hold at 12 °C. After comparisons were made, the one temperature regime was used for further studies. Electrophoresis was performed directly following completion of amplification, or stored at −20 °C.

2.6. Interpretation of RT-LAMP results

RT-LAMP amplification products (17 μL) were routinely visualized with agarose gel electrophoresis (1.5% agarose,

Table 1
Attributes of primers used for the RT-LAMP detection of *Plum pox virus*

Primer name	Type	Length	Genome position[a]	Sequence (5′–3′)
F3	Forward outer	19-mer	9153–9171	GGAATGTGGGTGATGATGG
B3	Backward outer	18-mer	9332–9349	AGGCTGTAGTCTGTCAGG
FIP	Forward inner (F1C + TTTT + F2)	48-mer	9218–9239, 9178–9199	TTGTCTAAAAGTGGGTTTCGCATTTT AAACACAAGTGGAGTATCCAAT
BIP	Backward inner (B1C + TTTT + B2)	46-mer	9243–9264, 9305–9324	ATGGCACATTTCAGTAACGTGGTTTT GAATCCCATACCTTGGCATG
F-Loop	Forward loop	14-mer	9203–9216	GATCCAACAATGGC
B-Loop	Backward loop	15-mer	9265–9279	CTGAAGCGTATATTG

[a] Genome position refers to reference molecule PPV Fantasia, accession AY912056.1.

Table 2
Components, concentrations, and supplier information of reagents used for the RT-LAMP amplification

Component (concentration) (supplier)	Volume (μL) per reaction
Autoclaved PCR-grade MilliQ™ water	1.98
[a]10× ThermoPol reaction buffer (New England Biolabs, Ipswich, MA)	2.5
[a]50 mM $MgSO_4$ (6 mM final) (Invitrogen, Burlington, Ont.)	3
5 M Betain (0.8 M final) (Sigma–Aldrich Canada Ltd., Oakville, Ont.)	4
25 mM dNTP's (1.4 mM final) (Invitrogen)	1.4
F3 5 μM (0.2 μM final) (Sigma–Genosys, Canada, Oakville, Ont.)	1
B3 5 μM (0.2 μM final) (Sigma–Genosys, Canada)	1
FIP 40 μM (1.6 μM final) (Sigma–Genosys, Canada)	1
BIP 40 μM (1.6 μM final) (Sigma–Genosys, Canada)	1
F-loop 20 μM (0.8 μM final) (Sigma–Genosys, Canada)	1
B-loop 20 μM (0.8 μM final) (Sigma–Genosys, Canada)	1
RNaseOUT™ recombinant ribonuclease inhibitor 40 U/μL (0.8 U) (Invitrogen)	0.02
SuperScript™ III reverse transcriptase 200 U/μL (final 20 U) (Invitrogen)	0.1
Bst DNA polymerase 8 U/μL (8 U final) (New England Biolabs)	1

[a] Total [$MgSO_4$] is 8 mM as the reaction buffer contains an additional 2 mM.

TBE). In addition, direct visual inspection of the reaction tube after the addition of SYBR green I® (Molecular Probes) (1:1000TE, v/v) under regular or UV light was evaluated, as well as examining the presence or absence of white pyrophosphate precipitate in the reaction tube following centrifugation.

2.7. Sensitivity comparison of RT-LAMP to real-time RT-PCR with SYBR green melt curve analyses

Sensitivity comparisons were performed between the accelerated RT-LAMP amplification and a real-time RT-PCR assay developed for PPV detection (see Varga and James, 2006 for reaction details). Briefly, a single extraction of PPV-Fantasia was used to make a dilution series (in water). This single dilution series was used as template for the two amplifications which were set-up sequentially. Results obtained from gel electrophoresis (run on the same gel) and melt curve analyses (for the real-time assay) were compared.

3. Results

3.1. Optimal conditions for RT-LAMP amplification

Table 2 summarizes the reagents and final concentrations that were used for reliable RT-LAMP detection of PPV in herbaceous and woody hosts. Serial dilutions of tRNA (neat/undiluted to $1{:}10^{-10}$) were used to identify the range of concentrations that may be used for reliable amplification. Although detectable at dilutions of 1:1000, 1:10 dilution was used as the default concentration for both woody and herbaceous hosts, since this dilution provided consistent results for all strains and isolates, in both host types. All reverse transcriptase enzymes evaluated in this study were similar in their activity so SuperScript™ III was selected for routine use (Table 2).

Comparisons were made between the one and two step temperature amplification regimes. As amplifications were similar, as determined by electrophoretic analysis (data not shown), the one step temperature regime was adopted for all subsequent studies. Of the range of temperatures assessed 63 °C was found to be optimal for *Bst* DNA polymerase activity and PPV amplification under the conditions of this study. RNaseOUT™ enhanced the activity of the reaction and was added to all subsequent reactions (Table 2). Also, addition of the forward and backward loop primers (Table 1), enhanced the reaction in both herbaceous and woody hosts, reducing the time required for reliable amplification of isolates of all five strains tested.

The typical ladder-like pattern of a RT-LAMP reaction was influenced by incubation time. Generally, shorter reactions showed more typical ladder-like patterns. Incubation times longer than an hour showed a reduced ladder-like pattern with a predominance of shorter fragments (Figs. 2 and 4). Longer incubation times also improved detection of more dilute samples (Fig. 4). However, incubation times that were greater than

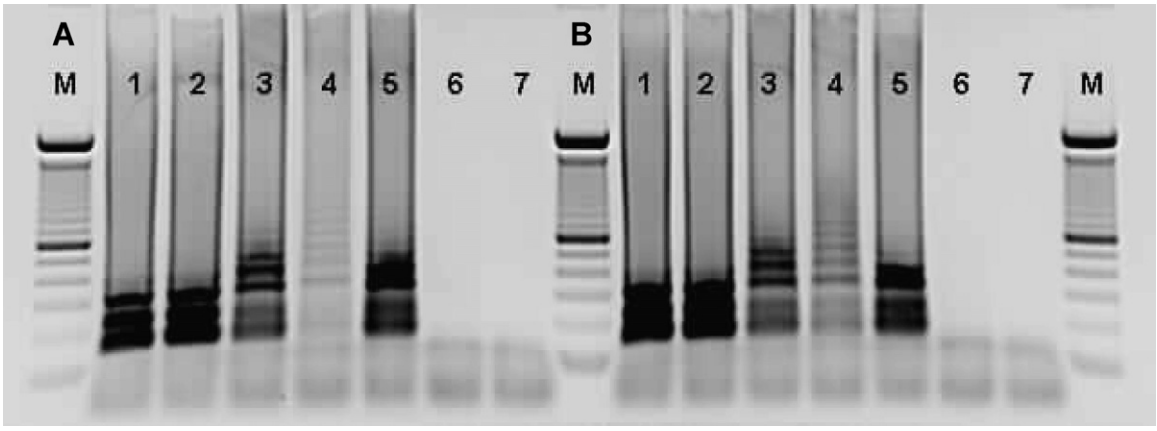

Fig. 2. Gel separation (1.5% agarose in TBE) of RT-LAMP amplicons for isolates of five strains of PPV, all in the herbaceous host *Nicotiana benthamiana*. The reactions were incubated at 63 °C, for 30 min (A), and at 63 °C for 40 min (B). Lanes M, 100 bp DNA ladder; lane 1, PPV D; lane 2, PPV M; lane 3, PPV C; lane 4, PPV EA; lane 5, PPV W; lane 6, healthy *N. benthamiana*; lane 7, water control.

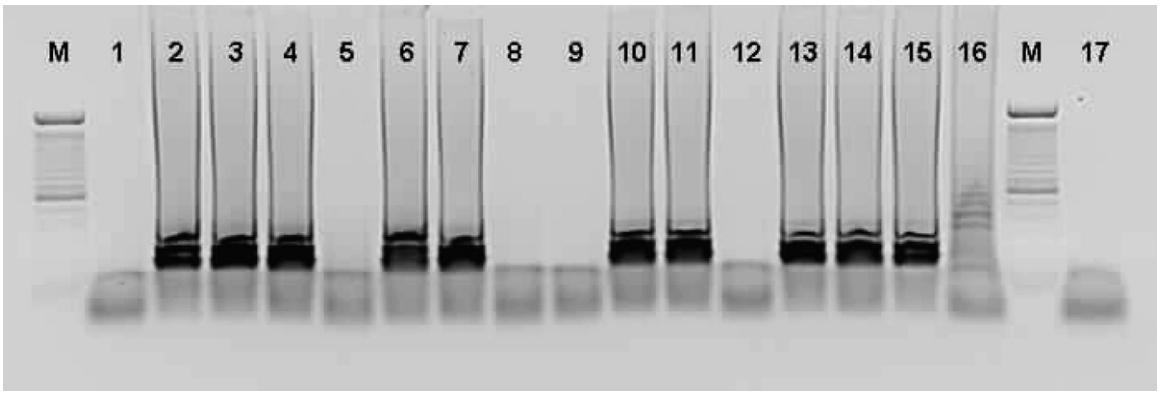

Fig. 3. Gel separation (1.5% agarose in TBE) of RT-LAMP amplicons showing typical patterns observed and some non-specific amplification in a healthy GF305 peach. Lanes M, 100 bp DNA ladder; lane 1, water control, no template; lane 2, PPV D in *Nicotiana benthamian*; lane 3, PPV M in *N. benthamiana*; lane 4, PPV C in *N. benthamiana*; lane 5, healthy *C. quinoa*; lane 6, PPV EA in *N. benthamiana*; lane 7, PPV W3174 in *N. benthamiana*; lane 8, healthy *N. benthamiana*; lane 9, healthy Shiro plum (*P. salicina*); lane 10, PPV Fan in Fantasia nectarine (*P. persica*); lane 11, PPV Vul in Vulcan clingstone peach (*P. persica)*; lane 12, healthy St. Julien plum (*P. instititia*); lane 13, PPV 48-922 in Redhaven peach (*P. persica*); lane 14, PPV W3174 in plum (*P. domestica*); lane 15, PPV 2630 in peach (*P. persica*); lane 16, healthy GF305 peach (*P. persica*); and lane 17, water control, no template.

optimal (2 or 3 h) resulted in an increase in non-specific amplification with healthy controls sometimes generating false positive amplifications (data not shown).

3.2. Interpretation of RT-LAMP amplification

The most reliable process for evaluating reaction results was by electrophoretic analysis (see Figs. 2–4). Visual inspection of a color change with SYBR green I addition (under visible or UV light) did occur with positive samples (data not shown), however, interpretation was sometimes difficult as primers would also interact with the stain, giving a weak signal. The presence of a white pyrophosphate precipitate following centrifugation was not as reliable or distinct as electrophoretic separation. As a result, all amplifications were run on a gel.

3.3. Specificity of RT-LAMP for PPV detection

This RT-LAMP assay was able to detect isolates of all five PPV strains tested in this study, in both herbaceous and woody tissue (Figs. 2 and 3). Isolates that were detected in woody tissue include PPV-Fan, PPV-Vul, PPV-48-922, and PPV-2630 (Fig. 3). These are all isolates of the strain D. Isolate W3174, strain W, was detected reliably in both herbaceous and woody tissue. Fig. 1, lane 5 shows the detection of isolate W3174 in *N. benthamiana*.

Several healthy hosts were included to assess amplification specificity. These healthy controls included the herbaceous species *Chenopodium quinoa*, *N. benthamiana*, and *N. occidentalis*. Healthy woody species included GF305 peach (*Prunus persica*), Bing cherry (*P. avium*), Colt (*P. avium x pseudocera-*

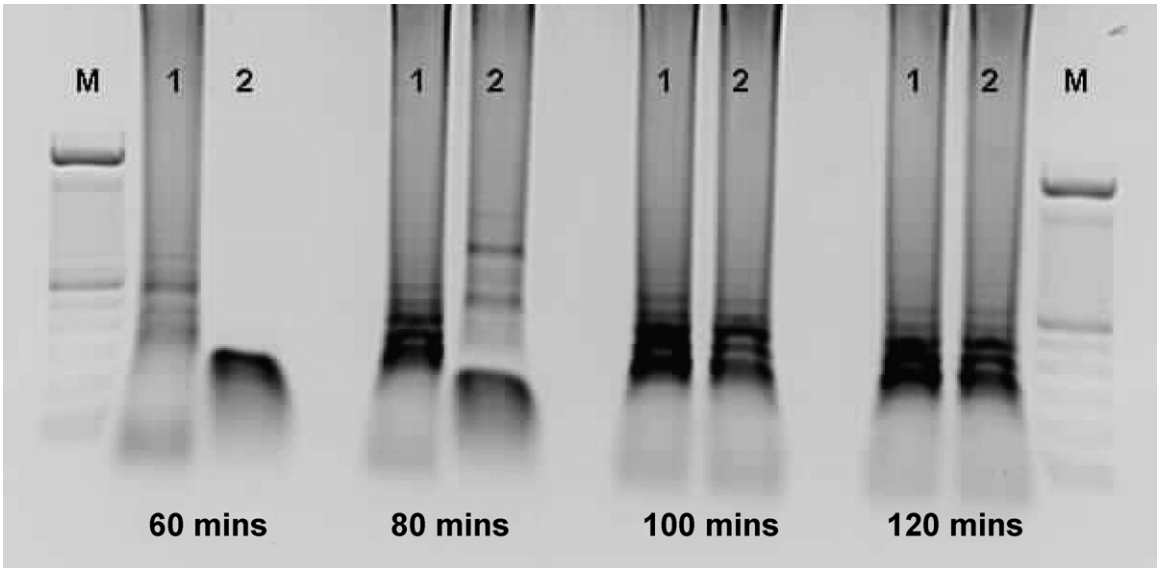

Fig. 4. RT-LAMP amplification of PPV Vul in the woody host Vulcan clingstone peach (*P. persica*), using 2 dilutions of PPV total RNA extractions, and incubated for 60, 80, 100, and 120 min. Lane M contains a 100 bp DNA ladder. Sample 1 (indicated as lane 1) consists of a 1:1000 dilution, and sample 2 (indicated as lane 2) consists of 1:10,000 dilution. A single master mix was used for all samples and reactions were set up at the same time. Samples were incubated during the time course and the reactions were stopped individually by incubation at 80 °C for 10 min, then stored on ice prior to electrophoresis.

sus), Shiro plum (*P. salicina*) and St. Julien plum (*P. institutia*). Some results are shown in Fig. 3. Specificity of the LAMP primers was high and healthy samples routinely showed no amplification. Infrequently a healthy sample would show weak amplification (Fig. 3, lane 16, healthy GF305 peach). Non-template controls (water) showed no amplification (Fig. 2A and B, lane 7; and Fig. 3, lanes 1 and 17).

3.4. Sensitivity of RT-LAMP amplification compared to real-time RT-PCR with melt curve analyses

Sensitivity comparisons between the RT-LAMP amplification (with loop primers) and real-time RT-PCR with melt curve analyses showed similar results, based on gel electrophoresis and melting curve analyses (data not shown). Using a dilution series of total RNA extracted from PPV-Fan infected Fantasia nectarine, positive RT-LAMP amplifications were reliably observed for dilutions of 1:100 with weak and inconsistent positives for some replicates at dilutions of 1:1000 and 1:10,000. Real-time melt curve analyses gave more consistent positive results up to a 1:10,000 dilution (data not shown).

3.5. Consideration of primer quality (synthesis, storage, and degradation)

This RT-LAMP assay was affected by primer quality, with variability from batch to batch. This variability seemed to be associated in particular with the purity of the FIP and BIP primers. The higher purity, HPLC purified FIP and BIP, primers gave more consistent and reliable amplification. Consistent and reliable results were achieved also by ensuring that aliquots (in TE) were stored at −20 °C and that working stocks were used for a maximum of 2 weeks.

4. Discussion

Despite a considerable increase in our understanding of the plum pox (Sharka) disease, increased knowledge of the mechanisms of distribution, and molecular characterization of the causal agent (PPV), the virus continues to spread or new regions of geographic distribution continue to be identified. Sensitive but accessible and cost effective diagnostic tools can contribute to more reliable routine diagnosis of PPV. This is true especially in areas where limited resources are available for plant virus indexing programs. Sensitive and cost effective tools would better assist in an accurate determination of the global distribution of PPV and speedier containment of this devastating virus. RT-LAMP is a good candidate since it meets both requirements. Parida et al. (2004) found that the accelerated RT-LAMP (Nagamine et al., 2002) was 10-fold more sensitive than regular RT-PCR for the detection of West Nile virus. Real-time RT-PCR is one of the most sensitive techniques for PPV detection (Schneider et al., 2004), and RT-LAMP was found to be as sensitive as real-time RT-PCR (Pham et al., 2005; Dukes et al., 2006). The results of this study support these findings. RT-LAMP was found to be a much more rapid technique of comparable sensitive to real-time PCR using TaqMan® probes for the detection of foot-and-mouth disease virus (Dukes et al., 2006). Accelerated RT-LAMP detected 10 copies of the foot-and-mouth disease virus template in 22.2 min, while it took 55 min by real-time PCR. The sensitivity of RT-LAMP resulted in infrequent false positive results, in the present study, with some healthy samples, presumably representing contamination events. Careful RT-LAMP analysis is required for accurate results.

An accelerated RT-LAMP procedure was developed for reliable PPV detection. The technique was used to detect isolates of five strains that included the strains D, M, EA, C, and W. The strain PPV Rec was not available for analysis. However, PPV Rec is the product of recombination between PPV D and PPV M (Glasa et al., 2004). The 3′ end of PPV Rec is derived from PPV M and this segment includes the coat protein coding region. The PPV specific RT-LAMP developed in this study targets a conserved region in the C-terminus of the coat protein coding region. M strain isolates included in this study were detected so it means that isolates of PPV Rec should be detected using this technique. This was confirmed by virtual analysis using GenBank sequence data (accession AY028309.2). The simplicity of the procedure was improved by showing that a one step RT-LAMP procedure (63 °C for both RT and LAMP) is just as reliable as a two step procedure with RT at 42 °C, and the LAMP reaction at 63 °C. This reduces time and simplifies the procedure.

RT-LAMP requires no complex or expensive thermocycler equipment for amplification (Notomi et al., 2000). A regular water bath or heat block capable of maintaining the isothermal conditions is all that is required (Pham et al., 2005). Amplification products may even be detected with the naked eye (Mori et al., 2001; Pham et al., 2005). This makes the LAMP technique a very accessible technology with primer design being the main challenge. The four basic primers required for LAMP amplification recognize six sites on the template for amplification, which confers great specificity to the technique (Notomi et al., 2000). This specificity increases with the addition of two loop primers for an accelerated LAMP reaction (Nagamine et al., 2002). The reliability of the LAMP amplification was enhanced by the addition of the loop primers. In this study, addition of the loop primers reduced reaction time with a concomitant increase in sensitivity.

Several factors affected the reliability of RT-LAMP analysis in this study. HPLC purified FIP and BIP primers were more efficient and reliable than FIP and BIP primers that were obtained with a desalted level of purity. The purity of the other primers was not as critical and desalting seems adequate. Incubation time affected the ladder-like pattern observed after agarose gel electrophoresis, with shorter incubation times (≤60 min) giving the typical patterns. This was dependent on template concentration. However, longer incubation times (over 60 min) gave more reliable results with dilute samples.

In summary, an accelerated RT-LAMP procedure was developed for the detection of PPV in herbaceous and woody hosts. Isolates of five strains of the virus were detected reliably using this assay. The sensitivity of the procedure was similar to that of a real-time PCR for PPV detection (Varga and James, 2006), which is in agreement with the findings of Pham et al. (2005) and

Dukes et al. (2006). Consequently, careful analysis is required to avoid false positive results because of this level of sensitivity. No significant differences were found between a two step procedure (different temperatures for RT and LAMP), versus a one step reaction (RT and LAMP reactions both at 63 °C). This indicates that SuperScript™ III is still functional at temperatures above 42 °C. This is similar to the findings of Parida et al. (2004), and Nie (2005). This relatively simple and sensitive technique should have wider application, because of its low cost and minimal equipment requirements, for more accurate indexing for PPV and other plant viruses. This could be invaluable in better understanding the distribution and limiting the spread of this undesirable virus. This simple yet very sensitive diagnostic tool is suitable for use with field-collected samples, and for use in developing countries with limited resources (Poon et al., 2004; Soliman and El-Matbouli, 2006).

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