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DETECTION OF POTATO VIRUS Y (PVY) BY REVERSE-TRANSCRIPTION LOOP-MEDIATED NUCLEIC ACID AMPLIFICATION (RT-LAMP)

ABSTRACT

Potato virus Y (PVY), a type member of the genus Potyvirus (family *Potyviridae*), is currently the most important virus infecting the potato crop. PVY is also a dangerous pathogen of the tomato, pepper, and tobacco. The reverse transcription loop-mediated amplification (RT-LAMP) is gaining recognition as a good alternative to RT-PCR in diagnosing plant viruses. Here, we provide a detailed description of a simple protocol for fast and sensitive detection of PVY by the RT-LAMP assay, which can be easily adapted to detect other plant pathogens, harboring both RNA and DNA genomes.

Key words: detection, plant pathogen, RT-LAMP

INTRODUCTION

The loop-mediated nucleic acids amplification (LAMP) is based on an isothermal amplification of the target nucleic acid by auto cyclic strand displacement at a single and constant temperature. The detection of RNA is accomplished with addition of a reverse transcriptase to the LAMP reaction (Notomi *et al.*, 2015). The method uses 4 or 6 primers that recognize 6 to 8 specific DNA regions. That makes LAMP highly specific. The primers include inner, external, and loop pairs. The inner primers are called FIP (Forward Inner Primer) and BIP (Backward Inner Primer). Their sequences are complementary to two different locations in the sense and antisense strand. The resulting primary product of their extension forms single-stranded loops on both ends. The external primers F3 (Forward) and B3 (Backward) are complementary to the regions sur-

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rounding the fragment amplified with the inner primers. These primers are shorter and their concentration is lower in the reaction. Thus, their hybridization to the template is slower than that of the inner primers. External primers initiate strand displacement in duplex DNA. Loop primers are used to increase the speed and sensitivity of the LAMP assay. The primers are designed for the DNA regions that oscillate between double-stranded and single-stranded state 60-65°C. Thus, the primers can anneal to the target strands without thermal denaturation of the DNA. The primer extension is performed by a DNA-dependent DNA polymerase with strand displacement activity. A simplified scheme of the LAMP reaction is shown in Fig. 1. The details of the molecular mechanism of LAMP can be learned from the website of the Eiken Chemical Co., Ltd. (http://loopamp.eiken.co.jp/e/lamp/anim.html).

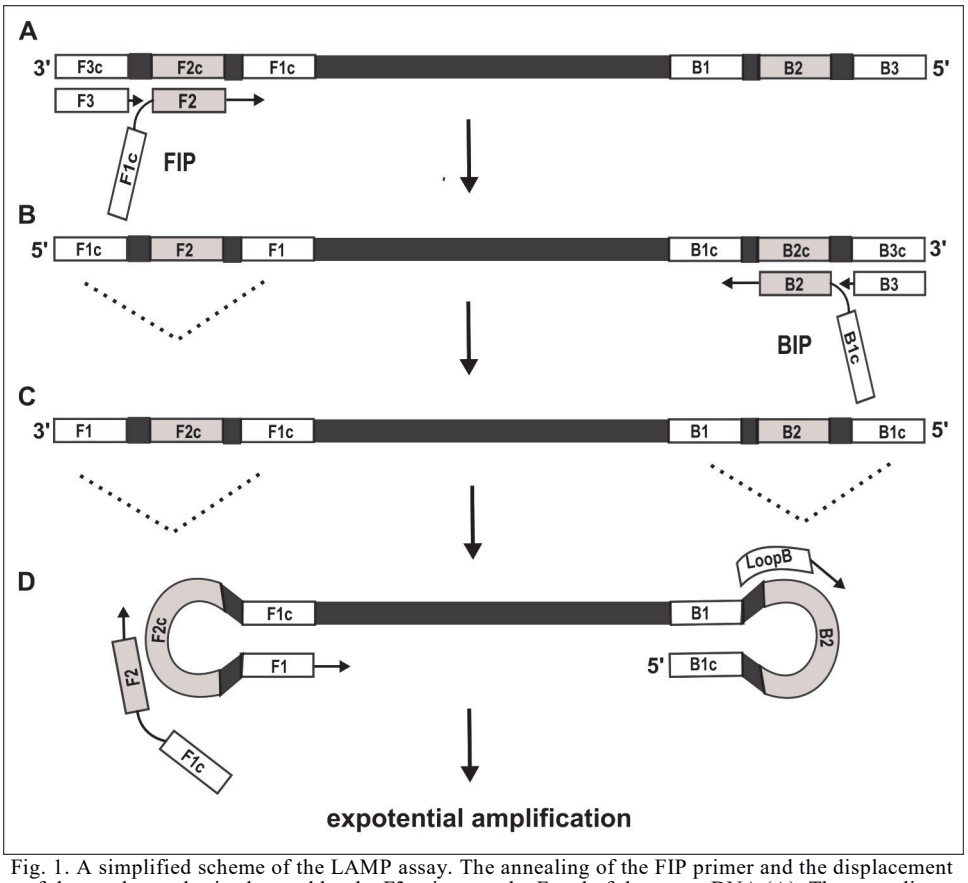

Fig. 1. A simplified scheme of the LAMP assay. The annealing of the FIP primer and the displacement of the newly synthesized strand by the F3 primer at the F end of the target DNA (A). The annealing of the BIP primer and the displacement of the nascent strand by the B3 primer at the B end of the DNA strand synthesized in A (B). The DNA strand synthesized in B before (C) and after (D) the formation of loop structures. The dotted lines indicate complementary regions in the DNA strand. The arrows indicate the direction of the strand extension. The structure shown in D is a basic unit undergoing further exponential amplification. An analogous process to that shown in steps A-D is initiated simultaneously at the B end of the target DNA.

LAMP allows rapid and highly efficient amplification of the DNA. Under optimal conditions, it can be accomplished in 15-30 min. The amount of DNA produced during LAMP is so high that the deposition of the byproduct of the reaction – magnesium pyrophosphate is visible to the naked eye as opaque turbidity in the positive samples. Consequently, the LAMP is suitable for developing a rapid test in which pathogens can be detected directly in the field. To further increase the sensitivity of the LAMP, many variants have been developed based on monitoring turbidity, color change or fluorescence, in both end-point and kinetic (real time) forms. In most of these tests, a positive result can be determined with the naked eye, but measuring instruments facilitate quantitative analysis of the results. The fluorescent real-time RT-LAMP has a number of advantages compared to the real-time RT-PCR. While providing a similar level of sensitivity of detection, it is more resistant to inhibitors of polymerases (Kaneko *et al.*, 2007), easier to perform, and faster (Tomlinson *et al.*, 2013, Przewodowska *et al.*, 2015). In the last two years, over 5,000 publications have appeared, describing the LAMP and RT-LAMP for detecting bacterial, fungal and viral pathogens of animals and plants (according to Google Scholar). During the last decade several variants of RT-LAMP have been developed to detect PVY (Nie, 2005; Almasi and Dehabadi, 2013; Hasiów–Jaroszewska *et al.*, 2015; Przewodowska *et al.*, 2015, Treder *et al.*, 2018) as well as other plant viruses (Varga and James, 2006; Ahmadi *et al.*, 2013; Tomlinson *et al.*, 2013; Hasiów–Jaroszewska and Borodynko, 2013; Shen *et al.*, 2014; Budziszewska *et al.*, 2016) and *Potato spindle tuber viroid* (Lenarčič *et al.*, 2012). The LAMP protocols have also recently been published for *Phytophtora infestans* (Hansen *et al.*, 2016; Khan *et al.*, 2017; Si Ammour *et al.*, 2017), *Pectobacterium carotovorum* (Yasuhara-Bell *et al.*, 2016) *Pectobacterium atrosepticum* (Hu *et al.*, 2016), *Dickeya* spp (Yasuhara-Bell *et al.*, 2017) and *Ralstonia solanacearum* (Lenarčič *et al.*, 2014).

Here, we describe a detailed procedure of an RT-LAMP assay for sensitive detection of the most important potato virus – PVY. The preferred format of the assay is fluorescent (Fig. 2), but an option for colorimetric detection (Photo 1) according to Goto *et al.* (2009) is also described. The provided conditions should work well with primer sets specific to other potato-infecting pathogens. In our laboratory, the protocol is routinely used not only to detect PVY but also for detecting PLRV, PVM, PVS, and PSTVd.

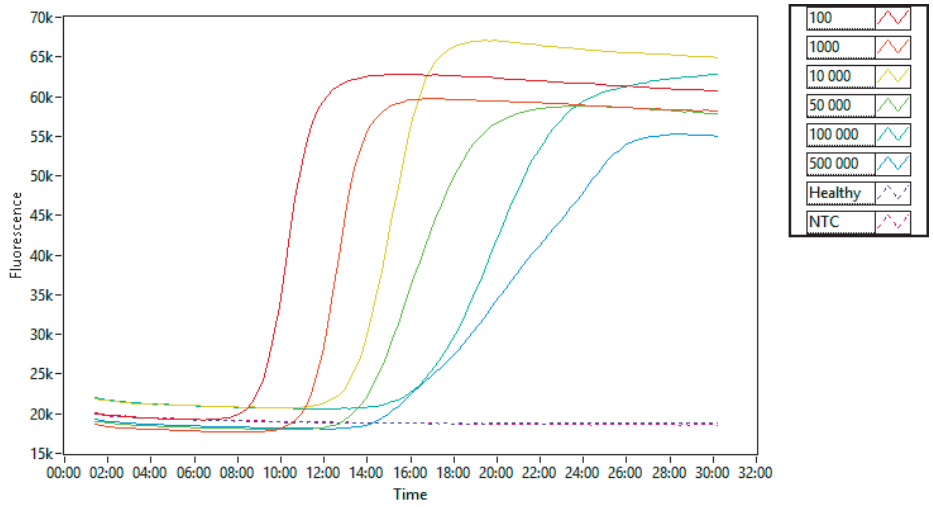

Fig. 2. Fluorescent detection of PVY by RT-LAMP. The amplification curves were recorded in Genie II. The total RNA was purified from dilutions of potato sap extracted from a virus-infected plant. The non-template control reaction (NTC) was supplemented with water instead of RNA. The negative control reaction (Healthy) was supplemented with the RNA isolated from 100-fold diluted virus-free sap. A PBS buffer was used to prepare sap dilutions in the range from 100-fold to 500 000-fold. The RNA was purified according to Zacharzewska *et al.* (2014). PVY was detected in all tested dilutions.

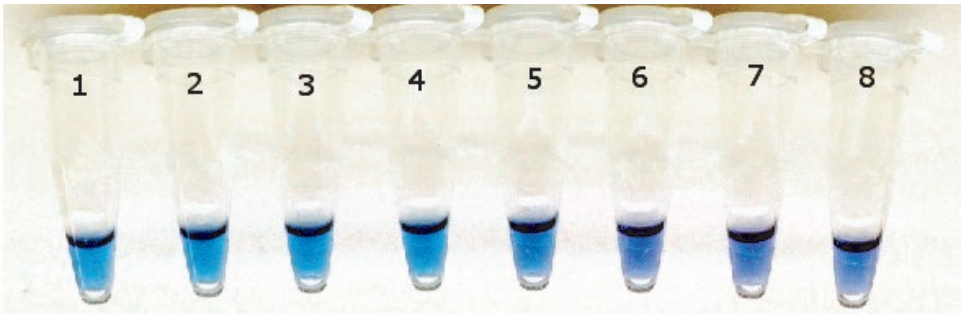

Photo 1. Colorimetric detection of PVY by RT-LAMP. The same samples as in Fig. 2 were amplifiedin the presence of HNB dye. The dilutions of sap in the range from 100-fold to 500 000-fold – tubes 1-6. A healthy control – tube 7. NTC – tube 8. PVY was detected in the sap diluted up to 50 000-fold (tubes 1-4).

MATERIALS AND REAGENTS

1) A source of ice or cooling stands to store the samples and mixes at 0°C during RNA isolation and setting up the reaction.
2) PCR plates (with a sealing film) or tubes appropriate for instrumentation.
3) Kit for isolation of RNA from plant tissue.
4) RT-LAMP Isothermal Amplification Kit. (e.g., Novazym Polska S.A.,

cat. No RT-LAMP-02). (see note 1).

5) PVY specific oligonucleotide primers (see note 2). Prepare 100 µM stocks in nuclease-free H_2O or nuclease-free 1× TE buffer:
   a) F3: 5'-TGC CAA CTG TGA TGA ATG G-3'
   b) B3: 5'-GTT CGT GAT GTG ACC TCA TAA-3'
   c) FIP: 5'-GCA TTC TCA ACG ATT GGT ACG GAG TTT GGG TTA TGA TG-3'
   d) BIP: 5'-GCA AAT CAT GGC ACA TTT CCG TGG CAT ATA TGG TTC CTT-3'
   e) LF: 5'-CAA TGG GTA TTC GAC TTG TTC A-3'
   f) LB: 5'-TCA GAT GTT GCA GAA GCG T-3'
6) Nuclease-free molecular-biology-grade water.
7) Hydroxynaphthol blue (HNB) for colorimetric detection (optional, see Recipes).

EQUIPMENT

1) A microcentrifuge with rotors for 0.2 ml tubes and/or PCR strips, 1.5-2 ml tubes and PCR plates.
2) A heating block, a thermal cycler, or a water bath for colorimetric detection.
3) A microplate spectrophotometer facilitating UV-Vis absorbance measurements in 1-2 µl samples (e.g., Epoch™ Microplate Spectrophotometer with Take3 plate, BioTek, or NanoDrop Thermo Fisher Scientific)
4) A real-time thermal cycler (e.g., CFX96 Touch™ Real-Time PCR Detection System, BioRad Laboratories, Inc., or Genie® II/III Ultra rapid amplification instruments, OptiGene Ltd.) for fluorescent detection.

PROCEDURE

1) Sample preparation
   a) Extract the total RNA from 50-100 mg of potato tissue (young leaves, rose end of tubers) according to the chosen procedure (see note 3).
   b) Use a microplate spectrophotometer or NanoDrop to test the quality and quantity of RNA.
   c) Use directly or store the RNA samples in -80°C for future use.
2) Preparation of LAMP primer mixes
   a) Pulse-spin the tubes containing freeze-dried oligonucleotides in a microcentrifuge and then reconstitute with nuclease-free water or nuclease-free 1xTE buffer to get 100 µM stocks. These stocks should be aliquoted and stored at -20°C for long-term storage (indefinitely).
   b) Prepare 20 µM stocks of each oligonucleotide primer by pipetting 20 µl of the 100 µM stocks into tubes (a separate tube for each oligonucleotide) containing 80 µl of nuclease-free water. Mix well and pulse–spin.
   c) Prepare the working mix of oligonucleotide primers for the RT-

LAMP reaction in one tube (final volume of the working mix for one RT-LAMP reaction equals to 2.63 µl):

• Reaction number	1	96*
• 20 µM Fip	0.750 µl	75.0 µl
• 20 µM Bip	0.750 µl	75.0 µl
• 20 µM LF	0.375 µl	37.5 µl
• 20 µM LB	0.375 µl	37.5 µl
• 20 µM F3	0.188 µl	18.8 µl
• 20 µM B3	0.188 µl	18.8 µl

* (see note 4)

d) Denature the working mix of primers prior to amplification by heating to 95°C for 3 min and then place on ice before adding to the RT-LAMP reaction.

3) RT-LAMP reaction

a) Prepare RT-LAMP reaction mix (on ice):

• Reactions number	1	96
• Primer working mix[a]	2.63 µl	263 µl
• H_2O nuclease-free[b]	0.27 µl	27 µl
• Reverse transcriptase[c]	0.10 µl	10 µl
• Isothermal Mastermix	6.00 µl	600 µl

[a] (see note 5), [b] (see note 6), [c] (see note 7)

b) Mix the components by pipetting or gentle vortexing, then briefly pulse-spin in a microcentrifuge. Excessively harsh mixing may denature the enzymes.

c) Aliquot 9 µl of the reaction mix into the wells of microplates or into tubes (keep on ice).

d) Add 1 µl of the RNA template into each well/tube (the final reaction volume: 10 µl). Include a negative control: 1 µl of RNA purified from a virus-free plant and a non-template control: 1 µl of water instead of RNA. Close the tubes/plates firmly using appropriate means e.g., lids, a sealing film (see note 8).

e) Mix the components by pipetting or vortexing, then briefly pulse-spin in a microcentrifuge. Make sure to eliminate any air bubbles in the reaction and ensure that the entire reaction mix is at the bottom of the reaction vessels and that the vessels are well sealed.

f) Place the RT-LAMP reactions in a thermal block/water bath or a thermal cycler preheated to 65°C.

g) Incubate at 65°C for the desired amount of time. Fluorescent real-time detection (Fig. 2) usually requires 30 min to detect the lowest detectable concentration of the virus. Optionally, incubate for 45 min for colorimetric end-point detection with HNB. A positive reaction is indicated by a color change from violet to sky blue (Photo 1).

DATA ANALYSIS

1) In the Genie II Ultra rapid amplification instrument (OptiGene Ltd.), conduct the amplification at 65°C for 30 min. with fluorescence monitoring. Analyze the specificity of the resulting amplification products by determining the annealing temperature (Ta) resulting from slow annealing (0.05 °C/s) for 5 min, starting at 95°C and ending at 80°C with fluorescence monitoring. The average Ta should be about 84.4°C for PVY strains with N-type, which include PVY^{N} and PVY^{NTN}, and 84.9°C for O-type coat protein coding genomes, which include $PVY^{N\square Wi}$ and PVY^{O} (Treder *et al.,* 2018).
2) Using a CFX96 Touch™ Real-Time PCR Detection System (BioRad Ltd), set the thermal profile to 60 cycles of 30 sec at 65°C. Follow the amplification by an analysis of melting temperature (65°C to 98°C, 0.5° C/sec). The PVY strains that harbor N-type coat protein coding sequences should have a Tm close to 84°C, while the strains harboring O-type coat protein - about 84.5°C (Treder *et al.,* 2018).
3) The exact values of Tm and Ta may shift, depending on the purity of the template and individual properties of the equipment used for amplification.

NOTES

1) The kit includes all enzymes and reagents necessary for fluorescent RT-LAMP so only target-specific primers and RNA templates must be provided by the user. To increase the sensitivity of fluorescent detection, the kit contains pyrophosphatase, an enzyme degrading magnesium pyrophosphate. Thus, for turbidimetric or visual detection
2) another kit should be chosen, e.g., Isothermal Mastermix – with neither dye, nor pyrophosphatase (Cat. No.: ISO-001t, Novazym Polska S.A.). In such a case, to detect RNA pathogens, thermostable reverse transcriptase should also be purchased and supplemented into the reaction mix. The detection of DNA does not require this enzyme.
3) To detect other potato pathogens use primers specific to the particular organism (see Background for references).
4) The total RNA of good quality can be purified from potato leaves using most commercial kits. In our laboratory, good results were achieved using kits sold by A&A Biotechnology, (Cat. No.: 031-100) and by Novazym Polska S.A. (Cat. No.: RA1000-31, requires magnetic stand). As a cost-saving option, home-made methods of RNA purification can also be used. In our laboratory, good RT-LAMP sensitivity was achieved using silica capture of the total RNA, performed according to Zacharzewska *et al.* (2014). However, for isolating good quality total RNA from potato tubers, commercial (e.g., RNA3-Zone, Cat. No.: RA1000-10, Novazym Polska S.A., or TRIzol™ Reagent, Cat. No.: 15596026, Thermo Fisher Scientific) or homemade Trizol protocols must be applied. For optimal results, the quality and quantity of the total RNA

should be tested before setting up the reaction. Excessive or insufficient RNA in the reaction will negatively impact detection sensitivity. The optimal final RT-LAMP reaction should contain no more than 10 ng and no less than 100 pg of the total RNA.

5) For a higher number of RT-LAMP reactions, multiply the volumes provided in column two for one reaction by the number of reactions including 4% excess of the reagents to compensate for the loss of liquid on the walls of the reaction vessels and pipette tips (in the example in column three for 96 reactions a single reaction is multiplied by 100, which equals to four reactions in excess). The working mix can be prepared prior to amplification and the aliquots stored at -20°C for long-term storage but multiple freezing/thawing should be avoided.
6) The final concentration of primers in the reaction is 1.5 µM for FIP/BIP, 0.75 µM for LF/LB and 0.376 µM for F3/B3.
7) For colorimetric detection instead of water add 0.2 µl of 6 mM stock of HNB dye and 0.07 µl of nuclease-free H_2O.
8) For detecting DNA pathogens, instead of reverse transcriptase, add water. To save costs, choose kits w/o reverse transcriptase.
9) Optionally, to eliminate secondary structures, denature the template RNA by heating to 95°C for 3 min and then place on ice before adding the RT-LAMP reaction mix. Denaturation after adding the reaction mix will denature reverse transcriptase and DNA polymerase, and no amplification will occur. The template RNA and the working mix of primers may be denatured together.

RECIPES

1) Hydroxynaphthol blue (HNB) for colorimetric detection (optional). Prepare a 6 mM stock in nuclease-free water (50x). Store at room temperature for up to 6 months or 2 years at −20°C. The final dye concentration in the reaction: 120 µM.

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