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Simple and Rapid Detection of *Potato leafroll virus* by Reverse Transcription Loop-mediated Isothermal Amplification

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A new reverse transcription loop-mediated isothermal amplification (RT-LAMP) method for the *Potato leafroll virus* (PLRV) was developed and compared with conventional reverse transcription polymerase chain reaction (RT-PCR) to address its advantages over RT-PCR. RT-LAMP primers were designed from the open reading frame 3 (ORF3) sequence of PLRV. The RT-LAMP reactions were conducted without or with a set of loop primers. By real-time monitoring using Turbimeter, the RT-LAMP (with loop primers) detects PLRV in less than 30 min, compared to 120 min of RT-PCR. By adding fluorescent reagent during the reaction, final products of the RT-LAMP were fluorescently visualized under UV light or could be differentiated by naked-eye inspection under normal light. The RT-LAMP was extremely sensitive, about 2000-fold more sensitive than RT-PCR. This study presents great potential of the RT-LAMP for diagnosis and PLRV epidemiology because RT-LAMP method is speedy, sensitive, inexpensive, and convenient.

Keywords **:** diagnosis, loop primers, real-time, RT-PCR, virus detection

Potato (*Solanum tuberosum*) belongs the Solanaceae, and is one of the most important crops worldwide ranking the world's fourth-largest crop, with production reaching 325 million tonnes in 2007 (FAO, 2008). Because of its importance, the whole genome was recently sequenced (The Potato Genome Sequencing Consortium, 2011). Potato is easy to cultivate and has a high level of nutritive contents such as carbohydrate, protein, and vitamins, resulting in continuous increased potato consumption and production, especially in developing countries where more than half of the global potato is produced (FAO, 2008). These characteristics have led to potato being a valuable cash crop for millions of farmers. However, in contrast to diploid wild species of potato (Birhman and Hosaka, 2000), most of the current potato cultivars are autotetraploid and encounter indreeding depression because of narrow genetic background, resulting in disease susceptibility (Bryan and Hein, 2008).

Although potatoes can be infected by over 35 viruses that may reduce quantity and quality of potato tubers (Salazar, 1996 and Wikipedia, 2011), *Potato leafroll virus* (PLRV) is one of the major viruses infecting potato (Harrison, 1984). The PLRV forms icosahedral particles containing a 5.8 kb single-stranded (+) sense RNA that consists of eight open reading frames (ORFs) (Harrison, 1984; Taliansky et al., 2003). As PLRV is a member of the genus *Polerovirus* (family *Luteoviridae*), unlike the majority of plant viruses PLRV is restricted to the phloem-associated tissues (Gray and Gildow, 2003; Nurkiyanova et al., 2000; Taliansky et al., 2003). It is transmitted by a limited number of aphid species in a non-propagative circulative manner from plant to plant (Gray and Gildow, 2003; Harrison, 1985; Taliansky et al., 2003).

PLRV infection can be diagnosed by visual examination of symptoms such as upward leaf rolling. Symptom expression depends on interaction between the plant and PLRV, and growing conditions such as fertility and the weather, or the age of the plant when it is infected, affect symptom development. Primary infection typically shows pallor and in some cultivars rosiness and leaves may become rolled. Secondary symptoms in potatoes cultivated from infected tubers are severe with stunting of the plant and rolling of the leaves (Harrison, 1984). PLRV infection and expression in *Nicotiana benthamiana* and hairy nightshade did not show leaf rolling symptoms, whereas they displayed inter-vein chlorosis (Peter et al., 2008). Like other viruses, PLRV infection sometimes displays no symptom or plants may develop symptoms similar to those caused by different viruses (Webster et al., 2004) or nonpathogenic disorders due to nutritional deficiency, water stresses, insect damages, or pollution. There are many possibilities that lead to visual examination resulting in misdiagnosis.

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Therefore, several techniques have been used to detect PLRV in host plants. Enzyme-linked immunosorbent assay (ELISA) is widely used worldwide as the predominant technique to detect PLRV in host plants due to its accuracy, simplicity, and low cost (Casper, 1977). Although ELISA has been widely used to detect virus in potato plants and it is suitable for high-throughput testing, this technique may yield false negative results because of the low amount of virus in potato tubers, meaning ELISA may not be suitable for virus detection in the tuber. To increase sensitivity of virus detection by ELISA, sprouts from tubers have been used (Mumford et al., 2000). Conventional polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR) which have high sensitivity and specificity over ELISA-based methods and have been used to detect DNA viruses or RNA viruses, respectively (Bostan and Peker, 2009; Stark et al., 2008; Zaghloul, 2011). RT-PCR and immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) have been employed for detection of PLRV in plant or in aphid vectors (Ahouee et al., 2010; Hadidi et al., 1993; Peiman and Xie, 2006; Singh et al., 1995; 1997). Notomi et al. (2000) developed loop-mediated isothermal amplification (LAMP) to amplify DNA with high specificity. Two additional loop primers improve the reaction kinetics in terms of time and sensitivity of the regular LAMP reaction (Nagamine et al., 2002). The LAMP method has the several advantages over RT-PCR. It does not need an expensive thermocycler machine since a constant temperature is applied for all reactions. It is highly specific because the LAMP requires four or six primers which recognize six or eight regions of a target sequence respectively. LAMP does not need the extra step of visualization by gel electrophoresis (Goto et al., 2009). Some plant viruses have been detected by LAMP, such as *Tomato spotted wilt virus* (Fukuta et al., 2004), *Plum pox virus* (Varga and James, 2006) , *Peach latent mosaic viroid* (Boubourakas et al., 2009), *Japanese yam mosaic virus* (Fukuta et al., 2003), *Rice black-streaked dwarf virus*, *Rice dwarf virus*, *Rice gall dwarf virus*, *Rice ragged stunt virus*, *Rice transitory yellowing virus*, *Rice stripe virus*, *Rice grassy stunt virus*, *Rice tungro spherical virus*, *Rice tungro bacilliform virus* (Le et al., 2010), and *Potato virus Y* (Nie, 2005). The objective of this study was to evaluate feasibility of RT-LAMP method for detection of PLRV.

PLRV was inoculated using *Agrobacterium tumefaciens* culture carrying the plasmid pBPY, full-length cDNA of the PLRV-Canada strain (GeneBank No: D13954.1) as described by Lee et al. (2005) and Peter et al. (2008). *A. tumefaciens* containing the plasmid pBPY was grown in LB broth with kanamycin (50 μg/ml) for 48 h at 28 °C. After centrifugation of *A. tumefaciens*, culture was resuspended to an optical density at 600 nm of 0.5 to 0.6 with autoclaved water and infiltrated with a needless syringe into fully expanded leaves (2 leaves per plant) of *Nicotiana benthamiana* plants that were the five- to seven-leaf stage. Inoculated *N. benthamiana* plants were grown in a greenhouse. Total RNA was extracted from *N. benthamiana* leaf (100 mg) frozen in liquid nitrogen and ground to a fine powder using easy-Blue RNA extraction kit according to the manufacturer's protocol (Intron, South Korea). RT-LAMP primers to detect PLRV were designed according to the published sequence of ORF3 (coat protein) (GeneBank No: D13954.1) (Table 1) with the LAMP primer designing software, Primer Explorer V4 (available at http://primerexplorer.jp/elamp4.0.0/index.html). RT-LAMP was conducted using an RT-LAMP kit (Eiken Chemicals Co., Tokyo, Japan) according to the manufacturer's protocol. Twenty μl of optimized master mix contained 12.5 μl of 2X reaction mixture, 40 pmol of inner primers (FIP and BIP), 5 pmol of outer primers (F3 and B3), and with 20 pmol of loop primers (LF and LB) to accelerate amplification kinetic or without those. The RT-LAMP reaction was performed at 63 °C for 60 min followed by 2 min at 80 °C to terminate the reaction by enzyme inactivation.

The LAMP reactions with and without the loop primers (LF and LB) were conducted to compare detection time and reaction kinetics by monitoring in real time (Fig. 1). For real-time monitoring of the RT-LAMP reaction, the

Table 1. Primers used for this study

Primer	Length	Sequence (5'-3')
F3	19-mer	GCGAAGAAGGCAATCCCTT
B3	20-mer	CCCGAAGGTGAAACTTCCTT
FIP	39-mer	GCCTCCTCTTCTGCGTCTTCGAGGCGCGCTAACAGAGTT
BIP	40-mer	TCGCCGCTCAAGAAGAACTGGCCACGAGGTTGTCCTTTGT
LF	20-mer	GTGACCATAACCACTGGCTG
LB	20-mer	CGAGGACGAGGCTCAAGCGA
RT-forward*	24-mer	ACTGGAATGTTGGTGAAGCGGATG
RT-reverse*	28-mer	CTCCACTACACAACCCTGTAAGAGGATC

*Primers used conventional one step RT-PCR.

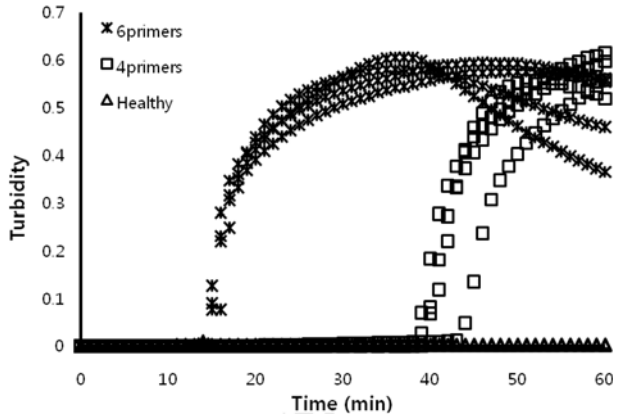

Fig. 1. Real time monitoring of turbidity in LAMP reaction with loop primers or without loop primers using total RNA extract from PLRV infected *Nicotiana benthamiana* plant (with loop primers-6 primers; without loop primers-4 primers) or using RNA extract from healthy *Nicotiana benthamiana* plant.

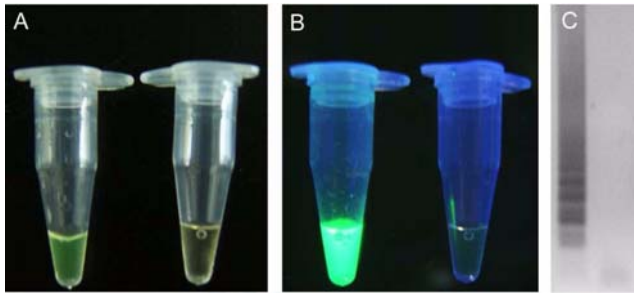

Fig. 2. Fluorescent dye mediated visual detection of RT-LAMP. (A) Naked-eye inspection under normal light, positive shows green (left) while negative remains yellow (right). (B) Fluorescent detection under UV irradiation from positive (left) but no fluorescent detection under UV irradiation from negative (right). (C) Agarose gel electrophoresis shows ladder-like pattern at RAMP reaction using total RNA extraction from PLRV infected *Nicotiana benthamiana* plant (positive; left) but no detectable production at RAMP reaction using total RNA extraction from healthy *Nicotiana benthamiana* plant (negative; right).

reaction mixture was incubated at Real Time Turbidimeter LA500 (Eiken Co. Ltd., Tokyo, Japan) which measures in real time the turbidity of the reaction mixture at the optical density at 400 nm. The analyzed data indicated that the turbidity increase by the LAMP reaction was detected at 15.4 ± 1.7 min when RAMP was conducted accelerated by the addition of two primers resulting in reducing detection time from 43.3 ± 3.5 min (without loop primers). This means that LAMP reaction with the loop primers provides greater sensitivity than LAMP reaction without the loop primers and is in agreement with a previous study carried out by Nagamine et al. (2002).

By adding a fluorescent agent during the RT-LAMP reaction, the RT-LAMP product could be detected visually by observing fluorescence emitted by the reaction solution under UV irradiation (Le et al., 2010; Tomita et al., 2008). In order to visualize the product of RT-LAMP in this study, 1 µl of fluorescent detection reagent (Eiken Chemicals Co., Tokyo, Japan) was added into the RT-LAMP reaction tubes. Even without UV irradiation, amplified product was visualized through naked-eye inspection under normal light (Fig. 2A). As shown at Fig. 2A, positive RT-LAMP product was detected through naked-eye inspection under normal light showing green color while negative appeared yellow instead of green. Under UV irradiation (Fig. 2B), positive samples were clearly detected by emitting fluorescent signal but negative samples showed no fluorescent light. Positive LAMP products could be also visualized by 1.5% agarose gel electrophoresis (Fig. 2C). As seen Fig. 2C, positive samples typically yielded a ladder-like pattern but negative did not show any signal.

In order to compare sensitivity of the RT-LAMP to that of conventional RT-PCR, total RNA from PLRV infected *N. benthamiana* plant was prepared in a 10-fold series dilution up to 10^{10} and subjected to both RT-LAMP and conventional RT-PCR. Since a 30 min reaction time was sufficient to yield visible detection of PLRV (Fig. 2), RT-LAMP reaction was performed for 30 min (Fig. 3 and 4). RT-PCR was conducted with Maxime RT PreMix Kit (Intron, South Korea) following the manufacturer's instructions. One µl of extracted total RNA, 10 pmol (1 µl) of primers (RT-forward and RT-reverse), and 17 µl of DEPC treated H_2O were added into a tube containing all components for RT-PCR experiment. The thermo-cycler for RT-PCR was programmed as follow: 1 cycle of 30 min at

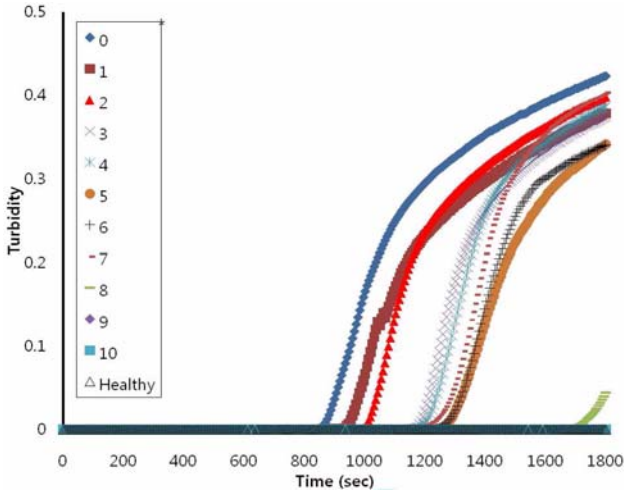

Fig. 3. Real-time monitoring of LAMP reactions conducted using a dilution series (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}) of total RNA extract from PLRV infected *Nicotiana benthamiana* plant or a total RNA extract from healthy *Nicotiana benthamiana* plant.

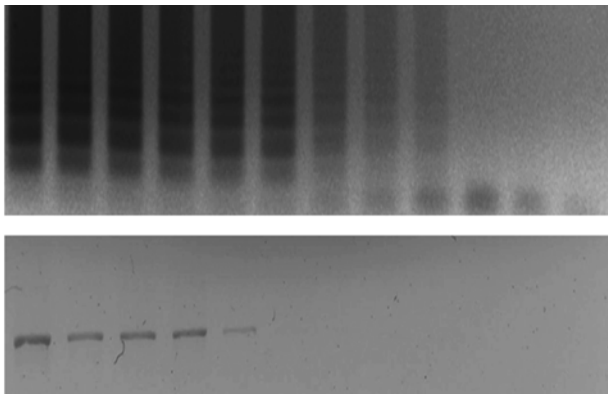

Fig. 4. Sensitivity of RT-LAMP (top) and RT-PCR (bottom) at serial dilutions (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, and 10^{10} from left to right) of total RNA extraction from PLRV infected or healthy *Nicotiana benthamiana* plants (most right lane).

45 °C and 5 min at 94 °C and 35 cycles of 30 s at 94 °C, 30 s at 54.7 °C, 1 min 20 sec at 72 °C, and final extension of the amplification time for 5 min at 72 °C. RT-LAMP reactions were monitored in real time using Turbidimeter LA500 (Fig. 3) and amplified products by RT-LAMP and by RT-PCR were analyzed by 1.5% agarose gel electrophoresis (Fig. 4). As shown in Fig. 3, dilution limit was determined as 10^8 fold for PLRV detection at RT-LAMP with 29 min detection time. The minimum threshold time to detect for positive RT-LAMP was 16.5, 18.1, 19, 21.5, 22.2, 24, 23.5, and 23.1 at 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 fold dilution, respectively. Data analysis by 1.5% agarose gel electrophoresis revealed that the maximum dilution threshold of total RNA extract for PLRV detection by the RT-LAMP was 10^8 fold dilution, whereas that of total RNA extract for PLRV detection by the RT-PCR was 10^4 fold dilution (Fig. 4). When noted that 1 µl of RNA was used for RT-PCR and 5 µl of RNA extraction was used for RT-LAMP, RT-LAMP was estimated to be about 2,000 times more sensitive than conventional RT-PCR for PLRV detection.

RT-PCR (Hadidi et al., 1993; Peiman and Xie, 2006; Singh et al., 1995; 1997) and IC-RT-PCR (Ahouee et al., 2010) have been developed for the rapid and accurate detection of PLRV in plants or/and aphids. Although these conventional PCR-based methods increased rapidness and accuracy for PLRV detection, these methods require expensive and sophisticated instruments for the PLRV amplification and detection of the PCR products. This study demonstrated advantages of RT-LAMP over RT-PCR assay for PLRV detection in *N. benthamiana* plants. RT-LAMP can be conducted using simple inexpensive instruments such as a water bath or heating block since PLRV RNA can be amplified at a constant temperature instead of using an expensive thermocycler. If fluorescent reagent is applied in the reaction tube, the gel electrophoresis step is not required to determine positive or negative results. With loop primers, RT-LAMP reaction time can be down to less than 30 min, one third of the time required for RT-PCR. The greater sensitivity of LAMP compared to RT-PCR and other methods means that false negative results in samples such as tubers with extremely low levels of PLRV could be reduced.

In conclusion, RT-LAMP is an extremely efficient, sensitive, accurate, simple, fast, and reliable diagnostic tool for PLRV detection in plants, in potato tubers, and in the aphid vector. Therefore, RT-LAMP can be useful for seed certification programs and epidemiological studies.

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