Detection of turnip mosaic virus (TuMV) in brassicaceous plants through colorimetric one-step reverse transcription loopmediated isothermal amplification (RT-LAMP) assay

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Supakitthanakorn, S. (2025). Detection of turnip mosaic virus (TuMV) in brassicaceous plants through colorimetric one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay. International Journal of Agricultural Technology 21(4):1491-1502.

Abstract This study developed a highly efficient colorimetric one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for enhanced turnip mosaic virus (TuMV) detection. LAMP primers were newly designed targeting the coat protein (*CP*) gene of TuMV, ensuring specific amplification. The optimized protocol achieved successful amplification after a 45-min incubation in a heat block incubator at 65°C, with results immediately visible through distinctive colorimetric changes: positive samples transitioning from pink to yellowish color, whereas negative samples retained pink. Importantly, the sensitivity of this colorimetric one-step RT-LAMP significantly surpassed conventional RT-polymerase chain reaction (RT-PCR) technique for TuMV detection. The assay demonstrated complete specificity without cross-reactivity observed when tested against non-target plant viruses, confirming the high selectivity of the designed primers for TuMV detection. By integrating reverse transcription and amplification into a single reaction, this streamlined approach eliminates separate processing steps, substantially reducing both detection time and associated costs. This advancement represents a valuable tool for efficient diagnosis of RNA plant viruses in resource-limited settings.

Keywords: Potyvirus, Plant virus disease, Brassicaceae, Molecular detection

Introduction

Brassicaceous plants belong to the family of Brassicaceae, commonly known as the mustard family or crucifers (Gupta, 2016). This diverse family includes several economically significant crops, such as mustard, cabbage, broccoli, cauliflower, radish, and other cruciferous vegetables (Franzke *et al.*, 2011). One of the primary challenges in cultivating brassicaceous plants globally is viral infections, particularly those caused by both DNA and RNA viruses causing substantial yield losses (Kamitani *et al.*, 2019; Li *et al.*, 2019).

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TuMV is one of members belonging to the genus *Potyvirus* and primarily infects brassicaceous plants, including key crops such as turnips, mustard, broccoli, and cabbage (Ohshima *et al.*, 2002). TuMV infection causes diverse symptoms consisting of mosaic on leaves, leaf deformation, vein banding, yellowing, and stunting. The symptom pattern and severity differ according to host plants and specific viral strains (Sanchez *et al.*, 2003; Guerret *et al.*, 2017).

The particle of TuMV is a flexuous rod measuring approximately 750 nm in length. Its genome consists of positive-sense, single-stranded RNA, approximately 9,835 nucleotides in length, which encodes a polyprotein cleaving into individual functional proteins essential for replication, encapsidation, and movement (Cuesta *et al.*, 2019). TuMV has wide host ranges which infect more over 300 species of plant, consisting of both dicotyledonous and monocotyledonous plants. It is regarded as the *Potyvirus* with the widest host range and is disseminated by numerous species of aphid (Gibbs *et al.*, 2008; Nellist *et al.*, 2021).

Detecting plant viruses presents several challenges due to various factors. Common issues include viral variability, low virus concentrations, co-infections, sample preparation difficulties, cross-reactivity, and field detection limitations (Rubio *et al.*, 2020). To overcome these challenges, researchers continue to develop more specific, sensitive, and rapid virus detection. Molecular techniques such as PCR, LAMP, and recombinase polymerase amplification (RPA), and serological assays are widely and commonly used in plant virus diagnostics. Advances in molecular biology further enhance virus detection and management strategies (Le and Vu, 2017; Wang *et al.*, 2022).

LAMP is a molecular method characterized by its rapidity, sensitivity, and specificity in amplifying target DNA under one stable temperature conditions, removing the necessity for thermal cycling machine required in conventional PCR (Notomi *et al.*, 2000). RT-LAMP allows for the amplification of RNA by integrating reverse transcription and amplification of DNA into a single-step, isothermal process, reducing contamination risks and accelerating detection (Notomi *et al.*, 2000; Budziszewska *et al.*, 2016). The results of LAMP amplification are often visually detectable, with a color change, called colorimetric LAMP, indicating the presence of the target sequence, making it particularly useful for field-based diagnostics (Bhat *et al.*, 2022).

Recently, colorimetric LAMP has gained widespread use for visual detection of plant viruses, enabling rapid and accessible identification of both RNA and DNA viruses. Many LAMP assays were successfully developed and applied to detect plant viruses such as bean common mosaic virus (BCMV), papaya ringspot virus (PRSV), tobacco mosaic virus (TMV) and zucchini yellow mosaic virus (ZYMV) (Kuan *et al.*, 2014; Shen *et al.*, 2014; Supakitthanakorn *et*

al., 2022a; Celik *et al.*, 2023). Therefore, this research focused on developing colorimetric one-step RT-LAMP assay for the rapid and efficient technique for TuMV detection.

Materials and methods

TuMV sample and total RNA extraction

TuMV which was detected and isolated from the mustard green (*Brassica juncea*) of the previous work (Supakitthanakorn and Ruangwong, 2021) was propagated in tobacco (*Nicotiana tabacum* cv. Xanthi). TRIzol[®] Reagent (Invitrogen, USA) was used for extracting total RNA from tobacco leaves following the method recommended by the manufacturer, subsequently extracted RNA was kept at -80°C until further use.

Designing LAMP primers

Six LAMP primers were obtained by designing in the NEB[®] LAMP Primer Design Tool (https://lamp.neb.com/) provided by New England Biolabs, USA. Six primers included forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP), backward inner primer (BIP), loop-forward primer (LF), and loop-backward primer (LB). The sequence of the coat protein (*CP*) gene of TuMV (GenBank accession no. LC537576.1) was obtained through the GenBank database and used as a template, with default settings applied during primer design.

Optimization of RT-LAMP amplification

The one-step RT-LAMP was evaluated to to establish the most suitable incubation conditions, including temperature and duration for effective LAMP product amplification. A total of 25 μ L reaction composed of 12.5 μ L WarmStart[®] Colorimetric LAMP 2X Master Mix with UDG (New England Biolabs, USA), 1.0 μ L RNA, 2.5 μ L10X LAMP primer mix, and 9.0 μ L nuclease-free water. Concentrations of each primer were 8 μ M for F3 and B3 primers, 16 μ M for FIP and BIP primers, and 4 μ M for LF and LB primers. The stock concentration of mixed LAMP primers was prepared at 10X. The LAMP reaction tubes were tested in the 1MJC-MD-MINI heat block incubator (Major Science, China), with incubation temperatures ranging at 61, 63, 65, and 67°C, each for 1 hour. The well-suited temperature for LAMP product amplification was determined and used to assess incubation times of 30, 45, and 60 min.

However, the primary incubation temperature was maintained at 65°C, as the recommendation provided by the manufacturer.

The amplified LAMP products were observed through gel electrophoresis with 1.5% agarose gel stained with RedSafeTM Nucleic Acid Staining Solution (iNtRON, Korea). For colorimetric interpretation, a color shift from the initial pink to yellow following completed incubation was indicated as a positive reaction (infected) for TuMV detection, whereas maintenance of the original pink color signified a negative result.

Sensitivity test of RT-LAMP

To determine the lowest RNA concentrations that could be amplified by RT-LAMP, RNA was serially diluted tenfold (10^0 to 10^{-10}). Concentrations of diluted RNA were subjected for measurement using a NanoDrop 2000c (Thermo Fisher, USA), ranging from 1 µg/µL to 100 ag/µL. However, the NanoDrop spectrophotometer could reliably measure RNA concentrations only down to 1 ng/µL. For dilutions below this threshold, each was further diluted tenfold sequentially. The one-step RT-LAMP was conducted under optimized conditions of temperature and time of incubation, and amplified LAMP products were subsequently detected using AGE.

The detection limit of the one-step RT-LAMP was comparative analyzed with RT-PCR using diluted cDNA derived from serially diluted RNA, identical to those used as templates for RT-LAMP. The RT-PCR reaction was amplified using 2X PCR Super Mix (Bio-Helix, Taiwan) with *CP* gene-specific primers which was shown in Table 1. PCR amplification was conducted in a Labcycler (SensoQuest, Germany). The reaction was conducted using the following thermal cycling protocol: pre-heating for 4 min at 94°C, followed by 35 cycles of denaturation (94°C for 30 sec), annealing (50°C for 30 sec), extension (72°C for 30 sec), with a final extension step at 72°C for 7 min. The amplified 345 bp amplicon was visualized and confirmed via 1.5% AGE.

Specificity test of RT-LAMP

To determine potential cross-reactivity with non-target viruses, positive RNA samples from cucumber mosaic virus (CMV), TMV, PRSV, and ZYMV were used. CMV isolate TB1 and TMV isolate TB4 were isolated from tobacco (unpublished), while PRSV isolate KPS and ZYMV isolate CM were isolated from pumpkin (unpublished). A healthy mustard green (*Brassica juncea*) leaf was used as a control. The one-step RT-LAMP was examined under well-suited amplification conditions, and amplified LAMP products were detected via 1.5% AGE, together with colorimetric observation.

Accessment of RT-LAMP performance

The efficiency of colorimetric one-step RT-LAMP to detect TuMV was assessed in newly collected brassicaceous plants displaying virus-like symptoms from various locations. Extraction of plant total RNA was conducted following the previously mentioned method. The extracted RNA was utilized as template material for the colorimetric one-step RT-LAMP assay according to the previously optimized reaction parameters. LAMP products were detected using 1.5% AGE and simultaneously evaluated via colorimetric assessment to confirm TuMV presence.

Results

LAMP primers

Six specialized LAMP primers were obtained by targeting to the *CP* gene of TuMV, with their respective sequences and complementary binding sites illustrated in Table 1 and Figure 1.

Detection technique	Primer name	Sequence (5'-3')	Position (5'–3')	Reference
PCR	Tu-F Tu-R	ATGTGGGTGATGATGGACGG GTTCTCTACCGTTGTGCCGA	454–473 779–798	Supakitthanakorn and Ruangwong (2022)
LAMP	FIP	CCACCTGATCATCGCCGTCC-	F2: 426–443	
		GAACGGAACCTCCCCGAA	F1c: 468–487	
	BIP	TCCCGATCAAACCGCTCATTGA-	B2: 534–553	
		TGAAATGGGCCGTTATCTGC	B1c: 491–512	This study
	F3	TGGTTTAATGGTCTGGTGCA	402-421	
	B3	GTACGCTTCAGCTACGTCAC	554-573	
	LF	ATCACCCACATTCCGTTTATG	444-464	
	LB	CCACGCCAAACCCACATTTAG	513-533	

Table 1. PCR and LAMP primers specialized to the CP gene of TuMV



Figure 1. Genomic structure of TuMV (top) and complementary binding sites of six LAMP primers (bottom)

Optimization of one-step colorimetric RT-LAMP

LAMP detection results showed a characteristic ladder-like pattern of amplified products when incubated at 61, 63, and 65°C for 60 min (Figure 2A). Nevertheless, no amplification was detected at 67°C. Based on these findings, the incubation temperature was fixed at 65°C, and the reaction time was varied to 30, 45, and 60 min, following the recommendation of the manufacturer. The results confirmed successful LAMP product detection for 45 and 60 min at 65°C (Figure 2B). In all positive LAMP reactions, color that was converted from pink to yellow was observed, while samples that showed negative results did not undergo a color change (Figure 2A and 2B). As a result, the well-suited amplification conditions were determined as incubation for 45 min at 65°C.



Figure 2. Amplification optimization of colorimetric one-step RT-LAMP for TuMV detection: (A) optimized temperature at 61, 63, 65 and 67°C for 45 min. (B) incubation period for 30, 45 and 60 min at 65°C. M is DNA ladder, and NC is a negative control (nuclease-free water)

Sensitivity and specificity tests of RT-LAMP

RNA diluted up to 10^{-7} (100 fg/µl) was the detection limit of one-step RT-LAMP which was more 10^3 -fold upper than the detection limit of RT-PCR (Figure 3A). In contrast, RT-PCR detected amplicons in cDNA dilutions was 10^{-4} (100 pg/µL) (Figure 3A). The colorimetric observation was consistent with gel electrophoresis analysis. In one-step RT-LAMP reactions, RNA dilutions from 10^0 to 10^{-7} exhibited a yellow color changed from pink, indicating positive amplification. Alternatively, the negative control (NC) and RNA dilutions from 10^{-8} to 10^{-10} retained their pink color, confirming the absence of amplification (Figure 3B).

The specificity assay revealed that the LAMP products were exclusively observed in the TuMV lane and were absent in the lanes representing other different viruses, including CMV, TMV, PRSV, ZYMV, and a healthy mustard green (*Brassica juncea*). This finding was consistent with the colorimetric results, where positive samples exhibited a yellow color change, while negative samples

did not change (Figure 3). This conclusion proved that the developed colorimetric one-step RT-LAMP, utilizing the newly designed LAMP primers, exhibited specificity exclusively to TuMV.



Figure 3. Sensitivity (left) and specificity (right) tests of colorimetric one-step RT-LAMP for detecting TuMV. (A) the sensitivity of RT-LAMP showed limit of detection at 10^{-7} (100 fg/µL) of diluted RNA compared to RT-PCR with limit of detection at 10^{-4} (100 pg/µL) of diluted cDNA and (B) the specificity of colorimetric one-step RT-LAMP. M is a DNA ladder, and NC is a negative control (nuclease-free water)

Evaluation of one-step colorimetric RT-LAMP performance

A total of 17 brassicaceous leaf samples, including mustard greens, radishes, and cos lettuce, were collected and total RNA was extracted. The results exhibited that 9 samples were positively tested for TuMV detection which were indicated by a changed yellow color and validated by the observation of LAMP products confirmed in 1.5% AGE (Figure 4). Negative samples retained their pink color, and no LAMP product was observed on agarose gel electrophoresis. Specifically, TuMV was detected in radish leaves exhibiting a mottled symptom, mustard green leaves showing a mosaic symptom, and cos lettuce leaves displaying malformation with mosaic symptoms (Figure 5).



Figure 4. Evaluation of the colorimetric one-step RT-LAMP assay for detection of TuMV in 17 symptomatic brassicaceous plant samples. Positive reactions were confirmed by the presence of amplified LAMP products exhibited in 1.5% AGE alongside with a yellow color of the tubes, while negative reactions were indicated by the lack of amplified LAMP product formation and retention of the pink color. M is a DNA ladder, PC is a positive control, and NC is a negative control (nuclease-free water)



Figure 5. TuMV-infected brassicaceous plants detected by colorimetric one-step RT-LAMP: (A) radish leaves exhibiting a mottled symptom, (B) mustard green leaves showing a mosaic symptom, and (C) cos lettuce leaves displaying malformation with mosaic symptoms

Discussion

Brassica crops are particularly susceptible to TuMV, and infections can result in economic losses for farmers (Guerret *et al.*, 2017). Prevention and management strategies include planting virus-resistant crop varieties, implementing regular crop rotation practices, controlling insect populations that act as disease vectors, and maintaining rigorous field sanitation protocols to prevent contamination. Recognizing the problem early and addressing it promptly is essential to mitigate the consequences of TuMV on brassica crops (Li *et al.*, 2019).

The RT-LAMP technique has been successfully developed and widely applied for detecting a range of plant pathogenic microorganisms, with completion times ranging from 30 to 60 minutes after incubation at temperatures ranged between 60 to 69°C (Koh *et al.*, 2020; Supakitthanakorn *et al.*, 2022a-c). This study successfully optimized a colorimetric one-step RT-LAMP assay that achieved complete amplification within 45 min of incubation at 65°C. The LAMP products were observed, and colorimetric observation were clearly visualized immediately following the incubation period, eliminating the need for additional processing steps or specialized equipment. The findings suggest that the designed LAMP primers are effective for detecting TuMV across different host plants, demonstrating the potential of the developed colorimetric one-step RT-LAMP as a detection tool for TuMV.

A previous RT-LAMP technique for detecting TuMV had been developed (Zhao *et al.*, 2014). The detection limit of the earlier RT-LAMP technique was 10 times greater than RT-PCR, with detection limits up to 100 fg/ μ L of cDNA. However, the previous RT-LAMP for detecting TuMV relied on turbidity for observation. This study presents a simplified approach to visualize the RT-LAMP reaction through color changes, referred to as colorimetric observation. This method eliminates laborious post-amplification work, and the results are easily distinguished (Alhamid *et al.*, 2022).

The one-step RT-LAMP has been developed and utilized to detect several plant-infecting viruses from various host plants, leveraging its advantages in avoiding cDNA synthesis. Examples include detection of tomato torrado virus (ToTV) (Budziszewska *et al.*, 2016), prunus necrotic ringspot virus (PNRSV) and apple mosaic virus (ApMV) (Wani *et al.*, 2023), and prune dwarf virus (PDV) (Celik, 2022). Furthermore, one-step RT-LAMP has shown its effectiveness in detecting viruses across various plant tissues, such as the detection of cucumber green mottle mosaic virus (CGMMV) from cucumber seeds (Kwon *et al.*, 2021). It can also be applied to detect tomato chlorosis virus (ToCV) from both plants and whiteflies (Karwitha *et al.*, 2015).

Furthermore, this study successfully introduces a colorimetric one-step RT-LAMP, offering enhanced convenience for virus detection by integrating reverse transcription and amplification into a single reaction. This innovative

approach not only enhances workflow efficiency but also demonstrates exceptional sensitivity for TuMV detection, potentially revolutionizing field-based diagnostics for plant pathogens.

Acknowledgements

The author extends sincere gratitude to the Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, for providing research facilities and support throughout the study.

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(Received: 12 March 2024, Revised: 2 January 2025, Accepted: 3 July 2025)