Advanced loop-mediated isothermal amplification method for sensitive and specific detection of *Tomato chlorosis virus* using a uracil DNA glycosylase to control carry-over contamination

Eui-Joon Kii,a, Sunhoo Kim4, Ye-Ji Leea, c, Eun-Ha Kanga, d, Minji Leea, Sang-Ho Choa, Mi-Kyeong Kimc, Kyeong-Yeoll Lee, Noh-Youl Heod, Hong-Soo Choi, Suk-Tae Kwon, a, Sukchan Leea, ∗

a Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
b The Institute of Life Science and Technology, Sungkyunkwan University, Suwon 440-746, Republic of Korea
c Crop Protection Division, National Academy of Agricultural Science, Rural Development Administration, Wanju 565-851, Republic of Korea
d Plant Quarantine Technology Center, Animal and Plant Quarantine Agency, Suwon 443-400, Republic of Korea

Abstract

In 2013, *Tomato chlorosis virus* (ToCV) was identified in symptomatic tomato plants in Korea. In the present study, a loop-mediated isothermal amplification (LAMP) method was developed using four specific primers designed against ORF6 in ToCV RNA2 to detect ToCV rapidly and with high sensitivity. The optimized reaction involved incubation of a reaction mixture containing 2 U Bst DNA polymerase and 4 mM MgSO4 for 1 h at 60–62 °C. Although specific and rapid detection of ToCV by LAMP was confirmed, false-positive reactions caused by carry-over contamination sometimes occurred because of the high sensitivity of LAMP compared with other detection methods. To prevent false-positive reactions, dUTP was substituted for dTTP and uracil-DNA glycosylase (UDG) was added to the LAMP reaction. First, the LAMP reaction was conducted successfully with substitution of dUTP for dTTP. Before the next reaction, LAMP products with incorporated dUTP were cleaved selectively by UDG without any effect on thymine-containing DNA (template DNA). This modified LAMP method complemented with UDG treatment to prevent carry-over contamination offers a potentially powerful method for detecting plant viruses.

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1. Introduction

*Tomato chlorosis virus* (ToCV), genus *Crinivirus* family *Crinoviridae*, is a plant virus that infects and damages cultivated tomatoes (*Lycopersicon esculentum*) (Wisler et al., 1998; Wintermantel and Wisler, 2006). ToCV is transmitted by greenhouse whitefly (*Trialeurodes vaporariorum*), banded wing whitefly (*T. abutilonea*), and sweet potato whitefly (*Bemisia tabaci*). ToCV-infected tomato plants show typical symptoms of ToCV disease in leaves including brittleness or bronzing and, interveinal chlorosis (Wintermantel and Wisler, 2006). ToCV has a bipartite genome of positive-sense single-stranded RNAs (RNA1 and RNA2) consisting of four and nine ORFs, respectively, that encode functional proteins (Lozano et al., 2006; Wintermantel and Wisler, 2006). In addition to tomato, many other cultivated crops such as sweet pepper (*Capsicum annuum*), potato (*Solanum tuberosum*), and zinna (*Zinnia elegans*) have been identified as natural ToCV hosts (*L. esculentum*) (Lozano et al., 2004; Tsai et al., 2004; Barbosa et al., 2010; Vargas et al., 2011; Fortes and Navas-Castillo, 2012), and approximately 25 species of weeds are also ToCV hosts (Font et al., 2004; Segev et al., 2004; Morris et al., 2006; Wintermantel and Wisler, 2006; Alvarez-Ruiz et al., 2007; Trenado et al., 2007; Solórzano-Morales et al., 2011). ToCV has been reported in many countries throughout Europe, the Middle East, the Americas, and Africa since the first report was published by US researchers in 1998 (Accotto et al., 2001; Font et al., 2004; Segev et al., 2004; Dalmont et al., 2005; Alvarez-Ruiz et al., 2007; Barbosa et al., 2008; Fiallo-Olivé et al., 2011). ToCV has also been detected in some Northeast Asian counties (China, Taiwan, and Japan) (Tsai et al., 2004; Hirota et al., 2010; Zhao et al., 2013), but the first outbreak of ToCV in Korea was not reported until 2013.
Reverse transcription-polymerase chain reaction (RT-PCR) has been used to detect ToCV in several previous studies (Louro et al., 2000; Morris et al., 2006; Wintermantel and Wisler, 2006; Trenado et al., 2007). Loop-mediated isothermal amplification (LAMP) was developed for simple nucleic acid amplification and has been applied to plant pathogen detection (Notomi et al., 2000; Fukuta et al., 2003; Tomita et al., 2008; Park et al., 2013). LAMP can amplify target DNA with high rapidity, efficiency, and specificity under isothermal conditions using four or six specific primers (Notomi et al., 2000; Nagamine et al., 2001). The final products from LAMP have stem-loop structures of various sizes that can be detected by a ladder pattern of bands on a DNA agarose gel or can be detected by addition of SYBR Green I and visualization under a UV lamp or visible light without the need for gel electrophoresis (Notomi et al., 2000; Tomita et al., 2008). A LAMP method for ToCV detection has not previously been developed; therefore, this is the first report of ToCV detection using the LAMP method.

Contamination of DNA is one of the most important issues affecting nucleic acid amplification reactions because it can cause false-positive results (Kwok and Higuchi, 1989; Borst et al., 2004). Cross-contamination between samples or contamination with recombinant plasmids containing the target sequence can occur in the laboratory (Longo et al., 1990). Carry-over contamination of amplified DNA from a previous reaction has also been a major problem (Kwok, 1990; Longo et al., 1990; van Pelt-Verkuil et al., 2008). If repeated detection of certain pathogens is conducted in the same laboratory, carry-over contamination could be generated through reagents, pipettes, work space surfaces, or the skin of experimenters (Kwok and Higuchi, 1989; Longo et al., 1990). Two changes have been introduced into PCR procedures to prevent carry-over contamination: (1) incorporation of dUTP into all PCR products and (2) applying uracil-DNA glycosidase (UDG) before the next PCR procedure (Longo et al., 1990). UDG is an enzyme that catalyzes the removal of uracil bases from uracil-containing DNA by cleaving the N-glycosidic bond between the uracil base and the sugar-phosphate backbone, but it cannot remove uracil from RNA or free nucleotides (Longo et al., 1990). In its natural condition, genomic DNA from plants and viral DNA do not contain uracil, but instead contain thymine. However, if dUTP is used instead of dTTP in the reaction mixture, PCR products containing uracil instead of thymine residues can be produced. Because PCR products containing uracil are targets for UDG, whereas natural DNA (template DNA) is not, carry-over contamination caused by products from previous reactions can be prevented by treatment with UDG (Longo et al., 1990). A PCR protocol using UDG to prevent contamination is recommended.
carry-over contamination has been developed and used widely but UD
G has not been applied further in other amplification methods un
til recently when simultaneous elimination of carryover contam
ination and detection of DNA with UDG-supplemented LAMP was
described (Hsieh et al., 2014).

LAMP can be particularly vulnerable to carryover contami
nation because of its high sensitivity. Therefore, when the LAMP
method is considered for detection of plant viruses, the possi
bility of carryover contamination should be eliminated. In this study,
we developed a carryover contamination-free detection method
for ToCV.

2. Materials and methods

2.1. Sample collection, total RNA extraction, polymerase chain
reaction, and sequence analysis

In 2013, ToCV-like symptoms, including brittleness or bronz
ing of leaves and interveinal chlorosis, appeared on the leaves of
tomato plants cultivated in Nonan, Iksan, Hwasun, Hampyeong,
and Jeju (all in the southwestern area of the Korean peninsula)
(Fig. 1(A)). To detect ToCV from symptomatic tomato plants, total
RNA was isolated and RT-PCR was performed. Total RNA was
extracted from the leaves of tomatoes cultivated in these five dif
ferent areas using the Viral Gene-spin™ Viral DNA/RNA Extraction
Kit (iNtRON Biotechnology, Seongnam, Korea). cDNA was prepared
from total RNA (1–2 μg) with 30 U of AMV Reverse Transcrip
tase (Promega, Madison, WI, USA) and 10 pmol of ToCV-specific
reverse primer (5′-ATTCGAGTATCTTCTCGAACT-3′). The primer set
for PCR was designed to amplify a 439-bp region in ORF 2 of the
RNA2 genome (forward: 5′-GCTTCCGAAACTCCGTCTTG-3′; reverse:
5′-TGTCGAAAGTACCGCCACCA-3′). PCR was conducted using 2×
Taq PCR MasterMix (Bioneer, Daejeon, Korea) in a T100™ Thermal
Cycler (Bio-Rad, Hercules, CA, USA) under the following conditions:
2 min at 95 °C for initial denaturation; 30 cycles of 1 min at 95 °C,
1 min at 60 °C, 1 min at 72 °C; and 5 min at 72 °C for final extension.

Target-specific amplification was confirmed by agarose gel elec
trophoresis. PCR products were ligated into pGEM-T easy vector
(Promega) and transformed into competent E. coli strain DH5α.
After cultivation at 37 °C for 10 h, three single colonies were picked
randomly from each plate and incubated at 37 °C for 8 h. Plasmid
DNA was isolated using AccuPrep® Nano-Plus Plasmid Mini Extrac
tion Kit (Bioneer) and sequenced (Macrogen, Seoul, Korea). The
results of PCR were confirmed in three repeated reactions.

2.2. Primer design

Because full-length viral RNA sequences of ToCV isolated from
Korea have not been confirmed, conserved sequences reported
previously for ToCV from China, Japan, and the USA (NCBI Gen
Bank accession numbers: KC887999, KC709510, AB513443 and
DQ234675) were used to design the LAMP primer set. The spe
cific LAMP primer set for detection of ToCV was designed using
PrimerExplorer V4 software (http://primerexplorer.jp/e, Table 1
and Fig. 2(A)). The target for amplification was ORF 6 in ToCV RNA2.
The six priming sites of four specific primers are depicted with
green or blue boxes in Fig. 2(A).

2.3. Optimization of LAMP conditions

To determine the optimized LAMP conditions, LAMP reactions
were performed with different concentrations of Bst DNA poly
merase (New England Biolabs, Ipswich, MA, USA) and MgSO4 at
different reaction temperatures. The reaction mixture consisted

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′−3′)</th>
</tr>
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<tbody>
<tr>
<td>ToCV-F3</td>
<td>GACGAGGTCGACAGACTTG</td>
</tr>
<tr>
<td>ToCV-R3</td>
<td>ATTCGAGTATCTTCTCGAACT</td>
</tr>
<tr>
<td>ToCV-FIP</td>
<td>AAGCCCAACTGGAAGATCGAGGACGATCCGCACT</td>
</tr>
<tr>
<td>ToCV-BIP</td>
<td>AAGCAGTTAACAGAAATTTGAAAGTACA-CTTCCTGAGTCAAGTGA</td>
</tr>
</tbody>
</table>
of 2 μl of 10 × ThermoPol® reaction buffer (New England Biolabs), 1.6 μl of 10 mM dNTPs, 1.6 μM each of the inner FIP and BIP primers, 0.4 μM each of the outer F3 and B3 primers, 1 μl of template DNA (ToCV cDNA prepared in Section 2.1), 0–16 U Bst DNA polymerase, and 0–5 mM additional MgSO₄ (2–7 mM final concentration) in a final volume of 20 μl. The reaction was conducted at 60, 62, or 65 °C for 1 h and heated to 80 °C for 10 min to stop the reaction. Real-time amplification was performed in a Rotor-Gene Q real-time PCR instrument (Qiagen, Valencia, CA, USA) after addition of 1 μl diluted SYBR Green I (Invitrogen, Carlsbad, CA, USA) to each tube. LAMP products were visualized by (1) gel electrophoresis using a 1.5% agarose gel stained with ethidium bromide and (2) observing fluorescence under UV light and natural light after addition of 1 μl diluted SYBR Green I.

2.4. Specificity test

The specificity of the LAMP reaction to detect ToCV without misdiagnosis of other tomato-infecting viruses was evaluated. Total DNA or cDNA (50–100 ng) prepared from tomato plants infected by Tomato yellow leaf curl virus (TYLCV), Cucumber mosaic virus (CMV), or Tomato spotted wilt virus (TSWV) was used as a template for a LAMP reaction with the ToCV-specific primer set.

2.5. Substitution of dUTP for dTTP in LAMP reaction

To remove uracil-incorporated LAMP products (carry-over contaminants) using UDG without affecting natural DNA (template DNA), dUTP should be substituted for dTTP in all LAMP products. To confirm that Bst DNA polymerase can incorporate dUTP instead of dTTP in the LAMP reaction, LAMP for ToCV was performed in the optimized reaction mixture containing 1.6 μl of 10 mM or 30 mM dUTP instead of 10 mM dTTP.

2.6. UDG treatment before LAMP reaction

To evaluate the ability of UDG to prevent carry-over contamination in a LAMP reaction, 5 U of UDG (New England Biolabs) was added to the optimized reaction mixture with 1 μl of LAMP product from a previous reaction performed using dUTP instead of dTTP. The added LAMP products were prepared at the original concentration and at 1/10 and 1/50 dilutions. The supplied 10× reaction buffer for UDG was not added to the reaction mixture, and the required reaction time and temperature for UDG were provided by adding one incubation for 2 min at 37 °C before the main LAMP reaction without opening the tubes.

3. Results

3.1. Detection of ToCV by RT-PCR

Leaf tissues of symptomatic tomato plants were used to detect ToCV by RT-PCR. In repeated experiments, ToCV was successfully detected by RT-PCR analysis with ToCV-specific primers (Fig. 1) in all symptomatic tomatoes collected from five different regions. Further sequence and phylogenetic analysis of ToCV strains isolated and identified in Korea will be described in a separate paper (unpublished data). cDNA prepared from samples that showed positive results in RT-PCR was used as the template for LAMP reactions.

3.2. Optimized conditions and specificity of LAMP reactions

Optimized concentrations of Bst DNA polymerase and MgSO₄ were determined using real-time detection of fluorescence in various reaction mixtures. Different amounts of Bst DNA polymerase from 0 to 16 U were used in each reaction mixture. As a negative control, no product was obtained with 0 U of polymerase. Even though reaction with 2 U Bst DNA polymerase showed the fastest results with respect to fluorescence rates, similar overall fluorescence was detected with 2, 4, 8, and 16 U of enzyme (Fig. 2(B)).

The original 1× ThermoPol® reaction buffer (New England Biolabs) already contained 2 mM MgSO₄, but additional Mg²⁺ was added for an optimized reaction. Therefore, various concentrations (0–5 mM) of Mg²⁺ ion were added to find the optimized
reaction condition. According to real-time detection results, amplification was first saturated after addition of 2 mM MgSO4 to the mixture (4 mM final concentration), but similar results were achieved with 3 mM and 4 mM MgSO4. Slow saturation was confirmed with 0 and 5 mM MgSO4 (Fig. 2(C)). The optimal temperature for isothermal amplification was determined to be between 60 and 62 °C (Fig. 3(A)). Taken together, the optimal conditions for ToCV detection by LAMP were established as 2 U Bst DNA polymerase and 2 mM MgSO4 (4 mM final concentration) at 60 or 62 °C.

The specificity of the LAMP reaction for ToCV detection was evaluated by reactions with other tomato infecting viruses (TYLCV, CMV, and TSWV). Clear fluorescence and laddering were observed only for ToCV; LAMP reactions with other viruses did not show any positive results, confirming the specificity of the LAMP reaction for ToCV detection (Fig. 3(B)).

### 3.3. Incorporation of dUTP instead of dTTP in LAMP products

A modified LAMP method with incorporation of dUTP instead of dTTP was experimentally verified (Fig. 4). The LAMP reaction with Bst polymerase progressed well using dUTP instead of dTTP (Fig. 5(B) and (C)), and the concentration of dUTP did not affect the results (Fig. 5(C) and (D)).
Fig. 6. Control of carry-over contamination in the loop-mediated isothermal amplification (LAMP) reaction with UDG treatment. To determine whether UDG can prevent false-positive results by cleaving contaminants that could act as templates in the LAMP reaction, two individual reactions were performed under different conditions: (A) UDG treatment and (B) No UDG treatment. To create conditions in which contaminants exist in the reactants, four different concentrations [non-diluted (lane 1), 1/10 dilution (lane 2) and 1/50 dilution (lane 3)] of LAMP products from a previous reaction using dUTP instead of dTTP were added to each tube. Each product was visualized by mixing with SYBR Green I [top (under natural light) and middle (under UV light)] and agarose gel electrophoresis (bottom). Lane N, no template control.

3.4. UDG treatment to prevent carry-over contamination

To confirm that UDG treatment can be used to prevent carry-over contamination in LAMP reactions, dUTP-incorporated LAMP products were added as substrates to a reaction mixture containing UDG. No amplification occurred when UDG was added to reaction mixtures containing undiluted or 1/10 or 1/50 dilutions of LAMP products because uracil-incorporated LAMP products were degraded in the UDG treatment step (Fig. 6(A)). However, amplification from contaminants was observed when UDG was not added to the reaction mixture (Fig. 6(B)).

4. Discussion

ToCV is a virus that infects tomatoes and affects their cultivation (Wisler et al., 1998; Wintermantel and Wisler, 2006). Many studies on ToCV have used RT-PCR for detection (Louro et al., 2000; Morris et al., 2006; Wintermantel and Wisler, 2006; Trenado et al., 2007). However, these protocols require a long reaction time, multiple steps, sophisticated machines, and specialists to run the experiments (Kil et al., 2014), therefore development of a time- and cost-efficient method for virus detection with high efficiency and specificity is essential to perform field studies. LAMP technology has been introduced because it yields specific and sensitive results from a small amount of sample without the need for specialized machines (Notomi et al., 2000; Kil et al., 2014). In this study, we apply the LAMP method to ToCV detection.

Optimization of reaction conditions is critical for efficient and accurate detection of ToCV by the LAMP method. Among several components in the reaction mixture, the DNA polymerase and divalent metal ions (magnesium ions in most cases) exert a strong influence on outcome and are the most important consideration when developing new detection methods using PCR or other nucleic acid amplification methods. According to our results, reactions performed with 2 U Bst DNA polymerase and 2 mM MgSO₄ (4 mM final concentration) at 60 or 62 °C yielded the best results (Figs. 2 and 3(A)). TYLCV-specific results were also confirmed using the optimized reaction condition (Fig. 3(B)).

Because of the high sensitivity of the LAMP reaction, a very small amount of contaminant can produce false-positive results (Kuboki et al., 2003); thus, preventing carry-over contamination is an important factor for accurate and reliable detection. In many laboratories, false-positive results caused by carry-over contamination are observed during optimization testing. Various technical approaches have been used to minimize PCR product carry-over, such as physical separation of pre- and post-PCR amplification samples, changing gloves frequently, and careful uncapping and closing of tubes to prevent aerosolization (Kwok, 1990). However, these methods do not remove contaminants and are therefore not ideal approaches to preventing carry-over contamination. Previous studies introduced the idea of incorporation of dUTP instead of dTTP and subsequent application of UDG to prevent carry-over contamination in PCR (Longo et al., 1990). The principle and experimental schemes behind advanced PCR combined with UDG treatment were summarized briefly in the Introduction section. In this study, we applied the modified method to the LAMP reaction for ToCV detection. A brief explanation of carry-over contamination-free LAMP is depicted in Fig. 4.

To prevent carry-over contamination using dUTP and UDG, dUTP was first incorporated instead of dTTP into all LAMP products.
While the first studies seeking to prevent carry-over contamination in PCR using Taq DNA polymerase were successful (Longo et al., 1990), some DNA polymerases that originate from hyperthermophilic archaea such as Pfu DNA polymerase recognize uracil and stall DNA synthesis to prevent mutagenesis caused by deamination of cytosine to uracil (Greagg et al., 1999, Fogg et al., 2002). Therefore it is important to verify that substitution of dUTP for dTTP does not affect amplification (Fig. 5A). We confirmed that preparation of all LAMP products with dUTP instead of dTTP was possible before treating the first LAMP reaction mixture with UDG (Fig. 4). Bst DNA polymerase originates from the prokaryote Bacillus stearothermophilus and, like Taq DNA polymerase, lacks 5′→3′ exonuclease activity. Therefore these results were natural outcomes reflecting the characteristics of the DNA polymerase used. In addition, according to results from experiments with UDG treatment before LAMP reaction, UDG treatment of dUTP-incorporated LAMP products can eliminate the possibility of re-amplification of products synthesized in previous reactions (Fig. 5).

In summary, a new ToCV detection method was developed using LAMP combined with UDG treatment to prevent carry-over contamination. This modified LAMP method provides rapid and accurate detection of ToCV without false-positive results.

Acknowledgements

This research was supported by a grant from the iPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries: no. 311058-05-3-HD140), Ministry for Agriculture, Food and Rural Affairs, Republic of Korea.

References


