Detection of tomato mosaic virus infecting tomato using realtime RT-PCR

Quyen N. D. Cao[1], Toan Q. Truong[2], & Biet V. Huynh[1,2*]

[1]Faculty of Biological Sciences, Nong Lam University, Ho Chi Minh City, Vietnam

[2]Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: August 31, 2024
Revised: November 02, 2024
Accepted: November 22, 2024

Keywords

Escherichia coli JM109
Realtime RT-PCR
RNA virus
Tomato mosaic virus

***Corresponding author**

Huynh Van Biet
Email: hvbiet@hcmuaf.edu.vn

ABSTRACT

Tomato mosaic virus (ToMV) is known as one of the most common and devastating tomato viruses worldwide. It causes mosaic disease, which significantly impacts the productivity and quality of tomatoes in Vietnam. Early and accurate detection of ToMV in tomatoes is essential for effective disease control. This study developed a detection and quantification procedure for ToMV based on realtime RT-PCR. In this study, a positive control carrying ToMV's target gene segment was amplified to a size of 595 bp, then cloned into pJET1.2 vector and transformed into *Escherichia coli* JM109. A realtime RT-PCR procedure was established using designed primers to amplify a 182 bp gene segment of the RdRP-ORF2 gene region. A calibration curve was created with the equation y = -3.777x + 41.973, resulting in a correlation coefficient (R^2) of 0.9939, which was used to quantify the ToMV virus. Additionally, the procedure quantified test samples with viral loads ranging from 1.7 x 10^4 to 9.5 x 10^6 copies/µL

Cited as: Cao, Q. N. D., Truong, T. Q., & Huynh, B. V. (2024). Detection of tomato mosaic virus infecting tomato using realtime RT-PCR. *The Journal of Agriculture and Development* 23(Special issue 2), 119-130.

1. Introduction

According to data from the Food and Agriculture Organization (FAO, 2022) approximately 189 million tons of tomatoes were produced worldwide in 2021. In Vietnam, tomatoes have been cultivated for over 100 years, with significant growth in various regions across the country. In 2011, the tomato cultivation area reached 23,083 ha, with an average yield of 25.55 tons per ha, resulting in a total output of 589.83 thousand tons. However, tomato diseases pose major challenges to production in Vietnam and globally. Tomato viral diseases, in particular, cause substantial damage to plants, leading to symptoms such as curled leaves, deformed flowers, leaf drop, and small, malformed fruits with poor quality. The tomato mosaic virus (ToMV) and tomato mottle mosaic virus (ToMMV) are among the most prevalent and significant viruses affecting tomato plants (Hanssen et al., 2010). The ToMV is found in tomato-growing regions worldwide and damages most commercial tomato varieties in the field,

with potential yield reductions of up to 25%. It is notable for its high survival rate outside plant cells and in dead tissues (Lanter et al., 1982). The virus's capacity for rapid spread complicates early disease detection and exacerbates the impact on crop productivity and quality. Currently, there is no effective treatment for ToMV, and management relies on controlling the source of infection and developing resistant varieties (Dhaliwal et al., 2019). Rapid and accurate diagnosis of ToMV is crucial for effective disease surveillance and management strategies to minimize damage to tomato crops. Various diagnostic methods are available, including symptom observation, electron microscopy, ELISA, RT-PCR, and realtime RT-PCR. While symptom-based diagnosis is quick and generally accurate, it can lead to confusion, particularly when distinguishing between diseases with similar external symptoms caused by different pathogens. Microscopic diagnosis is commonly used for viruses that form characteristic intracellular inclusions (Varma & Singh, 2020). However, viruses in host cells may exist in amorphous crystalline forms, making them difficult to observe. The ELISA is a straightforward and user-friendly method but often has low sensitivity and can be time-consuming (Hu et al., 1993). Reverse transcription (RT)-PCR has been widely employed to diagnose many plant diseases; while conventional PCR can detect plant pathogens, the advent of realtime PCR has enhanced the ability to identify and quantify them (Varma & Singh, 2020). Realtime RT-PCR demonstrates significantly higher specificity and sensitivity compared to traditional ELISA or RT-PCR methods (Kogovsek et al., 2008; Bertolini et al., 2010).

In this study, the realtime RT-PCR method was employed to develop an assay for the early and accurate diagnosis of ToMV disease in tomatoes. This approach aimed to support the management of viral diseases in tomato crops and facilitate research on the development of virus-resistant tomato varieties in Vietnam.

2. Materials and Methods

2.1. Materials

Tomato leaf samples suspected of being infected with the ToMV virus were collected from tomato gardens in Duc Trong district, Lam Dong province, Vietnam. The positive control for ToMV was provided by the Research Institute for Biotechnology and Environment at Nong Lam University in Ho Chi Minh City. The *E. coli* JM109 bacterial strain was used for gene cloning.

2.2. Methods

2.2.1. Sample collection method

Sampling methods were carried out in accordance with TCVN 9016:2011 (VS, 2011). Tomato leaf samples were collected diagonally to ensure inclusion of leaves exhibiting symptoms of ToMV disease, such as curled leaves, deformed flowers, and small, malformed fruits, in Duc Trong district, Lam Dong province, Vietnam.

2.2.2. Extracting viral RNA

Total RNA was extracted from 50 mg of leaf sample using the EZ-10 Spin Column Plant RNA Miniprep Kit (Biobasic), following the manufacturer's protocol. A volume of 1 µL of the extracted total RNA was used to determine the concentration (ng/µL) and assess RNA purity using a spectrophotometer (Biodrop, UK).

2.2.3. Synthesis of cDNA

cDNA was synthesized from RNA according to the manufacturer's protocol of the SensiFAST™

cDNA Synthesis Kit. The incubation steps were carried out using a thermal cycler (GeneAmp® PCR System 9700). The thermal cycling conditions for the reaction included denaturation at 25°C for 10 min, reverse transcription at 42°C for 15 min, incubation at 48°C for 15 min, and final denaturation at 85°C for 5 min.

2.2.4. PCR amplification of the target gene of ToMV

The 595 bp gene fragment was amplified using the primer pair ToMV-F (5'-AAGATGTCAAACCAACTTA-3') and ToMV-R(5'-GAAACATCCAACTCAAGTACG-3') (Sui et al., 2017). The reaction was conducted in a total volume of 50 µL, comprising 25 µL of MyTaq Mix (2X), 20 µL of nuclease-free water, 1 µL (20 µM) of each forward and reverse primer, and 3 µL of the DNA sample. The thermal cycling conditions included 1 cycle at 95°C for 5 min; followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 40 sec; and a final extension at 72°C for 7 min. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel for 30 min at 100 V.

The amplified gene segment was sent for sequencing to Nam Khoa Trading and Service Company Limited in Ho Chi Minh City. The sequencing results will be compared with virus sequences available in the NCBI GenBank database to assess their similarity and confirm the presence of the ToMV virus.

2.2.5. Creating a bacterial strain carrying the target gene segment of the ToMV virus

The target gene fragment, after amplification by PCR, was inserted into the pJET1.2 vector following the manufacturer's protocol of the CloneJET Cloning Kit (Thermo). The resulting vector was transformed into *E. coli* JM109 bacteria using the heat shock transformation method. The transformed *E. coli* JM109 cell solution was spread on a petri dish containing LB medium supplemented with 50 mg/L ampicillin and cultured overnight at 37°C. Colonies that grew on the antibiotic medium were then checked by colony PCR using the primers pJET1.2-F (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2-R (5'-AAGAACATCGATTTTCCATGGCAG-3').

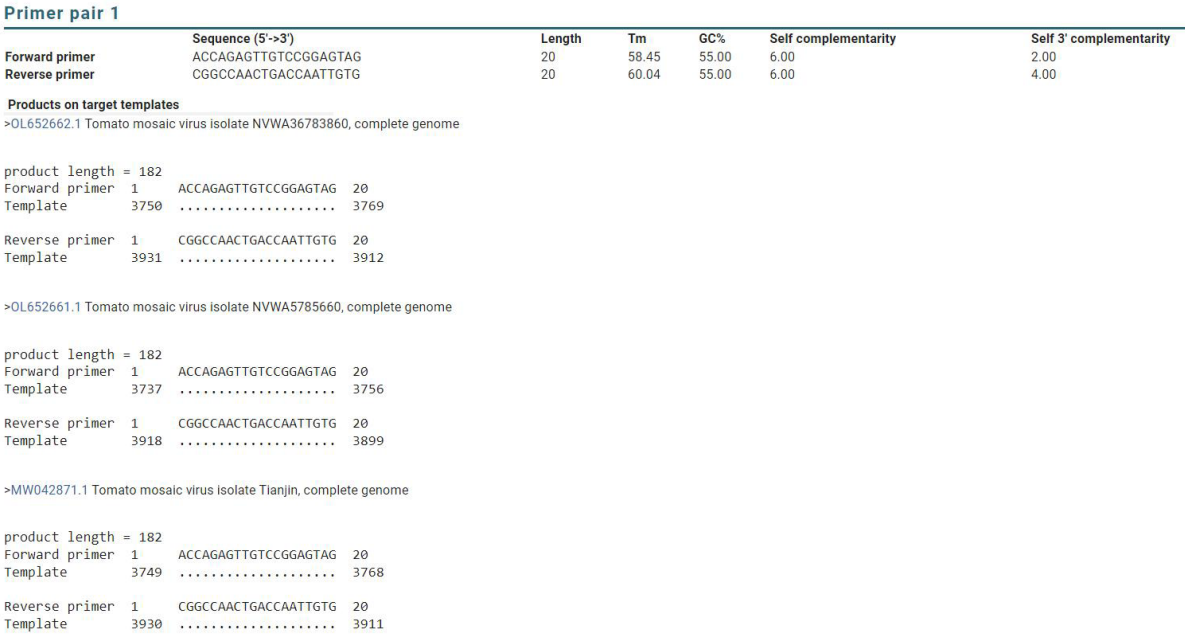
2.2.6. Extraction and purification of recombinant plasmid

Colonies containing the recombinant vector were grown in 5 mL of LB medium (supplemented with ampicillin) at 37°C for 16 h. Plasmid DNA was extracted and purified following the manufacturer's protocol of the TopPURE® Plasmid DNA Extraction Kit (ABT, Vietnam). The purity and concentration (ng/µL) of the plasmid DNA samples were determined using a spectrophotometer (BioDrop, UK). The presence of the target gene on the plasmid was confirmed by PCR using the primer pair p-JET1.2 F/R. The amplified product was analyzed by electrophoresis on a 1.5% agarose gel for 30 min at 100 V. Plasmid DNA samples were subsequently sequenced at Nam Khoa Trading and Service Company Limited (Ho Chi Minh City). The sequencing results were compared with the pre-cloning sequence and with virus sequences published on GenBank (NCBI).

2.2.7. Designing specific primer pairs for virus detection using realtime RT-PCR method

Primers used for realtime RT-PCR to detect ToMV virus were designed using Primer3 software to amplify a 182 bp gene segment. The forward primer was ToMVDQ-F (5-ACCAGAGTTGTCCGGAGTAG-3'), and the reverse was ToMVDQ-R (5-CGGCCAACTGACCAATTGTG-3').

The sequence of primers was checked for the non-specific binding in NCBI Primer Blast tool. This primer pair had a high specificity to only pair with ToMV (Figure 1).



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ACCAGAGTTGTCCGGAGTAG	20	58.45	55.00	6.00	2.00
Reverse primer	CGGCCAACTGACCAATTGTG	20	60.04	55.00	6.00	4.00

Products on target templates

>OL652662.1 Tomato mosaic virus isolate NVWA36783860, complete genome

```
product length = 182
Forward primer  1     ACCAGAGTTGTCCGGAGTAG  20
Template        3750  ....................  3769

Reverse primer  1     CGGCCAACTGACCAATTGTG  20
Template        3931  ....................  3912
```

>OL652661.1 Tomato mosaic virus isolate NVWA5785660, complete genome

```
product length = 182
Forward primer  1     ACCAGAGTTGTCCGGAGTAG  20
Template        3737  ....................  3756

Reverse primer  1     CGGCCAACTGACCAATTGTG  20
Template        3918  ....................  3899
```

>MW042871.1 Tomato mosaic virus isolate Tianjin, complete genome

```
product length = 182
Forward primer  1     ACCAGAGTTGTCCGGAGTAG  20
Template        3749  ....................  3768

Reverse primer  1     CGGCCAACTGACCAATTGTG  20
Template        3930  ....................  3911
```

Figure 1. The result of the primer was compared to the target sequences by using Primer Blast tool on NCBI Genbank.

2.2.8. Setting up realtime RT-PCR reaction

The realtime RT-PCR reaction was performed with standard samples on an Applied Biosystems® 7500 Realtime PCR machine, following the manufacturer's protocol for the SensiFAST™ SYBR® Lo-ROX Kit. The reaction components included 10 µL of 2x SensiFAST SYBR® Lo-ROX Mix (2X), 0.8 µL (10 µM) of each forward and reverse primer, 2 µL of DNA sample, and 6.4 µL of nuclease-free water. The thermal cycling conditions consisted of 1 cycle at 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec, and 72°C for 35 sec.

2.2.9. Generation of a standard curve

The number of copies of plasmid DNA was calculated according to the formula (Staroscik, 2004):

$$\text{Number of copies} = \frac{6{,}022 \times 10^{23} \times C}{650 \times 10^{9} \times L}$$

In there:

C: mass of plasmid sample (ng)

L: plasmid length (bp)

The plasmid DNA sample was diluted with nuclease-free water. Ten-fold serial dilutions from 10^7 to 10^3 copies of the recombinant plasmid were prepared using nuclease-free water and used as templates. Realtime PCR reactions were performed on standard samples with concentrations ranging from 10^7 to 10^3 copies/µL. The standard curve was constructed based on the threshold cycle (Ct) values and the corresponding log values (number of copies) of the standard samples. The equation of the standard curve is given by:

$$Y = aX + b$$

Where Y represents the Ct value, X represents the log of the copy number, a is the slope, and b is the y-intercept.

2.2.10. Evaluation of the specificity of the RT-PCR

The cDNA of other Tobamovirus including TMV (Tobacco mosaic virus), ToMMV (Tomato mottle mosaic virus), and ToBRFV (Tomato brown rugose fruit virus) were tested with ToMV primer to verify the specificity of the RT-PCR assay.

2.2.11. Diagnosis of ToMV in field samples using the newly established realtime RT-PCR process

The realtime RT-PCR procedure was employed to detect and quantify the infection levels in field samples. Following the reaction, the copy number of ToMV in the infected samples was determined by substituting the threshold cycle values into the standard curve equation for each gene.

3. Results and Discussion

3.1. Amplifying the target gene segment of the virus

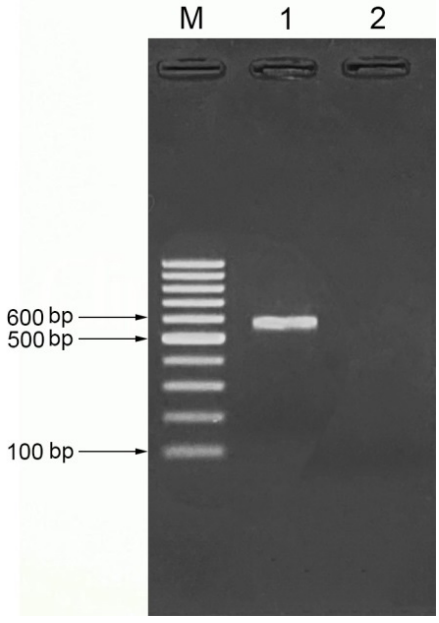


Figure 2. Electrophoresis results of PCR product of target gene segment of Tomato mosaic virus (ToMV) with primer pair ToMV-F/ToMV-R. DNA ladder (M), positive control (well 1), negative control (well 2).

The PCR reaction yielded a DNA band at nearly 600 bp, indicating successful amplification of the expected 595 bp gene segment of ToMV (well 1, Figure 2). This result confirms that the PCR reaction effectively amplified a gene segment of the anticipated size. To verify that the amplified sequences correspond to viral gene sequences, the DNA segment obtained from the amplification reaction was sequenced.

Comparison of the sequence with entries in NCBI GenBank revealed that the amplified gene segment shares 98.99% similarity with the published gene sequence MH393623.1 (Bae et al., 2019). This confirms that the PCR reaction developed in this study successfully amplified the correct gene segment of the ToMV virus.

3.2. Create clones and check the sequence of the amplified gene segment

Figure 3. Colony of *E. coli* bacteria after being transformed with a vector carrying the tomato mosaic virus (ToMV) gene.

The successfully cloned product resulted in white colonies on LB medium supplemented with 50 mg/L ampicillin (Figure 3). This outcome indicates that the target gene segment was inserted into the pJET1.2 vector, and the transformation process into *E. coli* was successful. The transformed bacterial strains are able to grow and form colonies on ampicillin-containing media because the pJET1.2 vector carries an ampicillin resistance gene. Additionally, the negative selection gene eco47IR helps eliminate strains containing self-ligated vectors.

To confirm the presence of the correct target gene, the colonies were tested by PCR using the primer pair pJET1.2. Theoretically, the expected amplification product size was approximately 713 bp. The results showed a DNA band of about 713 bp, positioned near the 700 bp marker, consistent with expectations (Figure 4). Therefore, it can be initially confirmed that the vector containing the target gene segment has been successfully transformed into the bacteria.

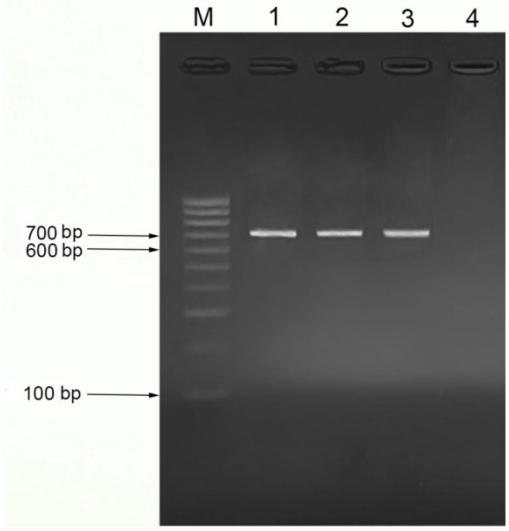


Figure 4. Electrophoresis results of PCR products of suspected colonies with primer pair PJET1.2. DNA ladder (M), Colony sample (wells 1-3), negative control (well 4).

3.3. Extraction and purification of recombinant plasmid DNA

The results of electrophoresis of the extracted and purified total plasmid DNA revealed a DNA band at 3687 bp, corresponding to the combined length of the vector and the target gene (Figure 5). This indicates that the plasmid sample was successfully inserted into the bacteria.

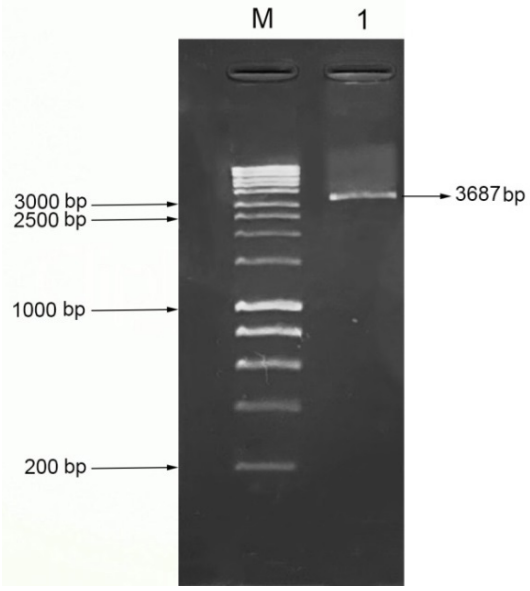


Figure 5. Electrophoresis results of total plasmid DNA of purified plasmid sample. DNA ladder (M), purified plasmid DNA sample (well 1).

The electrophoresis results of PCR products from the purified plasmid samples, using the primer pair PJET 1.2, showed a DNA band of approximately 713 bp, which is close to the expected size of 700 bp (Figure 6). However, to confirm the accuracy of this result, sequencing of the target gene segment inserted into the plasmid is necessary.

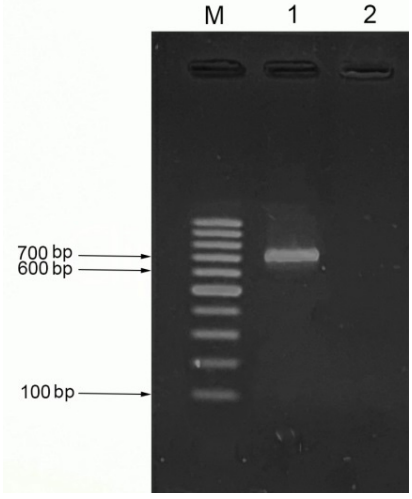


Figure 6. Electrophoresis results of PCR products of purified plasmid DNA samples with primer pair PJET1.2. DNA ladder (M), plasmid sample (well 1), negative control (well 2).

The comparison of the obtained sequence with NCBI GenBank revealed that the sequence of the amplified gene segment was 100% identical to the original gene segment before it was inserted into the plasmid. Additionally, it showed 98.99% similarity to the ToMV virus gene sequence with accession number MH393623.1 (Bae et al., 2019). This confirms that the gene segment inserted into the plasmid is indeed the ToMV viral gene segment and can be effectively used to detect the presence of the virus in tomato samples.

3.4. Generation of a standard curve

Table 1. Ct values of the realtime RT-PCR amplification reaction for the plasmid samples

Sample (copies/µL)	Log of copies number	Threshold cycle (Ct) (Mean ± SD)	Coefficient of Variation (%)
10^7	7	15.37 ± 0.28	1.79
10^6	6	19.27 ± 0.29	1.49
10^5	5	23.32 ± 0.34	1.44
10^4	4	27.15 ± 0.31	1.15
10^3	3	30.32 ± 0.74	2.42
(-)	0	_	_

_: Not detected; (-): Negative control.

Plasmid DNA samples with concentrations of 10^7, 10^6, 10^5, 10^4, and 10^3 copies were successfully amplified, as shown by the amplification chart (Figure 8) and the melting curve (Figure 9). The threshold cycle values (Ct) were obtained after performing the realtime RT-PCR reaction with samples at different concentrations, each repeated three times (Table 1). The standard curve equation is expressed as y = -3.777x + 41.973 with a correlation coefficient of R^2 = 0.9939 (Figure 7). The slope of the standard curve was 3.777 deducing the efficiency of the Realtime PCR reaction was 84%. The reaction efficiency was low but still acceptable with a value greater than 80%.

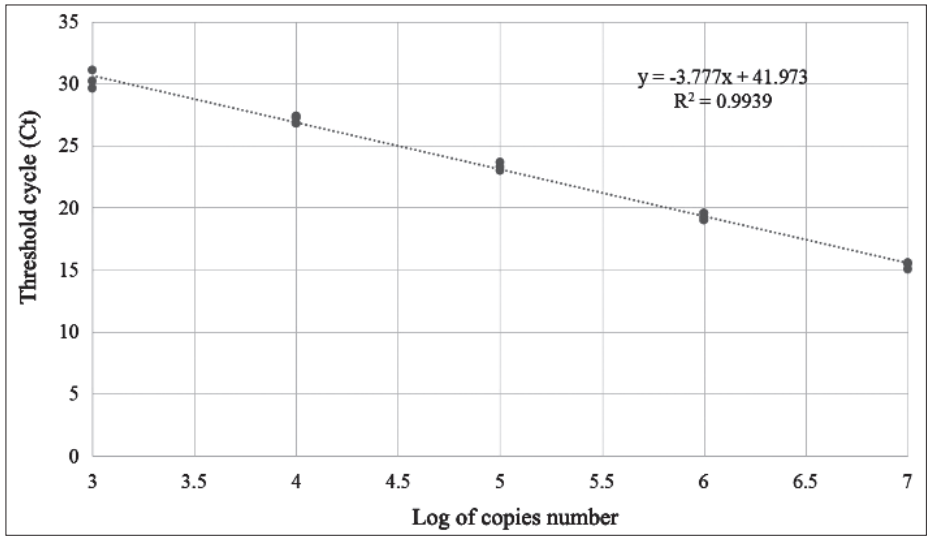


Figure 7. Standard curve generated from the plasmid containing the target gene.

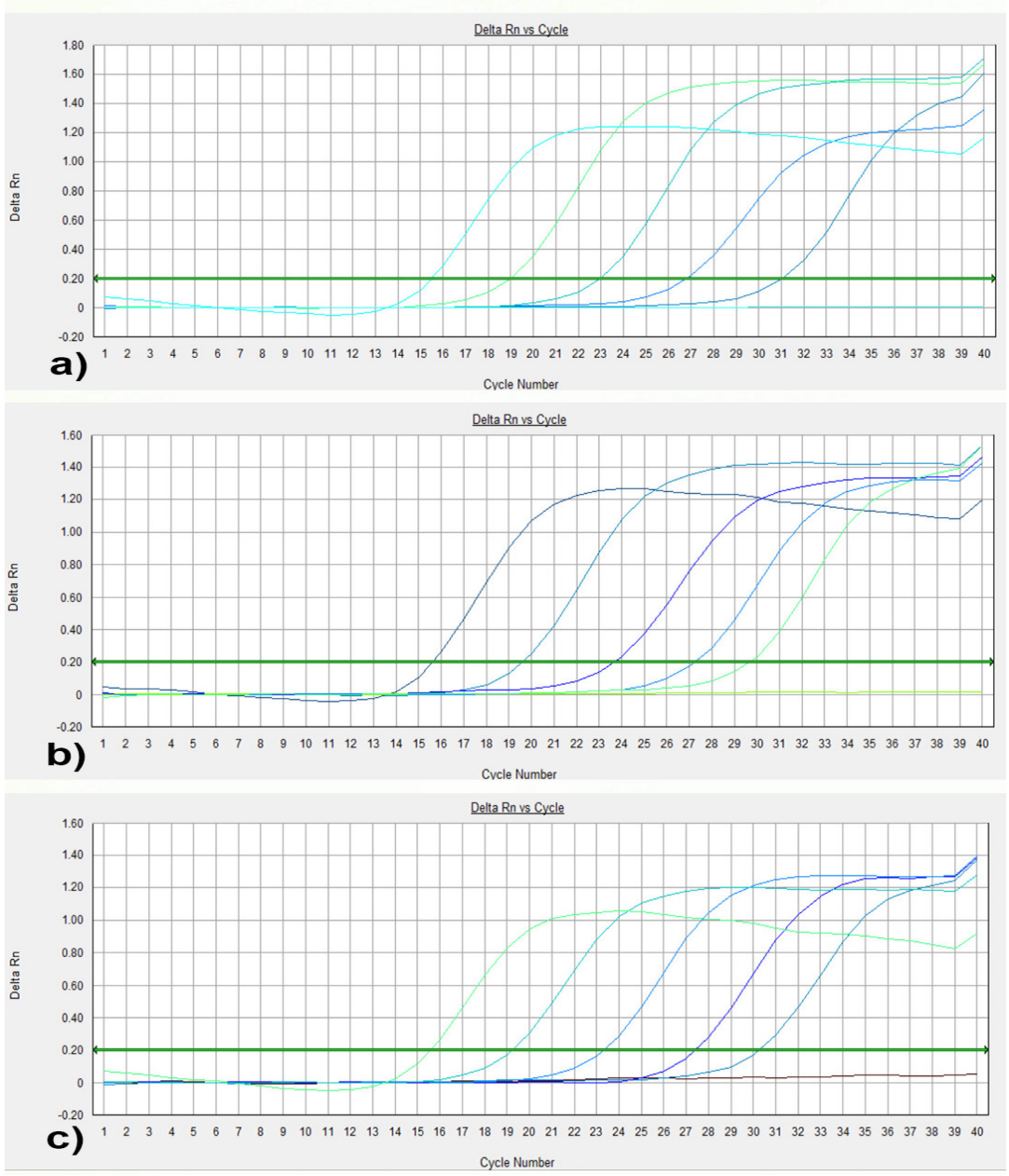


Figure 8. Gene segment amplification chart of tomato mosaic virus based on fluorescence signal. Fluorescence signal (Y vertical axis), threshold cycle (Ct) (X horizontal axis). first replication (a); second replication (b); third replication (c).

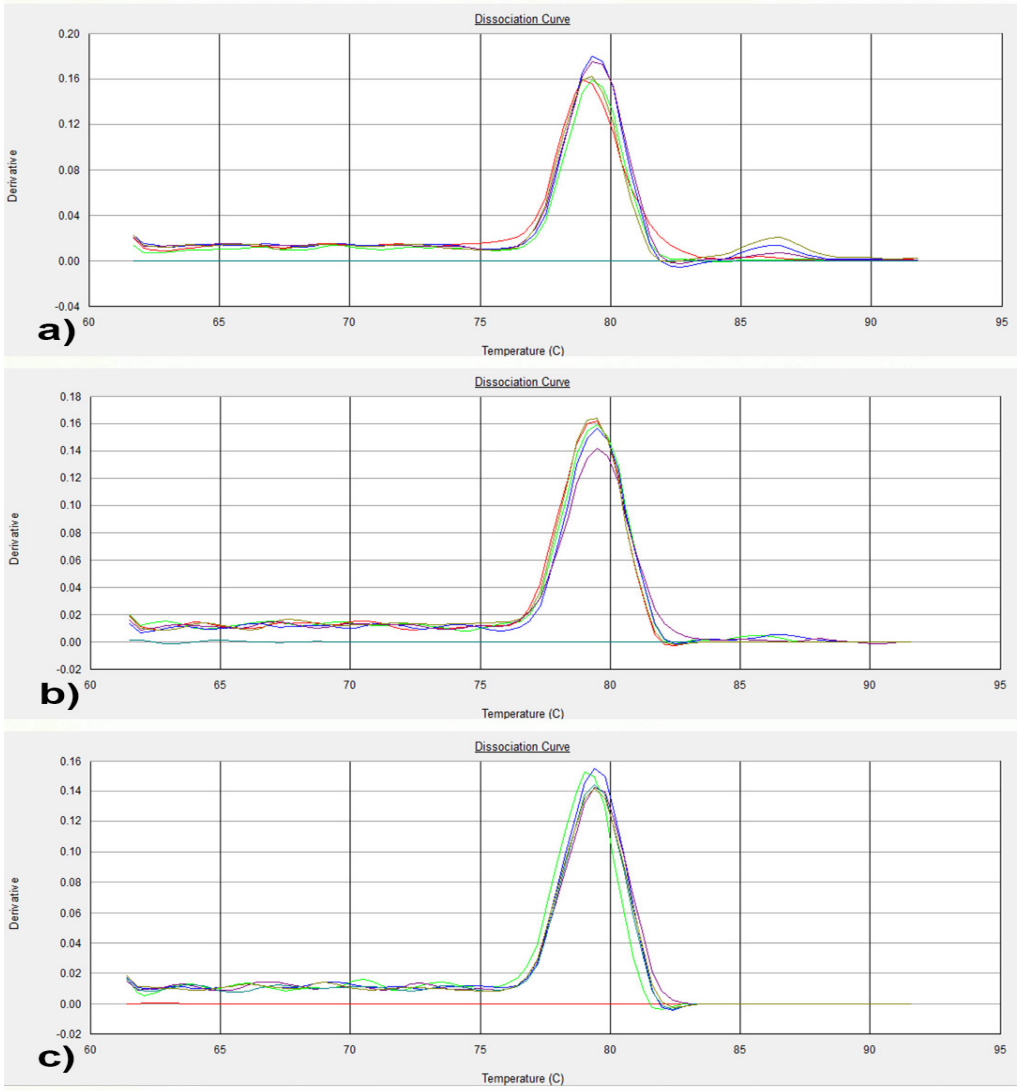


Figure 9. Melting temperature analysis chart of tomato mosaic virus gene segments. first replication (a); second replication (b); third replication (c).

3.5. Evaluation of the specificity of the realtime RT-PCR

Table 2. The results for the specificity evaluation of the realtime RT-PCR

	Threshold cycle (Ct)	Quantitative value (number of copies/ µL)	Qualitative
ToMV	16.40	5.9×10^6	+
ToMMV	_	_	-
TMV	_	_	-
ToBRFV	_	_	-

ToMV: Tomato mosaic virus; ToMMV: Tomato mottle mosaic virus; TMV: Tobacco mosaic virus; ToBRFV: Tomato brown rugose fruit virus. "+": positive , "-": negative, _ : not detected.

The result in Table 2 showed that the RT-PCR assay did not detect the virulence gene in the cDNA of TMV, ToMMV, and ToBRFV. This result suggested that the primer was specified with ToMV.

3.6. Diagnosis of ToMV on field samples with realtime RT-PCR procedure

Table 3. Tomato leaf samples were diagnosed using realtime RT-PCR and PCR methods

Sample symbol	Realtime RT-PCR method			PCR method
	Threshold cycle (Ct)	Quantitative value (number of copies/µL)	Qualitative	Qualitative
1.1	15.61	9.5×10^6	+	+
1.2	_	_	-	-
1.3	23.85	6.3×10^4	+	+
1.4	20.04	6.3×10^5	+	+
1.5	20.15	5×10^5	+	+
1.6	23.12	9.7×10^4	+	+
1.7	_	_	-	-
1.8	_	_	-	-
1.9	25.89	1.7×10^4	+	+
(+)	15.54	9.7×10^6	+	+
(-)	_	_	-	-

+: positive; -: negative; (-): negative control; (+): positive control ; _ : not detected; 1.1-1.9: Field samples 1-9.

The developed Realtime RT-PCR process detected 6 positive samples out of a total of 9 field samples. Additionally, the method quantified the field samples, with concentrations ranging from 1.7×10^4 to 9.5×10^6 copies/µL (Table 3).

The qualitative results showed 100% concordance between the realtime RT-PCR method and the RT-PCR method, which was explained by the fact that the field sampling process collected samples with symptoms of ToMV disease, so the positive samples had a high enough virus concentration to reach the detection threshold of both methods. In addition, the realtime RT-PCR method successfully quantified the ToMV virus concentration in tomato leaf samples. The realtime RT-PCR method also shows its superiority in terms of time savings compared to the RT-PCR method due to the omission of the PCR product electrophoresis step. This is important in detecting diseases early on tomatoes, thereby providing treatment and prevention measures to avoid disease outbreaks in order to reduce costs and improve crop productivity and quality for farmers.

4. Conclusions

A process for diagnosing the ToMV virus, which causes disease in tomato plants, has been established using the realtime RT-PCR method. This procedure successfully detects and quantifies the presence of the ToMV virus in tomato leaf samples, with virus amounts ranging from 1.7×10^4 to 9.5×10^6 copies/µL.

Conflict of interest

The authors declare that there are no conflict of interest to disclose related to this manuscript.

Acknowledgements

The authors send their sincere thanks to the Research Institute for Biotechnology and Environment, Ho Chi Minh City Nong Lam University for providing actual funding to carry out this research and to the farmers at the collection site. sample assisted in completing this study.

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