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Research Article

Using Multiplex RT-PCR Assay for Detection and Differentiation of Three Pepper-Infecting Viruses

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Abstract

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A multiplex reverse transcription-polymerase chain reaction (multiplex RT-PCR) method was developed to enable the simultaneous detection and differentiation of three tobamoviruses infecting peppers, namely pepper mild mottle virus (PMMoV), tobacco mosaic virus (TMV), and tomato mosaic virus (ToMV). The differentiation was achieved using 3 optimized specific oligonucleotide primer pairs, including 1 universal primer for detecting all tobamoviruses and 3 virus-specific primers as forward in the multiplex RT-PCR. The amplification of these three target viruses was finely tuned by analyzing the PCR primer ratios. The multiplex RT-PCR products generated distinct fragments: 519 base pairs (bp) for PMMoV, 228 bp for TMV, and 177 bp for ToMV. Importantly, this method exhibits a high level of specificity, as no products were amplified from non-target pepper virus RNAs as templates. Additionally, it has been demonstrated that multiplex RT-PCR is a virus-specific, sensitive, and cost-effective method for the multiple detection of pepper-infecting tobamoviruses in the field.

Key words: Tobamoviruses, Pepper mild mottle virus, Tobacco mosaic virus, Tomato mosaic virus, Detection.

INTRODUCTION

Pepper (*Capsicum annuum* L.), a member of the Solanaceae family, is widely cultivated in Taiwan. However, viral diseases can result in severe symptoms and cause epidemics and significant economic losses (Jones 2021). Among the various viruses affecting peppers, aside from insect-transmitted ones, mechanical and seed-born tobamoviruses, as highlighted by Moury & Verdin (2012), are particularly prevalent. Tobamoviruses lack insect vectors but can easily be transmitted through plant saps and mechanical tools. Tobamoviruses are highly infectious through mechanical means and can be found in seeds and plant debris (Spence *et al*. 2001), and even in wastewater (Rosario *et al*. 2009). Traditionally, tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) are the predominant tobamoviruses in pepper fields, but their management has been successful through resistance breeding utilizing resistance genes. Tobamovirus genomes consist

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of single-stranded RNA (ssRNA) encapsulated in stable, rod-shaped particles, approximately 300 nm long, known for their remarkable stability (Knapp & Lewandowski 2001). Generally, TMV-infected pepper plants exhibit chlorotic mosaic leaves, and stunted growth with distortion of younger leaves and fruits. In the case of pepper mild mottle virus (PMMoV) infection of pepper, leaf symptoms are typically mild, and may remain asymptomatic, but the virus causes striking symptoms on the fruit. These tobamovirus epidemics significantly reduce both the yield and quality of pepper production. ToMV has a broad host range, including members of the Solanaceae family such as tomatoes and peppers (Broadbent 1976).

Multiplex reverse transcription-polymerase chain reaction (multiplex RT-PCR) is a widely adopted technique known for its speed, reliability, and cost-effectiveness in simultaneously detecting multiple viruses (Viganó & Stevens 2007; Deb & Anderson 2008; Wei *et al*. 2009). In this study, we present a specific and sensitive multiplex RT-PCR method capable of simultaneously detecting and differentiating three tobamoviruses, PMMoV, TMV, and ToMV in pepper crops.

MATERIALS AND METHODS

Virus sources and cDNA clones

Virus isolates of PMMoV, TMV, ToMV, potato virus Y (PVY), pepper mottle virus (PepMoV), potato virus X (PVX), and cucumber mosaic virus (CMV) were kindly provided by Dr. Ting-Ching Deng (former virus specialist in Plant Pathology Division, Taiwan Agricultural Research Institute), and were maintained in tobacco (*Nicotiana benthamiana*) in the greenhouse (Cheng *et al*. 2013). The experiments were conducted at the Virus and Bacteria Laboratory, Division of Plant Pathology, Taiwan Agricultural Research Institute, Taiwan. The viruses were introduced to tomato or bell pepper plants (*Capsicum annuum*) by grinding with ten times the amount (w/v) of potassium phosphate buffer (0.01 M $KHPO_4$, pH 7.0). Disease symptoms were observed, and leaves were collected 6 wk after virus inoculation and used for confirmation via RT-PCR. RT-PCR products that were amplified using primers designed for the detection of PMMoV, ToMV, and TMV (Table 1, referred to as tobamo3/tobamo2 or tobamo2.1R) underwent purification. This purification step was carried out using a PCR DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer's instructions. Subsequently, these purified products were cloned into the pGEM-T vector (Promega, Madison, WI, USA). The generated plasmids were named pEASY-PMMoV, pEASY-ToMV, and pEASY-TMV. Each plasmid contained inserts of the expected size corresponding to PCR products PMMoV, TMV, and ToMV. These plasmids were used in multiplex PCR experiments.

RNA extraction

One hundred milligrams of fresh tomato or bell pepper leaves were ground in liquid nitrogen and used to extract total RNA according to the operation manual (Trizol reagent, Invitrogen, Carlsbad, CA, USA). The extracted total RNA was resuspended in 50 µL of diethyl pyrocarbonate (DEPC)-treated water and stored at -80℃ and was used for subsequent experiments.

Table 1. Nucleotide sequences of primers use in this study[z].

Primer/probe	Sequence (5′-3′)	Product (bp)	Tm (℃)	Reference
Tobamo3 (F)	5′-CAR ACN ATW GTB TAY CA-3′	540	50	Gibbs *et al*. 2004
Tobamo2 (R)	5′-TTB GCYTCR AAR TTC CA-3′	540	50	Gibbs *et al*. 2004
Tobamo2.1R	5′-TTB GCY TCA AAA TTC CA-3′	540	50	This study
PMMoV-Rp-0521-F	GCTTTTTGGTCCTGTATTTTCAGAAT			This study
ToMV-Rp-0521-F	TACAACTTTTATCGGTAATACC			This study
TMV-Rp-0521-F	TACCGCAGGTATAAAAACTTGCATC			This study

[z] bp: base pairs; PMMoV: pepper mild mottle virus; ToMV: tomato mosaic virus; TMV: tobacco mosaic virus.

Primer selection and optimization of multiplex RT-PCR

To ensure the specificity and efficiency of the multiplex RT-PCR method, previously published tobamovirus primers, tobamo2, and tobamo3 (Gibbs *et al*. 2004), were initially utilized. However, for increasing specificity, new species-specific primers, PMMoV-Rp-0521-F, TMV-Rp-0521-F, and ToMV-Rp-0521-F, were designed based on the alignment of the RNA-dependent RNA polymerase (RdRp) gene sequences of PMMoV, TMV, and ToMV obtained from the National Center for Biotechnology Information (NCBI) GenBank (Fig. 1, Fig. 2, and Fig. 3). These primers were combined with tobamo2.1, modified from tobamo2, for multiplex RT-PCR

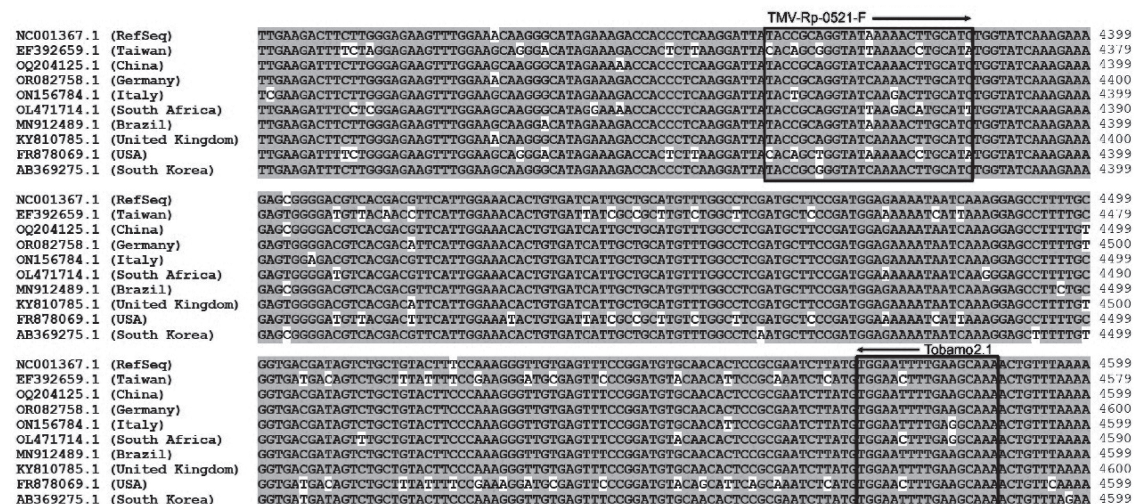


Fig. 1. Sequences alignment of tobacco mosaic virus and the position of the designed primers (shown in black box). Accession numbers for the virus isolates (top to bottom) are NC001367.1, EF392659.1, OQ204125.1, OR082758.1, ON156784.1, OL471714.1, MN912489.1, KY810785.1, FR878069.1, and AB369275.1. The design of the primers was based on the conserved sequences within the RNA-dependent RNA polymerase (RdRp) gene.

Fig. 2. Sequences alignment of tomato mosaic virus and the position of the designed primers (shown in black box). Accession numbers for the virus isolates (top to bottom) are NC002692.1, KJ207374.1, KR537870.1, ON156781.1, OL652662.1, MW848529.1, MW012409.1, LC650928.1, and KU321698.1. The design of the primers was based on the conserved sequences within the RNA-dependent RNA polymerase (RdRp) gene.

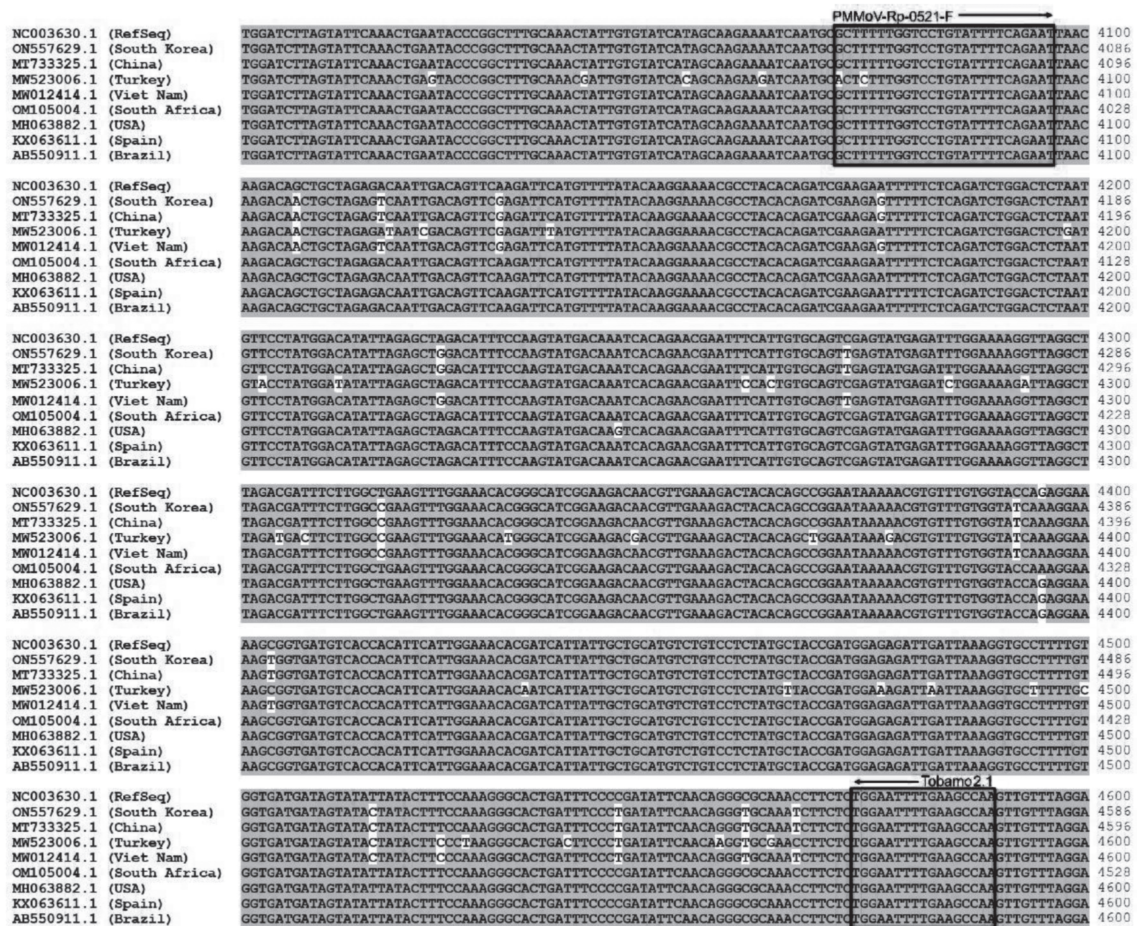


Fig. 3. Sequences alignment of pepper mild mottle virus and the position of the designed primers (shown in black box). Accession numbers for the virus isolates (top to bottom) are NC003630.1, ON557629.1, MT733325.1, MW523006.1, MW012414.1, OM105004.1, MH063882.1, KX063611.1, and AB550911.1. The design of the primers was based on the conserved sequences within the RNA-dependent RNA polymerase (RdRp) gene.

(Fig. 4). To optimize the multiplex PCR reaction, different combinations of primer ratios and concentrations were tested. The concentration ranges for each primer were as follows: 0.1–0.15 μM of PMMoV-Rp-0521-F, 0.2–0.4 μM of TMV-Rp-0521-F, 0.1–0.4 μM of ToMV-Rp-0521-F, and 0.4 μM of tobamo2.1R. In a 25 μL RT reaction, 2 μg of total RNA extracted from bell pepper or tomato leaves and 1 μL of random hexamer primers (50 μM) were mixed thoroughly and incubated at 70℃ for 5 min, then placed on ice. Subsequently, 5 μL of 5× first-strand buffer (Promega, Madison, WI, USA), 5 μL of 10 mM dNTPs, 1 μL of Moloney murine leukemia virus (MMLV) reverse transcriptase (200 U) (Promega), and 1 μL of recombinant ribonuclease (RNasin) ribonuclease inhibitor (25 U) (Promega) were added. Make up the volume to 25 μL with sterile water, and allow the reaction to proceed for 60 min at 42℃. In the PCR reaction, 2.5 μL of 10× PCR buffer (Protech, Taipei, Taiwan), 0.5 μL of 10 mM dNTPs, 0.5 μL each of the primers: PMMoV-Rp-0521-F (0.1–0.15 μM), TMV-Rp-0521-F (0.2–0.4 μM), ToMV-Rp-0521-F (0.1–0.4 μM), and tobamo2.1R (0.4 μM), and 2 μL of the cDNA template were mixed, and adjust the volume to 25 μL with ddH$_2$O. The PCR reaction

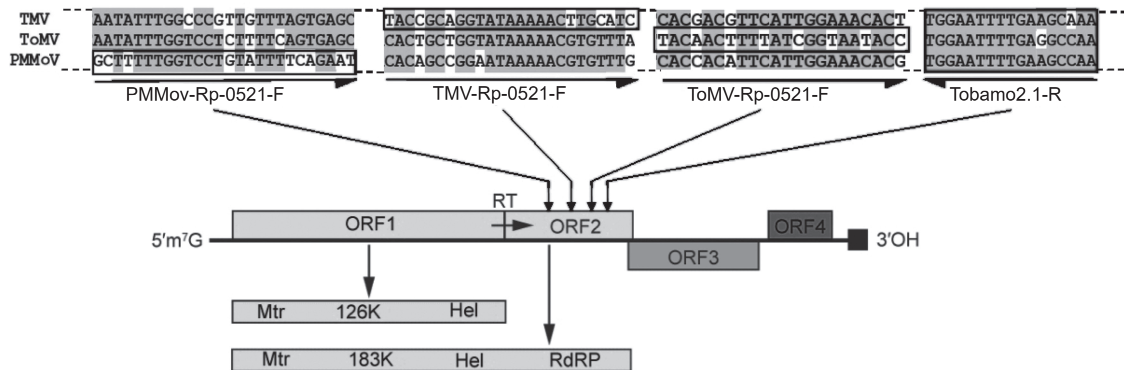


Fig. 4. A schema showing the genome organization of the four predicted open reading frames (ORFs) of a tobamovirus and the positions of the designed primers. RT: reverse transcription.

conditions are set as initial denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s. Finally, the reaction was extended at 72℃ for 5 min and held at 16℃ to complete the reaction. To analyze the PCR products, 5 μL of the reaction mixture was subjected to 1.5% agarose gel electrophoresis. A gel imaging system (Bio-Rad, Hercules, CA, USA) was used to visualize and document the electrophoresis results.

Specificity assay of multiplex RT-PCR

To assess the specificity of multiplex RT-PCR for PMMoV, ToMV, and TMV, samples collected from bell pepper or tomato plants infected with PMMoV, ToMV, TMV, PVY, PepMoV, PVX, or CMV, along with samples from healthy bell pepper or tomato plants were tested. Total RNA extracted from these samples was subjected to RT-PCR using mixed primers under the same reaction conditions as described above.

Sensitivity assay of multiplex PCR

To evaluate the sensitivity of multiplex RT-PCR in detecting PMMoV, TMV, and ToMV, this study employed a methodology involving cloned plasmid DNA containing fragments of these viral genes. The plasmids were mixed in equal proportions, and sensitivity was assessed using a series of 10× dilutions ranging from 1×10^2 to 1×10^{10} copies. These dilutions were created by combining the viral gene-selected plasmids with pEASY-PMMoV, pEASY-ToMV, and pEASY-TMV at individual concentrations of 50 ng μL^{-1}. Subsequently, 5 μL of the PCR product was extracted for analysis through 1.5% agarose gel electrophoresis. The electrophoresis results were visualized using a gel imaging system (Bio-Rad, Hercules, CA, USA).

Evaluation of mixed viruses in pepper samples using multiplex RT-PCR

Bell peppers infected with PMMoV and TMV, and tomatoes infected with ToMV were utilized in this study. Approximately 0.1 g of leaf tissue from each infected sample was collected and subjected to RNA extraction following the total RNA purification described above. These extracted total RNAs were divided into 3 sets: one for each virus (PMMoV, TMV, and ToMV), another set combining 2 viruses (PMMoV + TMV, TMV + ToMV, and ToMV + PMMoV), and the third set containing all 3 viruses (PMMoV + TMV + ToMV). These RNA sets were subjected to RT-PCR reactions as described above.

RESULTS

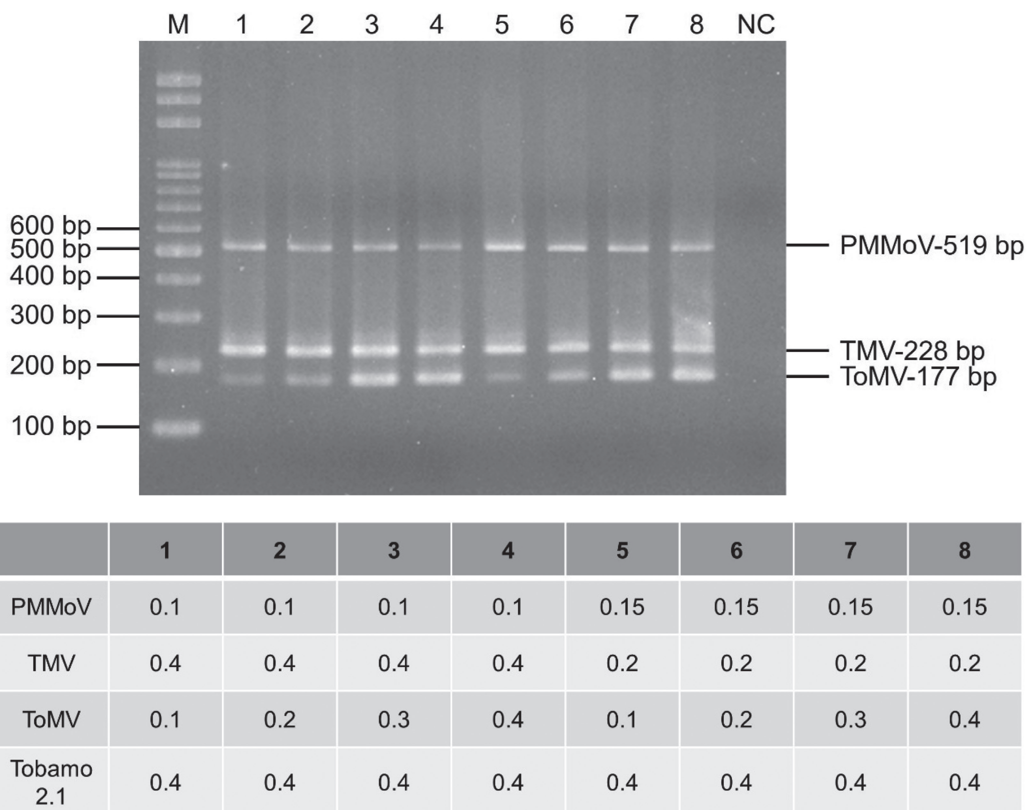
Optimization of the RT-PCR assay

To assess the suitability of the newly designed species-specific primers (PMMoV-Rp-

0521-F, TMV-Rp-0521-F, and ToMV-Rp-0521-F) in combination with the universal *Tobamovirus* primer (tobamo2.1), a series of experiments were conducted. Initially, it was confirmed that PMMoV, TMV, and ToMV could be all amplified by PCR using the universal *Tobamovirus* primer pair tobamo2/tobamo3 (data not shown) (Gibbs *et al*. 2004). Subsequently, the newly designed species-specific primers were tested in combination with tobamo2.1 for multiplex RT-PCR. Several primer ratios at different mixing concentrations were explored to optimize the PCR reaction conditions (Fig. 5). The optimal primer concentration combination for multiplex RT-PCR was determined to be PMMoV : ToMV : TMV : tobamo2.1 = 0.15 : 0.2 : 0.3 : 0.4. Using this combination, the PCR products for PMMoV, ToMV, and TMV were specifically amplified, resulting in amplicon sizes of 519 base pairs (bp) for PMMoV, 228 bp for TMV, and 177 bp for ToMV (Fig. 5).

Specificity of the RT-PCR for tobamovirus detection

The findings indicated that the merged primers could generate a 519-bp product when subjected to PCR with PMMoV. Additionally, a 228-bp product was yielded when employed in PCR with TMV, and a 177-bp product was obtained when utilized in PCR with ToMV. Each multiplex RT-PCR did not react with PVY, PepMoV, PVX, or CMV as well as healthy bell



	1	2	3	4	5	6	7	8
PMMoV	0.1	0.1	0.1	0.1	0.15	0.15	0.15	0.15
TMV	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2
ToMV	0.1	0.2	0.3	0.4	0.1	0.2	0.3	0.4
Tobamo 2.1	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

Fig. 5. Optimization of multiplex reverse transcription-polymerase chain reaction (multiplex RT-PCR) with different concentration ratios of primer set. Total RNA of mix-infected sweet pepper plants was used for multiplex RT-PCR amplification. The primer concentration ratio of each treatment is shown at the lower panel of the figure. The number indicates the concentration (μM) of each primer pair in a PCR reaction. The RT-PCR products of tobamoviruses identified by 1.5% agarose gel electrophoresis are indicated by arrows. Lane M: 100 base pairs (bp) DNA ladder (Invitrogen, Carlsbad, CA, USA); Lane NC: no template control.

peppers and tomatoes. Furthermore, no primer dimers were formed in the nucleic acid amplification products (Fig. 6).

Sensitivity of the multiplex PCR

To determine the detection limit of multiplex PCR, the products of PMMoV, TMV, and ToMV amplified by PCR were cloned into plasmid DNA. A plasmid mix containing these clones was prepared, and a 10× serial dilution, ranging from 10^9 to 10^0 copies in 10 sets, along with a no template control (NC), was used to assess the sensitivity of the multiplex PCR. Sensitivity was tested using the PCR method, and the results showed that the sensitivity could reach as low as 10^4 copies (Fig. 7).

Detection of the three tobamoviruses in plant samples

To evaluate the performance of the established multiplex RT-PCR, artificially mixed viral RNAs extracted from infected plants

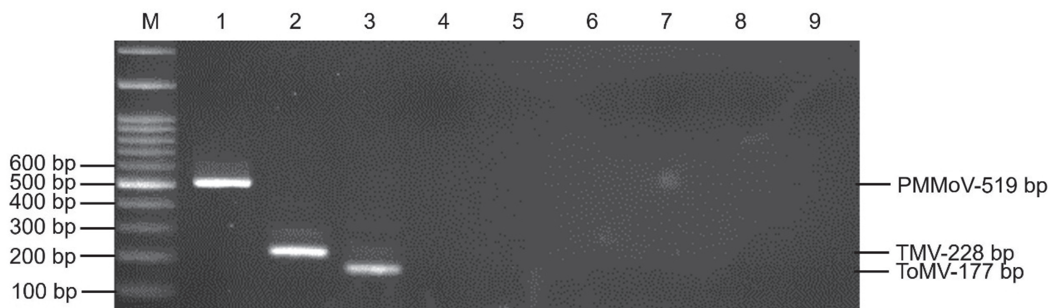


Fig. 6. Specificity of multiplex reverse transcription-polymerase chain reaction (multiplex RT-PCR) for detection of pepper mild mottle virus (PMMoV), tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV). Lane M: 100 base pairs (bp) DNA ladder; Lane 1: PMMoV; Lane 2: ToMV; Lane 3: TMV; Lane 4: potato virus Y (PVY); Lane 5: pepper mottle virus (PepMoV); Lane 6: potato virus X (PVX); Lane 7: cucumber mosaic virus (CMV); Lane 8: healthy bell pepper; Lane 9: healthy tomato.

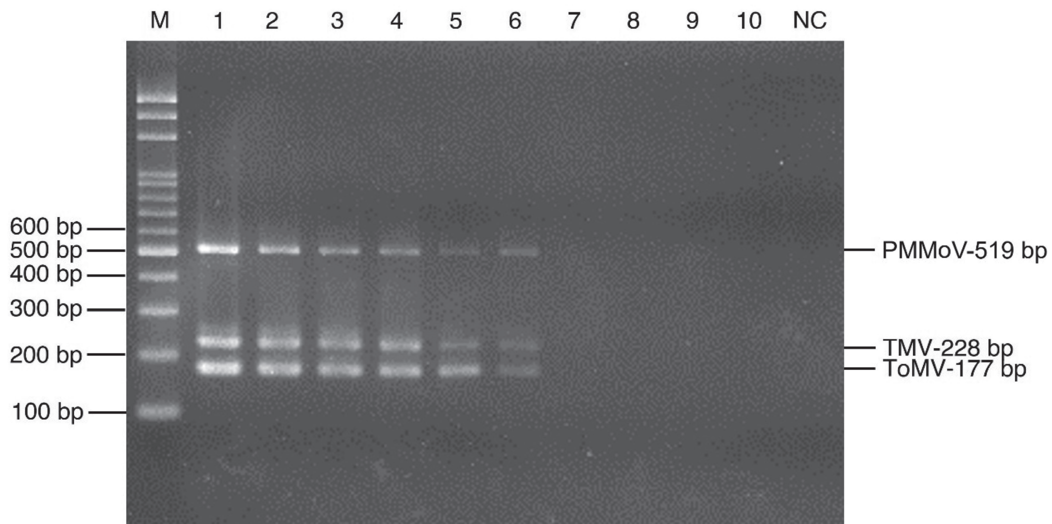


Fig. 7. Estimation of the detection limit of multiplex PCR for pepper mild mottle virus (PMMoV), tobacco mosaic virus (TMV), and tomato mosaic virus (ToMV) using 10× serial dilutions of viral clones. Lane M: 100 base pairs (bp) DNA ladder; Lane NC: no template control; Lane 1–10: 10^9–10^0 copies of viral RNA-dependent RNA polymerase (RdRp) genes.

were tested. The results demonstrated that the multiplex RT-PCR could detect various combinations of mixed virus RNA extracts. Specifically, it successfully detected the presence of PMMoV RNA, TMV RNA, ToMV RNA, PMMoV + TMV RNAs, TMV + ToMV RNAs, and ToMV + PMMoV RNAs, as well as the combination of PMMoV + TMV + ToMV RNAs. These detections resulted in the generation of target fragments of 519 bp (PMMoV), 228 bp (TMV), and 177 bp (ToMV). Importantly, no false-positive reactions were observed when healthy bell pepper plants were tested (Fig. 8).

DISCUSSION

The PCR-based method has become a general and routine virus diagnostic method, capable of confirming specific viral infections. If combined with multiplex RT-PCR, it can be used to detect multiple viruses at the same time, offering advantages of speed, reliability, cost reduction, and shorter processing time (Viganó & Stevens 2007; Deb & Anderson 2008; Wei *et al*. 2009). Our study marks the first instance of using the RdRp gene to identify three different tobamoviruses. In general, detecting multiple viruses simultaneously requires 6 primers (3 primer pairs), but our assay only requires 4 primers while maintaining specificity for distinguishing and identifying these three viruses.

Gibbs *et al*. (1998) developed an RT-PCR detection method using a pair of RdRp region-specific primers based on the conservative RNA sequence of tobacco mosaic virus. However, their evaluation was limited to the use of a hybridization-based method. Vinayarani *et al*. (2011) previously established a multiplex RT-PCR detection method for distinguishing TMV and ToMV in chili varieties. Nemes & Salánki (2020) simultaneously detected five viruses (CMV, TMV, PMMoV, PVY, and tomato spot wilt virus (TSWV)) in sweet peppers, but they did not simultaneously detect PMMoV, TMV, and ToMV. Moreover, their results could only detect 2 tobamovirus-related viruses. Currently, differentiating these three viruses by conventional methods remains challenging, except through nucleic acid sequencing.

Another approach employs a universal RT-PCR assay using 2 degenerate primers (Letschert *et al*. 2002), but it can only detect members of tobamoviruses (i.e., TMV, ToMV, PMMoV, Odontoglossum ringspot virus, and tobacco mild green mosaic virus). Dovas *et al*. (2004) described a point-nested reverse transcriptase-polymerase chain reaction-restriction fragment length polymorphism (RTPCR-RFLP) method for TMV detection. However, this

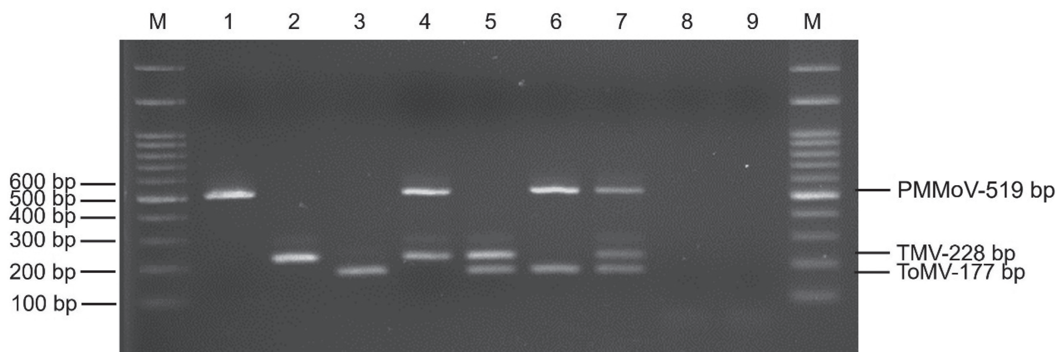


Fig. 8. Multiplex RT-PCR detection of pepper-infecting viruses, pepper mild mottle virus (PMMoV), tobacco mosaic virus (TMV), or tomato mosaic virus (ToMV) from pepper leaves in artificial combinations of infections using mixed RNAs. Lane M: DL2000 DNA Marker; Lane 1: PMMoV RNA; Lane 2: TMV RNA; Lane 3: ToMV RNA; Lane 4: PMMoV + TMV RNAs; Lane 5: TMV + ToMV RNAs; Lane 6: ToMV + PMMoV RNAs; Lane 7: PMMoV + TMV + ToMV RNAs; Lane 8: healthy bell pepper RNA; Lane 9: no template control (NC).

method is time-consuming, labor-intensive, and costly due to the repeated PCR amplification and restriction enzyme digestion. Therefore, new primer designs were tested for multiplex RT-PCR. Primer ratios are crucial factors affecting PCR specificity and amplification efficiency (Ma & Michailides, 2007; Wei *et al*. 2009). Hence, primer ratios were optimized, and our results indicated that well-designed primers can avoid the generation of primer dimers. From the above results, it can be concluded that the minimum detectable virus concentration using RT-PCR is 10^4 copies in a 25 uL reaction.

In our study, the newly developed multiplex RT-PCR can simultaneously detect and differentiate PMMoV, TMV, and ToMV using three specific forward primers PMMoV-Rp-0521-F, TMV-Rp-0521-F, and ToMV-Rp-0521-F, combining a universal reverse primer tobamo2.1R. This multiplex RT-PCR method could be used for virus detection in single virus infection as well as in mixed infection. This indicates that the multiplex RT-PCR is capable of accurately identifying various mixed virus infection scenarios. In addition, the use of artificially infected bell peppers with three viruses also showed that this combination of primer pairs can simultaneously detect the three viruses. The method we developed offers a simple and convenient approach to developing multiplex assays based on previously published primers and can be supplemented with newly designed primers when necessary. This specific and sensitive method for detecting multiple tobamoviruses in the Solanaceae family is suitable for large-scale sampling efforts to study the distribution of tobamoviruses in Taiwan and other regions worldwide. This detection technique can facilitate research in tobamovirus epidemiology, outbreak monitoring, and the study of interactions between viruses, hosts, and vectors.

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使用多重 RT-PCR 檢測和區分 3 種感染甜椒病毒

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摘要

關政平、蕭崇仁、鄭櫻慧、陳述。2024。使用多重 RT-PCR 檢測和區分 3 種感染甜椒病毒。台灣農業研究 73(2):89–99。

本研究利用多重反轉錄聚合酶鏈鎖反應 (multiplex reverse transcription-polymerase chain reaction; multiplex RT-PCR) 方法，同時檢測與區分感染甜椒的 3 種 tobamoviruses，分別是辣椒輕斑駁病毒 (pepper mild mottle virus; PMMoV)、菸草嵌紋病毒 (tobacco mosaic virus; TMV) 及番茄嵌紋病毒 (tomato mosaic virus; ToMV)。使用 1 種用於檢測所有 tobamoviruses 的通用引子與 3 種病毒專一性引子透過分析 PCR 引子比率，對這 3 種目標病毒的擴增進行調整。產生的 PCR 產物由不同的片段組成：PMMoV 為 519 base pairs (bp)，TMV 為 228 bp 及 ToMV 為 177 bp。本方法表現出高度的專一性，沒有與其他非目標甜椒病毒擴增產生特定的產物。此外，multiplex RT-PCR 已被證明是對檢測甜椒植物中多種 tobamoviruses，為一種特異性、靈敏且經濟有效的方法。

關鍵詞：辣椒輕斑駁病毒、菸草嵌紋病毒、番茄嵌紋病毒、偵測。

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