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Protocols

A multiplex reverse transcription PCR assay for simultaneous detection of six main RNA viruses in tomato plants

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ABSTRACT

Tomato virus diseases occur all around the world, causing serious yield losses. To detect these viruses quickly and provide a basis for disease control, a multiplex reverse transcription polymerase chain reaction system was established for simultaneous detection of *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), *Tomato chlorosis virus* (ToCV), *Potato virus Y* (PVY) and *Potato virus X* (PVX) in tomato plants, with 6 pairs of specific primers being designed based on the coat protein (CP) genes of these viruses. *Transcriptional elongation factor-1α* (EF-1α) from tomato was added to the multiplex RT-PCR reaction system to prevent false negatives. The concentration of the primers, annealing temperature, annealing time, extension time and amplification cycles were optimized. Expected fragments of 159 bp (ToCV), 262 bp (PVY), 362 bp (EF-1α), 430 bp (TMV), 500 bp (TSWV), 600 bp (CMV) and 705 bp (PVX) were amplified by this multiplex RT-PCR system, and their origin was confirmed by DNA sequencing. This method will have a wide application in virus detection of field samples.

1. Introduction

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops. The total production of tomatoes worldwide reached 170 million metric tons in 2014 (Food and Agricultural Organization, United Nations; http://www.fao.org/home/en/). However, many tomato viruses occur commonly and it is difficult to distinguish between them based on symptoms alone, especially in mixed infections, which commonly occur in the field. In China, the main RNA viruses in tomato crops include Tobacco mosaic virus (TMV), Cucumber mosaic virus (CMV), Potato virus X (PVX) and Potato virus Y (PVY); (Feng et al., 1987). In addition, Tomato spotted wilt virus (TSWV) and Tomato chlorosis virus (ToCV) have become important viruses in tomato production in China (Mo et al., 2016; Zhou et al., 2014), with serious loss of yield, quality and other economic issues.

TMV, the type member of the genus *Tobamovirus* (Creager et al., 1999), was the first tomato virus pathogen found in the USA (Pelham, 1972). TMV infects many plant species, especially members of the family *Solanaceae* including tomato, tobacco and pepper (Alonso et al., 2013).

CMV, the type member of the genus *Cucumovirus,* has a tripartite, positive-sense, single-stranded RNA genome (Katoch et al., 2009). Up to 85 families, including > 1,100 species can be infected by CMV, and most of them are crop plant. CMV infection induces a variety of symptoms on plants, resulting in mosaics, dwarfing, and systemic necrosis. CMV mainly relies on aphids to spread in a non-persistent manner, and about 75 species of aphids are known to transmit this virus (Xu and Barnett, 1984).

PVX and PVY are type members of the genera *Potexvirus* and *Potyvirus*, respectively. PVX is mechanically transmitted and PVY is transmitted in the field non-persistently by aphids (Harrison, 1985).

TSWV, the type member of the genus *Tospovirus* (Parrella et al., 2003), belongs to the family *Bunyaviridae*. This virus causes damages on vegetable and horticultural crops, including *Solanaceae*, *Compositae*, and *legumes*. It is estimated that TSWV infections of vegetable crops result in losses of billions of dollars every year (Adkins, 2000). This virus is transmitted by *Frankliniella occidentalis*. The main symptoms caused by TSWV infection are abnormal coloring on leaves, with black spots and deformities.

ToCV belongs to the genus *Crinivirus*, and causes damage on a global scale. This viral disease was first found on tomatoes in the USA in 1989 (Bese et al., 2011; Fiallo-Olivé et al., 2011). It cannot be transmitted mechanically, but instead by 4 types of whitefly (Karwitha et al., 2014; Zhao et al., 2013). ToCV can infect at least 30 plant species, including

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Table 1
Virus-specific primers for multiplex RT-PCR.

Primer	Sequence (5'-3')	Length (bp)	T_m (℃)	Products size (bp)	Primer binding site[a]
TMV-F	TCTTGTCATCAGCGTGGGC	19	57.3	430	38-56
TMV-R	CCAGAGGTCCAAACCAAACCA	21	57.6		447-467
CMV-F	TGGACAAATCTGAATCAACCAGTG	24	56.2	600	2-25
CMV-R	GTACTAGCTCGTCCGTCTCG	20	59.5		582-601
PVX-F	TCAGCACCAGCTAGCACAAC	20	57.4	705	167-186
PVX-R	TGGTGGGAGAGTGACAACAGC	21	59.5		851-871
PVY-F	ATACTCGGGCAACTCAATCAC	21	55.6	262	75-95
PVY-R	GCTTCTGCAACATCTGAGAAATG	23	56		314-336
TSWV-F	CAGAATCTGGTAGCATTCAACTTCA	25	56.3	500	33-57
TSWV-R	ACTTTTCCTAAGGCTTCCCTG	21	55.6		512-532
ToCV-F	AGGGACCTCAGTTAAAGCAGC	21	57.6	159	372-392
ToCV-R	TCATGACTTCTGGCGTACCG	20	57.4		511-530
EF-1α-F	GGCTGACTGTGCTGTTCTCA	20	57.4	362	384-403
EF-1α-R	GCCTCTTGGGCTCGTTAATCT	21	57.6		725-745

[a] Primer binding site was according to GenBank AM412008.1, AB448696.1, Z29333.1, AJ890332.1, KF006260.1, KC311375.1 and X14449.1 respectively.

vegetables, horticultural crops and weeds (Fortes et al., 2012; Lozano et al., 2004; Vargas et al., 2011; Vargas-Asencio et al., 2013). The symptoms on infected tomato are very similar to those associated with plant malnutrition.

Currently, detection methods of tomato viruses include reverse transcription polymerase chain reaction (RT-PCR) ; (Dai et al., 2013; Du et al., 2013; Gil-Salas et al., 2007; Kil et al., 2015), enzyme-linked immunosorbent assay (ELISA; Hu et al., 1995; Kapoor et al., 2014; Sun et al., 2000; Zahn et al., 2011), reverse transcription loop-mediated isothermal amplification (RT-LAMP) ; (Karwitha et al., 2016; Zhao et al., 2012) and electron microscopy (Ie, 1971). RT-PCR has the characteristics of high sensitivity and specificity, and it is widely used in plant virus detection. Serology methods are often used for the detection of large numbers of samples, though with low sensitivity. Electron microscopy, which is time-consuming and technically complicated, is often adopted for virus identification. Traditional RT-PCR methods can only detect one virus species in one reaction, with a specific primer pair for one target sequence. Separate amplifications need to be set up for multiple viruses. Multiple set ups for amplification are cumbersome and increase the risk of cross contamination.

Multiplex RT-PCR is a convenient detection method that uses multiple pairs of primers in 1 reaction tube for simultaneous amplification of multiple gene sequences (Casas et al., 1999). This technique reduces the reagents and time used in setting up reactions, which makes it more efficient, fast and economical, and also improves detection accuracy at the same time (Henegariu et al., 1997). Plant viral infections are often complex in the field. Multiplex RT-PCR has been developed as a crucial detection method that is widely used for detecting a great variety of plant viruses (Adeyemi et al., 2017; Tao et al., 2012). In this study, a high-efficiency multiplex RT-PCR system was developed, which could simultaneously detect 6 tomato viruses, CMV, TSWV, TMV, ToCV, PVX and PVY from tomato samples in the field. As such, it was also capable of detecting mix infections of up to 6 tomato viruses in a single plant.

2. Materials and methods

2.1. Plant and viral materials

The virus-infected tomato leaf samples were collected from different tomato fields of Shaanxi, Gansu and Shandong provinces in China. Healthy tomato leaves from the laboratory and infected leaves with viruses were stored at −80℃ for later use. The viruses were detected in these samples by RT-PCR.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted from healthy and infected tomato leaves with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the product manual. The first-strand cDNA was synthesized using PrimeScript™ II 1 st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China).

2.3. Primer design

The specific primers were designed based on nucleotide sequences of viral coat protein (CP) genes for the specific viruses. All primers for multiplex RT-PCR were obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast; Ye et al., 2012). To distinguish the differentiation of multiplex RT-PCR results, the optimal primer pairs were selected with similar annealing temperatures, no non-specific amplification and optimal size of the respective amplicons. In addition, CP sequences were downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov). Primer sequences were blasted against these sequences using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software to ensure the primers were specific to their respective virus, but also located in conserved regions with regard to strains of the same virus. Ten pairs of specific primers were designed as candidates for each targeted virus, and the best primers for multiplex RT-PCR are shown in Table 1. These primers were synthesized and purified by PAGE at the Tsingke Biological Technology Company (Beijing, China).

2.4. Simplex RT-PCR

For the optimization of multiplex RT-PCR, simplex RT-PCR was carried out to ensure the material is positive. Six tomato viral cDNA solutions were mixed equally and 4 μL was taken as the template for simplex RT-PCR. Total of 50 μL PCR reaction volume included 25 μL of 2 × Es Taq MasterMix (CW Biotech, Beijing, China), 4 μL of forward and reverse primer mixtures (10 μmol L^{-1} each) and 17 μL of ddH_2O. The PCR reaction protocol was: pre-denaturation at 94℃ for 3 min; followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 54℃ for 30 s and extension at 72℃ for 30 s; with a final extension at 72℃ for 10 min. Five μL of the PCR products were electrophoresed on a 2.5% agarose gel with 0.01% Goldview (HEART, Xi'an, China). Results were observed with a UV transilluminator (Bio-Rad, Hercules, CA, USA).

2.5. Cloning and sequencing of RT-PCR products

To confirm that the RT-PCR amplification products were of the correct origin, they were purified with a gel extraction kit (BioTeke Corp., Beijing, China) and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). After identifying positive clones by PCR, the extracted plasmids were sequenced by the Tsingke Company (Beijing, China).

2.6. Optimization multiplex RT-PCR

Multiplex RT-PCR was optimized to enable synchronous amplification of 7 target regions in 1 reaction. Six tomato viral cDNA solutions were mixed equally as template for multiplex RT-PCR. The optimized factors of the multiplex RT-PCR were: the ratio of the 7 pairs of primers, annealing temperature (44 - 65℃, in 3℃ increments), annealing time (10–60 sec, in 10 s increments), extension time (20–70 sec, in 10 s increments), and number of cycles (25–45 in 5 cycle increments). Fifty μL multiplex RT-PCR reactions were amplified using a Multiplex PCR Assay Kit (TaKaRa, Dalian, China).

2.7. Evaluation of the sensitivity of multiplex RT-PCR

An equal mixture of 6 tomato virus cDNAs was used as a template. After a 10-fold serial dilution (10° – 10^{-6}), the individual virus sequences were detected by simplex RT-PCR and multiplex RT-PCR. For quantification of multiplex RT-PCR detection using plasmid clones of the PCR fragments, the initial plasmid amount was set to 4 pg. Systematic comparisons were made between different concentrations of virus species to test the limits of detection, where EF-1α and one virus were in high concentration (4 ng) while the others were at their lowest detectable concentrations.

2.8. Stability of multiplex RT-PCR

The stability of multiplex RT-PCR was detected using various combinations of cDNA solutions. Each one of the 6 different virus cDNAs was individually removed to assess the multiplex RT-PCR system for accuracy.

2.9. Application of multiplex RT-PCR

Field tomato samples with unknown virus infections were collected in Shaanxi, Gansu and Shandong provinces and tested by multiplex RT-PCR. Total RNA extraction and reverse transcription were as described above; 4 μL of RT products were used for multiplex PCR and 5 μL of amplification product for electrophoretic analysis.

3. Results

3.1. Specificity of primers

In this study, all primers were designed from multiple alignments of 6 CP gene sequences and an internal control gene. Simplex RT-PCR amplified products of 705 bp, 600 bp, 500 bp, 430 bp, 362 bp, 262 bp and 159 bp corresponding in size to the expected target sequence from PVX, CMV, TSWV, TMV, EF-1α, PVY and ToCV, respectively (Fig. 1). In the simplex RT-PCR experiment, despite a mixed solution of 6 viral cDNAs being used as template, only the band specific to the primer set was amplified. This demonstrated the specificity of the designed primers and suggested they could be successfully incorporated in a multiplex RT-PCR.

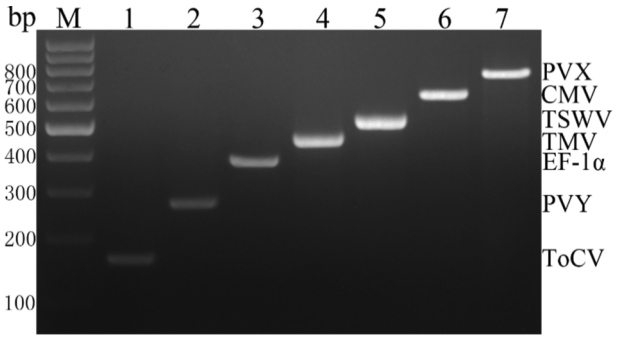

Fig. 1. The specificity of the primers was confirmed by simplex RT-PCR. Lanes 1 - 7, simplex RT-PCR to detect ToCV, PVY, EF-1α, TMV, TSWV, CMV and PVX, respectively. Lane M, 100 bp DNA Ladder.

3.2. Confirmation of PCR amplification products by DNA sequencing

The sequences from cloned PCR fragments were aligned with those of viruses in GenBank, confirming the validity and specificity of the PCR. The sequences showed a high level of homology with the coat protein genes of the 6 viruses and EF-1α.

3.3. Optimization of multiplex RT-PCR

Many gradient parameter tests, including annealing temperature, annealing time, extensive time and cycle times were conducted to determine the optimal combination for multiplex RT-PCR. Firstly, 7 pairs of primers with different proportions were evaluated, and the best primer concentrations were 2 μL, 4 μL, 1 μL, 1 μL, 2 μL, 1 μL and 2 μL of each10 μM solution corresponding to PVX, PVY, CMV, TMV, ToCV, TSWV and EF-1α, respectively. This optimized mixture of primers was used for multiplex RT-PCR.

The annealing temperature was increased from 44 to 65℃, in increments of 3℃. With an annealing temperature of 44 - 47℃, the PVY target fragment was blurred with non-specific fragments. When the annealing temperature was higher than 62℃, the TSWV target band brightness was reduced or disappeared. The optimum annealing temperature was found to be 56℃ for multiplex RT-PCR (Supplementary Fig. S1).

The annealing time was increased from 10 s to 60 s in increments of 10 s. Some target bands increased in brightness with an increase in annealing time. The optimum annealing time was found to be 50 s for multiplex RT-PCR (Supplementary Fig. S2).

When the extension time was increased from 20 s to 70 s in increments of 10 s all the bands were amplified to a greater extent; however, the brightness of some target bands decreased when the time was > 60 s. It was concluded that the best extension time for the 6 virus targets was 60 s (Supplementary Fig. S3).

The amplification cycle number was increased from 25 to 45 in increments of 5 cycles. When the cycle number was less than 30, the ToCV and PVY bands could not be observed on gel electrophoresis. As increasing the number of cycles to > 40 did not noticeably increase the yield of the PCR target products, 40 was selected as the optimum cycle number (Supplementary Fig. S4).

Overall, the optimal reaction system was established, which included 25 μL 2 × Multiplex PCR Buffer, 0.25 μL Multiplex PCR Enzyme Mix, 6 μL mixed cDNA as the multiplex RT-PCR template, 13 μL primer mixture, 5.75 μL ddH_2O, in a total volume of 50 μL. The optimized program was 94℃ for 1 min, followed by 40 cycles of denaturation at 94℃ for 30 s, annealing at 56℃ for 50 s, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min.

3.4. Sensitivity of multiplex RT-PCR

The detection limits for simplex RT-PCR and multiplex RT-PCR were compared using a 10-fold serial dilution (10° – 10^{-6}) of mixed cDNA. The results showed that the highest dilution of multiplex RT-PCR assays for electrophoretic detection of PVX, CMV, TSWV and TMV was 10^{-4}, while for EF-1α, PVY and ToCV it was 10^{-3} (Fig.2). Quantitative test results show that the detection lower limit for multiplex RT-PCR of ToCV, PVY, TMV, TSWV, CMV and PVX were 1.15×10^4, 1.39×10^4, 8.48×10^4, 7.30×10^5, 6.08×10^4 and 5.17×10^2 copies, respectively (Supplementary Fig. S6). The results of systematic comparison displayed that the detection lower limit of TSWV was reduced by an order of magnitude when PVX or CMV were at high concentrations, that of CMV and TSWV was reduced by an order of magnitude when TMV

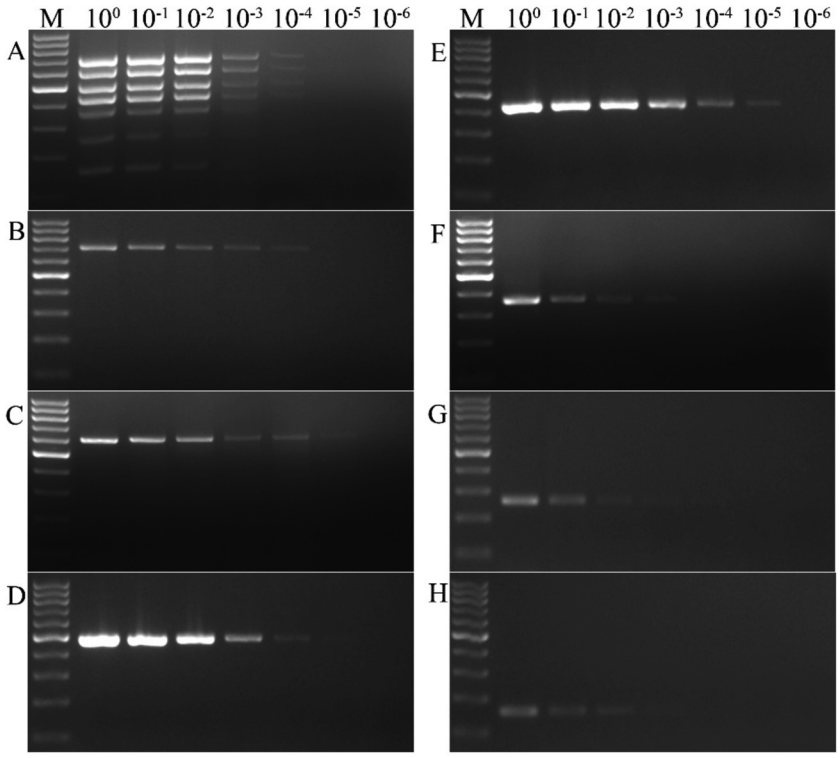

Fig. 2. Comparison of the sensitivity of the simplex RT-PCR and multiplex RT-PCR assays in detecting PVX (705 bp), CMV (600 bp), TSWV (500 bp), TMV (430 bp), EF-1α (362 bp), PVY (262 bp) and ToCV (159 bp) with specific primers. (A) Multiplex RT-PCR with mixed cDNA for the 10-fold serial dilution, simultaneously amplifying 7 target fragments. (B-H), simplex RT-PCR with mixed cDNA for the 10-fold serial dilution, detecting PVX, CMV, TSWV, TMV, EF-1α, PVY and ToCV, respectively. Lane M, 100 bp DNA Ladder.

was at high concentrations, while other viruses remained unchanged when TSWV, PVY and ToCV were at high concentrations (Supplementary Fig. S7). Conservatively speaking, the multiplex RT-PCR detection sensitivity of TSWV and CMV was 100 times lower than that for simplex RT-PCR, and the multiplex RT-PCR detection sensitivity of TMV was 10 times lower than that of a simplex RT-PCR. But for the other 3 viruses, the detection sensitivity of multiplex RT-PCR and simplex RT-PCR assays were at the same level。

3.5. Stability testing of multiplex RT-PCR

In order to test the stability of multiplex RT-PCR, assays were performed where one of the 6 viruses was omitted from the mixed cDNA solution. The results indicated that on removal of any one viral cDNA, the other viruses were still detected, including the internal reference gene (Fig. 3).

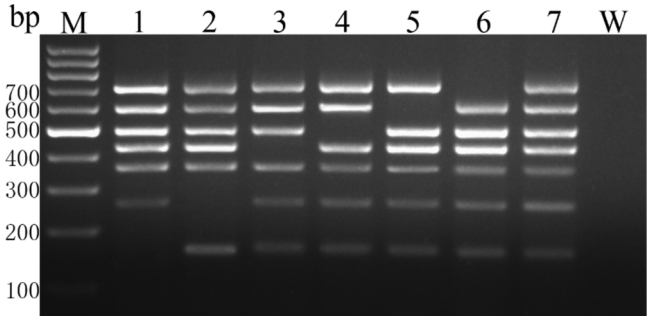

Fig. 3. Stability testing for multiplex RT-PCR. Lanes 1 - 6. Multiplex RT-PCR to detect all viruses in a mixed cDNA solution minus ToCV, PVY, TMV, TSWV, CMV and PVX, respectively. Lane 7, positive control of 6 viruses and EF-1α amplified simultaneously; Lane M, 100 bp DNA Ladder; Lane W, water blank control.

3.6. Application of multiplex RT-PCR in detecting viruses in tomatoes

Twenty-two tomato leaf samples with unknown viruses were collected from greenhouses and fields in Shaanxi, Gansu and Shandong provinces. All samples were assayed by multiplex and simplex RT-PCR. The results of multiplex and simplex RT-PCR were consistent (Fig. 4).

In addition, another 15 tomato leaf samples collected in the field from Shaanxi, Gansu and Shandong provinces were tested by optimized multiplex RT-PCR. The results showed that samples often had mixed virus infections; ToCV and TMV occurred in 6 samples, PVY in 4 samples, PVX in 7 samples, CMV in 10 samples and TSWV in 9 samples (Table 2).

4. Discussion

Tomato plants are often infected by multiple viruses in the field. It is crucial to distinguish between viruses for crop protection. However, it is hard to identify an individual virus infection based only on symptoms, as similar symptoms are often induced by different viruses. In order to recognize virus pathogens early, it is important to be able to identify them in field tomato plants. In this study, a multiplex RT-PCR assay was developed for the simultaneous detection of 6 major viruses of tomato. *EF-1α* was chosen as the internal reference gene to avoid false negatives. Although Solanaceae plants have many housekeeping genes including actin, aprt, 18S rRNA, EF-1α, tubulin and cyclophilin (Gigliotti et al., 2004), some research indicated that EF-1α was the most stable in solanaceous plants after virus infection and other adverse conditions (Nicot et al., 2005).

A few multiplex RT-PCR detection methods for tomato viruses have been published, but only 2–4 species of viruses could be detected in one reaction (Kumar et al., 2011; Panno et al., 2012; Quintero-Vásquez et al., 2013; Wieczorek and Obrępalska-Stęplowska, 2013). As far as we know, there are no multiplex RT-PCR assays for simultaneous detection of more than 4 tomato viruses at the same time. In this study, a multiplex RT-PCR assay for simultaneous detection of 6 tomato viruses was achieved, taking account of the present situation with regard to tomato virus diseases. This method greatly improved the detection efficiency

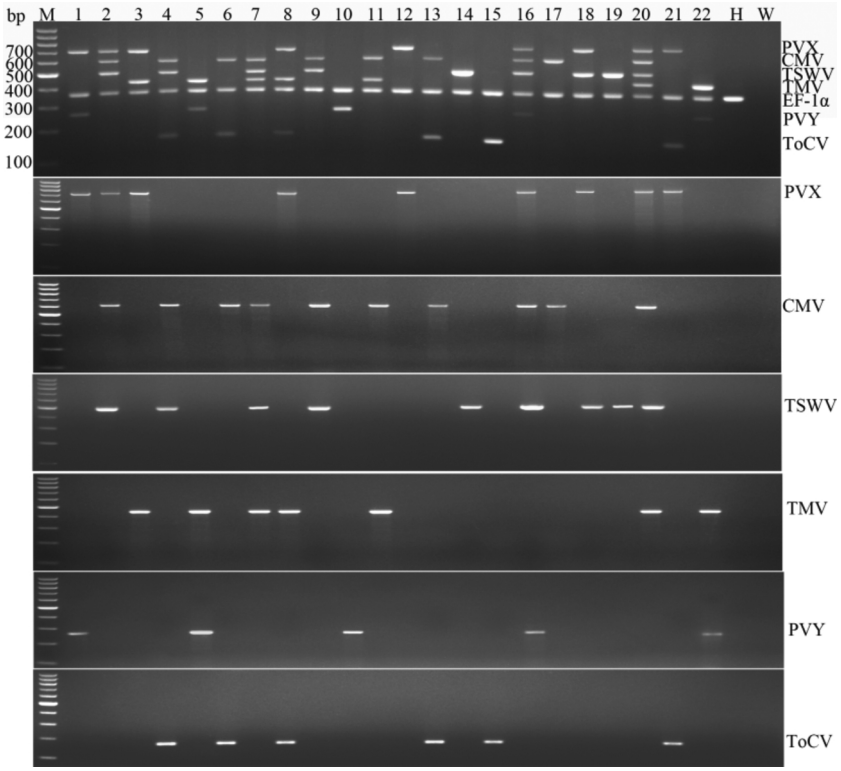

Fig. 4. Multiple viruses from greenhouse and field tomato samples were detected by optimized multiplex RT-PCR (top panel) and simplex RT-PCR systems (lower 6 panels). Lanes 1 - 2 2, leaf samples from diseased tomatoes; Lane M, 100 bp DNA Ladder; Lane H, healthy tomato leaf control; Lane W, water blank control.

Table 2
Results for field samples collected in Shaanxi, Gansu and Shandong provinces which were tested by multiplex RT-PCR.

Sample	Location	Virus name					
		ToCV	PVY	PVX	TMV	CMV	TSWV
TB-1	Taibai, Shaanxi Province	–	+	+	–	–	–
TB-2	Taibai, Shaanxi Province	–	–	+	–	+	–
TB-3	Taibai, Shaanxi Province	–	–	–	–	+	+
TB-4	Taibai, Shaanxi Province	–	+	–	–	+	+
YL-1	Yangling, Shaanxi Province	–	–	+	+	+	–
YL-2	Yangling, Shaanxi Province	+	–	+	+	+	–
YL-3	Yangling, Shaanxi Province	–	–	+	–	+	+
MJ-1	Maiji, Gansu Province	+	+	–	–	+	–
MJ-2	Maiji, Gansu Province	–	+	–	+	–	+
MJ-3	Maiji, Gansu Province	–	–	+	–	–	+
MJ-4	Maiji, Gansu Province	–	–	–	+	+	+
SG-1	Shouguang, Shandong Province	+	–	+	–	+	+
SG-2	Shouguang, Shandong Province	+	–	–	+	–	–
SG-3	Shouguang, Shandong Province	+	–	–	–	+	+
SG-4	Shouguang, Shandong Province	+	–	–	+	–	+

+, positive results for test; −, negative results for test.

and reduced the detection cost. It was successfully applied to the detection of tomato samples with mixed infections.

RT-PCR has the advantages of sensitivity, rapidity and specificity. Multiplex RT-PCR requires multiple site-specific amplifications in one reaction tube, but cannot just be a simple mix of simplex RT-PCRs. It needs repeated trials to establish the most appropriate reaction conditions (Thompson et al., 2003). This study has optimized the primer concentration, annealing temperature, annealing time, extension time and number of cycles.

Primers design was important for multiplex RT-PCR, as improper design might lead to some target sequences not being amplified. In this study, primers were designed based on conserved regions of CP and transcriptional elongation factor genes (Supplementary Fig. S5). More than one pair of primer was designed for each virus. A suitable combination of primers allowed synchronous amplification within a multiplex RT-PCR. Thus, the design and ratio of primers was critical, and inappropriate proportions resulted in unsynchronized amplification, which might lead to false negatives. This problem could be figured out by optimizing the ratio of the primers based on the brightness of the amplified bands on agarose gel electrophoresis, increasing the quantity of primer for “weak amplifiers” and reducing it for “strong amplifiers”. In addition, primers were selected with similar annealing temperature to ensure that the primers and templates could be effectively combined at the same temperature.

It was also important to select the appropriate size of the DNA amplification fragment. The amplified products in our multiplex RT-PCR assay were 705 bp, 600 bp, 500 bp, 430 bp, 362 bp, 262 bp and 159 bp corresponding to PVX, CMV, TSWV, TMV, EF-1α, PVY and ToCV, respectively. In order to distinguish the amplified products, they needed to be readily differentiated by electrophoresis. This was achieved by electrophoresing samples in parallel with a 100 bp DNA ladder.

We compared the sensitivity of detection of multiplex RT-PCR with that of simplex RT-PCR. We found it to be the same for PVX, ToCV, PVY and EF-1α, but was slightly lower for CMV, TSWV and TMV. Although multiplex RT-PCR has the advantage of saving time and cost, the sensitivity of detection of some target genes was lower than that of simplex RT-PCR. The reason may be that the substances in one reaction are consumed to simultaneously amplify multiple genotypes. We believe the decreased sensitivity, in some cases of 1–2 orders of magnitude in comparison to simplex RT-PCR, was not a limiting factor in using multiplex RT-PCR for virus detection in tomato plants (Roy et al., 2005).

In conclusion, a highly efficient multiplex RT-PCR assay was

established to detect 6 major tomato viruses, including ToCV, PVY, PVX, TMV, TSWV and CMV. Multiplex RT-PCR had advantages over simplex RT-PCR reactions in saving templates, time and cost. There were obvious advantages for detecting complex infections in tomato samples, and it should be helpful for studies on the distribution, epidemiology and control of virus diseases by these 6 tomato viruses. This method could be used as an efficient tool for detecting tomato viruses, and has prospects for broader application.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.12.011.

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