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Original Article

Multiplex RT-PCR detection of *Cucumber mosaic virus* subgroups and *Tobamoviruses* infecting Tomato using 18S rRNA as an internal control

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A multiplex reverse-transcription polymerase chain reaction (RT-PCR) protocol was developed for simultaneous detection and discrimination of subgroups of *Cucumber mosaic virus* (CMV), including its satellite RNA, *Tomato mosaic virus* (ToMV) and *Tobacco mosaic virus* (TMV), using 18S rRNA as an internal control. Species- and subgroups-specific primers designed to differentiate CMV subgroups I and II, ToMV and TMV, were assessed using the cDNA clones of viral genomes, CMV satellite RNA and 18S rRNA gene from tomato (*Solanum lycopersicum L.*) or tobacco (*Nicotiana tobacum*). Using total RNA extracted from artificial mixture of tomato leaf tissues infected by each virus, the reaction components and cycling parameters were optimized and a multiplex RT-PCR procedure was established. Six fragments of 704, 593, 512, 421, 385, 255 bp, specific to CMV subgroup II, CMV subgroup I, ToMV, TMV, satellite RNA and 18S rRNA, respectively, were simultaneously amplified. The sensitivity of the multiplex RT-PCR method for detecting CMV was 100 times higher than that of double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA). This method was successfully used for field detection. Among 141 samples collected from East China through tomato growth seasons, 106 single infections with one of the above isolates were detected and 13 mixed infections were found. The results showed the potential use of this method for investigating the epidemiology of viral diseases infecting tomato.

Keywords detection; virus; tomato; *tobacco mosaic virus*; *tomato mosaic virus*

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Introduction

Tomato, *Solanum lycopersicum L.*, is an important economic crop all over the world. Viral diseases often lead to yield and fruit quality losses. Several viruses, including *Cucumber mosaic virus* (CMV), *Tomato mosaic virus* (ToMV), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Tomato spot wilt virus* (TSWV), and *Tomato ring spot virus* (ToRSV) have been found as the main epidemic viruses in many places [1]. Viruses infecting tomato in China have frequently been investigated over the last few decades, with CMV and ToMV being considered as the most important viruses of this crop [2–5]. Single or mixed infection of the two viruses may potentially be devastating to field tomato crops in China, especially in East China, where tomatoes are grown on a large scale.

The type member of *Cucumovirus*, CMV has a worldwide distribution. To date, CMV isolates are classified into subgroups I and II according to their serological relationships and nucleotide sequence identity [6]. Based on the analysis of the RNA3 open reading frames, subgroup I is divided into subgroup IA and subgroup IB. The existence of the above clustering has been confirmed by some reports [7,8]. Many CMV isolates contain additional RNA molecules of 333–405 nt classified as satellite RNA (satRNA), which can modify CMV symptoms on tomato plants. Based on recent reports, CMV subgroup I was found to be predominant in tropical and subtropical regions, whereas subgroup II was more frequently found in temperate regions [9]. Since two isolates of CMV subgroup II were first identified from tomato in China in 2000 [5], many more subgroup II isolates have been detected [10].

Genomic recombination of CMV has been observed in many areas. Analysis of CMV collected from Spain showed that the frequency of reassortants among subgroups IA, IB, or II was about 5%, and the recombinant frequency between these groups was 17%. Recombinants at RNA3 were significantly more frequent than recombinants at RNAs 1 and 2 [11].

Tobacco mosaic virus (TMV), the type species of *Tobamovirus* is closely related to ToMV. As a distinct

species, ToMV was separated from TMV early in 1971, according to the serological relationship and nucleotide sequence. Although the two viruses have been historically considered synonymously, the predominant virus in tomatoes is considered to be ToMV [12]. Mixed infection of CMV and ToMV on tomatoes has frequently been reported. A severe necrotic disease due to mixed infection of the above two viruses arose in East China in 2007. The typical symptoms displayed at fruiting season were top necrosis, plant stunting and fruit distortion, in combination with brown necrotic strikes among stems and leaf stalks, which ultimately developed into death of the whole plant [13]. These damages occurred more severely when high temperatures prevailed. Understanding the occurrence of CMV and differentiating *Tobamoviruses* in tomatoes are required for epidemiological investigations aimed at management and control of the principal viruses. Hence, an accurate, reliable and effective method for routine diagnosis of above viruses is desirable.

Several methods have been described to detect and identify plant viruses. Many CMV isolates have been characterized using RT-PCR, triple antibody sandwich-ELISA and immunocapture (IC) RT-PCR methods [10,14,15]. In addition, a combination of virus-specific antibody capture and specific primers for a multiplex IC-RT-PCR assay to differentiate TMV and ToMV has been described [16]. However, a simultaneous detection method that can differentiate the above viral species and subgroups is still unavailable, by which the complex epidemiology of viruses infecting tomato can be understood.

We report here a multiplex RT-PCR method that can quickly and simultaneously detect and differentiate CMV subgroups I and II including their satellite RNA, TMV and ToMV. As an internal control, 18S rRNA primers were included. After optimizing the parameters, we also used this detection method to examine the epidemiology of viral disease in commercial tomato crops.

Materials and Methods

Virus isolates and cDNA clones

The virus isolates used in this study were: A-TMV representing TMV, N5-ToMV representing ToMV, Fny-CMV representing CMV subgroup I, Tsh-CMV representing CMV subgroup II, and Sat-Yn12 representing CMV-satRNA. A-TMV was the first isolated from *Solanum macrocarpon* in Africa [17]. N5-ToMV and Tsh-CMV were isolated from tomatoes in Shanghai. They were maintained on *Nicotiana tabacum L.*, *Solanum lycopersicum L.* and *Nicotiana glutinosa L.*, respectively. Fny-CMV was kindly provided by Dr. Peter Palukaitis, Scottish Crop Research Institute, Dundee, UK, and revived from infectious clones as a strain of CMV subgroup IA [18]. Sat-Yn12 for CMV satellite RNA was transcribed from an infectious clone, pseudorecombined with CMV-Fny and maintained on *N. glutinosa*. All viruses were inoculated onto tomato plants by mechanical transmission.

The cDNA from the above virus/satRNA was prepared by RT-PCR, cloned into plasmid pUC18, transformed into *E. coli* DH5α and verified by sequencing. The accession numbers in GenBank for Fny-CMV, Tsh-CMV, N5-ToMV, A-TMV, SatRNA and 18S rRNA are D10538, EF202597, GQ280794, GQ280795, EF363688 and GQ280796, respectively.

Design of primers

Primers used in this study are shown in **Table 1**. Except for 18S rRNA and CMV satRNA, four sets of primers were designed for simplex and multiplex RT-PCR using

Table 1 Sequence of primers used in the multiplex RT-PCR

Virus	Code no.	Primer sequence (5′–3′)	Position	Product size (bp)
CMV subgroup II	CMV II (F)	CTACGTTTATCTTCC	970–984	704
	CMV II (R)	AACCGGTGATTTACCATCGC	1655–1674	
CMV subgroup I	CMV I (F)	GCCACCAAAAATAGACCG	1484–1502	593
	CMV I (R)	ATCTGCTGGCGTGGATTTCT	2057–2076	
TMV	TMV (F)	CGATGATGATTCGGAGGC	5664–5681	512
	TMV (R)	GAGGTCCARACCAAMCCAG	6157–6175	
ToMV	ToMV (F)	CATCTGTATGGGCTGAC	5746–5762	421
	ToMV (R)	GAGGTCCARACCAAMCCAG	6148–6166	
satRNA	satRNA (F)	GTTTTGTTTGTTGGAGAGTTGCG	1–22	385
	satRNA (R)	GGGTCCTGTAGAGGAATGTGACATT	369–385	
18S rRNA	18S (F)	GAGAAACGGCTACCACATCCA	399–419	255
	18S (R)	CGTGCCATCCCAAAGTCCAAC	633–653	

F, forward; R, reverse

DNAstar-lasergene (DNASTAR, Madison, USA) and Primer Premier 5.0 (Premier Biosoft International, Palo Alto, USA) based on sequences published in GenBank. Multiple sequences were aligned using Clustal X, and the conserved and variable regions of these different viruses were examined. Percent identity matrices were generated using Genedoc. Specific sense primers were designed to differentiate TMV and ToMV in the variable regions, and a degenerated antisense primer was designed in a commonly conserved region of coat protein gene. For CMV subgroups I and II, the specific antisense and sense primers were designed within the coat protein gene for differentiating them. The primer pairs for CMV satRNA and for 18S rRNA, used as internal control from *N. tabacum* (AJ236016) and tomato (X51576), were both used as described previously [19,20].

Simplex and multiplex PCR

In order to confirm the specificity of the primers, and to establish the fundamental conditions for amplification of all the target fragments simultaneously, simplex and multiplex PCRs were carried out and cDNA clones of Fny-CMV, Tsh-CMV, N5-ToMV, A-TMV, SatRNA and 18S rRNA were used as templates. All PCRs were performed in a thermocycler (Biometra, Göttingen, Germany) using *Taq* DNA polymerase (Beijing BioDev-Tech, Beijing, China), according to the manufacturer's instructions. The PCR products were visualized after electrophoresis in 2.5% agarose gels by staining with ethidium bromide.

Simplex PCR was performed in a 25-μl of reaction mixture consisting of 19.25 μl sterile H_2O, 2.5 μl 10 × PCR buffer (100 mM Tris−HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 μl dNTPs (2.5 mM each), 0.5 μl forward and reverse primer (10 μM each), 0.25 μl *Taq* (5 U/μl), and 1 μl cDNA. The PCR reaction was carried out as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 45 s, with an additional cycle of 72°C for 10 min. The six cDNA clones were used to test the specificity of different primer combinations.

Multiplex PCR was performed with all six sets of primers using the mixture of the six cDNA templates. Several parameters, such as primer concentration, template concentration, annealing temperature and elongation duration (from 30 to 150 s), were tested to optimize conditions for the simultaneous amplification of target fragments. PCR products were identified after electrophoresis in 2.5% agarose gels.

RNA extraction and multiplex RT-PCR

Total RNAs were extracted from healthy and infected tomato leaf tissues (100 mg, frozen at −80°C) using Trizol Reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol, and dissolved in 35 μl of diethyl pyrocarbonate-treated water.

First-strand complementary DNA (cDNA) was synthesized with specific antisense primers in 10 μl volume using AMV reverse transcriptase (TaKaRa, Dalian, China) according to the manufacturer's instructions. The subsequent PCR in 25 μl volume was performed using 1 μl RT product as a template.

A mixture of the five viruses was prepared by mixing equal amounts (50 mg) of tomato leaf tissues infected by each virus for multiplex RT-PCR. At the RT stage, six antisense primers were mixed with 2 μl total RNA. Other reagents and reaction conditions were used as described for the single RT experiments. Multiplex RT-PCR was carried out and optimized similar to the conditions of simplex RT-PCR.

DAS-ELISA

Portions of 0.1 g of tomato leaf tissues, frozen at −80°C, were used for DAS-ELISA which was carried out in 96-well plates using commercially available alkaline phosphate compound ELISA test kits (Agdia Inc., Elkhart, USA) for CMV, ToMV, PVX, PVY, TAV, TSWV and ToRSV according to the manufacturer's instructions. Controls were included systematically and each sample was loaded in duplicate. The optical density (OD) was measured at 405 nm with a spectrophotometer (Spectra Max Plus384; Molecular Devices, Sunnyvale, USA). A sample was considered positive when its OD_{405} value was two times greater than that of negative/healthy controls.

Sensitivity determination of multiplex RT-PCR and DAS-ELISA

Purified viral RNA of Fny-CMV was serially diluted with extracts of healthy tomato leaf tissue and used for measuring the sensitivity of multiplex RT-PCR. The DAS-ELISA was performed with the same amount of virions. Dilutions, ranging from 1 to 1×10^{-7} μg/μl viral RNA, were subjected to multiplex RT-PCR, while virions of Fny-CMV diluted with extracts from healthy tomato leaf, ranging from 100 to 100×10^{-7} μg, were subjected to DAS-ELISA.

Detection of field samples

Field tomato samples with typical viral symptoms were collected in the suburbs of Shanghai, Hangzhou, and Jiashan in East China, during the growing seasons in 2007 and 2008. Leaf samples were kept at −80°C and subjected to multiplex RT-PCR and DAS-ELISA tests within 10 days.

Results

Multiplex PCR

Using the cDNAs described above, simplex and multiplex PCRs were carried out to evaluate the feasibility of each primer pair and the expected amplified fragments were yielded in all cases when each primer pair was tested with its specific cDNA template. No cross-amplification was observed when the primer pairs were used against other cDNA templates. Subsequently, relevant fragments were achieved by multiplex PCR without additional products, when all the templates were added into the reaction mixture (**Fig. 1**). The results showed that the primer pairs were target specific, and the multiplex PCR could amplify all the six fragments simultaneously under the optimized conditions.

For simplex PCR, annealing temperature gradients were from 48 to 60°C at 2°C intervals, and all target fragments could be amplified accordingly. But for multiplex PCR, all target fragments could be amplified at low annealing temperatures only, especially for the CMV subgroup II fragment, for which amplification failed when the annealing temperature was >56°C.

The relative concentrations of primers were determined after testing. When primers of equal amount were added in the mixture, only the fragments of CMV subgroup I, satRNA, and 18S rRNA were amplified. The final concentration of each primer pairs was determined as follows: CMV subgroup II F/R, 10 pM; CMV subgroup I F/R, 1.25 pM; TMV F/R, 5 pM; ToMV F/R, 3.5 pM; satRNA F/R, 1.25 pM, and 18S rRNA F/R, 1.25 pM. The template concentration of each cDNA clone was adjusted empirically, which was CMV subgroup II 1.5 ng/μl, CMV subgroup I 11.5 ng/μl, TMV 3 ng/μl, ToMV 2 ng/μl, satRNA 1.8 ng/μl, and 18S rRNA 1 ng/μl.

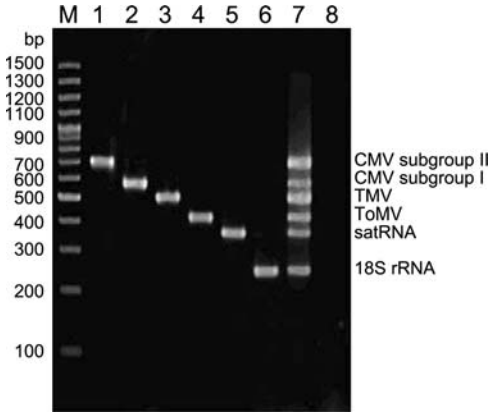

Figure 1 Results of multiplex PCR using the plasmid cDNAs of each virus Lane M, 1-kb DNA ladder; lane 1, CMV subgroup II F/R against RNA3 of Tsh-CMV; lane 2, CMV subgroup I F/R against RNA3 of Fny-CMV; lane 3, TMV F/R against A-TMV; lane 4, ToMV F/R against N5-ToMV; lane 5, satRNA F/R against CMV subgroup I + satRNA; lane 6, 18S rRNA F/R against total RNAs extracted; lane 7, mixed primer pairs against a mixture of all the above plasmids; lane 8, negative control using pUC18 plasmid.

Optimization of multiplex RT-PCR

To develop the multiplex RT-PCR system, samples of tomato leaf tissues separately infected by five viruses were mixed and total RNA was extracted. The RT and PCR steps of the multiplex RT-PCR system were optimized. For the RT step, all antisense primers of different relative concentrations were added, and the results showed that there was no apparent impact on the multiplex RT-PCR products and all reactions resulted in the expected amplification. The final concentrations of viral antisense primers were: CMV subgroup II 4 pM, CMV subgroup I 1 pM, TMV (ToMV) 6 pM, satRNA 1 pM, and the concentration of the antisense primer for 18S rRNA in total RNA was 1 pM. The multiplex RT product was amplified separately, with the fragments of amplification in accordance with the expected sizes, which were 704, 593, 512, 421, 385 and 255 bp, respectively, for CMV subgroup II, CMV subgroup I, TMV, ToMV, satRNA, and 18S rRNA (**Fig. 2**).

The optimum protocol was performed in final volume of 10 μl containing: 2 μl total RNA, 2 μl 5 × buffer, 0.25 μl AMV Reverse Transcriptase (5 U/μl), 2 μl dNTP (2.5 mM), 0.25 μl RNase inhibitor, and 0.4, 0.1, 0.6, 0.1, and 0.1 μl of primer (10 μM) CMV subgroup II R, CMV subgroup I R, TMV (ToMV)R, satRNAR, and 18S rRNAR, respectively. RT product was used as a cDNA template for multiplex RT-PCR. Multiplex PCR components, including $MgCl_2$, dNTPs, and DNA polymerase, as well as reaction parameters such as annealing temperature, extension time, and cycling numbers, were optimized (data not shown).

The optimal reaction mixture consisted of 2 U *Taq* DNA polymerase, 100 μM dNTPs, 2.0 mM $MgCl_2$, with addition

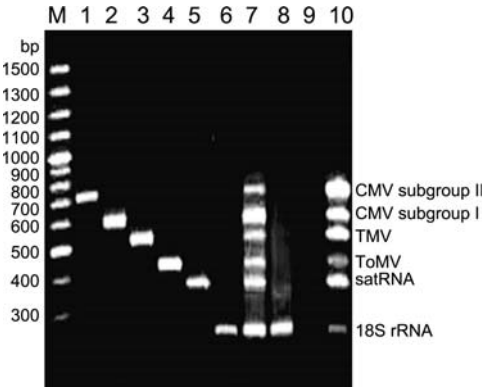

Figure 2 Detection of viruses from a mixture of leaf tissues individually infected using simplex or multiplex RT-PCR Lane M, 1-kb DNA ladder; lane 1, CMV subgroup II F/R; lane 2, CMV subgroup I F/R; lane 3, TMVF/R; lane 4, ToMV F/R; lane 5, satRNA F/R; lane 6, 18S rRNA F/R; lane 7–10, mixed primer pairs; lane 8–10, multiplex PCR control (healthy leaf tissue, ddH2O, six cDNA clones, respectively). A total of 0.1 g of leaf tissue was used for each.

of 1, 0.125, 0.5, 0.35, 0.125, 0.25 μl of CMV subgroup II, CMV subgroup I, TMV, ToMV, satRNA, and 18S rRNA primer pairs into a total volume of 50 μl. The optimal multiplex PCR reaction was conducted with one cycle at 94°C for 3 min, followed by 35 cycles: 94°C for 30 s, 52°C for 30 s, 72°C for 105 s, and one additional cycle at 72°C for 10 min. All the expected products, including six corresponding fragments, were specifically amplified by multiplex RT-PCR (**Fig. 2**). In addition, combinations of tomato leaf tissues infected by each virus were specifically differentiated (**Fig. 3**).

Sensitivity of multiplex RT-PCR and DAS-ELISA

The multiplex RT-PCR sensitivity for detection of CMV was determined using a 10-fold dilution series of a mixture of viral RNA using total RNA extract from healthy tomato leaves. The ELISA detection limit was determined using 10-fold dilution of virions of Fny-CMV diluted with extract from healthy tomato leaf. The multiplex RT-PCR showed detectable results until 10^{-6} (≈1 pg) for Fny-CMV (**Fig. 4**). The detection limit for ELISA was 10^{-3} (≈1000 pg) (**Fig. 5**).

Determination of the specificity of genome amplification

To assess the specificity of the primers, all positive amplicons from simplex and multiplex RT-PCR were sequenced. Sequences of amplified products showed 91.1−99.0% identity to the deposited sequences in the GenBank.

Analysis of field samples by multiplex RT-PCR and DAS-ELISA

The field tomato leaf tissues with symptoms of mosaic, local necrotic, or stunted growth were collected in 2007 and 2008. In total, 159 samples were analyzed by multiplex RT-PCR and ELISA. Using ELISA, PVX was detected in one sample and PVY in one sample. TMV and ToMV, as well as CMV subgroups I and II can be detected by ELISA, but there are cross-reactions between TMV and ToMV, as well as between CMV subgroups I and II. Using multiplex RT-PCR, viruses were detected in 128 samples, including some mixed infections of viruses (**Table 2**). No virus was detected by multiplex RT-PCR in some samples, which may be due to the fact that virus concentrations were below the detection limit, or other may contain viruses in these samples, such as PVX and PVY, which were able to be detected by ELISA but not by multiplex RT-PCR. Partial multiplex RT-PCR results are shown in **Fig. 6**.

Discussion

The detection of the type of CMV, as well as TMV and ToMV, is important for elucidating the epidemiology of these viruses, and also for suggesting control management

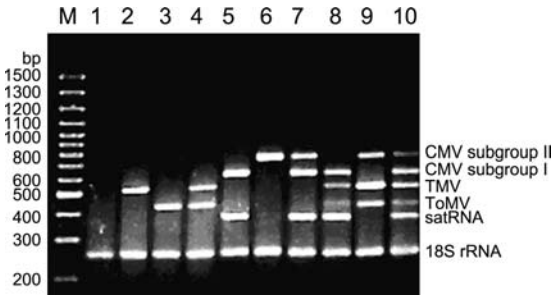

Figure 3 Detection of virus combinations from mixture of infected leaf tissues using multiplex RT-PCR Lane M, 1-kb DNA ladder; lane 1, healthy leaf tissue; lane 2, TMV; lane 3, ToMV; lane 4, TMV and ToMV; lane 5, CMV subgroup I + satRNA; lane 6, CMV subgroup II, lane 7, CMV subgroup I + satRNA, CMV subgroup II; lane 8, TMV, ToMV and CMV subgroup I + satRNA, lane 9, CMV subgroup II, TMV and ToMV; lane 10, all the viruses.

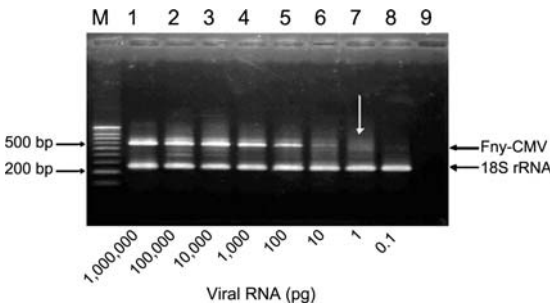

Figure 4 Sensitivity of multiplex RT-PCR for detecting Fny-CMV Lane M, 100 bp marker; lanes 1–8, RT-PCR products of virion RNA of a 10 fold serially diluted in total RNA of a healthy plant; lane 9, blank. The detection limit of multiplex RT-PCR is indicated with arrowhead.

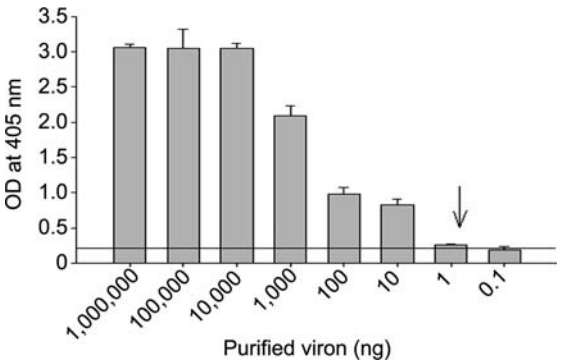

Figure 5 Sensitivity of ELISA for detecting Fny-CMV Absorbance value (OD405) and standard error are presented. The ELISA detection limit was indicated with arrowhead. The line showed the 2 fold of absorbance value of health, which interpret the positive results.

strategies for viruses infecting tomato. ELISA methods have been widely used for detecting and subgrouping the CMV isolates [9,21,22]. In addition, both RT-PCR and multiplex IC-RT-PCR assays were reported for differentiation of CMV subgroups I and II [10,14]. A multiplex IC-RT-PCR assay has also been developed for differentiation of TMV and ToMV [16]. However, an internal control procedure for interpretation of negative results, as

Table 2 Results of field samples detection by multiplex RT-PCR

Year	2007 (69 samples)	2008 (90 samples)	Total (159 samples)
Combination			
CMV subgroup I	29	35	64
CMV subgroup II	9	4	13
TMV	1	0	1
ToMV	12	15	27
CMV subgroup I + ToMV	3	6	9
CMV subgroup II + ToMV	4	0	4
CMV subgroup I + Sat RNA	5	2	7
CMV subgroup I + SatRNA + ToMV	3	0	3

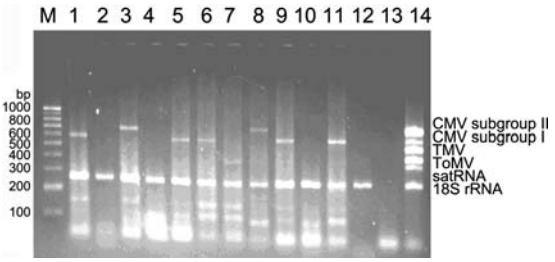

Figure 6 Analysis of field samples by multiplex RT-PCR Lane M, 100 bp marker; lanes 1–12, field samples; lane 13, blank; lane 14, six cDNA clones as the positive control.

well as for detecting the satellite RNA of CMV was not included in the IC-RT-PCR and ELISA methods. ELISA methods have failed to reliably differentiate TMV and ToMV because of the cross-reaction between the two viruses [16]. Multiplex RT-PCR is a useful technique for detecting more than one plant virus using different sets of primers. Such methods have been developed for detection of many plant viruses [15,23–26]. Using 18S rRNA as an internal control, we successfully developed a multiplex RT-PCR system to simultaneously detect and differentiate TMV, ToMV, and CMV subgroups and CMV-satRNA in tomato plants. The newly developed multiplex protocol will provide a convenient means for investigating the epidemic situations of CMV subgroups with its satRNA and also different *Tobamoviruses*.

The multiplex RT-PCR method also can be used to test field samples. A total of 159 field samples with mosaic symptoms were collected in different regions of East China. The detection results showed that these viruses represented complicated infections in tomato plant. CMV subgroup I, CMV subgroup II, CMV (I, II)-satRNA and ToMV infect tomato plants either alone or in combination with other viruses of different virus strains. TMV was detected only in one sample. This observation supports Broadbent's conclusion that TMV is competed out of tomato populations since ToMV is more aggressive and adaptable to the host plant [11]. The combination of two viruses was not detected in the field samples, even though the multiplex RT-PCR method was able to detect TMV/ToMV combination in artificially mixed tomato leaf tissues. This suggests that TMV is not a main pathogenic virus in tomato.

Even though most of the marketed tomato seeds are considered to be resistant to ToMV, infection was found in some field plants. During our survey in 2005–2008, 100% of infection by a top necrosis strain of ToMV was observed in Shanghai. A new strain of ToMV overcoming Tm-2^2 resistance gene has been reported in Spain [27], and the genotype of ToMV evolved gradually from 1, 2 and 1.2 strains along with the resistant varieties in one area [4,28]. Therefore, it is important to investigate the epidemic situation of ToMV for the control of viral diseases, and it seems that variations of this virus could overcome the resistance commonly encountered. Continuous inspection of emerging ToMV diversity using reliable detection methods is required to gain better knowledge of resistance forms and mechanisms.

In our present work, CMV was detected as the most dominant virus-infecting tomato, with both subgroups detected. Among 159 field samples randomly collected, 13 were found infected with CMV subgroup II, while 64 were found infected with CMV subgroup I. Although subgroup I was reported to be predominant in the tropics and subtropics, results from our study also showed that CMV subgroup II is now more prevalent than ever in East China. Strains of multiple viruses existing in one host can bring about recombinant and reassortant forms, which are frequently observed in natural CMV populations [8,29]. In fact, a new strain of CMV (Tsh-CMV) identified as the reassortant between CMV subgroups I and II was found to be coexistent with both ToMV and a CMV-satRNA from the same area in our previous work [30]. Thus, existence of coat protein gene or RNA3 cannot represent the whole genome of CMV subgroup I or II. Simultaneous detection

of the whole genome is therefore required to identify possible recombinant and reassortant forms.

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