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Development of a multiplex reverse transcription-PCR assay for simultaneous detection of pepper viruses

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ABSTRACT

The aim of the present study was to establish a fourplex RT-PCR method for examining the infecting peppers to improve the efficiency of pepper virus detection, which includes *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Broad bean wilt virus*- 2 (BBWV-2) and *Potato virus* Y (PVY). According to the genome sequences of TMV, CMV, BBWV-2 and PVY published by GenBank, four specific pairs of primers were designed to verify the specificity and sensitivity of detection system by simplex and multiplex RT-PCR method. In addition, using set multiplex RT-PCR method pepper plant samples of two geographic locations in China were tested and verified by simplex RT-PCR. The anticipated amplicon sizes of four primer pairs targeting TMV, CMV, BBWV-2 and PVY in both simplex and multiplex RT-PCR were 237 bp, 323 bp, 480 bp and 1057 bp, respectively, and established fourplex RT-PCR system was able to check out all sorts of viruses accurately from the pepper leaves of two geographic locations in China, which was 37.21%, 81.4%, 23.26%, and 2.33% respectively, and two or more mixed infections viruses sample accounted for 48.83% of the pepper samples. The research results show a detection technique of multiplex RT-PCR method for major peppers' viruses, which is a rapid, sensitive and reliable method and examines CMV, TMV, PVY and BBWV-2.

Key words: Multiplex RT-PCR, Pepper, Virus

Pepper (*Capsicum annuum* L.) being planted widely in middle and South of America, Europe, Asia and other regions, is an important vegetable crop and condiments, can be eaten in fresh and processed into a variety of chili products. Pepper contains high vitamin C, so people are crazy about it. Besides, it possesses red pigment that is applied widely for the natural food coloring agent. China was the main dry pepper producer in the 1990s, and now, pepper even is vegetable leading industry in some cities. However, in the procedure of production, a variety of diseases of pepper have negative effect on farmers' income. Especially, virus disease, one kind of wide spread and great detrimental disease category with more than 40 kinds of viruses, infect peppers, which often lead to pepper leaves, flowers and fruit left, and production reduction by 30%-50% or even failure, and is the main factor of impacting pepper production, but CMV, TMV, BBWV-2 and other viruses are more harmful. *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family *Bromoviridae*), can be spread by aphid and is one of the most significant economic value plant virus at present. TMV (*Tobacco mosaic virus*), a tobacco mosaic pathogen and other pathogens, belonges to *Tobamovirus* group and can infect 36 families of 350 species of plants, so that leaves appear mosaic symptoms and be abnormal, which is less harmful than CMV for chili, and often complex infection with CMV. *Broad bean vascula wilt virus*, BBWV-2, belong to bean virus genus, which infect 44 families of 46 genera of 328 kinds of plants and spread by aphids, agricultural operations contact friction. The sixth report of the International Virus Classification Committee divides BBWV-2 into two types, BBWV-1, and BBWV-2. At present, BBWV-1 has not been found in China, but BBWV-2 widely present. BBWV-2 causes harm to the production of pepper, and can cause pepper buds turned yellow, dried top, leaf system mottled, chlorosis, damaged stem or even whole plant wilted [20,21]. *Potato virus* Y, (PVY), is the potato Y virus genus, can be transmitted by aphids, mechanical inoculation and sick seed propagation which often lead to pepper appear systemic mosaic and mottled, manifested as mosaic, dwarf, fruit less embolism, but the PVY invasion research of pepper is less research in domestic.

Plant virus detection methods include biological methods, electron microscopy, serological methods and molecular biology methods. Biological method of

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measurement is time-consuming, and has poor sensitivity, so there are many restrictions in the area of application. Electron microscopy detection method is expensive, and operating difficulty making its, application limited. The serological method is adopted extensively, but the method may give false negative and false positive and the cost of kit is high for some little detection sample experiment. Molecular biology is the preferred method of current pathogen detection and is widely used in plant virus detection. RT-PCR is the most common molecular biology method, the single-RT-PCR of chili virus is more reported, but the double-RT-PCR is less reported. Zhang Jianyun determined pepper BBWV-2 infection by RT-PCR method, Wang Jianhua established ChiRSV detection by RT-PCR, Zhang Qiang *et al*. (2014) analyzed Xinjiang pepper PMMoV of its pathotype by RT-PCR, Yao Yu-rong *et al*. (2013) used RT-PCR method in the pepper and test the toxic resource both CMV and PMMoV and so on. Guo Siyao *et al*. (2015) detected CMV, TMV and other viruses by single RT-PCR; Huang Ya (2015) established the detection system of the five-RT-PCR, PMMoV, ChiVMV, PVY, TSWV and TuMV, but did not include most common CMV, TMV, and BBWV-2 in the field of production.

For four common virus, CMV, TMV, PVY, and BBWV-2, there is no established targeted detection multiplex RT-PCR test technique. So adopted former simplex or multiplex RT-PCR method need multiple reaction to complete and time-consuming and will be costly. But according to the multiplex RT-PCR detection method, there will be a high detection efficiency and low cost. In order to identify the four viruses of pepper efficiently, viz. CMV, TMV, PVY and BBWV-2 a multiplex RT-PCR detection method must be set up rapidly to test four viruses contemporarily.

In this study, CMV, TMV, BBWV-2 and PVY were used as the object in the capsicum plants of China pepper. Specific primers were desgned, based on a single RT-PCR assay, built up a simultaneous and contemporary multiplex RT-PCR detection technique for CMV, TMV, BBWV-2 and PVY to improve detection efficiency, reduce detection cost, and provide a referenced evidence for rapid detection of viral disease in the future.

MATERIALS AND METHODS

Young leaves with yellow, mosaic, shrinkage symptoms which were suspected of virus diseases were collected from 43 strains of pepper plants (Fig. 1) in several pepper field in China. Collected leaves were put in liquid nitrogen immediately, then stored at -85ºC. The pathogen cause symptoms were confirmed as CMV, TMV, PVY and BBWV 2 respectively by RT-PCR.

According to the reported sequence in Gene Bank, specific primers for CMV, TMV, PVY, BBWV-2 were designed by Primer 5 (Table 1).

The total RNA was extracted by using Tranzol up extraction kit and then stored at -20°C.

Reverse transcription reaction mix included: 2 µl of 5 × RT buffer, 1 µl of 0.1 M DTT, 0.5 µl of 100 µg/l RT reverse primer, 2 µl of 5 mM dNTPs, RNasin enzyme (10 U/µl), M-MLV RT enzyme (100 U/µl), at last,adding ultra-pure water to 7.5 µl.

RT reaction program was: 2.5 µl of RNA extractions, degenerating at 65°C for 8 min, ice bathing 3 min, adding 7.5 µl of RT reaction system, bathing for a 1 hr in 42°C water, then bathing in 95°C for 2 min to

Fig 1 Pepper leaves exhibiting different symptoms

Table 1 Primer pairs sequences for simplex and 4-plex RT- PCR assay of pepper

Primer name	Forward primer sequence (5'→3')	Base	T_m	Length (bp)
TMV-F	TAGACCCGCTAGTCACAG	18	48	237
TMV-R	CAGAGGTCCAAACCAAAC	18	49	
CMV-F	CCCACTCTTAACCACCCAACC	21	60.8	323
CMV-R	GACGCAGCATACTGATAAACCAA	23	60	
PVY-F	ACGTCCAAAATGAGAATGCC	20	57.4	480
PVY-R	TGGTGTTCGTGATGTGACCT	20	56.2	
BBWV-2-F	GCCAGAACTTGGGAGTGATAC	21	58.8	1057
BBWV-2-R	ACTTGTTAGCCATTCGGGTC	20	59.2	

terminate the reaction, finally, obtaining the cDNA product at -20°C.

The PCR products were prepared by PCR amplification reagent of 2× Taq Master Mix, and the specific operation steps were carried out according to the instructions. PCR reaction mix included: 1 µl of forward primer (positive primer) (10 µmol/l), 1 µl of reverse primer (10 µmol/l), 8.5 µl of ddH_2O, 12.5 µl of 2× Taq PCR mix, 2 µL of cDNA. PCR reaction program: The PCR amplification was set as a hot start at 94°C for 5 min; 35 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 1 min; and a final extengion at 72°C for 10 min.

The RT-PCR product was recycled by agarose gel recycle kit and ligated into pMD18-T vector. The ligation product was transformed into *Escherichia coli* DH5α competent cells by conventional methods. Positive colonies were screened and then coated with ampicillin-containing LB. The recombinant plasmids were identified and sequenced. The results were compared with the reported viral gene sequences in GenBank.

Total RNA samples containing CMV, TMV, PVY and BBWV-2 were mixed to obtain total RNA containing 4 viruses, and 2.5 µl (1 µg) of the total RNA was mixed.

Main influencing factors of multiple RT-PCR were optimized. PCR amplification was performed using the first strand of the cDNA synthesized as a template. PCR reaction system: 1 µ of CMV-F (10 µmol/l), 1 µl of CMV-R (10 µmol/l), 1 µl of TMV -F (10 µmol/l), 1 µl of TMV -R (10 µmol/l), 1 µl of BBWV-2-R (10 µmol/ l) of BBWV-2-R (10 µmol/l)), 2.5 µl of 10 × PCR Buffer, 3 µl of 50 mM Mg^{2+}, 3 µl of 2.5 mM dNTP, 5.5 µl of ddH_2O, 0.5 µl of Taq Enzyme (0.625 U/µl, 2 µl of cDNA, 4 µl of ddH_2O. PCR reaction program extension was start at 94°C for 5 min; 35 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 1 min; and a final extension was at 72°C for 10 min.

The temperature gradient was from 49.0 to 60.0°C, according to the T_m value of the four pairs of primers. The temperature gradient was 49.0, 49.3, 50.1, 51.2, 52.5, 53.8, 55.2, 56.5, 57.8, 58.9, 59.7 and 60.0°C and they were subjected to single and quadruple PCR amplification to determine the appropriate annealing temperature.

After determining the optimum annealing temperature for the PCR reaction, the total RNA of the four viral materials was diluted 10 times, and then diluted into 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} times, as a reverse transcription template synthesis of cDNA, respectively, single and quadruple PCR.

A total of 43 pepper plant leaf samples were collected from the field of Liling County, Changsha City, Hunan Privince, China, and tested by single RT-PCR.

Taking 8 µl of PCR product to be tested with 1.5% agarose gel electrophoresis, and with ethidium bromide (EB) staining on the gel imager results were observed.

RESULTS AND DISCUSSION

Establishment of Tetra RT-PCR reaction system

In order to rapidly identify four viruses of pepper, TMV, CMV, PVY, and BBWV-2, a multiplex RT-PCR technique was established to detect the simultaneous detection of four viruses. Based on the TMV, CMV, PVY and BBWV-2 genomic sequences, the primers of the four RT-PCR (Table 1) were designed by considering the specificity of the amplified product, the Tm value and the fragment size of the amplified product. The size of the singlet RT-PCR amplification product of PVY and BBWV-2 was the same as expected. (Fig 2, 1-4 lane). According to recovered, purified, cloned and sequenced format of TMV, CMV, PVY and BBWV-2 single RT-PCR products, the total length of the sequences was 237 bp, 323 bp, 480 bp and 1057 bp respectively. The virus gene sequence is highly homologous. Sequencing results confirm the reliability of RT-PCR results.

Two-fold RT-PCR (Fig 2, 5-10 lanes), triplet RT-PCR (Fig 2, 11-14 lanes) and quadruplex RT-PCR (Fig 2, 15 lanes). The results showed that single, double, triple and quadruple RT-PCR reactions could achieve the purpose of detecting these four viruses.

Optimization of annealing temperature for single and quadruple RT - PCR system

In order to optimize the design reaction system, 12 kinds of annealing temperature gradients were designed according to the Tm value of the four pairs of primers. The gradient range was from 49.0 to 60.0°C. The PCR amplification instrument generated 12 kinds of temperature gradients automatically. Fig. 3 illustrates that different annealing temperatures had little effect on single and multiple PCR amplification efficiency of CMV, TMV, PVY, and BBWV-2 viruses, and the amplification efficiency was stable in the temperature range from 49.0 to 60.0 °C, but the four-fold PCR system, CMV amplification efficiency rate was the highest. The results of the single and quadruple PCR and the Tm values of the four pairs of primers were used to finally set the annealing temperature of the quadruple PCR to 57 °C.

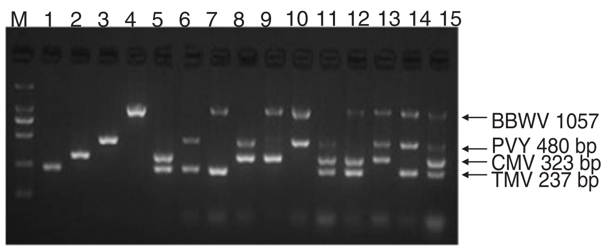

Fig 2 Simplex and multiplex RT-PCR (mRT-PCR) detection of viruses including CMV, TMV, BBWV-2 and PVY of pepper. Lane M, low DNA mass ladder (Invitrogen) (top to bottom: 2,000, 1,000, 750, 500, 250, and 100 bp); lanes 1 to 4: Simplex RT-PCR detection of positive controls for the TMV, CMV, PVY, BBWV-2; lanes 5 to 10: Double RT-PCR detection of positive controls; lanes 11-14: Triplex RT-PCR detection of positive controls: lane 15: Fourplex RT-PCR detection of positive controls.

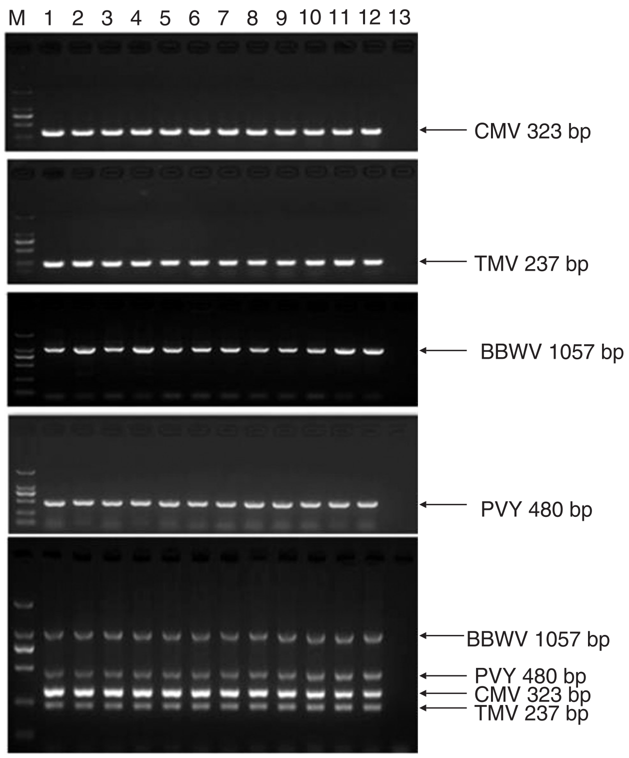

Fig 3 Different annealing temperatures in simplex and 4-plex RT-PCR. Lane M, low DNA mass ladder (Invitrogen) (top to bottom: 2,000, 1,000, 750, 500, 250, and 100 bp); lanes 1 to 12: correspond to annealing temperatures of 49.0, 49.3, 50.1, 51.2, 52.5, 53.8, 55.2, 56.5, 57.8, 58.9, 59.7, 60.0°C; lane 13 is negative control.

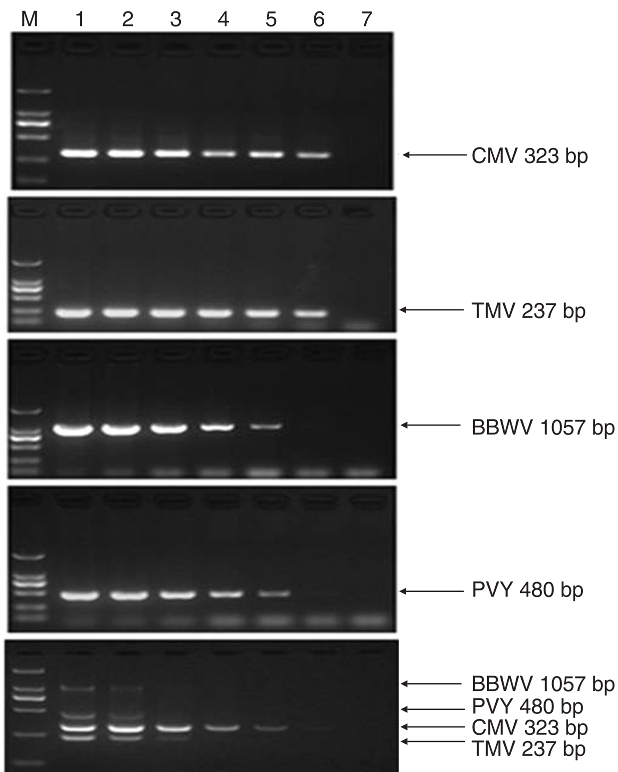

Fig 4 The sensitivity of simplex RT-PCR and 4plex RT-PCR. Lane M, low DNA mass ladder (Invitrogen) (top to bottom:2,000, 1,000, 750, 500, 250, and 100 bp), lanes 1 to 7: correspond to different RNA dilutions of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} of 2.5 µg of RNA.

Determination of sensitivity of single and multiplex PCR reactions

To determine the sensitivity of single RT-PCR and multiple RT-PCR, the total RNA extracted from the four viral materials was diluted 10 times, respectively as 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} µg of RNA (2.5 µl). cDNA was synthesized as a reverse transcription template, and single and quadruple PCR were performed. Fig. 4 shoued that, CMV, TMV detection sensitivity was 10^{-5} µg of RNA, PVY and BBWV-2 detection sensitivity is 10-^{4} µg of RNA. Four-fold PCR amplification for detection of 4 virus, the detection sensitivity of CMV was 10^{-5}, followed by TMV, (10^{-4}), PVY and BBWV-2 detection sensitivity was 10^{-1}, so the detection sensitivity of the four kinds of viruses was 10^{-1} at the same time, which was lower than single RT-PCR, and CMV, TMV datection efficency was slightly lower than the single-PCR method. It is probably due to the completion of simultaneous amplification of four pairs of primers.

Application of Tetra-RT-PCR Detection System

A total of 43 suspected pepper leaves were collected from the main vegetable producing areas of China. The results showed that the target bands of CMV, TMV, PVY and BBWV-2 were clear and specific (Fig 5). The detection rate was 97.67% in 42 kinds of samples from 43 kinds of total samples. The detection number of CMV, TMV, BBWV-2 and PVY were 35, 16, 10 and 1 respectively, the detection rates were 81.40%, 37.21%, 23.26%, and 2.33% respectively. The number of virus-infected samples was 21, accounting for 48.83% of all samples. The results were consistent with the results of single RT-PCR and the positive coincidence rate was 100%. The accuracy of multiple RT-PCR was further verified, which indicated that the reaction system could be effectively applied to the detection of these four viruses. Virus detection provides a quick and easy way.

CMV, TMV, PVY, and BBWV-2 are the main viruses that infect pepper. Production to strengthen the TMV, CMV, PVY, and BBWV-2 virus prevention and control, reduce the damage caused by the virus; understanding the main producing areas of the prevalence of viral disease is the basis of the prevention and control of pepper virus disease in the region. RT-PCR has become the most preferred method of pathogen detection because of its specificity and sensitivity, and it is widely used in plant virus detection. For multiple virus complex infection, multiple RT-PCR method can detect multiple viruses simultaneously, which can detect a large number of samples in a short time, improving the detection efficiency, and reducing the detection cost. Capsule virus single RT-PCR detection used much more than multiple RT-PCR research. Guo *et al*. (2008) used RT-PCR to detect PMMoV by real-time fluorescence quantitative RT-PCR, Wang Jianhua *et al*. (2012) established RT-PCR method for ChiRSV, Yao Yu-rong *et al*. (2013) detected CMV and

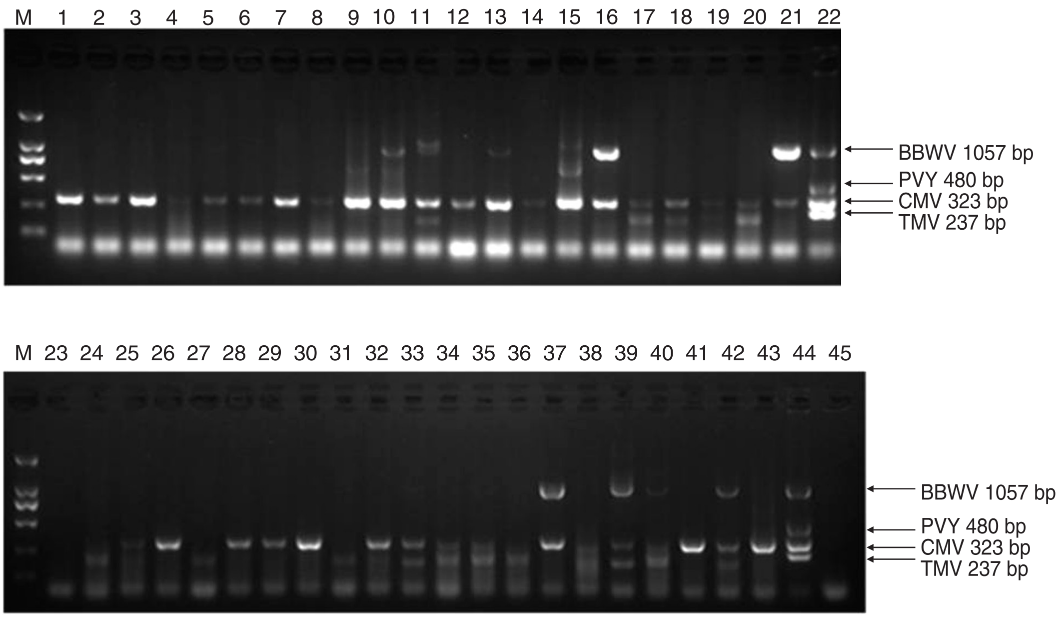

Fig 5 4-Plex RT-PCR detection of viruses of suspected pepper in Hunan. Lane M, low DNA mass ladder (Invitrogen) (top to bottom: 2,000, 1,000, 750, 500, 250, and 100 bp); lanes 1-43: Sample of pepper leaves in Hunan province; lane 44: Positive control; lane 45: Negative control.

PMMoV on pepper by RT-PCR, Guo Siyao, *et al*. (2015) detected CMV, TMV and other virus by single RT-PCR. Although Huang Ya (2015) established the five-fold RT-PCR detection system of pepper PMMoV, ChiVMV, PVY, TSWV and TuMV, it did not include the most common CMV, TMV and BBWV-2 in China's pepper production. Therefore, single RT-PCR detection of TMV, CMV, PVY and BBWV-2 virus needs multiple reactions to complete, is time-consuming, laborious, wasting kit supplies, high cost, but also causing environmental pollution. In this paper, four viruses, CMV, TMV, BBWV-2 and PVY, were detected simultaneously. Four viz. plex novel RT-PCR detection systems was used to detect the four viruses. At last, the detection rate of CMV was 81.40%, and the detection rate of TMV, BBWV-2 and PVY was 37.21%, 23.26% and 2.23%, respectively. The detection rate of CMV was the highest in the main producing areas of Hunan Province CMV, TMV and BBWV-2 were the main viral diseases of pepper in Hunan province. Therefore, the method of multiple RT-PCR was highly targeted, and this result was consistent with the results before. With a high sensitivity, the use of the detection method can greatly improve the detection efficiency.

Conclusion

In this study, a four-step RT-PCR method for simultaneous detection of CMV, TMV, PVY and BBWV-2 viruses was established, and the effectiveness and reliability were analyzed. The detection method was rapid and effective, and the sensitivity was high, which provides a technical reference for studying the epidemic law of pepper virus disease and a prevention and control of virus disease.

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