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ORIGINAL ARTICLE

Detection of *Tobacco mosaic virus* and *Tomato mosaic virus* in pepper and tomato by multiplex RT–PCR

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Abstract

Aims: To develop a highly sensitive and rapid protocol for simultaneous detection and differentiation of *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) in pepper and tomato. In this study, we use the multiplex PCR technique to detect dual infection of these two viruses.
Methods and Results: A multiplex RT–PCR method consisting of one-tube reaction with two primer pairs targeted to replicase genes was developed to simultaneously detect TMV and ToMV in seed samples of pepper and tomato. Specific primers were designed from conserved regions of each of the virus genomes, and their specificity was confirmed by sequencing PCR products. RT–PCR detected up to 10^{-6} dilution of total RNA extracted from infected leaves. Multiplex RT–PCR revealed the presence of both TMV and ToMV in three of 18 seed samples of tomato and one of 18 seed samples of pepper.
Conclusions: The multiplex PCR assay was a cost effective, quick diagnostic technique, which was helpful in differentiating TMV and ToMV accurately.
Significance and Impact of the Study: The multiplex PCR assay described in this study is a valuable tool for plant pathology and basic research studies. This method may facilitate better recognition and distinction of TMV and ToMV in both pepper and tomato.

Introduction

Tobamoviruses have become a serious constraint on tomato and pepper production in many parts of the world. Tobamoviruses have many host species, and they are readily spread mechanically (Spence *et al.* 2001). Tobamoviruses persist in seeds, plant debris and on glasshouse benches. Several tobamoviruses are seed-borne, which contributes to disease spread. *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) infect several solanaceous species. Both viruses produce local lesions on *Nicotiana glutinosa* and *Nicotiana tabacum* cv. Xanthi. The two viruses could be differentiated based on the symptoms on *Nicotiana sylvestris* and *N. tabacum* cv. White Burley: TMV produces systemic infection in both these hosts, whereas ToMV produces only local lesions (Fletcher and MacNeill 1971; Green *et al.* 1987). Cross-reaction between ToMV and TMV antisera limits serological differentiation of two viruses by enzyme-linked immunosorbent assay (ELISA) (Jacobi *et al.* 1998). For epidemiological investigations, resistance breeding and plant virus collections, the determination of the species or even the strains is desirable. Reverse transcriptase–polymerase chain reaction (RT–PCR) is used to identify RNA plant viruses. This procedure is rapid, specific and sensitive, and PCR-based methods are useful in high-throughput applications (Walsh *et al.* 2001).

Jacobi *et al.* (1998) developed the multiplex immunocapture reverse transcription–polymerase chain reaction (IC–RT–PCR) technique for the detection and differentiation of ToMV and TMV in spruce and pine extracts using primer pairs designed for the 3′ UTR, the movement protein and the coat protein gene for ToMV and the movement protein gene for TMV. Multiplex RT–PCR

is a simple and easy method to differentiate two closely related viruses like TMV and ToMV. Spence *et al.* (2001) designed primers for the CP gene of TMV for detection in trailing Petunias. Availability of complete genome sequence of tobamoviruses helps in designing specific primers for different gene targets, which also helps in multiplexing for differentiation of viruses. The tobamovirus genome codes for four proteins: the 126- and the 183-kDa proteins which are required for virus replication, the 30-kDa movement protein and the 17·5-kDa coat protein. The replicase is the first gene expressed in the replication cycle of the tobamovirus. The replicase gene of TMV and ToMV is conserved among the viruses of tobamovirus group. Primers were designed for the replicase gene of TMV and ToMV to detect the two viruses individually as well as in mixed infection in pepper and tomato seeds.

Material and methods

Virus isolates and sources

Tobacco mosaic virus (Isolate DAB TMV-1) and *Tomato mosaic virus* (Isolate DAB ToMV-1) were maintained on pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) plants by mechanical inoculation, respectively. Initially, isolates DAB TMV-1 and DAB ToMV-1 were confirmed by indicator plant test and ELISA. Additionally, TMV and ToMV were also maintained on *N. tabacum* cv. Samsun. TMV or ToMV infected tomato and pepper seedlings were maintained in screen house. For maintenance of virus, sap from infected plants was extracted in 100 mmol l^{-1} phosphate buffer (pH 7·5) at 1 : 50 w/v dilution and used to mechanically inoculate young leaves predusted with carborundum (600 mesh).

PCR Primers

Primer sequences were designed based on published sequence of genomes of TMV (GenBank accession number V01408) and ToMV (GenBank accession number DQ873692). Replicase regions with conserved and species-specific sequence for each virus were identified using multiple sequence alignment software (Corpet 1988), and primers were designed using the Primer3 software (Rozen and Skaletsky 2000). The uniqueness of the primer sequence was checked using Basic Local Alignment Search Tool (Altschul *et al.* 1990) provided online by the National Center for Biotechnology Information. One primer pair was chosen for specific amplification of TMV (5′-CGACATCAGCCGATGCAGC-3′; forward primer, corresponding to nucleotides 630–648 and 5′-ACCGTTTTCGAACCGAGACT-3′; reverse primer, corresponding to nucleotides 1491–1510 of the TMV genome) amplifying a 880-bp PCR product representing part of the replicase gene, For specific amplification of ToMV, the region corresponding to 527–546 nucleotides 5′-CGAGAGGGGCAACAAACAT-3′ forward primer and 5′-ACCTGTCTCCATCTCTTTGG-3′; reverse primer, corresponding to nucleotides 825–843 that amplified a 318-bp PCR product, within the replicase gene was selected.

Detection of TMV and ToMV from infected leaf samples

TMV, ToMV infected leaves and healthy leaves of pepper and tomato, respectively, were harvested and macerated immediately in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RT–PCR was carried out for TMV and ToMV individually using specific primers. cDNA synthesis was carried out using Enhanced Avian RT-PCR kit (Sigma-Aldrich, St Louis, MO, USA). The RT reaction mixture in a total volume of 20 μl contained 100 ng RNA template, sterile water, 125 μmol l^{-1} of each of the dNTPs and 2·5 μmol l^{-1}random nanomers. Initially, the reagents were incubated at 70°C for 10 min followed by addition of 1× buffer, 4 U RNase inhibitor and 20 U enhanced AMV-RT and incubation at 25°C for 15 min and 45°C for 50 min. The PCR mixture in a total volume of 50 μl consisted of following: 1× PCR buffer, 0·5 mmol l^{-1} $MgCl_2$, 5 U *Taq* DNA polymerase, 100 pmol each of forward and reverse primers of both TMV and ToMV and 3 μl cDNA. PCR conditions were as follows: initial denaturation at 94°C for 3 min followed by 31 cycles at 94°C denaturation for 30 s, annealing at 66°C for 30 s, extension at 72°C for 40 s and a final extension at 72°C for 10 min. PCR was carried out in a gradient Mastercycler (Eppendorf, Hamburg, Germany).

Multiplex RT–PCR of TMV and ToMV from seeds

One-hundred-milligram seeds of pepper or tomato was crushed in liquid nitrogen, and total RNA was extracted using RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. For multiplex detection of TMV and ToMV, cDNA produced from individually seed samples was used. Amplification of viral cDNA was carried out keeping the total reaction volume of 50 μl. The RT–PCR reaction was carried out as mentioned above.

Sensitivity of RT-PCR

To determine the detection limits of single-virus RT–PCR and multiplex RT–PCR, the total RNA extracted from infected leaves was serially diluted tenfold (10^0–10^{-6}) in

total RNA extracted from healthy leaves, which was later subjected to single-virus RT–PCR and multiplex RT–PCR using designed species-specific primers for TMV and ToMV.

Results

Symptoms of virus infected plants

Pepper and tomato plants were inoculated with DAB TMV-1 and DAB ToMV-2 isolates, respectively. The inoculated plants of both species displayed symptoms of leaf mosaic, leaf curling and stunted growth (Fig. 1a–d). In addition, the viruses produced systemic mosaic symptoms in *N. tabacum* cv. Samsun (Fig. 1).

Detection of TMV and ToMV from infected leaf samples

The RT–PCR method using specific primers designed for the replicase gene of TMV and ToMV detected the tobamovirus infection in tomato, pepper and *N. tabacum* cv. Samsun. The primers amplified fragments of 880 and 318 bp in the replicase region of the TMV and ToMV genomes, respectively, thus confirming the presence of these two tobamovirus species in tomato and pepper (Fig. 2).

Detection of TMV and ToMV from seeds

Total RNA was extracted from 18 seed samples each of tomato and pepper, using RNeasy Plant Mini kit (Qiagen) as per the manufacturer's protocol. The isolated total RNA from tomato and pepper seeds was subjected to RT–PCR. Employing specific primers individually or in multiplex, the ToMV-specific replicase primers amplified a 318-bp segment of the genome in seven seed samples of tomato (Fig. 2). Similarly, the presence of TMV in nine seed samples of pepper was confirmed with the TMV-specific primers for the replicase gene, which amplified an 880-bp amplicon (Fig. 2). Three seed samples of tomato and one seed sample of pepper were found infected with both TMV and ToMV (Fig. 2). Eight seed samples each of pepper and tomato were not infected with either TMV or ToMV.

Sensitivity and specificity of single-primer pair RT–PCR and multiplex RT–PCR

The primers detected TMV and ToMV when total RNA from virus-infected plants was diluted up to 10^{-6} fold with total RNA from healthy plants (Fig. 3a–c). PCR products were sequenced using the primers described

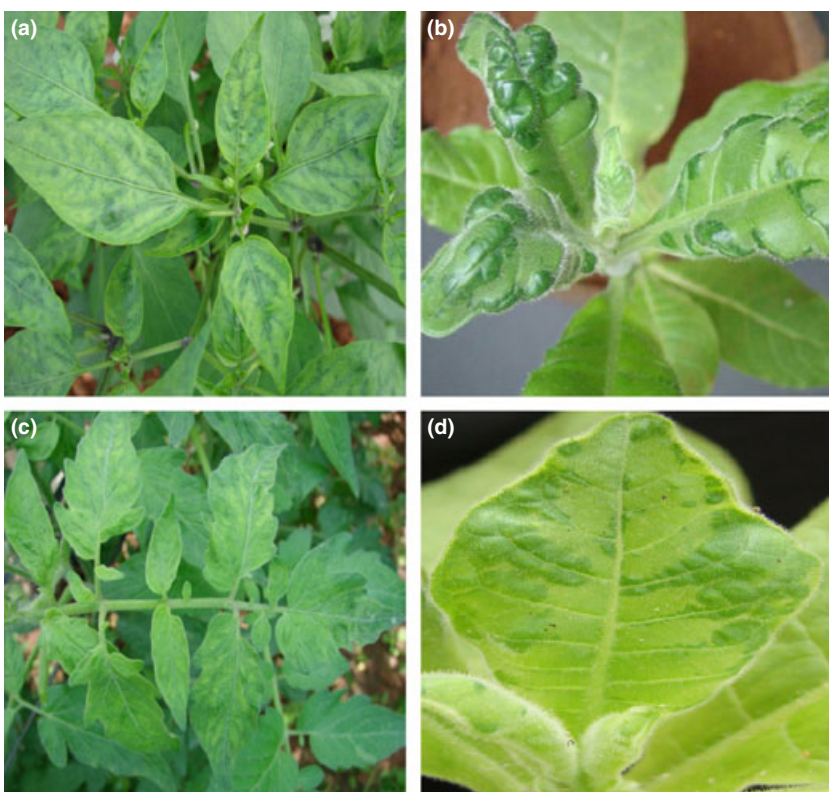

Figure 1 (a) Symptoms of *Tobacco mosaic virus* (TMV) infection on chilli leaves. (b) TMV infection in *Nicotiana tabacum* cv. Samsun showing mosaic. (c) Symptoms of *Tomato mosaic virus* (ToMV) infection on tomato leaves. (d) ToMV infection on *N. tabacum* cv. Samsun leaf.

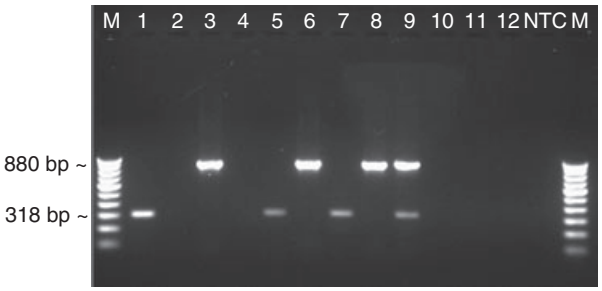

Figure 2 Detection of *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) by RT-PCR. Amplified cDNA fragments (*c.* 880 and 318 bp) were analysed by electrophoresis on 2% agarose gel. M: 100 bp DNA ladder, lane 1 – ToMV-infected total RNA with ToMV primers; lane 2 – ToMV-infected total RNA with TMV primers; lane 3 – TMV-infected total RNA with TMV primers; lane 4 – TMV-infected total RNA with ToMV primers; lane 5 – doubly infected total RNA with ToMV primers; lane 6 – doubly infected total RNA with TMV primers; lane 7 – ToMV-infected total RNA with multiplex primers; lane 8 – TMV-infected total RNA with multiplex primers; lane 9 – doubly infected total RNA with multiplex primers; lane 10 – healthy control with ToMV primers; lane 11 – healthy control with ToMV primers; lane 12 – healthy control with multiplex primers; lane 13 – non template control (PCR reaction without cDNA).

above (GenBank accession numbers GU723495 and GU723496).

Discussion

TMV and ToMV are closely related. Both viruses infect pepper, potato, tomato and several other hosts including weeds. Diagnosis of the pathogen plays an important role in crop improvement and disease management strategies. The PCR-based molecular detection of phytopathogenic viruses is gaining importance in recent years. Biological assays, serological detection and identification of species within the genus tobamovirus can be time consuming and might lead to ambiguous results, especially for TMV and ToMV. Differentiation problems are caused by heterologous reactions of antisera detecting common epitopes as well as by antisera produced against virus mixtures (Letschert *et al.* 2002). The multiplex RT–PCR technique allows rapid detection and differentiation of TMV and ToMV.

Chitra *et al.* (1999) reported the seed transmission of ToMV and TMV from seed to seedlings to be 1–13% and 1–10%, respectively. Traditionally, ToMV and TMV are detected by inoculating herbaceous indicator plant species that produce typical symptoms. ToMV and TMV could be differentiated based on differential reaction on *N. sylvestris* or on *N. tabacum* cv. White Burley. Few indicator plants distinguish reliably between ToMV and TMV: *Phaseolus vulgaris* cv. Black Turtle 1 is susceptible to infection by TMV and some isolates of

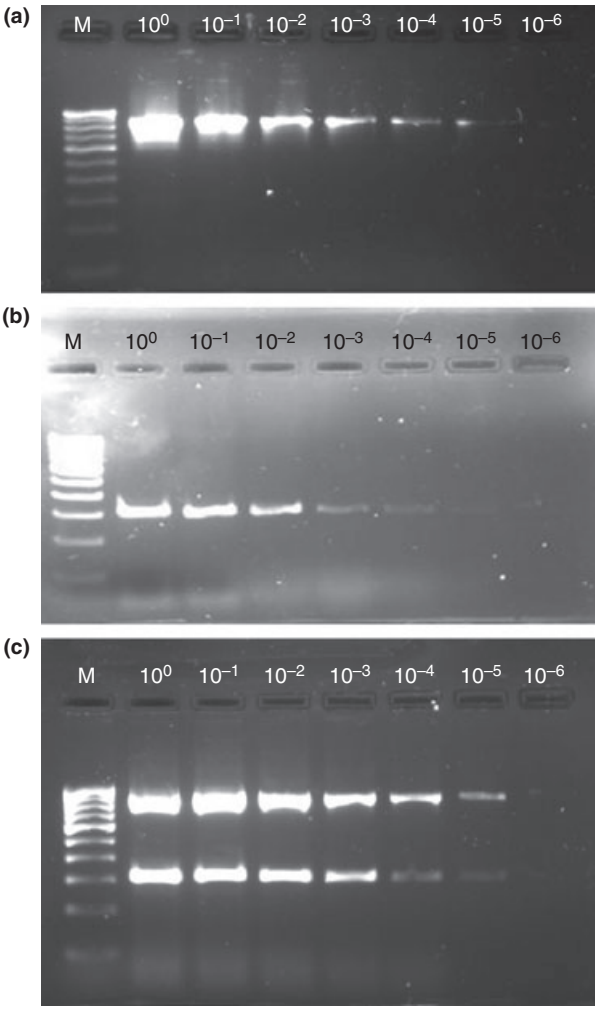

Figure 3 (a) Sensitivity of RT-PCR in the detection of *Tobacco mosaic virus* (TMV) – 1·5% Agarose gel electrophoretic analysis of PCR products amplified by single reverse transcription-polymerase chain reaction (sRT-PCR). Lane from 10^0 to 10^{-6} indicates tenfold serial dilutions of TMV infected leaf; lane M: 100-bp DNA ladder. (b) Sensitivity of RT-PCR in the detection of *Tomato mosaic virus* (ToMV) – lane from 10^0 to 10^{-6} indicates tenfold serial dilutions of ToMV infected leaf; lane M: 100-bp DNA ladder. (c) Sensitivity of mRT-PCR in the detection of TMV and ToMV – lane from 10^0 to 10^{-6} indicates tenfold serial dilutions of TMV and ToMV infected leaf; lane M: 100-bp DNA ladder.

ToMV but only TMV infection will result in the production of red, necrotic local lesions; *Cucumis sativus* L. produces local lesions upon inoculation with ToMV, and no symptoms were observed upon inoculation with TMV (Jacobi and Castello 1991; Fillhart *et al.* 1998). It takes 3–7 days for symptoms to develop, whereas multiplex RT–PCR can be performed in only 1 day including gel analysis.

In this study, RT–PCR was carried out on 18 seed samples of pepper and tomato, respectively, using specific

primers designed for the replicase gene of TMV and ToMV, respectively. Nine seed samples were found infested with TMV, whereas ToMV infestation was seen in seven seed samples and dual infestation of TMV and ToMV was observed in four seed samples.

Jacobi *et al.* (1998) employed a multiplex IC–RT–PCR method to detect TMV and ToMV in spruce and pine extracts and reported that IC–RT–PCR is 10^4 times more sensitive than ELISA. TMV and ToMV antibodies were used to perform IC–RT–PCR. Detection of these viruses by antibody is not reliable as possibility of cross-reaction may occur. Multiplex IC–RT–PCR is a more time-consuming procedure than multiplex RT–PCR. There are examples of simultaneous detection of mixed virus infection, and their amplification by multiplex RT–PCR. RT–PCR using a single primer pair was developed for the detection of five tobamovirus species, which are related serologically (Letschert *et al.* 2002). In this study, multiplex RT–PCR detected ToMV and TMV RNAs in up to 10^{-6} dilutions.

The multiplex RT–PCR is also applicable to detect and differentiate TMV and ToMV in major solanaceous hosts such as tobacco, tomato and pepper. In addition, it is a quick screening mechanism for unknown isolates of TMV, ToMV or both and verifies the authenticity of virus stock cultures in leaf material or purified virus preparations.

Earlier Jacobi *et al.* (1998) used primer from the coat and movement protein coding regions to detect and differentiate ToMV and TMV. In the present study, we targeted the replicase gene of tobamovirus to detect and differentiate TMV and ToMV in pepper and tomato. The replicase genes of TMV and ToMV are highly conserved, and only small region of variability are present; however, differentiation between the two viruses was possible.

Molecular techniques such as multiplex RT–PCR and RT–PCR using species-specific primers were proven to be useful for the diagnosis and control of the disease and will also be beneficial for resistance breeding, epidemiological investigations and plant virus collections. Hence, the technique developed in this study could be a useful tool for the preliminary rapid screening of a large number of seed samples for seed-borne TMV and ToMV. The samples testing positive by this method may be further examined by other confirmatory technique such as growing on test.

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