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Detection of Mixed Infection of Tobamoviruses in Tomato and Bell Pepper by using RT-PCR and Duplex RT-PCR

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ABSTRACT

The aim of the present study was to detect the mixed infection of the tobamoviruses (TMV and ToMV) simultaneously. The primers were designed for the movement protein regions of TMV and ToMV and synthesised. The primers were used to detect the TMV and ToMV in infected leaves. To confirm the results of molecular detection, Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA) was used to detect the viruses. Based on the results of the present study, the movement protein gene may be used as candidate gene for the identification of plant viruses in plant pathology laboratories. Mixed infection of both viruses to same host can be detected by using duplex RT-PCR method. The assay could be performed in a single tube for simultaneous detection of TMV and ToMV.

Key words: Tobamoviruses, movement protein gene, molecular detection, enzyme linked immunosorbent assay, mixed infection

INTRODUCTION

Plant viruses cause serious economic losses in many major crops by reducing yield and quality. Viruses are among the most agriculturally important and biologically intriguing groups of plant pathogens (Asano *et al.*, 2005). The detection of plant viruses is becoming major thrust area in the plant pathology research. Considerable efforts have been made to understand the detection, epidemiology of these viral diseases and to devise control measures (Makkouk and Kumari, 2006).

Viruses are being increasingly recognized as a major constraint to crop improvement as they are highly infectious and may attain epidemic proportions resulting in economic loss. Tobacco Mosaic Virus (TMV) and Tomato Mosaic Virus (ToMV) belongs to the tobamovirus group. TMV causes heavy yield losses for tobacco, tomato and pepper worldwide. The tomato mosaic virus has been reported to cause reduction in weight of tomato fruits up to 59% with a mean disease incidence of 55% (Cherian and Muniyapppa, 1998). Yield losses in bell pepper due to TMV have been reported to be 1-90% (Chitra *et al.*, 2002). Chitra *et al.* (1999) reported the transmission rate of TMV to the seedlings to be 1-10%.

The determination of virus infection in plants using serological or biochemical techniques is important for the prevention of virus diseases (Muthomi *et al*., 2009). Serological techniques such as ELISA are more convenient and inexpensive than existing technique, although they require some specialized equipment. However, cross-reaction between tobamoviruses and their antisera limits serological differentiation of theses viruses by ELISA. The technique is therefore unsuitable for detecting mixed infections in tissues and monitoring viruses separately in infected fields. Recently, molecular tools have been developed to detect the viruses especially tobamoviruses like RT-PCR, Multiplex PCR are available. Multiplex IC-RT-PCR had been used for detection and differentiation of tomato and tobacco mosaic tobamoviruses (Jacobi *et al*., 1998). Similarly, several viruses were detected by using RT-PCR, IC-RT-PCR and Multiplex IC-RT-PCR techniques in different plants (Gillaspie *et al*., 2007; Safaeizadeh, 2008; Capote *et al*., 2009; Al-Saleh *et al*., 2009).

Cell-to-cell movement of Tobacco Mosaic Virus (TMV) is mediatedby a 30kDa Movement Protein (MP) encoded by TMV RNA. It has been shown that P30 accumulates in plasmodesmata of TMV-infected and MP-transgenic plants to increase their permeability (Ashby *et al*., 2006). Hence, movement protein gene can be used for the detection of the tobamoviruses.

The present study developed a duplex RT-PCR assay for the simultaneous detection of both TMV and ToMV by using movement protein gene.

MATERIALS AND METHODS

Maintenance of host plants and viruses: Seeds of tomato cv. PKM-1 and bell pepper cv. California wonder were sown in earthen pots containing 1:2:1 ratio of sand, soil and farmyard manure. The pots were maintained in insect-proof screen house conditions. The Tobacco Mosaic Virus (TMV) and Tomato Mosaic Virus (ToMV) were maintained in bell pepper and tomato plants, respectively, in the screen house. The experiments were conducted at Seed Virology Laboratory, Department of Studies in Applied Botany and Biotechnology, University of Mysore, Mysore, India during the period November, 2006 to September, 2007.

Inoculation of tomato and bell pepper plants with ToMV and TMV: Fifteen days old tomato and bell pepper seedlings were inoculated with ToMV and TMV, respectively. The standard virus inoculum was prepared by using the leaves showing mosaic symptoms harvested from the pre-maintained ToMV-infected tomato and TMV infected bell pepper plants. The leaves (1 g) were homogenized in 5 mL of the phosphate buffer (pH 7.2, 0.1 M) in a pre-chilled pestle and mortar. After homogenization, the extract was filtered through muslin cloth and the supernatant was used as the source of inoculum. The cotton swab was dipped in the virus inoculum and swabbed over carborundum pre-dusted leaves. After 10 min, inoculated leaves were washed with distilled water. The seedlings were maintained in a screen house.

Mixed inoculation of TMV and ToMV to tomato plants: Both ToMV and TMV were inoculated to fifteen days old tomato seedlings. The inoculated seedlings were maintained in insect proof screen house condition.

Reverse transcriptase-PCR for movement proteins of TMV and ToMV: After 21 days of inoculation of TMV and ToMV to bell pepper and tomato plants, the third leaves of plants were harvested for RNA extraction. RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. RNA samples were digested with

Dnase (DNA-free, Ambion, Austin, TX, USA). Specific primers for movement protein genes of TMV and ToMV were designed for movement protein of TMV and ToMV with primer 3 software (Table 1).

Reverse transcription was performed using RETRO Script Kit (Ambion) as per manufacturer's instructions. Reverse transcription was carried out in Master Cycler Gradient (Eppendorf, France). The RT mixture contained 2 μL of reverse primer (50 μM), 1μg equivalent RNA, 5 μL of nuclease-free water, 2 μL of RT buffer, 2 μL of DNTPs, 1 μL of RNase inhibitor and 1 μL of Reverse transcriptase (RETRO Script Kit, Ambion). RNA was denatured by initial denaturation at 75°C for 3 min. The RT mixture was added to the denatured RNA. The reaction was carried out at 42°C for 60 min and the process was terminated 92°C for 10 min.

The cDNA synthesized from the RT reaction was taken for the amplification of movement protein of ToMV. The PCR reactions were carried out in a 50 μL mixture containing 1 μL of cDNA, 2.5 units of *Thermus aquaticus* DNA polymerase, 125 μM of each dNTPs (1 μL), 1 μL BSA (0.1%), 5 μL of buffer with $MgCl_2$ (Sigma) and 0.2 μM of primer of forward and reverse primers. The details of PCR conditions were provided in Table 2. The obtained products were loaded to 2% agarose gel. The gel was trans-illuminated with ultraviolet light and photographed or analyzed using an imaging system (Infinitii, Vilberlourmat, France). Using DNA elution kit purified the obtained products. The purified products were sequenced.

Duplex RT-PCR: The leaves from the tomato plants which were inoculated with both TMV and ToMV, were used for Multiplex RT-PCR. Multiple species or strains are detected in a single reaction by combining oligonucleotide primers specific for different viruses. It is important that the amplicons are of different lengths and that there is no cross-reactivity among them. The Movement Protein primers were used for multiplex PCR was done for the detection of TMV and ToMV by following same procedure of PCR mentioned earlier. The PCR condition used for Multiplex PCR is summarized in Table 2.

Detection of TMV and ToMV by using indirect ELISA: The Indirect ELISA was used for the detection of viruses to support the data of RT-PCR for the presence of TMV and ToMV. After 21 days of inoculation of ToMV and TMV to tomato were and bell pepper, respective the third leaves

Table 1: Details of the primers used in the study.

Name of the gene	Primers designed	Amplicon (bp)
TMV movement protein	Forward-5' TGAAAATGAATCATTGTCTG 3'	454
	Reverse-5' ACTCATCAACAACTTCTTCC3'	
ToMV movement protein	Forward-5' TGAAAATGAATCATTGTCTA 3'	623
	Reverse- 5'CATCTTCAATCAAATTATC 3'	

Table 2: PCR conditions for amplication of TMV, ToMV (RT-PCR) and both viruses (Multiplex PCR)

Conditions	MP-TMV	MP-ToMV	Duplex-PCR
Initial denaturation	94°C for 3 min	95°C for 3 min	94°C for 3 min
Annealing temperature	54°C for 30 sec	50°C for 30 sec	54°C for 30 sec
Initial extension	72°C for 1 min	72°C for 1 min	72°C for 1 min
Final extension	72°C for 5 min	72°C for 5 min	72°C for 5 min
No. of cycles	35	40	35

MP-TMV: Movement protein of tobacco mosaic virus, MP-ToMV: Movement protein of tomato mosaic virus, Duplex-PCR: Duplex polymerase chain reaction

harvested and the virus concentration was quantified by using ELISA. The leaves (0.1 g) harvested from 15-day-old untreated or treated plants were crushed in a mortar and pestle in antigen buffer and the extract was centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant extracted from bell pepper and tomato was subjected to indirect ELISA against a-TMV and a-ToMV (kindly provided by DGISP, Denmark), respectively (Hobbs *et al*., 1987).

Data analysis: For data analysis the statistical computer application package SPSS 10.0 was employed. The data generated were average of three independent experiments. Data were subjected to Analysis of Variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT; p = 0.05) (Duncan, 1955).

RESULTS AND DISCUSSION

Tobamovirus movement proteins play a determinant role in the establishment of infections in plants, allowing the local movement of viral RNA genome through plasmodesmata. Viral movement is an active process mediated by a specific virus-encoded P30 protein. P30has at least two functions, to cooperatively bind single-stranded nucleic acids and to increase plasmodesmatal permeability. Cell-to-cell movement of Tobacco Mosaic Virus (TMV) is mediated by a 30 Kilo Dalton (kDa) Movement Protein (MP) encoded by TMV RNA (Tzfira *et al*., 2000). With this background we targeted movement protein genes for the detection of TMV and ToMV in infected leaves.

As such many reverse transcription polymerase chain reaction (RT-PCR) protocols have been developed to individual viruses from crop plants (Grotzinger and Will, 1992; Pappu *et al*., 1993). *Alfalfa Mosaic Virus* (AMV) was detected in potato fields in several provinces in Canada and characterized by bioassay, enzyme-linked immunosorbent assay and reverse-transcription polymerase chain reaction (RT-PCR). The identity of eight Canadian potato AMV isolates was confirmed by sequence analysis of their Coat Protein (CP) gene (Gillaspie *et al*., 2006; Xu and Nie, 2006). Reverse transcription polymerase chain reaction (RT-PCR) analysis of the samples revealed that movement protein gene was amplified to detect the presence of tobomoviruses. The TMV and ToMV primers designed for movement proteins amplified 454 and 623 bp, respectively (Fig. 1, 2). The amplified MP gene in bell pepper and tomato by TMV and

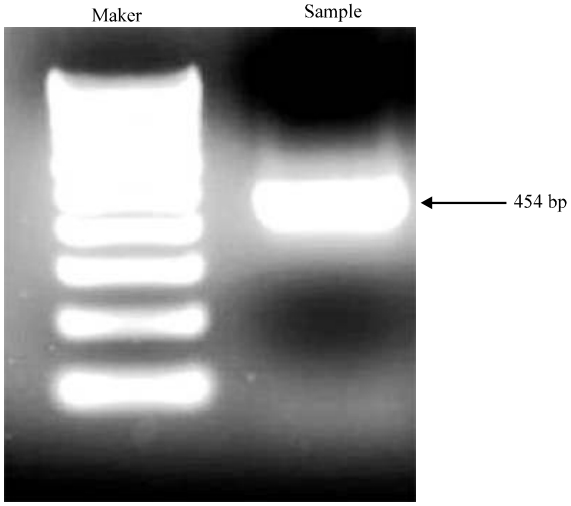

Fig. 1: TMV movement protein gene amplification

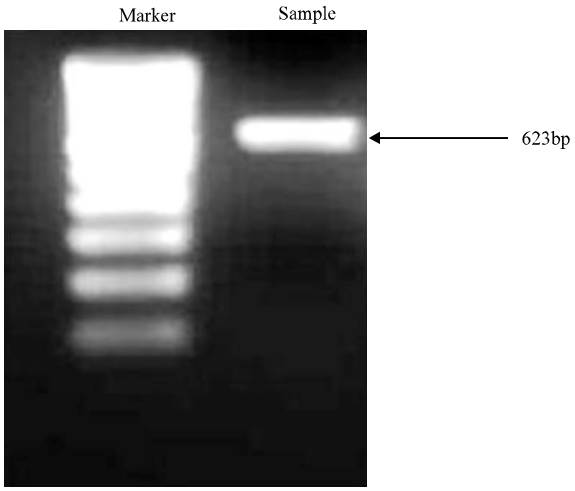

Fig. 2: ToMV movement protein gene amplification

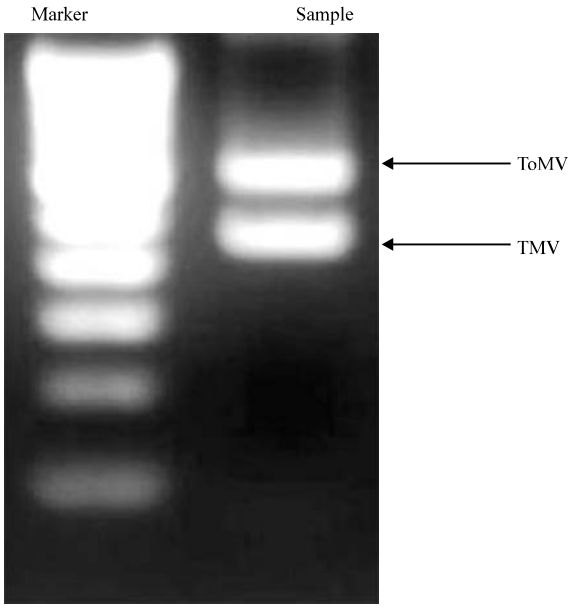

Fig. 3: Duplex RT-PCR of movement protein of TMV and ToMV

ToMV MP primers was the evidence to detect both TMV and ToMV. The gel eluted and purified PCR products were sequenced and the obtained sequences were submitted to NCBI gene bank (GU213293 and GU213294).

Multiple species or strains are detected in a single reaction by combining oligonucleotide primers specific for different viruses by using multiplex PCR. It is important that the amplicons are of different lengths and that there is no cross-reactivity among them. To reduce the cost, a duplex RT-PCR has been developed (Osiowy, 1998; Rosenfield and Jaykus, 1999; Nassuth *et al.,* 2000). Two molecular procedures, RT-PCR and restriction fragment length polymorphism (RFLP) were developed by Xu *et al.* (2010) for the detection and identification of Potato Virus M in potato tubers. RT-PCR was highly specific and only amplified PVM RNA from potato samples. PVM RNAs were

Table 3: Detection of TMV and ToMV in bell pepper and tomato plants (Indirect ELISA) (Absorbance at 410 nm)

	Absorbance at 410 nm	
Sample	TMV	ToMV
Positive control	0.415±0.47[a]	0.397±0.39[a]
Negative control	0.041±0.19[c]	0.047±0.27[c]
Test samples (inoculated leaves)	0.319±0.27[b]	0.334±0.29[b]

Every Value represents the mean of three replicates ± SE. The values followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

easily detected in composite samples of 400 to 800 potato leaves or 200 to 400 dormant tubers. Similarly, present results also confirmed that, RT-PCR and ELISA results confirmed the presence of the TMV and ToMV in the infected plants. The primers designed for both TMV and ToMV movement protein genes was able to amplify the both the viruses in single tube (Fig. 3).

Enzyme Linked Immunosorbent Assay (ELISA) was introduced in plant virology mainly by Clark and Adams (1977) and has become the most widely used serological method for detecting and identifying plant viruses. A direct comparison of detection sensitivity with ELISA in the presence of both viruses was not possible as the ELISA assay could not reliably distinguish between TMV and ToMV. But in present study we have employed to confirm the presence of TMV and ToMV in inoculated leaves by using polyclonal antibodies raised against viruses. The results obtained in this study confirmed the presence of TMV in bell pepper and ToMV in tomato. The results of ELISA confirmed the presence of TMV and ToMV in virus inoculated leaf. The positive control samples showed absorbance of 0.415 and 0.397 for TMV and ToMV, respectively. Similarly, samples from TMV and ToMV inoculated plants showed absorbance of 0.319 and 0.334 absorbance, respectively at 410 nm (Table 3).

CONCLUSION

Based on present research findings, major tobamoviruses infecting tomato and bell pepper can be identified by using RT-PCR method by using movement protein gene. Mixed infection of both viruses to same host can be detected by using Duplex PCR method. In contrast with ELISA which could not discriminate between TMV and ToMV, the experiments confirmed the specificity of primer designed for movement proteins of respective target viruses in both single-virus and Duplex RT-PCR assays. Based on the finding, the duplex system can be used for survey of virus infecting tomato and bell peppers plants.

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