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Reverse Transcription-Polymerase Chain Reaction Mediated Detection and Identification of *Tobacco mosaic virus*- Egyptian Strain

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A procedure involving reverse transcription followed by polymerase chain reaction (RT-PCR), with availability of the database sequences (GenBank) provided a sensitive method for the detection of *Tobacco mosaic virus*- Egyptian strain (TMV-E). Total RNA extract of infected plant and RNA of purified TMV particles were subjected in this method with four degenerate and undegenerate primers. The primer pairs generated two specific PCR fragments of 3428 base pair (bp) and 3836 bp of the whole TMV genome. The intensity of these RT-PCR amplified products was estimated and it indicated relatively to the amount of the virus in the infected plant. The specificity of amplification was verified using internal primers through nested–PCR (n-PCR). To demonstrate the specificity of this technique, the n-PCR-expected product (869bp) was sequenced and this nucleotide sequence was analyzed using soft ware program. Sequence analysis of the amplified fragment revealed a high conserved sequence homology located at the 3'-terminal region of the 126KDa (ORF1) sequence of the published TMV strains. Detection of the TMV-E using RT-PCR raise the availability of species-specific primers for the TMV-E that will be helpful for diagnosis in mixed infection. In addition it will be helpful in further molecular characterization of this strain.

INTRODUCTION

Tobacco mosaic virus (TMV) has enjoyed a storied history in its first century of research. Beside its prominent role in the birth of virology, TMV has been at the centre of many firsts (Zaitlin, 2002). Many important discoveries have been made with TMV, where it had a significant impact on our understanding of the concept of viruses as a significant infectious agent (Harrison and Wilson, 1999)

TMV is the type species of the *Tobamovirus* genus, expressed its genetic information from a positive–sense monopartite RNA molecule 6.395 nucleotide (nt) in length, containing four open reading frames (ORFs) (Goelet *et al.*, 1982). The 5'-terminus of the TMV genomic RNA is capped with 7-methyl guanosine (Zimmern, 1977). The 5'-proximal ORFs that encode the overlapping 126 and 183 KDa replication proteins initiate at nt 69 and terminate with amber and ochre stop codons at nt 3417-3419 and nt 4917-4919, respectively. Both proteins are translated directly from the genomic RNA and are required for efficient replication (Lewandowski and Dawson, 2000). The 30 KDa movement protein (MP) at nt 4903-5709 and the 17.5 KDa capsid protein (CP) at nt 5712-6191 are expressed from individual 3'-co-terminal subgenomic mRNAs (Saito *et al.*, 1990)

The available viral sequence data made possible the development of a method for the identification of related strains based upon the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1985). Universal oligonucleotide primers complementary to conserved genomic sequences shared by all

known members of a virus group have been shown to enable the identification of a new member of the different virus groups: animal hepadnavirus group (Mack and Sninsky, 1988), geminiviruses (Rybicki and Hughes, 1990) and potyviruses (Langeveld *et al* 1991). Sensitivity and specificity of the PCR also made it more easier and reliable method to distinguish between different strains of one virus. Rowhani *et al.* (2000) & Nie and Singh, (2002) investigated the strain diversity among different *Grapevine fleck virus* (GFKV) isolates and *Potato virus* Y (PVY) strains respectively, by design and use specific primers for each strain as well as more degenerate primers that could detect all or groups of strains at a time. Moreover, RT-PCR is commonly used for virus detection and characterization especially in sporadic cases. It enabled detection of *Tomato infectious chlorosis virus* (TICV) from 104 field samples in different regions of Italy (Vaira *et al.*, 2002).

Investigation concerning the differentiation between eight–Egyptian isolates of TMV was anciently undertaken by observing the symptoms elaborated in a variety of differential hosts (Allam *et al.*, 1978). Two tomato strains of Egyptian TMV were characterized by biological, biochemical, and immunological techniques (El-Ahdal *et al.*, 1984). The biological tests were indicated on the basis of symptoms observed on differential hosts and differences between the two strains. However, detailed studies with the other techniques didn't reveal significant differences. Developing a more accurate identification method for the Egyptian strain of TMV, local conserved sequence were selected from alignment of other published world wide TMV sequences to provide primers used for application in combined assay of reverse transcription (RT) and Polymerase Chain Reaction (PCR). Since little molecular data is available on this strain, sequence of the PCR-amplified product was carried out to characterize it more fully and to demonstrate the utility of PCR-technique.

MATERIALS AND METHODS

Materials

Chemicals and reagents used throughout this study were purchased from Sigma Chemical Company, unless otherwise indicated. The selected primers were synthesized by TMG Biotech (Germany).

Source of virus strain

The study was conducted mainly on Egyptian strain of TMV. This strain is one of eight isolates and differentiated as tomato strain (Allam, *et al.*, 1978). Virus was provided as infected tissue and as purified preparation from Virology lab., Microbiology Dept., Faculty of Agriculture, Ain Shames Univ., Cairo, Egypt.

Isolation of viral RNA

Total RNAs were extracted from 1 gm TMV-infected tobacco leaf tissues by using guanidinum isothiocyanate method according to Wilkinson (1988). The final RNA pellet was dried, resuspended in 50 µl of DEPC-treated water and stored at -80 °C till used.
Viral RNA was isolated from purified virus using the phenol-SDS method (Wilcockson and Hull, 1974). Following phenol-chloroform extraction, the viral RNA was precipitated with 1/10 volume sodium acetate and 0.8 volume of cold ethanol. The concentration of both isolated RNAs (total and viral) was determined photometrically at A_{260nm}.

Design of oligonucleotide primers for RT-PCR

The sequence alignment of the different TMV-strains (EMBL data base) facilitated the choice of the two upstream and downstream degenerate and undegenerate primers for the detection and identification of the Egyptian strain of TMV. These four oligonucleotide primers were derived from the conserved regions of 5'-terminal of TMV genome (TMVP1) and the 3'-terminal sequence of the replicase genes (TMVP2), while the downstream primers derived from the 5'-terminal of RNA polymerase gene is (TMVM1) and the 3'-terminal non coding sequence of the TMV genome (TMVM2) (Figure 1). This would result in the whole genome but with varying lengths of molecules. TMVP2 and TMVM1 primers would have very useful. since it could have potentially annealed overlapping in the two previous molecules. The nucleotide sequence and position of these four primers were shown in Table (1).

Reverse transcription and PCR amplification:

DNA double strand of TMV-E was synthesized using Titan one tube RT-PCR system (Roche Applied Science, Penzberg, Germany). In a single vial, two stage protocols were followed with RT and PCR reagents combined in a single mixture. The first strands cDNAs synthesis was accomplished in two separate vials by incubation each 10 µg total RNA or 1 µg viral RNA in the reaction with TMVP1/ TMVM1, TMVP2/ TMVM2 primer pairs at 50 °C for 30 minutes before amplification. The amplification conditions were started with 5 minutes at 94 "C followed by 35 cycles of 94 °C for 10 seconds; 60 °C for 30 seconds; 68 °C for 3 minutes then the final prolonged elongation time up to 7 minutes at 68 °C.

Nested-PCR

Two µl of the initial DNA amplicons amplified by RT-PCR were used as templates in the nested PCR with a second set of internal primers TMVP2 and TMVM1. The PCR was performed in 50 µl total reaction volume containing 10mM Tris-HCl pH 8.8, 50mM KCl, 1.5mM $MgCl_2$, 20µg/ml gelatin, 1.25 U *Taq* polymerase (Promega), 1µm TMVP2 primer and 1µm TMVM1 primer. Mixture was layered with 50µl mineral oil and the reaction was repeated 35 cycles under the following conditions: 1 minute at 93 °C, 2 minutes at 55 °C, and 1 minute at 72 °C.

Both RT-PCR and nested PCR products were analyzed by electrophoresis of 15 µl aliquot from each reaction mixture on 1% agarose gel in TAE buffer. Bands were visualized after soaking for 15 minutes in ethidium bromide (1 µg/µl) and destained in water for 15 minutes. The DNA Ladder (Promega) was used as a molecular weight markers (Sambrook *et al.*, 1989).

DNA sequence determination:

The amplified nested-PCR products of approximately 869 bp was purified with Qiaquick PCR purification kit (Qiagen, Eton Avenue, CA) and sequenced at gene analysis unit (VACSERA, Agouza, Cairo, Egypt). Sequencing was performed by using a Big Dye terminator cycle sequencing kit (Applied Biosystem, Foster city, CA). The sequencing products were purified by using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ) and were resolved on an Applied Biosystem model 3100 automated DNA sequencing system. Sequence analysis was carried out using DNA star programs (MegaAlign Expert sequence analysis software ver. 4.00 from DNA Star Inc).

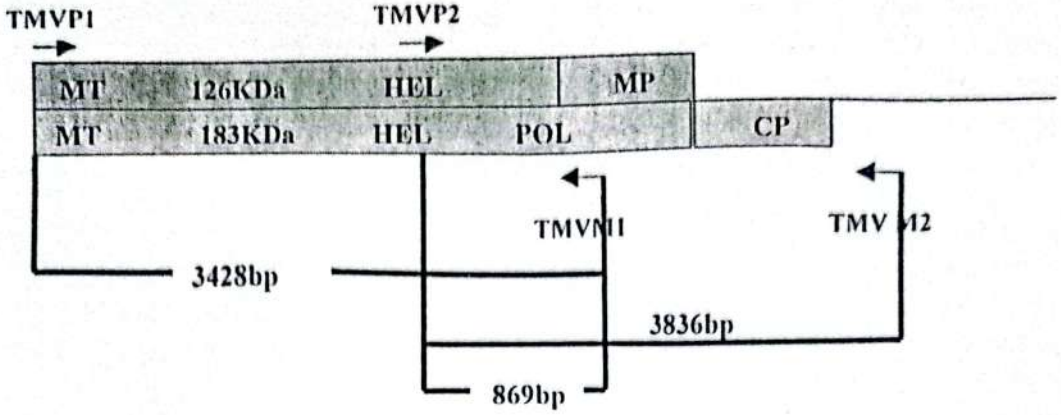

Figure (1): Schematic diagram of TMV genomic RNA. Long boxes represent open reading frames that contain domains similar with methyltransferase (MT), Helicase (HEL), and RNA-dependent RNA polymerase (POL). Small boxes represented the encoded movement protein (MP) and capsid protein (CP). Diagram represents the location of the oligonucleotides primers, the arrows indicate their directions, and the length of both RT-PCR and nested PCR amplified products.

Table (1): Nucleotide sequences of the primers used for detection of TMV - Egyptian strains (TMV-E).

Primer	Sequence	mers	Position+
TMVP1	GTATTTTTACAACAATTACC	20	1-20
TMVP2	GTGGACGGAGTTCC(G/A)GG	17	2559-2575
TMVM1	CTGTAATTGCTATTGTGTTCC	21	3407-3428
TMVM2	TGGGCCCCTACCGGGGGT	18	6377-6395

P and M indicated to the plus and minus primers respectively. + the Position of the primers in the published TMV nucleotide sequences

RESULTS AND DISCUSSION

RT-PCR for the detection of Egyptian TMV-RNA sequence

The biological assays as well as serological detection and identification of viral species might lead to ambiguous results, especially for the new virus isolates (Lin *et al.*, 1990). To circumvent this problem, procedures based upon the plolymerase chain reaction allowing rapid detection and identification of Egyptian strain of TMV (TMV-E) even in 1 gm of the infected plant tissue. The TMV sequences availability enabled us to design four primers for the successful identification. When testing these selected primers in the detection of the TMV-E RNA using RT-PCR, the total RNA isolated from TMV infected tobacco plants and viral RNA from purified particles were used as templates in RT-PCR. After 35 cycles of PCR, the amplified products were analyzed on 1% agarose electrophoresis. The expected length of the amplified fragments was corresponding in all reactions to the position of the four primers. With viral RNA, the combination of the TMVP2 and TMVM2 yield a single fragment of

about 3836 bp (Figure 2 Lane V1). However a second fragment of about 3428 bp was obtained with TMVP1 and TMVM1 primer set (Figure 2 Lane V2), hence they produced specific two DNA fragments covering the whole TMV-genome with expected molecular sizes. This proves the use of these primers in RT-PCR is preferable for the detection of the TMV-E. Jacobi *et al.* (1998) used RT-PCR to distinguish TMV and ToMV that are members of the subgroup1. Letschert *et al.* (2002) also developed the same technique involving RT followed by PCR using single primer pair for the detection and differentiation of the five tobamovirus species which are related serologically. Using the same PCR-conditions and primer sets (TMVP2/TMVM2) and (TMVP1/TMVM1), the visibly less intense 3836bp and 3428 bp fragments were respectively obtained with RNA from infected plant material ((Figure 2 Lane P1, P2). Comparing the RT-PCR products of viral RNA with those of total RNA using gel analyzer software, enabled us to evaluate the concentration of viral RNA present in plant tissue. It was estimated to be 0.7 µg in 10µl sample of leaf extract, and 3.5 µg/gm leaf tissue, where the higher virus titer was in plant- the stronger band showed up in the agarose gel. This result is in accordance with the hypothesis of Willment *et al.* (2001) indicated that, the relative proportion of the component of the mixed infections could be analytically deduced from the intensity of the different banding pattern.

Verification of the RT-PCR products by nested –PCR

Based upon the position of the four primers, two internal primers TMVP2 and TMVM1 were used in a hemi nested-PCR. The four RT-PCR amplified products from both the viral and plant total RNA were used at 4% of the final volume reaction. As shown in figure (3), the size of the nested-PCR products obtained from DNA amplification was in consistent with the expected size (869bp). This was resulted with DNA amplification products of viral RNA (Figure 3A, lanes nV1, nV2) and plant total RNA (Figure3B, lanes nP1, nP2). These results indicate to the validation of the RT-PCR products, moreover the specificity of the chosen primers in this study as Castello *et al.* (1999) where, the nested –PCR was applied with different samples to validate the detection of TMV in the ancient glacial ice.

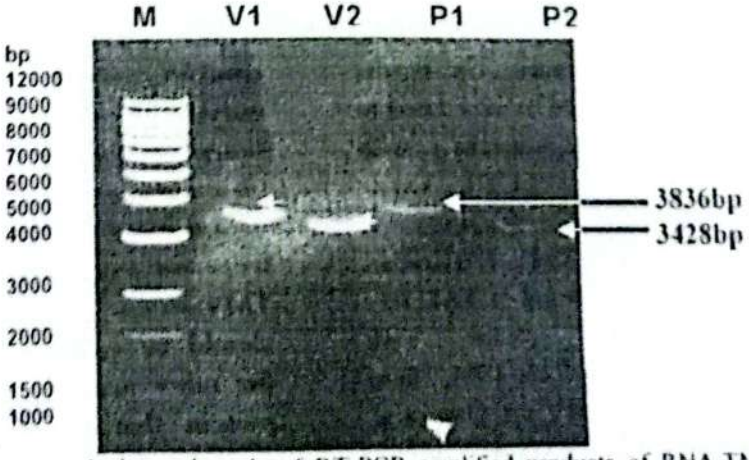

Figure (2): 1% agarose gel electrophoresis of RT-PCR amplified products of RNA-TMV genome fragments using TMVP1, TMVM1 and TMVP2, TMVM2 primers. Lanes (V1, V2) amplified produc of viral RNA, lanes (P1, P2) amplified products of plant total RNA using the same pairs of primers respectively. M: DNA molecular weight marker in base pair.

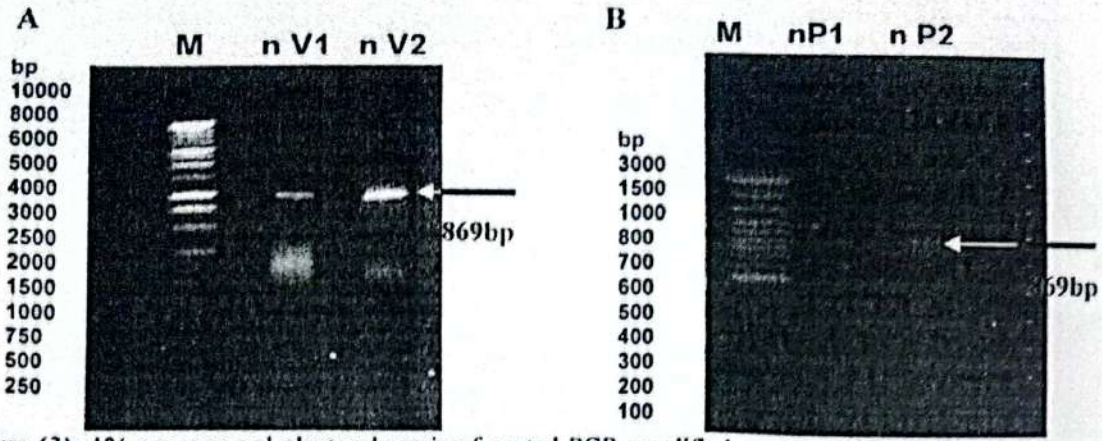

Figure (3): 1% agarose gel electrophoresis of nested-PCR amplified products of DNA -TMV genome fragments using TMVP2, TMVM1 primers. A: Lanes (nV1, nV2) amplified products of viral DNA. B: lanes (nP1, nP2) nested PCR amplified products of plant viral DNA. M: DNA molecular weight marker in base pair.

Sequence analysis of the 869bp fragment of TMV-E

The direct sequencing of the PCR amplified products (869bp) using TMVP2, TMVM1 primers resulted in sequencing of about 839 nt with specific sequence pattern (Figure 4). The sequence was aligned with full length of six different TMV-strains. The homologous sequence was started at nt 28 of TMV-E and nt 2580 of the six strains. It was extended up to 787 nt as a highly conserved sequence while, the 5' and 3' remaining sequence of TMV-E was dispersed as sequence homologous clusters through the sequence of the different TMV-strains. Table (2) shows the percentage of sequence identity among the seven TMV strains when aligned according to the DNA star program. The results imply that TMV-E was more related to TMV-152 (Chinese strain with accession number AF395129), TMV-fuj (Chinese strain with accession number AF395127), TMV-K (Korean strain with accession number X68110), TMV-ch (Chinese strain with accession number AF165190), than other two strains TMV-cg (Japanese strain with accession number D38444), and TMV-U2 (Tobacco mild green mosaic with accession number GI:335243). Sequence analysis of 869 bp fragment of TMV-E revealed that this region located at the 3'-terminus of the ORF1 (126KDa) of TMV-genome. 126KDa protein was encoded by the 5'-proximal region of the viral genome and it includes the methyltransferase and RNA helicase motifs (Kadare and Haenni, 1997). Osman and Buck (2003) also stated that the c-terminal domain of the 126KDa protein contain motif that is highly conserved in known helicases. The sequence alignment of TMV-E fragment with TMV-strains showed the apparent of pairwise sequence similarities especially from nt 2580 to nt 3419. This position is in consistent with the position of the selected primers in this study. The TMV-E fragment sequence possibly located in the helicase sequence which, explain the sequence similarity of this region, where each enzyme containing domain with several conserved sequence segments (Watanabe *et al.*, 1999). However, the presence of varying degree of this fragment sequence identity among the TMV-strains, the more investigation should be carried out as sequencing the other genes of TMV-E like coat protein that reveal more about the molecular characterization of this strain and its relationship with other known TMV published strains.

1	GTGGACGGAG	TTCCGGGCTG	TGGAAAAACT	AAAGAAAGAT	CTTAATTTTA
50	GTAACCCGGG	AAGCAAGCCG	CGGAAATGAT	CAGAAGAGTG	CGAATCCTAG
100	GGATACTTGT	GGCCACGAAG	GATAACGCTT	AAAACCGTTG	ATTCTTTATG
150	ATGAATTTTG	GGAAAAGCAC	ACGCTGTCAG	TTTCAAGAGG	TTATTCATTG
200	ATGAAGGGTT	GATGTTGCAT	ACGTGGTTGT	GTTAATTTTC	GTTGTGGCGA
250	TGTCATTGTG	CGAAATTGCA	ATATGATTTA	CGGGAAGACA	CACAGCCAGA
300	ATTCCGATAC	TATCAATAGA	GTTTTAGGAT	TCCCCGTTAC	CCCGCCCATT
350	TTTGCCAAAT	TGGAAGTTGA	CGAGGGGAAA	CACGCAGAAC	TCTTCTCCGT
400	TGGTCCAGCC	CGATGTCACA	CATTATCTGA	ACAGGAGATA	TGAGGGGCTT
450	TGTCAATGAG	CACTTCTTCG	GTAAAAAAAG	TCTGTTTCCG	CAGGAGATGG
500	TCGGCGGAGC	CGCCGTGATC	AATCCGATCT	CAAANCCCTT	GCATGGCAAG
550	ATCCCTGACT	TTTACCCAAT	CCGGATAAGA	AGCTCTGCCT	TTCAAGAGGG
600	TATTCAGATG	TTCACACTGT	GCATGAAGTG	CAAGGCGAGA	CATACTTGAT
650	GTTTCACTAG	TTAGGTTAAC	CCCTACACCG	GTCTCCATCA	TTGCCAGGGA
700	GACAGCCCAC	ATGTTTTGGT	CGCATTGTCA	AGGCACACCT	GTTCGCTCAA
750	GTACTACACT	GTTTGTTATG	GATCCTTTAG	TTAGTATCAT	TAGAGATTCA
800	GAGAAACTTA	GCTCGACATG	GTTCATGCGT	CGAGTCGA	

Figure (4): Nucleotide sequence of the amplified 869bp fragment of the TMV-Egyptian strain (TMV – E).

The nucleotide sequence data reported is specific for TMV-Egyptian strain and was submitted in the EMBL, GenBank and DDBJ nucleotide sequence Databases under the accession number AY596920.

Table (2): The percentage of Nucleotide sequence similarity of gene fragment (839 nt) of the TMV-E with those of the other world wide published TMV-strains:

TMV-strains	TMV-E	TMV-152	TMV-cg	TMV-Ch	TMV-fuj	TMV-k	TMV-U2
TMV-E		65.5	32.7	67.1	65.5	66.3	25.2
TMV-152			32.8	97.7	55.6	97.7	40.7
TMV-cg				50.5	33.9	50.5	42.3
TMV-Ch					89.1	75.0	52.9
TMV-fuj						95.3	45.2
TMV-k							53.2
TMV-U2							

Numbers denote to the percentage of identical nucleotides

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