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# Real-Time PCR of cDNA of Tomato Mosaic Virus (ToMV) RNA

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## Abstract

A protocol for successful detection of ssRNA from infected leaf tissue always yielded high quality RNA. In this project, a combination of degenerate primers was used to amplify part of the polymerase gene of the ToMV viral genome, followed by nested PCR that resulted in increased amplicon specificity and sensitivity of detection. This project allowed overcoming problems of generic detection and identification of ToMV RNA by nested Real-Time PCR involving DI-containing primers. The Real-Time PCR of the cDNA generated by reverse transcription was done using DNA Master SYBR Green I from Roche in an Eppendorf RealPlex© Real-Time PCR Thermocycler. The amplicon profile of the various treatments will be presented. Little or no amplification was detected in the negative controls. The cT values were used as a measure to test the specificity of the primer design. The non-specificity cT value was close to 27.5. All Real-Time PCR results that had stable PCR products resulted in melting temperature ™ values of close to 84.5 $^{0}$C.

**Keywords: Tomato mosaic virus, epidemiology, PCR RNA**

## 1. Introduction

Tomato Mosaic Virus (ToMV) is an important member of the *Tobamovirus* group. Viruses belonging to this group have a wide host-range infecting several chlorophyllous plant species. It is a world-wide plant pathogen known to cause infection in more than 150 types of economically important crop species, including vegetables and ornamental flowers[1]. The virus is highly infectious and is very easily transmitted mechanically. In the literature there are no known invertebrate insect vectors for transmission[8]. We purified a strain of ToMV causing severe systemic mosaic and necrosis from tomatoes *(Lycopersicon esculentum)* grown in Central United States and identified the virus as a member belonging to the *Tobamovirus* group by host range, electron microscopy, serology, and nucleic acid hybridization[5]. Typically mosaic symptoms, characteristic of ToMV infection is shown in assay host *Nicotiana clevlandii* (Figure 1) and necrotic local lesions *Nicotiana tabacum* cvs *Xanthi* (Figure 2) when mechanically inoculated with ToMV. ToMV which belongs to Subgroup I also includes other viruses such as Tobacco mosaic virus (TMV), Pepper mild mottle virus (PPMV), Paprika mild mottle virus (PaMMV) and Odontoglossum ring spot virus (ORSV), which mainly infects solanaceous and orchid plants[8]. The other viruses like Cucumber green fruit mottle virus (CGMMV) and Sun-hemp mosaic virus (SHMV) which infect cucurbits and legumes belong to Subgroup II[8]. However, these classifications are based on the natural host these viruses infect, the positions of the origin of virus assembly, and viral gene specificity.

Immunological assays and nucleic acid hybridization techniques are one of the many conventional methods in identifying these viruses[4]. Most recently, generic PCR methods capable of amplifying specific regions of a virus gene from diverse species has been developed.

Figure1. Typical mosaic symptoms of *Nicotiana clevlandii* mechanically inoculated with ToMV

Figure 2. Necrotic local lesions in *Nicotiana tabacum* cvs *Xanthi* when mechanically inoculated with ToMV

Attempts will be made to test specific nucleic acid signatures that which can be used to discriminate between determined strains of the same virus. We report results from spot nested Real-Time PCR-detection method of a strain of ToMV. This method involves a one-step Real-Time PCR using a combination of several degenerate deoxyinosine (dI)-substituted primers that amplified part of the polymerase region of the putative viral RNA, followed by a nested PCR.

## 2. Methodology

Total RNA from infected leaf tissue was purified as described previously (Ambion). The RT-PCR Analysis Tomato Mosaic Virus (ToMV) was done using a one-step-RT-PCR protocol, in which the combination of degenerate deoxyinosine (dI)-substituted primers were designed to from a previously published report to amplify part of the polymerase region of the ToMV viral RNA genome. This was followed by a nested PCR amplification step that would increase specificity and sensitivity of detection. The highly degenerate primers (Tobamo 1 and Tobamo 2) were designed from conserved motifs of the RdRp genes of tobamoviruses[2]. All primers were synthesized by Integrated DNA technologies and Invitrogen.

### 2.1 Reverse Transcription PCR

Reverse transcription (RT) PCR of the purified ssRNA from ToMV was done using the Ambion Retroscript© Kit with few modifications. Approximately 8.0 µg of the ssRNA or ToMV were denatured at 97°C for 90 seconds in the presence of 1% DMSO and degenerate primers (see below) were used. After denaturation, the tubes were immediately put on ice, allowed to equilibrate for one minute, and then were spun briefly. To the denatured nucleic acid, the rest of the RT-PCR components from the kit were added, including:

2µL 10X RT Buffer,
4 µL dNTP mix,
1 µL RNase Inhibitor,
1 µL of MMLV-RT Reverse Transcriptase to a final volume of 20µL.

The reaction was mixed gently by pipetting, spun briefly, and placed in a Bio-Rad MyCycler Thermocycler programmed for the following: 44°C for 1 hr., followed by 92°C for 10 min. The newly generated cDNA-RNA hybrids were then either stored at -20°C or used immediately for the 2nd round PCR.

### 2.2 Conventional 2nd Round PCR of cDNA of ToMV ssRNA

Conventional PCR of the cDNA from the RT-PCR was conducted using Ambion SuperTaq™ Plus DNA Polymerase.

The reaction was set up as follows:

5µL of RT-PCR reaction,
5µL of 10X PCR Buffer (100 *mM* Tris-HCl pH 9.0, 500 *mM* KOAc, 15 *mM* MgSO4),
2.5µL dNTP mix (2.5 *mM* each dATP, dCTP, dGTP, dTTP),
33.5µL of nuclease- free water,

5 *µM* of primers (TOBRTUP1 (GAGTACGCIGCITICAGAC, where I denotes deoxyinosine), and TOBRTD02 (CGCTTCAAAGTTCCA) for ToMV ssRNA; and 1 U of SuperTaq DNA polymerase.

The reactions were placed in a Bio-Rad MyCycler programmed for the following:

Denaturation at 94°C for 4 minutes, 30 cycles of denaturation (94°C for 30 seconds), annealing (46°C for 30 seconds), and extension (72°C for 4 minutes), followed by a final extension of 72°C for 10 minutes.

The PCR products were analyzed by 1% agarose- gel electrophoresis in 1X TAE buffer. Agarose-gels were run at 37 V/cm.

The PCR product was purified from the PCR tube with GeneJET™ PCR Purification kit (Fermentas). The purified product was then cloned into pDrive cloning vector (Qiagen) and transformed into *E. coli* competent cells. Next, the cloned plasmids were purified with GeneJET™ Plasmid Miniprep kit (Fermentas)[3,6]. All procedures followed the manufacturer's instructions. The purified plasmids were cut with Fermentas FastDigest® EcoRI and analyzed on a 1% agarose gel stained with ethidium bromide[7]. After screening the correct orientation of the cloned inserts, the plasmids were sequenced using M13 forward primer and M13 reverse primer at Retrogen, Inc., USA.

## 2.3 DNA Sequencing and Alignment

A positive clone was picked and sent for sequencing by Retrogen, Inc. The sequence was BLAST searched in the NCBI database. The complete sequence was formed by manual defining the overlap common sequence between the forward and reverses scanned sequence through Microsoft word "Find" program. First, the reverse complementary sequence of forward sequence was determined by using the Reverse Complement - Bioinformatics.org server (http://www.bioinformatics.org/sms/rev_comp.html). Then copy the last row of ~ 50 nucleotide sequences and paste on the "Find" program to find out the overlap sequence, repeated until the whole overlapping sequence has been identified. Multiple alignments of complete formed sequences were carried out using both UniProt - Sequence alignments and Clustal Omega.

## 2.4 Design of Primer

The final sequence was used to design the primer through Primer 3 Input version 0.4.0 (http://frodo.wi.mit.edu/). The chosen forward and reverse primers sequences were sent to Biosearch Technologies (http://www.biosearchtech.com/) to be synthesized.

## 2.5 Real-Time PCR Analysis of ToMV ssRNA Cloned Plasmid

Real-time PCR of the cDNA from the RT-PCR reaction was done using DNA Master SYBR Green I from Roche in an Eppendorf RealPlex© Real Time PCR Thermocycler. The reactions were set up as follows:

13.6µL of nuclease- free water, 0.4µL of MgCl2 stock (25 *mM*), 5 *µM* each of the primersTOBRTD02 and TOBRTUP1 (for ToMV); 2µL of the SYBR Green Master Mix (already containing *Taq* DNA polymerase, reaction buffer, dNTP mix, and SYBR Green dye), and 2µL of template from the previous RT-PCR reaction.

The reactions were placed in the thermocycler programmed for the following: initial denaturation at 94°C for 3 minutes, 40 cycles of denaturation (94°C for 15 seconds), annealing (46°C for 15 seconds), and extension (72°C for 1 minute), followed by a final extension at 72°C for 5 minutes. Fluorescence readings were taken at the extension phase of each cycle, and the samples were ultimately subjected to a melting curve analysis.

## 3. Results

The results from viral and total RNA are shown in figure 3 lanes 1-4.


Figure 3. Lane 1: purified viral RNA; lane 2: total crude RNA from ToMV infected leaf; lanes 3 and 4 represent total RNA from non-inoculated tobacco leaf

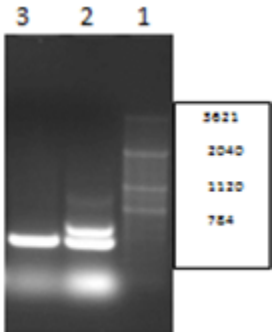



Figure 4. ToMV PCR products of various RNA samples; lane 1: marker; lanes 2 and 3 represent PCR products from the RNA from infected leaf tissue and purified ToMV viral ssRNA

Real-Time PCR of total RNA from infected leaf tissue was completed. RNA was extracted from infected leaf tissue and purified (Figure 4). Additionally, RT-PCR of purified RNA was done with Retroscript© Kit with some modifications to generate cDNA-RNA hybrids of ToMV. Conventional PCR of the cDNA was conducted using SuperTaq™ DNA polymerase (Figure 5).

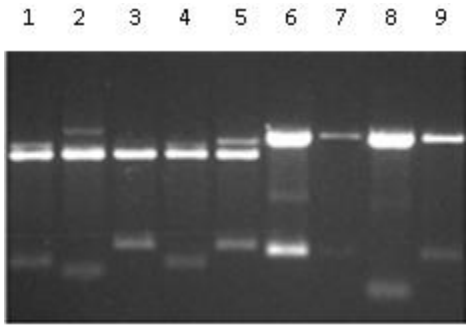
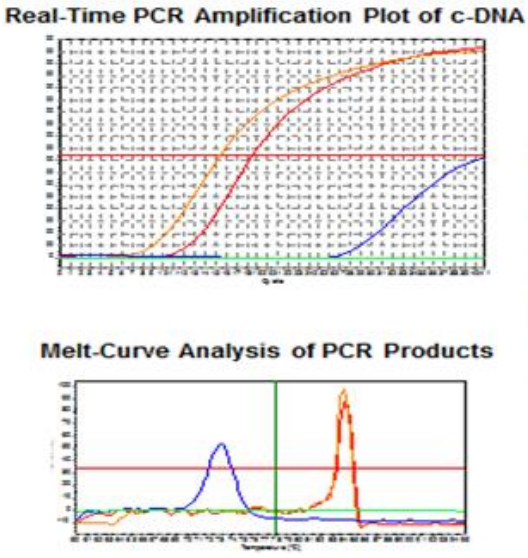



Figure 5. Gel picture of plasmid DNA stained with ethidium bromide that were fast digested with EcoRI DNA analysis of cloned fragments of ToMV ssRNA, following enzyme digests

Table1. sizes of the cloned fragments of ToMV from fast EcoRI digests

| TOMV Plasmid | TOMV Plasmid | TOMV Plasmid | TOMV Plasmid | TOMV Plasmid | TOMV Plasmid | TOMV Plasmid | TOMV Plasmid | TOMV Plasmid |
|---|---|---|---|---|---|---|---|---|
| Lane 1 | Lane 2 | Lane 3 | Lane 4 | Lane 5 | Lane 6 | Lane 7 | Lane 8 | Lane 9 |
| MW (bp) | MW (bp) | MW (bp) | MW (bp) | MW (bp) | MW (bp) | MW (bp) | MW (bp) | MW (bp) |
| 8436.1 | 4314.5 | 3289.8 | 3309.1 | 3655 | 3683.7 | 4022 | 3622.3 | 3837.5 |
| 3251.2 | 3270.5 | 877.9 | 1003.1 | 3289.8 | 1913.7 | 843.9 | 359.2 | 756.5 |
| 955.1 | 574.4 | | 668.2 | 866.8 | 843.9 | | | |
| 579.2 | | | | | | | | |
| | | | | | | | | |

Gel electrophoresis was used to analyze the plasmid DNA of the cloned ToMV PCR products (Figure 6). Specific sizes of the cloned fragments were determined (Table 1).




| Name | Ct value RT-PCR | Tm Value PCR Products |
|---|---|---|
| Primers with SYBR (no ToMV template) | 32.7 | 73.6 |
| ToMV Infected UP3 and DO4 | 6.97 | 84.2 |
| Tomv Infected UP3 and DO4 II | 6.21 | 84.0 |
| Water with SYBR | 0 | 0 |

Figure 7. Real-Time PCR of ToMV with cDNA, amplification, melt-curve analysis of PCR products, and $C_t$ and Tm values

When specific primers were used for Real-Time PCR there was amplification for both ToMV infected samples, and no amplification for the negative control.

## 4. Discussion

ToMV RNA was consistently detected by gel electrophoresis (Figure 3) and by Real-Time PCR of Total RNA from infected tissues using degenerate primers, Table 1 and Figure 4 (lanes 2 and 3). Approximately 0.01 µg of the total RNA from the infected tissue was consistently amplified by degenerate primers. The PCR products thus generated were cloned into a pDrive cloning vector and all such colonies were analyzed by fast digest followed by gel electrophoresis (Figure 5, lanes 1-9)[7]. The sizes of the cloned fragments are shown in Table 2. The PCR sequencing of the cloned fragments was done by Retrogen, and PCR primers were designed for the specific ToMV isolates used in the study. Primers for the isolates used in the study were designed by IDT-Technology and subsequently used for all Real-Time PCR analysis. The primers were very specific to the ToMV RNA isolate with

little or no amplification to ToMV non-infected tobacco tissue (Figure 7). The positive ToMV RNA had PCR products that had Tm Values that ranged between 84 and 84.2, whereas primers with SYBR Green with no template had a Tm Value of 73.6 (Figure 7). The $C_t$ values of ToMV infected samples ranged between 6.21 and 6.97, whereas the non-infected sample had a $C_t$ value of 32.7. The $C_t$ value is a reflection of the overall performance of the reaction system. The efficiency of the PCR reaction can affect the $C_t$ values. Generally, the efficiency of our reaction ranged between 90 and 110 percent (data not shown). The specificity and precision of the BHQ probe is currently being tested for sensitivity of detection. With this strategy we hope to effectively improve detection technologies for diagnosis, characterization of known and unidentified *Tobamovirus* species, and shed light on the detection method to examine epidemiology of virus disease in crop plants and its distribution in the Allegheny forests of western Pennsylvania.

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