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Molecular Identification of ToMV Isolates Distributed in Uzbekistan and Study of Some Biological Properties

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Abstract The vegetables consumed by humans, tomato products occupy a special place among vegetables rich in minerals and vitamins. Viral diseases are the main limiting factor in the cultivation of tomatoes and other cultivated plants. Tomato mosaic virus, a member of the genus *Tobamovirus* of the family *Virgaviridae*, is the main and most common pathogen of tomato, whose virions are rod-shaped, harboring positive single-stranded RNA. In this study, ToMV was molecularly identified by QT-PCR method, and disease symptoms of ToMV in indicator plants and their appearance periods were determined.

Keywords *Tobamovirus*, ToMV, RNA, QT-PCR, Tomato, Mosaic, Necrosis, Nicotiana tabacum, *N. glutinosa* L., *Solanaceae, D. stramonium* L., *C. annum* L., *Ch. quinoa* L., *Ch. amaranticolor* L., *Ch. album* L.

1. Introduction

Among the vegetables consumed by humans, tomato products occupy a special place among vegetables rich in minerals and vitamins [1,2]. According to FAO (2009), most of the tomato crops are China, 45 million per year. tons (920.8 thousand ha), USA - 14.14 mln. tons (175.44 thousand ha), Turkey - 10.75 mln. tons (32.46 thousand ha), Ukraine and the south of Russia (from 35-36%), Moldavia (8.4%) and Azerbaijan (5%) from the CIS countries [2].

Most of the crops grown as food, fodder, technical and decorative plants around the world are infected with phytopathogenic viruses, which cause significant yield losses and significantly deteriorate the quality of agricultural products. Almost half of the new diseases detected in plants in the last decade are of viral nature [3].

Viral diseases are the main limiting factor in the cultivation of tomato and other cultivated plants [4,5]. Virus diseases of the tomato (*Lycopersicum esculentum* Mill.) plant are one of the main factors that reduce its yield, as a result of infection with viruses, a sharp decrease in yield, as well as a decrease in tomato quality, shelf life, transportability and other properties have been determined [6].

Tomato mosaic virus, a member of the genus *Tobamovirus* of the *Virgaviridae* family, is the main and most widespread pathogen of tomato, with a rod-shaped, positive single-stranded RNA virion [7]. The ToMV genome, like the genome of other tobamoviruses, consists of four protein-expressing genes, ORF 1 (130 kDa) and ORF 2 (180 kDa) genes are responsible for the synthesis of the replicase, ORF 3 (30 kDa) for the movement protein, and ORF 4 (18 kDa) for the synthesis of the protein coat [8,9,10].

ToMV is spread mechanically, through pollen and seeds, or by various virus-carrying vectors (plant aphids, aphids, whiteflies) [11,12]. ToMV causes a number of disease symptoms in plants: mosaic, yellow spotting, curling of leaves, shrinking of leaves, internal necrosis of fruits, etc. [11,13]. Because of ToMV's biological and serological affinity with TMV, Tomato mosaic virus has been considered the "Tomato strain" of Tobacco mosaic virus [13]. *Tobamovirus*, the so-called "tomato strain" of TMV, with approximately 80% nucleotide sequence similarity to TMV, has been shown to be actually Tomato mosaic virus [14].

Based on this, the main goal of this study was to identify ToMV isolates distributed in Uzbekistan based on the replicase gene and to study some of their biological properties.

2. Materials and Methods

Virus transmission by mechanical inoculation method. Mechanical transmission of viruses was performed according to generally accepted methods [15]. For this, 100 g of the initially infected plant. and added 0.1 M phosphate

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buffer (pH 7.2) in a ratio of 1:1 and ground it in a porcelain mortar [16]. The liquid part of the homogenate, which has reached the same mass, is rotated for 15 minutes at 7000-8000 rpm. spun in a centrifuge at 1000 rpm, the supernatant was separated. The isolated supernatant was dusted with carborundum (400 mesh) powder on the leaves of the plant intended for infection, and the virus was introduced into the leaf cells by mechanical microinjury by rubbing the inoculum [17]. After one hour, excess carborundum powder and plant sap were washed off with distilled water. The infected plant was kept in the shade for a day and then brought out into the light. From 2-3 days to 15-20 days, depending on the type of plant, the development of virus-specific disease symptoms was visually monitored from the moment of mechanical transmission of the virus [15,17].

Molecular identification of ToMV. The genome of most plant viruses consists of 1-stranded RNA, and their diagnosis by QT-PCR method is carried out in the following steps.

Isolation of total RNA. Isolation of total RNA from virus-infected samples was carried out using "PURELINK RNA MINI KIT" kits from "Invitrogen" (ThermoFisher, USA) according to the attached instructions. To do this, samples of tomato plants with ToMV disease symptoms were collected and extracted by placing 5 g in a porcelain mortar and adding liquid nitrogen. 200 mg of the prepared extract was taken, placed in a 2 ml Eppendorf test tube and thoroughly mixed in a vortex (Vortex MX-S, DLAB) after adding 1.5 ml of the lysis buffer (prepared in advance by adding 10 µl of 2-mercaptoethanol to 1 ml of the lysis buffer). Then, it was incubated at room temperature for 3 minutes and spun in a centrifuge for 5 minutes (2600xg rpm). The supernatant was separated and placed in a new test tube. 96% ethanol was added at a ratio of 1:1.5 to the volume of the supernatant and vortexed. It is poured into a filter tube included in the kit, then centrifuged for 15 seconds (12000xg rpm), the precipitate is discarded. 700 µl of washing buffer (Wash buffer I) is added and centrifuged again for 15 seconds (12000xg rpm). The bottom of the column tube was discarded and replaced with a new tube. Then 500 µl of the second wash buffer (Wash buffer II) was added and centrifuged for 15 seconds (12000xg rpm). The filter part of the test tube is removed and placed in a new sterile test tube. 100 µl of RNAse-free sterile water (RNase-Free water) was slowly added from the central part of the test tube and incubated at room temperature for 2 minutes. After the specified time, it was centrifuged for 2 minutes (12000xg rpm) and the filter part of the tube was removed. In this way, total RNA was extracted in the lower tube.

Preparation of cDNA by reverse transcription. Invitrogen (ThermoFisher, USA) reagent kit was used for cDNA synthesis based on the isolated RNA matrix. For this, a reaction mixture was prepared by adding ddH2O (2 µl), 5 µl of isolated RNA, reverse primer (GGTGGTGTGCCTCTCTGT) (Integrated DNA Technologies, Belgium) with a concentration of 10 pM/µl and at 70℃ for 2 minutes. was incubated. At the end of the incubation time, the mixture was quickly transferred to an ice-cold container. The reaction mixture for obtaining cDNA from ToMV RNA by reverse transcription method includes: reaction buffer-8 µl, dNTP-2 µl, revertase-1 µl, RNA 9 µl. The prepared reaction mixture was placed in the amplifier at 37℃ for 60 minutes and 70℃ for 10 minutes [14]. Diagnosis of ToMV by QT-PCR method. A reaction mixture (25 µl) for amplification of the synthesized cDNA was prepared as follows: primers selected at 10 pM/µl each based on the NCBI database

ToMV-Forward primer-GAGCYTTCACTGAAAGATG (0.5 mkl)

ToMV-Reverse primer-GGTGGTGTGCCTCTCTGT (0.5 mkl)

Master mix 7.5 µl, ddH2O 12.5 µl, synthesized cDNA 4 µl. PCR amplification was programmed as follows (Table 1) [18]:

Table 1. Temperature-time mode of amplification according to M. Chikh-Ali [18]

Name of stage	Temperature	Time	Duration
Basic denaturation	94 °C	2 minutes	1 cycle
Denaturation	94 °C	30 sec.	45 cycle
Location of the primer to the DNA (annealing)	56 °C	30 sec.	
Elongation	72 °C	45 sec.	
Finally elongation	72 °C	2 minutes	1 cycle

PCR products were detected by horizontal gel electrophoresis (Helikon, Russia) on a 2% agarose gel prepared in 1x TBE buffer (ThermoFisher, USA), using ethidium bromide (3 µl). 3 µl of dye (BlueJuice Gel Loading Buffer, ThermoFisher, USA) was added to 10 µl of PCR product and placed in an agarose well. A mixture of DNA molecules of known size (GeneRuler Plus 100 bp DNA ladder) was used as a marker. Electrophoresis was performed at a current of 80 mA and a voltage of 220 W. The gel was analyzed on a transiluminador Kvant 312B (Helikon, Russia) at a wavelength of 312 nm and imaged using a gel recording system "Vzglyad" (Helikon, RF). The list of primers, the conditions for determining the temperature-time mode of PCR amplification were made based on literature analysis [14,18].

3. Results and Discussion

Samples of tomato grown in Andijan, Samarkand and Tashkent regions of Uzbekistan were collected from different varieties and hybrids with mosaic, deformations, virus-like streaks, spots, hardening of the fruit, change of the growth cone of the infected plant, and a number of minor disease symptoms. (Figure 1).

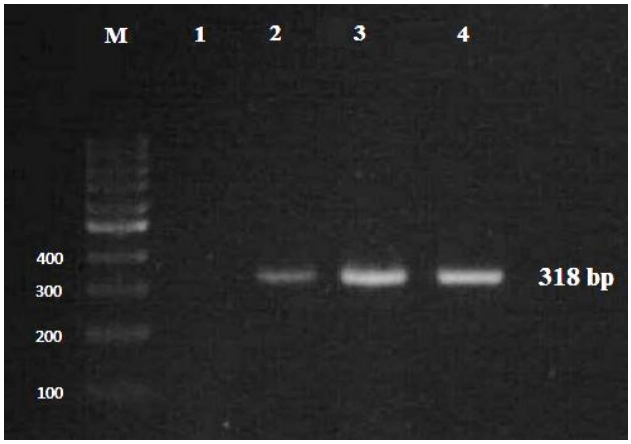
1-intervascular mosaic; 2-necrosis of the leaf; 3-leaf roughening and mosaic; 4-fruit spotting and "stone-like" hardening; 5- spotting of tomato fruits

Figure 1. Typical signs of viral diseases in tomato plants

ToMV detection was performed using the QT-PCR method. Specific primers were selected for QT-PCR. To do this, ToMV primer sequences were designed based on ToMV genome sequences included in the NCBI database. Universal nucleotides with ToMV-specific sequences were identified using alignment software, and primers were designed using Primer-3 software [19]. Primer sequence specificity was checked online using the Basic Local Alignment Search Tool provided by the NCBI database [20].

Forward primer: 5-CGAGAGGGGCAACAAACAT-3 corresponds to the sequence of nucleotides 527-546 of the genome; and the *reverse* primer: 5-ACCTGTCTCCATCTCTTTGG-3 was chosen to correspond to the sequence of nucleotides 825-843 of the genome. The PCR product, representing a part of the gene responsible for replicase synthesis, is equivalent to a sequence of 318 pairs of nucleotides [14]. During the monitoring, total RNA was isolated from the samples collected in laboratory conditions as described in the materials and methods section, and cDNA was synthesized from the RNA based on the established protocol, and QT-PCR was performed. Using specific primers separately, primers selected for the ToMV specific replicase gene amplified a 318 bp segment of the genome in tomato samples (Figure 2).

The presence of ToMV was proved by the QT-PCR method in the samples taken from the varieties "Yusupov", "Sultan", "Volgograd" with symptoms of the virus.



M-1000 bp DNA ladder Plus. 1-control sample without cDNA, 2-from "Yusupov" variety of tomato; 3 - "Sultan" tomato variety; 4- Tomato "Volgograd" variety

Figure 2. Diagnosis of ToMV using QT-PCR method

To determine the disease symptoms caused by ToMV in indicator plants, a number of indicator plants grown in specially isolated experimental rooms were mechanically infected with plant sap isolated from ToMV-bearing tomato plants under laboratory conditions and monitored until the appearance of disease symptoms [15,17].

ToMV causes 1-2 mm red necroses on the surface of the leaf in *Chenopodium amaranticolor* L., *Ch. quiena* L. and *Ch. album* L. caused yellow chlorotic necrosis of different sizes in L. species (Figure 3).

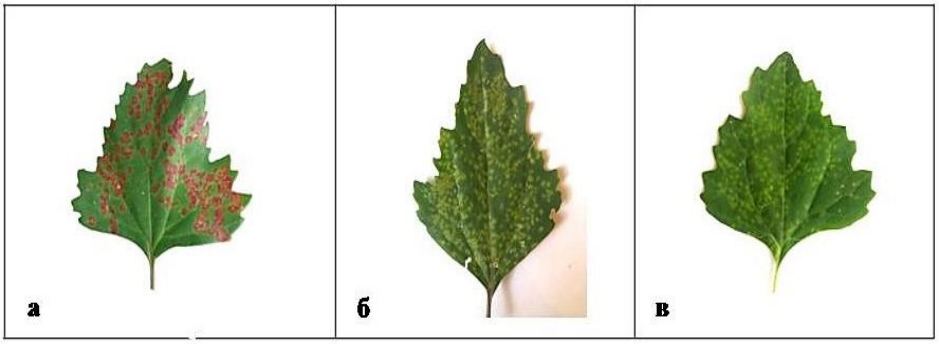
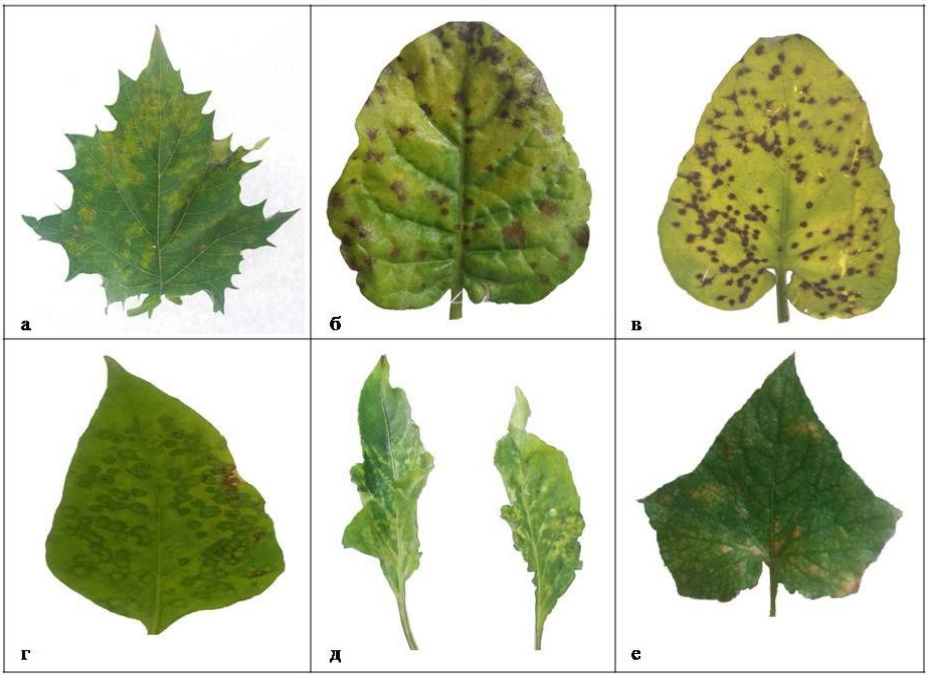


Figure 3. Disease symptoms of ToMV on leaves of *Ch. amaranticolor* (a), *Ch. quinoa* (b) and *Ch. album* (v) plants

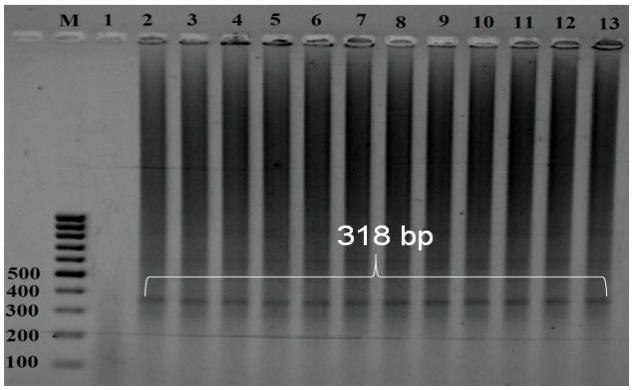


a-Necrosis on the leaf of *D.stramonium* L.; b-Necrosis in *Nicotiana tabacum* L. Barley variety; v- necrosis that appeared in *N. glutinosa* L. plant; g- Necrosis that appeared in *C. annum* L. plant; d- Deformed mosaic leaf of *Solanum melongena* L. plant; e- Disease symptom of ToMV in cucumber

Figure 4. Disease symptoms of ToMV in indicator plants

Small black necrosis appeared on the leaf surface 4 days after mechanical inoculation of ToMV *Datura stramonium* L., and systemic mosaic symptoms were observed by 6-7 days after inoculation (Fig. 4, a) in Samsun and Barley cultivars of *Nicotiana tabacum* L. in 3 days large 3-4 mm necroses and after 4-5 days a systemic mosaic symptom, it was found that necrosis was formed in 2 days in the leaf of *N.glutinosa* L. (Fig. 4, b, c). Total vegetation of the plant as a result of the appearance of mosaic symptoms after 5-7 days in *Capsicum annum* L. (bell pepper) (Fig. 4, g), *Solanum melongena* L. (eggplant) (Fig. 4, d) and *Cucumis sativus* (cucumber) plants it was observed that it was retarded compared to a healthy plant (Fig. 4, e).

QT-PCR was performed to prove that the disease symptoms on the indicator plants were specific to ToMV (Figure 5).



M-1000 bp DNA ladder Plus. 1-control (healthy tomato); 2-*Ch. quinoa;*3- *Ch.album*; 4-*D. stramonium*; 5- *N. tabacum* Burley variety; 6-*N. glutinosa*; 7- *C. annum*; 8- *Solanum melongena*; 9-*Cucumis sativus; 10- Capsicum frutescens;* 11- *N. tabacum* Samsun variety; 12- *Ch. amaranticolor*; 13- "TMK" variety of tomato

Figure 5. QT-PCR analysis of ToMV-infected indicator plants

In order to reduce damage caused by viruses and develop control strategies, it is important to first identify viruses in the cultivated plant [21]. Evidence of symptomological and biological studies conducted in the detection of viruses using molecular genetic methods increases the level of reliability of the results. For example, in Iran, the high prevalence of ToMV virus, which was not recorded by classical methods until 2014 in cabbage, was detected by QT-PCR method, due to the exchange of infected material between *solanaceous* and *brassicas* cultivation [22]. Although indicator plants and IFA methods are convenient methods for virus detection and analysis, RT-PCR is increasingly used for virus detection and identification due to its high sensitivity [23]. Among the molecular methods, RT-PCR is the most widely used method [24].

Mosaic, deformations, virus-like stripes on fruits, spots, hardening of fruits, changes in the growth cone of the infected plant, and a number of disease symptoms observed in the studies conducted in the tomato fields corresponded to the disease symptoms reported in the scientific works of other researchers [14,25]. Disease symptoms in mechanically infected indicator plants and their QT-PCR results indicate the presence of ToMV, consistent with previous research findings. The indicator plant method used to study the biological properties of ToMV is used for the biological identification and differentiation of strains of plant viruses [26,27].

In this study, the disease symptoms observed in indicator plants were confirmed by QT-PCR method to be specific to ToMV. The results of the study were carried out using ToMV-specific remer pairs [14], in accordance with the study by Kumar et al. All indicator plants mechanically infected with ToMV were found to contain 318 bp of the genome in all QT-PCR results. amplified segment.

4. Conclusions

The presence of ToMV in tomato fields grown in Andijan, Samarkand and Tashkent regions of Uzbekistan was determined using biological and molecular methods. By mechanically infecting ToMV to indicator plants, ToMV infected plants such as *D. stramonium* L., *C.annum* L., *N.tabacum* L., *N.glutinosa* L. from representatives of the *So*lanaceae family, and a number of representatives of the *Chenopodiaceae* family: It was found to cause necroses and chlorotic spots of different sizes in *Ch. quinoa* L., Ch. amaranticolor L., *Ch.album* L. species. This study was the first molecular genetic identification of ToMV in Uzbekistan.

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