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Detection, Identification and Molecular Characterization of Tomato Mosaic Virus (ToMV) Isolates from Pakistan

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ABSTRACT

Tomato mosaic virus (ToMV) is one of the lethal tomato pathogens. In this study, we collected tomato leaves exhibiting interveinal chlorosis from farmer's fields in district Lahore, Pakistan. The presence of tobamoviruses was confirmed through extracting RNA, cDNA synthesis, PCR amplification and subsequent sequencing of capsid protein (CP) gene of ToMV that contained 480 nucleotides (encoded for 160 amino acids making ~17 kDa protein) located at 5703rd to 6182nd nucleotide position in ToMV genome. In sequence analysis, Pakistani isolates shared 98.9-99.5% and 97.3-99.3% nucleotide and amino acid homologies with each other and 96-99% and 90-98.6% with other isolates respectively. In phylogenetic analysis the Pakistani isolates grouped with Iranian and Chines isolates, exhibiting higher nucleotides and amino acids identities as well. This research reveals a geographical association among Pakistani, Chines and Iranian ToMV isolates. The study suggests extensive surveys of tobamoviruses infecting field crops for their detailed genetic information.

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INTRODUCTION

Tomato is a vital of our daily diet and because of its utmost utility, it is broadly demanded around the globe. Above 136 viruses infect tomatoes worldwide. In Pakistan reported tomato infecting viruses are *Tomato spotted wilt virus* (TSWV) (Ashfaq and Ahmad, 2018), *Potato virus X* (PVX), *Tomato leaf curl virus* (TLCV), *Cucumber mosaic virus* (CMV) and *Tomato mosaic virus* (ToMV) (Riaz *et al.,* 2021). Tomato mosaic virus is a tobamovirus that belongs to family *Virgaviridae*. The virgaviruses are plant infecting, rod shape, positive-sense single stranded RNA (+ssRNA) viruses. *Tobamovirus* is the largest genus of family *Virgaviridae* (Gibbs *et al.,* 2015). Tobamoviruses are mechanically and/or seed transmitted monopartite, unsegmented particles with approximately 300 nm length and 18 nm radius. These viruses own 6.3-6.6 kb genomic RNA with four open reading frames (ORFs). ORF1 and ORF2 both are involved in RNA replication and encode the translational read through product of 130K protein (130K and 180K protein) of the genomic RNA from the 5' proximal open reading frame. ORF3 encodes the 30K movement protein, and the ORF4 carries information for the synthesis of 17.8 kDa capsid protein that is considered as crucial for disease spread and symptom development (Hanssen *et al.,* 2010; Ishibashi and Ishikawa, 2013).

Tomato mosaic virus (ToMV) is prevalent in all over the Pakistan (Huaasin *et al.,* 2022) and possessing the

capability to infect the host plants in tropical as well as temperate climates. ToMV infected plants exhibit the characteristics symptoms of corky ring, light and dark green patches of mosaic and mottling, yellow streaks, curling and deformation of leaves. If the infection occurs in the early stages of plants yellowing and stunting occurs in the whole plant. Fruit ripening occurs unevenly and are reduced in size having necrotic spots hence corky fruit development occurs (Ullah *et al.,* 2017).

Basic requirements for the development of long-lasting management approaches like virus resistant varieties are dependent on correct identification of these viruses. For the classification of various viral variants like species and strains, currently the molecular characterization is the tool that is most broadly used worldwide. The molecular information related to major viruses found in Pakistan is unknown and deficient for the development of appropriate management approaches. This study was focused to characterize the CP gene of Pakistani isolates of ToMV.

MATERIALS AND METHODS

Collection of Samples and Bioassay

In 2019, leaf samples of tomato plants showing foliar chlorosis especially in interveinal areas were sampled in a randomized way from farmer's fields from district Lahore using stratified sampling method stored in ice in plastic bags and brought to molecular plant pathology laboratory FA&ES IUB. Sterilized and distilled water was used to remove dirt and cleaning of samples. The samples were kept at 4^{o}C for further processing. The ToMV isolates were biologically purified by mechanically inoculating ToMV infected leaf sap (Homogenized in 0.1 M phosphate buffer) on *Chenopodium quinoa* leaves (at 5-6 leaf stage), pre-dusted with 600 mesh carborandom. Process was repeated to three consecutive transfers. Plants maintained in glasshouse at 25^{o}C and ~70% RH were observed for local lesions and/or symptoms after 1-2 weeks.

Molecular Characterization

The copies of capsid protein gene of *Tomato mosaic virus* (ToMV) were synthesized by using a ToMV-specific primer pair, developed in this study; ToMVCPF: TTCTCCCGTCGATGTTCACG and ToMVCPR: CAGGTGCAGAGGTCCAGAC. The direct sequencing of PCR amplified and purified *Tomato mosaic virus* (ToMV) isolates gave full sequence of capsid protein gene. The sequences sent by sequencing company were translated to amino acid sequences and both forms of sequences were compared with *Tomato mosaic virus* (ToMV) isolates by using BLAST, RDP4, MEGA software and tools of bioinformatics. Additionally, submission of all sequences to GenBank was done after careful analysis.

Total plant RNA Separation

The RNA content was separated from 0.1g of collected tomato leaf samples by Sigma Tri-Reagent Kit as per protocol given by manufacturer. Leaf powder was obtained by crushing the leaves by sterilized pestle mortar in liquid nitrogen and poured into DNase and RNase free microcentrifuge tubes of 2 mL capacity. One mL of trizole reagent was added into each sample and mixed well by vertexing each tube and incubated for few minutes at 25ºC. Then, after adding the 200 µL of chloroform tubes were vortexed for 15 seconds to mix well and kept at 25 ºC for 5 minutes. A ''g'' value of 12,000 was given to each sample at 4 ºC for 15 minutes, which separated the RNA in upper most aqueous phase. This aqueous phase was shifted into new DNase and RNase free microcentrifuge tubes of 2 mL capacity. Later, 500 µL of isopropanol precipitated the collected RNA which was palletized by giving a g value of 12,000 at 4 ºC for about 10 minutes. The collected pallet was exposed to 1 mL of very cold 75% ethanol to wash it off the remaining impurities and again palletized at g = 12,000 for 5 min. Each pallet was air dried at 25 ºC for 5 min. RNA suspension was prepared by mixing through gentle pipetting the collected pallet into 50-100 µL DNase and RNase free water. For further support to suspension process tubes were heated to 60 ºC for 15 minutes. For future use, the separated RNA was kept at -80 ºC.

cDNA Synthesis and RCR

The complementary DNA of *Tomato mosaic virus* genomic RNA was synthesized by thermoscientific revert aid kit containing reverse transcriptase enzyme and random hexamer reverse primers. A mixture comprising 5 µL of RNA template, 6 µL of water and I uL of 20 pM down primer was heated at 65 ºC for 5 minutes. After that a quick cold-shock was given to the samples by shifting them in ice. Afterwards, 1 µL, 4 µL, 1 µL and 1 µL of RNase-inhibitor, 5X reaction buffer, dNTPs 10 Mm and enzyme RT were added respectively and incubated at 42 ºC for 60 minutes. The reaction was terminated at 70 ºC for 10 minutes.

The capsid protein (CP) gene amplification of *Tomato mosaic virus* (ToMV) was done by polymerase chain reaction in a PCR reaction mixture of 50 uL which contained 10X PCR buffer (5 µL), cDNA template (4 µL), 25mM MgCl2 (3 µL), 10 Mm dNTPs (1 µL), water free of

nuclease (35 µL), 1 µL of each 20 pM Up and down primers and Taq polymerase; 500 units. The reaction was carried out at following temperatures; 5 minutes of 95°C, 35 cycles of 94°C, 58°C and 72°C for 60 seconds each, and in the end 72°C for 10 min. The amplified PCR products were separated on 1% agarose gel and observed under UV light.

Sequence Analysis

By using MEGA 6 software, sequences of upper and lower strands were aligned to observe the sequencing accuracy. Necessary corrections in misread sequences were made by keen observation of upper and lower strand sequencing peaks. Poor sequences from 3' and 5' ends were trimmed off and a clear sequence of each amplicon was taken. These sequences were checked for protein formation by using an online tool for translation; ExPASy (https://web.expasy.org/translate/) (Gasteiger *et al.,* 2003). The sequences of nucleotides and translated amino acids were used for BLAST analysis and the isolates showing maximum and minimum sequence identities were downloaded from NCBI Genbank (Daugelaite *et al.,* 2013). The nucleotide sequences of Pakistani isolates and those downloaded from GenBank were subjected to alignment by using ClustalX sequence alignment tool in MEGA 6 (Tamura *et al.,* 2013).

Phylogenetic Analysis

Analysis of phylogeny of aligned sequences were performed by using MEGA 6 software (Tamura *et al.,* 2013). The phylogenetic trees were constructed by using neighbor-joining method. In numerous parsimonious rooted trees, the 1000 replications of bootstraps were used to place the confidence estimation on groups.

Analysis of Recombination

In order to detect the probable recombination in nucleotide sequences of *Tomato mosaic virus* (ToMV), the RDP package was used (Martin *et al.,* 2010). The Chimaera, Bootscan, RDP, 3SEQ, Lard, MaxChi, Siscan and GENECONV were used by following the corrected Bonferroni cut-off P-value (a = 0.05) and default settings. Events of recombination were taken as true if their detection was confirmed by more than four of the above-mentioned methods.

RESULTS

In course of sample collection and field surveys the remarked symptoms concerning with ToMV were foliar chlorosis especially in interveinal areas of leaves.

PCR Amplification and Sequence Analysis

The PCR using designed primers amplified the fragments of DNA of ~600 base pairs (Figure 1). From each amplified fragment a sequence of 480 nucleotides was obtained and in BLASTn analysis identified as capsid protein (CP) gene of ToMV starting from 5703rd nucleotide and ending at 6182nd nucleotide of ToMV genome. ExPasy translate tool revealed that each each nucleotide sequence encodes for 160 amino acids making ~17 kDa protein (Table 1). The nucleotide sequences of pakistani isolates were submitted in GenBank with accession numbers mentioned in Table 2.

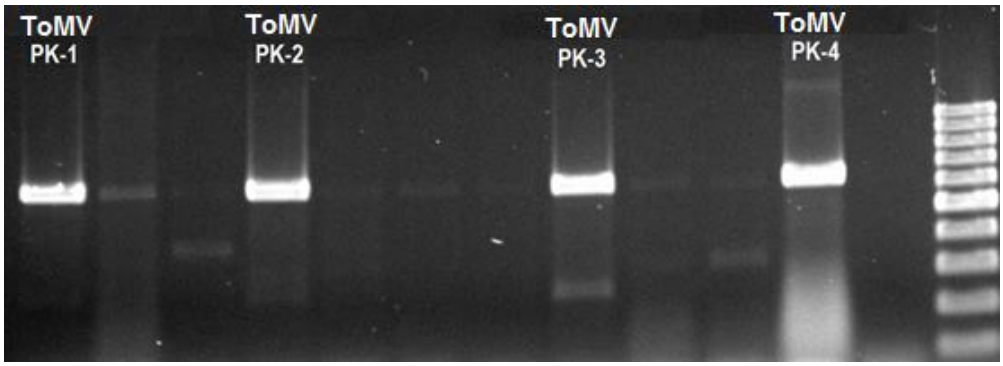

Figure 1. Amplified fragments of 600 bases by designed primers.

Table 1. Nucleotide sequence properties of capsid protein genes of Pakistani ToMV isolates.

Isolate	Characteristics					
	(nt)	(aa)	A %	C %	G %	U %
ToMV PK1	480	160	34	20	24	22
ToMV PK2	480	160	33	20	24	23
ToMV PK3	480	160	34	20	23	23
ToMV PK4	480	160	33	20	24	23

ToMV PK5	480	160	33	20	24	23

After a careful analysis submission of five isolates of *Tomato mosaic virus* (ToMV) was done in GenBank. Each sequence contained 28-29% Adenine, 20-21% Cytosine, 22-23% G and 29-30 % Uracil content (Table 1). Results of BLASTn analysis revealed that the sequences of nucleotide and amino acid of all new isolates were 98.9-99.5% and 97.3-99.3% homologues to each other and 96-99% and 90-98.6% homologues to other isolates respectively. Pakistani isolates held higher percentages of nucleotides and amino acids identities with KY977427 and KY629784, KY629782, AJ429084 isolates respectively. While lower nucleotide and amino acid identity percentages were observed with HM623426, EU885417 and MH230113 isolates respectively. Nucleotides (Figure 2) and amino acids (Figure 3) Multiple sequence alignments of all new sequences was also done.

Figure 2. Alignment of nucleotide sequences of *Tomato mosaic virus* isolates.

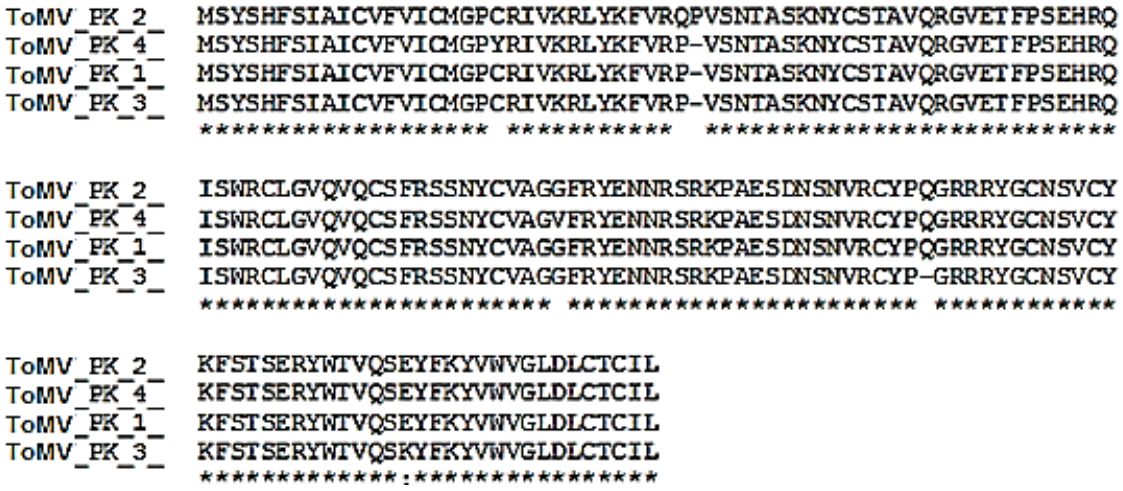

Figure 3. Alignment of amino acid sequences of *Tomato mosaic virus* isolates.

Table 2. List of *Tomato mosaic virus* (ToMV) isolates downloaded from NCBI-Genbank.

Acc. No.	Isolate	Country	Year	Host
MW051889	ToMVPK1	Pakistan	2019	Tomato
MW051890	ToMVPK2	Pakistan	2019	Tomato
MW051891	ToMVPK3	Pakistan	2019	Tomato
MW051892	ToMVPK4	Pakistan	2019	Tomato
MH230113	AWZHR-S91	Iran	2017	Hibiscus
MH230112	AWZHR-S87	Iran	2017	Hibiscus
KY977427	ToMV-X1-3	China	2016	River Water
KY629784	Tai'an	China	2016	Tomato
KY629782	Shouguang	China	2016	Tomato
KY629780	Shanxi	China	2016	Tomato
KY652976	Beijing	China	2016	Tomato
JX982095	RS8	China	2016	Pepino
JQ085379	Penghu	Taiwan	2011	Pepino
HM623426	Ls-K	Korea	2010	Tomato Seed
EU885417	ToMV-tom	Korea	2008	Tomato
MG018614	105-2	Serbia	2012	Tomato
KY629783	Liaocheng	China	2016	Tomato
KY629781	Neimenggu	China	2016	Pepino
JQ966552	HX-T3	China	2012	Capsicum
JQ966551	HX-T3	China	2012	Capsicum
HQ593624	G3	Iran	2010	Eggplant
AJ429084	DSMZ PV-0135	Germany	2002	Tomato
AJ429083	437	Germany	2002	Tomato
AY313136	ToMV	China	2003	Tomato
LN827931	AH2	Egypt	2015	Tomato
AF155507	ToMV Full	China	1999	Tomato
KC914400	Kh-C239	Iran	2012	Cucumis Sativus
KC914398	Sm-E59	Iran	2011	Solanum melongena
JX101616	Palmira	Colombia	2012	Tomato

Phylogenetic Reconstruction

Nucleotides and amino acids sequences of CP gene of 29 isolates comprising four Pakistani and 25 of earlier published ToMV isolates (Table 2) were subjected to phylogenetic reconstruction. All of the Pakistani isolates grouped with capsicum infecting Chines isolate HX-T4 (JQ966551) and tomato infecting Chines isolates Neimenggu (KY629781) and Beijing (KY652976). While, in case of amino acid sequence based phylogenetic reconstruction, one Pakistani isolates viz; ToMV-PK2 clustered with an eggplant infecting Iranian isolate G6 (HQ593624) and the rest of the Pakistani isolates clustered with JQ96551 and KY652976 (Figure 4, 5).

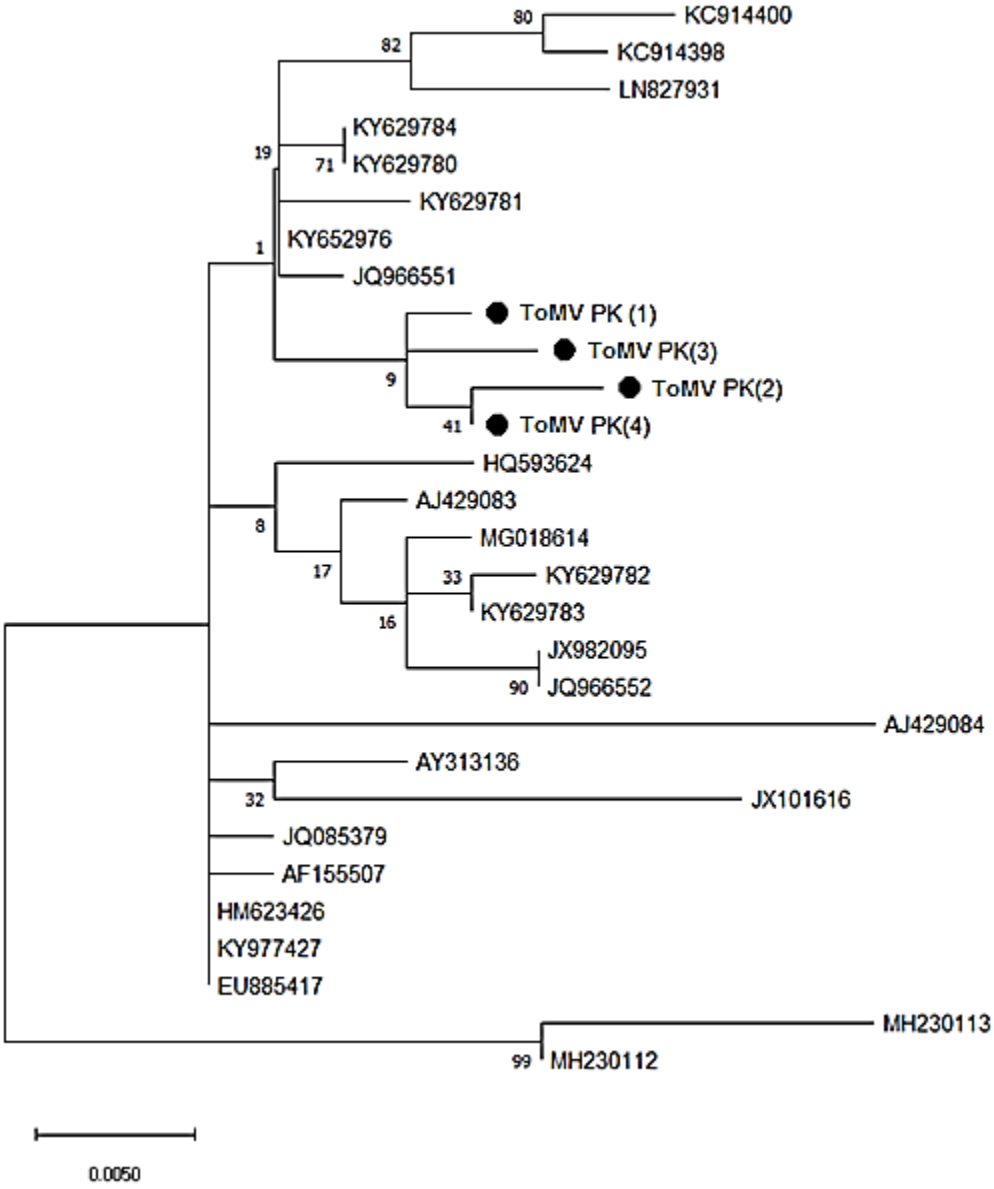

Figure 4. Phylogenetic tree of Pakistani and world isolates base on CP gene nucleotide sequences of *Tomato mosaic virus.*

DISCUSSION

A lot of work is reported on molecular diversity studies of various plant viruses all around the world (Rosenberg, 2005; Tsompana *et al.,* 2005; Moury and Simon, 2011; Balasubramanian and Selvarajan, 2014; Ahmad and Ashfaq 2018). Capsid protein (or coat protein) gene accounted as a benchmark for the classification and identification of plant viruses, viral life span and virus to vector transformation. Plant viruses with higher than 80% of CP amino acid entities

can be placed in the same genus (Fauquet *et al.,* 2003). Present study was aimed to find out the net molecular variability of capsid protein gene sequences undertaking 29 ToMV isolates comprising of 4 newly assimilated from Pakistan and 25 earlier reported from other parts of the world. BLAST analysis suggested that, capsid protein gene of our isolates shared more than 80% amino acid as well as greater than 90% nucleotide identities resembling to other ToMV isolates, designating their taxonomic status as isolates of ToMV. In present study, a geographical relationship was observed between Pakistani and Chinese isolates as they clustered with capsicum infecting Chines isolate HX-T4 (JQ966551) and tomato infecting Chines isolates Neimenggu (KY629781) and Beijing (KY652976). While, in case of amino acid sequence based phylogenetic reconstruction, they also clustered with chines isolates JQ966551 and KY652976. Meanwhile this topographical relation can be caused by massive exchange of fruits, seeds, vegetables and seeds of ornamental plants between adjacent countries. The present study reveals a less molecular variance of Pakistani ToMV isolates. The work done can be a key point for the future perspectives to sort out genetic mutations and understanding the genetic arrangement of Pakistani isolates. The venture of resistance malfunction towards ToMV in CP mediated tomato varieties is proposed by the possible existence of recombinant strain of ToMV, but yet in Pakistan, unluckily no local resistant varieties of tomato has been introduced. Present study being the only report of capsid protein gene on the basis of molecular divergence of ToMV isolates from Pakistan can be a core for not only the understanding of tomato isolates but all kinds of vegetable, fruits and crops in Pakistan. Based on the findings of this research, it is suggested that comprehensive research should be conducted regarding RNA viruses infecting vegetable and major crops. So that sustainable and comprehensive management approaches can be developed and applied in future against these lethal viruses that cause huge economic losses to our crops each year.

CONCLUSION

Likewise, countries around the globe, in Pakistan too, the tomato is infected by *Tomato mosaic virus* with typical interveinal foliar symptoms. In this research, Pakistani isolates of ToMV were observed to be in geographical relatedness with isolates from Iran and China, as they phylogenetically grouped with isolates from Iran and China and showed higher nucleotides and amino acids identities with them. *Tomato mosaic virus* (ToMV) is easily transmittable by infected plantlets and seeds over long distances and Pakistan continuously exchanges planting material/vegetables/seeds with neighboring countries with poor quarantine facilities. This recommends a strict constant and quarantine on trade of agricultural commodities. This research is first attempt to study molecular diversity of capsid protein gene of Pakistani *Tomato mosaic virus* (ToMV) isolates and this research can be extended to tomato, potato and other crops in Pakistan and around the globe.

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