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PHYLOGENETIC ANALYSIS OF TOMATO MOSAIC VIRUS ISOLATED FROM SEEDS OF *LYCOPERSICON ESCULENTUM L.*

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Tomato seeds of different cultivars were checked for contamination with Tomato mosaic virus (ToMV), typical pathogen of these vegetable crops in Ukraine. The incidence of viral contaminations was determined using ELISA. Approximately 40% of seed samples were contaminated with ToMV. The coat protein (CP) gene sequence of Ukrainian isolate (ToMV-ukr3) was obtained and analyzed. Phylogenetic tree based on complete CP sequences of Ukrainian isolate and strains available in Genbank was constructed in Mega 6. Comparison of nucleotide sequences of obtained isolate with those of other ToMV strains in Genbank confirmed that detected pathogen is isolate of Tomato mosaic virus. CP sequence of ToMV-ukr3 revealed 96-99% nucleotide homology with existing ToMV strains. ToMV-ukr3 was the most related to strains ToMV-1-2, ToMV-G26 and ToMV-G6.

Key words: ToMV, *Lycopersicon esculentum L.*, seeds, phylogenetic analysis

Tomato (*Lycopersicon esculentum L.*) is important crop for human nutrition and farming in Ukraine. These vegetables are highly susceptible to viral diseases, in particular, caused by *Tomato mosaic virus* [1, 8]. It belongs to genus *Tobamovirus*, family *Virgaviridae* [11]. ToMV is one of the most troublesome viruses infected tomato. The effects of this virus may be devastating and lead to serious losses of yield and fruit quality in tomatoes.

Seed transmission is considered the key point, which determines the high rates of ToMV dissemination over long distances. Therefore, control over seeds is the only practical and effective measure for preventing ToMV outbreaks [7]. The success of

modern agriculture depends on virus-free seed usage and development of seed certification programs.

The aim of this study was to detect the contaminations of seeds of *L.esculentum L.* with ToMV and perform the phylogenetic analysis of coat protein gene of obtained isolate.

Materials and methods. We randomly selected commercially available tomato seeds of 10 different cultivars produced by Ukrainian manufacturers and certified for sale. The following tomato cultivars were checked: pertsevydnyy, cherri, vedmezha lapa, rozhevi shchichky, ukrayins'kyy veleten', novynka Prydnistrov′ya, oberih, san'ka, vohni Moskvy, volove sertse rozheve. Before serological testing, seeds were germinated in sterile Petri dishes at 20^0 C for the period of 7 days. Then erminated seeds were homogenized in 0.1M phosphate buffered saline (PBS), pH7.4, 1:3 (m/v). Plant components were removed by centrifugation at 5000 g for 20min at 4^0 C. The supernatant was taken for serological tests.

For detection of viral antigens, double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) [3] was carried out using test systems of Loewe (Germany) according to manufacturer's recommendation. Standard positive and negative commercial controls were used. In order to obtain statistically significant results three times repeated testing of each sample was performed. The ELISA results were read at 405nm using microplate reader Dynatech (USA) with Dynex Revelation Quicklink software [3]. Morphology of virions was assessed under electron microscope Jeogs (JEM-1400) using 2% uranyl acetate as contrasting agent [4]. Total RNA extraction using RNeasy Plant Mini kit (Qiagen, UK) was carried out from ToMV-contaminated seeds. The results were checked by electrophoresis of nucleic acids in 1.5% agarose gel using TBE buffer. Obtained RNA was used as a template in reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed using ToMV-specific primers covering coat protein (CP) gene (product size – 700bp) [5]:

forward primer – CGGAAGGCCTAAACCAAAAAG;

Tob-Uni1 primer – ATTTAAGTGGAGGGAAAAACACT.

The amplified cDNA of expected 700 bp was visualized by electrophoresis. The purified amplicons were sequenced. A fragment of 480bp corresponding to the complete coat protein (CP) gene of Ukrainian isolates was compared with CP sequences of previously characterized strains available in Genbank using NCBI/BLAST (http://www.ncbi.nlm.nih.gov). The phylogenetic analysis was performed using Maximum likelihood algorithm in MEGA 6 software. For calculation of the nucleotide distances, the Tamura 3-parameter model [10] was used.

Results and discussion. The ELISA results showed that four seed samples out of ten were contaminated with ToMV antigens. Seeds of "vedmezha lapa" cultivar showing the highest virus titer in DAS-ELISA was chosen for further particularized investigation.

For confirmation the ELISA results, indication of ToMV morphology and size of viral particles electron microscopy was conducted. The rod-shaped particles of 300±3 x 19±3 nm in size were detected that corresponded to published ToMV dimensions (Fig.1).

RT-PCR with extracted total RNA from contaminated seed of "vedmezha lapa" cultivar resulted a cDNA of expected size (700 bp) (Fig.2).

Comparison of 480bp-length CP sequences using NCBI/BLAST (http://www.ncbi.nlm.nih.gov) showed that obtained isolate (ToMV-ukr3) shares the highest identity with the group of tobamoviruses mainly infecting solanaceous plants (*Tomato mosaic virus*, *Tobacco mosaic virus* (TMV), *Tomato mottle mosaic virus* (ToMMV), *Pepper mild mottle virus* (PMMoV)). The criteria for *Tobamovirus* species differentiation determines that less than 90% nucleotide sequence identity is considered a new species [6]. Ukrainian isolate showed minor differences and revealed 96-99% nucleotide identity with different ToMV strains, whereas ToMV-ukr3 shared much less identity with TMV (74-79%), PMMoV (71-73%), and ToMMV (85%). These results confirmed the attribution of obtained isolate to ToMV.

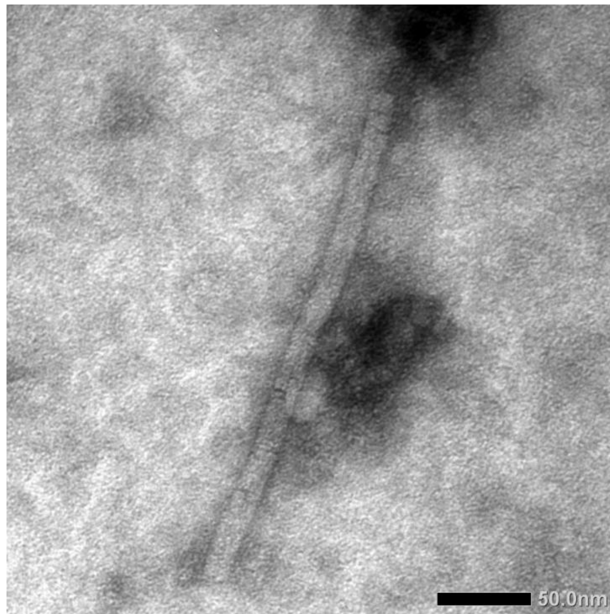

Fig. 1. Electron microphotograph of Tomato mosaic virus

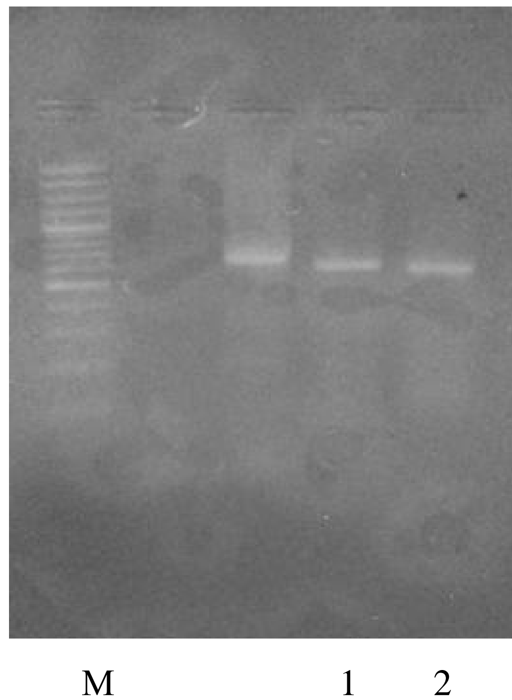

Fig. 2. The electrophoregram of ToMV cDNA obtained in RT-PCR: M – markers (100 bp, Fermentas), 1,2 – cDNA of coat protein gene. Product size - 700 bp

Phylogenetic tree was built using Maximum-Likelihood method Mega 6 software (Fig. 3).

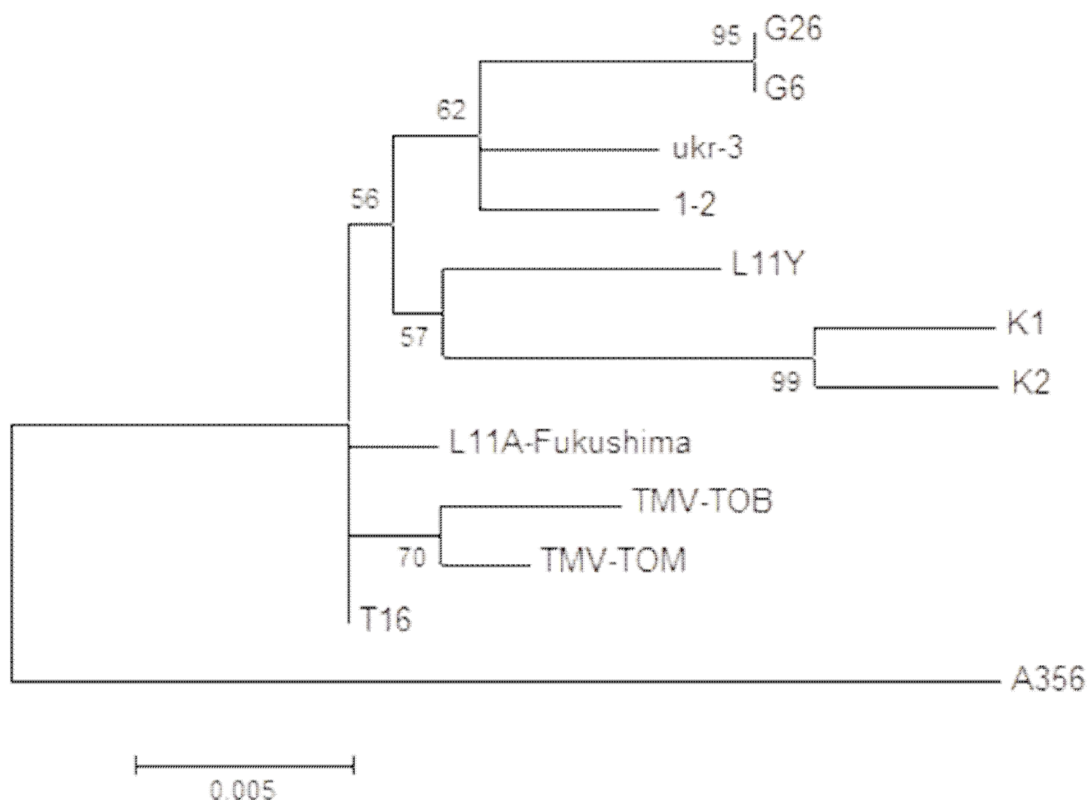

Fig. 3. Phylogenetic tree based on entire coat protein gene sequence of selected ToMV strains and Ukrainian isolate.

On the ML tree, Ukrainian isolate and all strains under study excepting strain ToMV-A356 were clustered together. They shared a common ancestor and close phylogenetic relationships. High homology percentage values were determined between these sequences: 98-99% (Table). Ukrainian isolate were grouped with strains ToMV-1-2, ToMV-G26 and ToMV-G6, homology values with them were approximately 99% at nucleotide level. Strain ToMV-A356 represents the lowest homology percentage (96%) with ToMV-ukr3. Tobamoviruses demonstrate strong correlation with their angiosperm hosts [9] supporting the suggestion that this low rate of homology could be explained by the fact, that strain ToMV-A356 was extracted from *Centaurea sp.*, member of *Asteraceae* family.

Nucleotide homology between coat protein gene sequences of Ukrainian ToMV isolate and existing strains from Genbank

Strains from Genbank database	Host (if known)	Accession number	Homology percentage

ToMV-K2		Z92909	98,1%
ToMV-K1		AJ243571	98,1%
ToMV-L11Y	*Nicotiana sp.*	AB355139	98,74%
ToMV-G26	*L.esculentum L.*	HQ593627	98,94%
ToMV-1-2		DQ873692	99,16%
ToMV-G6	*Solanum melongena L.*	HQ593624	98,94%
ToMV-L11A-Fukushima		AB083196	99,16%
ToMV-T16		HQ593626	99,37%
ToMV-A356	*Centaurea sp.*	KF527464	96,37%
TMV-TOB	*Nicotiana sp.*	AF103780	98,7%
TMV-TOM	*L. esculentum L.*	AF103779	98,9%

Conclusions

From this study, we can infer that obtained isolate is not an emergent strain with ability to break resistance in plants [12]. Thus, known strategies for control of tomato diseases caused by ToMV may be used [2]. The high incidence of ToMV contamination in checked tomato seeds points on importance of conducting preventive measures before planting. Possible approaches for ToMV control may include using of virus-free seed material and resistant tomato cultivars.

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