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Experimental Infection of Different Tomato Genotypes with *Tomato mosaic virus* Led to a Low Viral Population Heterogeneity in the Capsid Protein Encoding Region

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The complete genome sequence of a Slovak SL-1 isolate of *Tomato mosaic virus* (ToMV) was determined from the next generation sequencing (NGS) data, further confirming a limited sequence divergence in this tobamovirus species. Tomato genotypes Monalbo, Mobaci and Moperou, respectively carrying the susceptible tm-2 allele or the Tm-1 and Tm-2 resistant alleles, were tested for their susceptibility to ToMV SL-1. Although the three tomato genotypes accumulated ToMV SL-1 to similar amounts as judged by semi-quantitative DAS-ELISA, they showed variations in the rate of infection and symptomatology. Possible differences in the intra-isolate variability and polymorphism between viral populations propagating in these tomato genotypes were evaluated by analysis of the capsid protein (CP) encoding region. Irrespective of genotype infected, the intra-isolate haplotype structure showed the presence of the same highly dominant CP sequence and the low level of population diversity (0.08-0.19%). Our results suggest that ToMV CP encoding sequence is relatively stable in the viral population during its replication in vivo and provides further demonstration that RNA viruses may show high sequence stability, probably as a result of purifying selection.

Keywords **:** tobamovirus, diversity, haplotype, resistance gene

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Tomato mosaic virus (ToMV) belongs to the genus *Tobamovirus* in the family *Virgaviridae*. The virus is easily transmitted by mechanical inoculation and plant-to-plant contact (Hollings and Huttinga, 1976). The tobamovirus genome encodes at least four proteins. Open reading frame (ORF) 1 and ORF2 code for the 130K protein and the 180K protein (translational read-through product of the 130K protein), both involved in RNA replication. The 30K protein translated from ORF3 is required for cell-to-cell movement. The ORF4 encodes the coat protein (CP) essential for systemic spread of the virus and which has been shown to be involved in symptom development (Ishibashi and Ishikawa, 2016; Ohnishi et al., 2009).

Generally, ToMV-affected plants display a light or

dark green mottling or mosaic with distortion of younger leaves, and/or stunting to varying degrees. Fruits may be severely reduced, showing internal browning of the fruit wall, yellow blotches or necrotic spots (Broadbent 1976; Hollings and Huttinga, 1976).

One of the methods to control ToMV is the cultivation of tomato genotypes with major resistance gene(s). So far, three genes of ToMV resistance, namely Tm-1, Tm-2 and Tm-2^2 have been identified and used in tomato breeding programs (Hall, 1980; Pelham, 1966). The Tm-1 gene, whose product binds ToMV replication proteins and inhibits RNA replication, was originally identified in the ToMV-resistant wild tomato *Solanum habrochaites* (Ishibashi and Ishikawa, 2014; Pelham, 1966). The Tm-2 and Tm-2^2 genes, introgressed from *Solanum peruvianum*, are allelic and belong to the CC-NBS-LRR class of plant resistance genes (Lanfermeijer et al., 2003). However, after the introduction of resistant tomatoes in the field, resistance-breaking virus isolates have been documented (Meshi et al., 1989; Strasser and Pfitzner, 2007).

RNA viruses evolve as complex viral populations because of the rapid accumulation of mutations due to their high mutation rate and their large population size. This dynamic genetic structure of virus populations has a significant role in the epidemiology of the viruses as it may lead to the selection of variants with increased pathogenicity (Moury et al., 2006). The host and its environment represent a source of selection pressures for a viral pathogen for the majority of its life cycle (Elena and Sanjuan, 2007).

In this work, the intra-isolate heterogeneity was evaluated after the experimental infection of three different tomato genotypes with a full-length genome-characterised ToMV isolate.

Determination of the ToMV SL-1 full length genome sequence by next generation sequencing (NGS). ToMV SL-1 was obtained from the collection kept at NAFC-RIPP Piešťany. The isolate was originated from a local tomato plant grown in western Slovakia and the infected leaves were stored at -80°C prior to experiment.

Total RNAs from infected tomato leaves were extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel, Duren, Germany) and ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA, USA). The rRNA-depleted total RNAs sample was used for double stranded cDNA synthesis using the SuperScript II (Thermo Fisher Scientific, Waltham, USA) kit. The cDNA was then column-purified with the DNA Clean & Concentrator™-5 – DNA kit (Zymo Research, Irvine, USA) and quantified with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The sample was then processed with the transposon-based chemistry library preparation kit (Nextera XT, Illumina) as previously described (Sihelská et al., 2016). The fragment size structure of the DNA library was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The indexed denatured DNA library was sequenced (200-bp paired-end sequencing) on the Illumina MiSeq platform (Illumina).

Trimmed reads were used for de novo assembly and contigs aligned to the viral genomes database (ftp://ftp.ncbi.nih.gov/genomes/Viruses/all.fna.tar.gz/, downloaded on July 2016) using Geneious v.8.1.9.

The accuracy of the NGS-based full-length genome sequence obtained was verified by direct Sanger sequencing of PCR products encompassing various genome portions (data not shown, sequence of the ToMV-specific primers used available upon request).

Complete and partial ToMV sequences used for comparison and phylogenetic analyses were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). Phylogenetic analyses and comparisons were performed using the MEGA v.6 (Tamura et al., 2013) and DnaSP v.5 (Librado and Rozas, 2009) programs.

Inoculation and testing of tomato plants. Three different tomato genotypes, i.e. Monalbo (sensitive), Mobaci (Tm-1) and Moperou (Tm-2) were tested for their susceptibility to the experimental mechanical inoculation with ToMV SL-1 isolate. Homogenous lots of 10 plants per genotype were inoculated at the 3-4 true leaves stage using the same inoculum prepared by grinding SL-1-infected tomato leaves 1:10 (wt/vol) in Norit buffer (https://www.dsmz.de/uploads/media/Inoculation_01.pdf/). Inoculated plants were grown in an insect-proof cultivation room under controlled conditions (14 h light/10 h dark photoperiod, 55 µmol $m^{-2}s^{-1}$ photon flux density, day/night temperature: 25/18°C). Development of symptoms was monitored visually. ToMV presence was verified and the estimation of its accumulation were performed 21 and 48 days post inoculation (d.p.i.) by semiquantitative double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) performed following a standard protocol (Glasa et al., 2003) and a polyclonal antiserum (Loewe Biochemica, cat. no. 07047). Standardised plant extracts were obtained by homogenisation of leaf discs (0.15 g) taken from the two upper fully developed leaves of each of the inoculated plant and of uninfected control plants. All the samples were homogenised in PBS buffer (1/25 w/v) containing 0.05% Tween-20 and 2% polyvinylpyrrolidone and subsequently applied to a same ELISA plate, so that absorbance values measured at 405 nm for different genotypes could be compared. The experiment was

repeated two times.

DAS-ELISA- negative tomato plants were retested by RT-PCR using primers ToMV5119F (5′-CTTGTTGT-GTCCGGTGAGTG-3′, sense) / ToMV5465R (5′-ATG-GGTCCTCCATCGTTCAC-3′, antisense), designed from the SL-1 sequence.

Analysis of ToMV intra-plant diversity. The diversity and polymorphisms in the ToMV population present in one ToMV-positive plant per genotype was further assessed. Total RNAs were isolated from the upper leaf of plants at 21 and 48 d.p.i. The complete ToMV ORF4 sequence was amplified using a two-step reverse-transcriptase polymerase chain reaction (RT-PCR) protocol. The first-strand cDNA was synthesized by reverse transcription of total RNAs using random hexamer primers and the Avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI, USA). A 480-bp fragment (corresponding to positions 5703-6182 of the complete ToMV reference sequence, NC_002692) was amplified using primer pair ToMV-F (5′-ATGTCTTACTCAAT-CACTTC-3′, sense) and ToMV-R (5′-TTAAGATG-CAGGTGCAGAGG-3′, antisense) and the TaKaRa LA Taq polymerase (TaKaRa, Bio Inc., Shiga, Japan). PCR amplification was performed under the following cycling conditions: initial denaturation at 94°C for 5 min; 25 cycles of 94°C for 45 s, 54°C for 30 s, and 72°C for 45 s; followed by a final extension step at 72°C for 10 min. The RT-PCR products obtained from each plant were cloned into the pGEM-T Easy cloning vector (Promega Corp.) according to the manufacturer's instructions and for each PCR product, 12 randomly chosen cDNA clones were sequenced on both strands. The haplotypes genetic network was reconstructed using a statistical parsimony procedure implemented in the TCS 1.21 program (Clement et al., 2000).

To confirm the true-to-typeness of the infected tomato plants, the presence or absence of the susceptible tm-2 allele and of the resistant Tm-2 allele was determined by dominant allele-specific PCR amplification using the primers of Shi et al. (2011) and DNA isolated from leaves of the various tomato plants using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) as described (Hudcovicová et al., 2015).

The complete genome sequence of the SL-1 isolate was determined from the NGS data. After adaptor and low quality reads removal, a total of 3,236,162 Illumina MiSeq reads remained; of these 1,191,490 mapped on the ToMV genome (accession number NC_002692), yielding 100% coverage of the ToMV viral genome, including the 5' and 3' ends. The sequence obtained was validated by the Sanger sequencing of RT-PCR products representing various genome regions (about 60% of the total genome). The SL-1 genome is 6383 nucleotides long and its sequence has been deposited in the GenBank database under accession number KY912162. The overall genomic organization of SL-1 is similar to that of other tobamoviruses (Ohno et al., 1984). The four genome encoded ORFs have the same location and length as in the reference ToMV isolate (NC_002692), yielding products of 1616, 1116, 264 and 159 amino acids. As for other ToMV isolates, the examination of sequence confirmed the existence of an internal read-through stop codon (Pelham, 1978) at position 3420-3422. Comparative analysis showed that the SL-1 isolate sequence was 98.6-99.1% identical to the 15 ToMV complete genomes available in Genbank, confirming a close phylogenetic relationship and a limited diversity in the ToMV species (Fig. 1). Pairwise comparisons of SL-1 and of database isolates showed that the highest degree of nucleotide diversity (exceeding 3%) was observed in two genomic regions, around positions 2950-3200 (ORF1, encoding part of the viral helicase) and 5850-6000 (central part of the ORF4). Based on the analysis of complete genomes (n=16), the average nucleotide divergence between ToMV isolates reaches only 0.9% (± 0.1%), despite their different geographical origins and isolation hosts (tomato, pepino, tobacco, jasmin, camellia; for isolate characteristics see Fig. 1). This limited sequence divergence is about half of that observed in other tomato-infecting tobamoviruses. Indeed, the mean nucleotide divergence between the 21 available complete *Pepper mild mottle virus* genomes reaches 1.9% (± 0.1%) and that for *Tobacco mosaic virus* (74 genomes) is 2.1% (± 0.1%).

At the protein level, the SL-1 ORFs1-4 aminoacid sequences show divergence levels of respectively 0.2-0.9%, 0.1-1.1%, 0.4-1.5% and 1.3-3.1% with the corresponding proteins encoded by the available ToMV genomes.

To include a larger sequence dataset for comparison, 110 complete ToMV capsid protein gene sequences were retrieved from Genbank (accessed on January 2017). The SL-1 isolate is closest to the DSMZ PV-0135 isolate (differing in the CP gene by a single nucleotide mutation), forming a slightly divergent branch within the major ToMV cluster (Supplementary Fig. 1).

Tomato genotypes Monalbo, Mobaci and Moperou respectively carrying the susceptible tm-2 allele or the Tm-1 and Tm-2 resistant alleles, were repeatedly tested for their susceptibility to ToMV SL-1 by mechanical inoculation. Depending on the genotype, differences in the rate of infection were observed by DAS-ELISA at 21 d.p.i. (Table 1). While 100% of cv. Monalbo and Mobaci plants

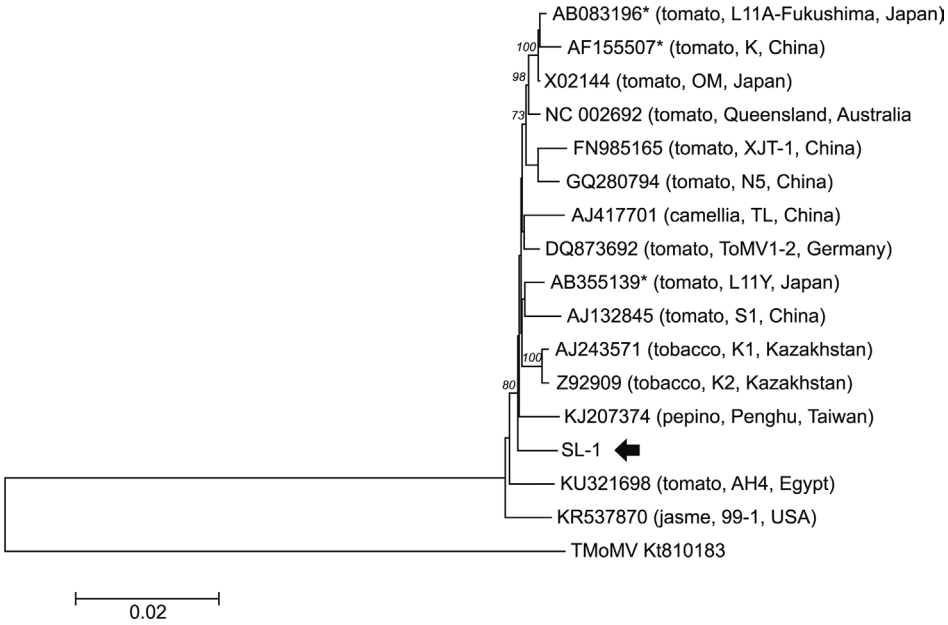

Fig. 1. Phylogenetic tree of ToMV isolates for which the complete genome sequence is available. The sequences are identified by their accession numbers, original host, isolate name and country of origin. Asterisk indicates the laboratory isolates or mutated genomes. The scale bar represents a distance of 0.01 substitutions per site. Bootstrap values (1,000 bootstrap resamplings) are indicated on the branches. SL-1 isolate is highlighted by an arrow. An isolate of *Tomato mottle mosaic virus* was used as an outgrup.

Table 1. Semiquantitative comparison of virus accumulation, measured as the absorbance at 405 nm in DAS-ELISA, in three different tomato genotypes 21 days p.i. (*values calculated only from positive plants)

genotype		Monalbo	Mobaci	Moperou
Assay 1	No. of inoculated/positive plants	10/10	10/10	10/2
	Absorbance after 1h (mean ± SD)	0.813 ± 0.139	0.928 ± 0.191	0.959 ± 0.097*
Assay2	No. of inoculated/positive plants	10/10	10/10	10/1
	Absorbance after 1h (mean ± SD)	0.700 ± 0.129	0.658 ± 0.068	0.684 ± 0.113*

tested positive in both inoculation experiments, only 1/10 and 2/10 inoculated Moperou plants showed ToMV accumulation. No additional infection was identified following a retesting of negative plants by RT-PCR. Interestingly, infected plants of the three tomato genotypes accumulated ToMV SL-1 to similar amounts as judged by semi-quantitative DAS-ELISA (Table 1), despite the differences observed in symptomatology. Although ToMV-infected Monalbo plants displayed vein clearing and mild leaf narrowing and deformations, the infected Mobaci plants remained symptomless, while the few infected Moperou plants exhibited stunting and severe leaf deformations (Supplementary Fig. 2).

Several types of symptoms have been associated with ToMV infection on tomato, including mottling, rough downturned edges or shoestring-like elongation of leaves, plant stunting (Broadbent, 1976; Hollings and Huttinga, 1976). However, symptoms of nutrient deficiency, physiological disorders or symptomless reaction (as observed on cv. Mobaci) can complicate the visual diagnostic especially in early stages of infection. Our data further support the observations that Tm-1 resistance gene is of a limited practical use because it can be overcome by naturally occurring ToMV strains (Meshi et al., 1989; Strasser and Pfitzner, 2007).

Plant viruses have the ability to evolve rapidly and consequently to adapt to their host organisms. Since viruses multiply within a host cell, they evolve under selective pressure imposed by their hosts (Dawson, 1967; Elena and Sanjuan, 2007). Theoretically, resistance-breaking virus mutants can emerge by mutations that escape the inhibitory interaction with the host resistance machinery. Such virus evolution poses a challenge to the durable and effective virus control. Possible differences in the intra-isolate variability and polymorphism between viral populations were determined following propagation of the same SL-1 ToMV inoculum in the 3 different tomato genotypes grown in parallel under the same conditions. RT-PCR amplicons generated from different ToMV-infected tomato genotypes and spanning the entire ORF4 (CP gene) were cloned and 12 individual sequences determined for each RT-PCR product. The analysis of these sequences shows that the average intra-isolate pairwise genetic distance (diversity) was low and comparable be-

tween the three plant genotypes between two time points (Table 2).

The intra-isolate haplotype structure showed the presence of the same highly dominant sequence in the three tomato genotypes. At both time points (21 and 48 d.p.i.), the minor haplotypes differed from the dominant one by only 1 to 2 mutations (Fig. 2). Even if several approaches were used (Predajňa et al., 2012) to try to reduce the introduction of artefactual mutations, we cannot exclude that some of the observed mutations may have been artificially introduced by the enzymes used in RT-PCR amplification procedure.

However, the spectra of variants found around the dominant sequence in the tomato samples at the two time points were totally different. In addition, although more than half of the mutations identified at 21 d.p.i. (13 out of 21) were non-silent, leading to an aminoacid change, none of them was observed again at the later time point. The low level of population diversity (0.08-0.19%, Table 2) and the distribution of mutations over the analyzed region determined in the present work suggests that ToMV CP encoding sequence is relatively stable in the viral population during its replication in vivo and provides further demonstration that despite their high mutation rates, RNA viruses may show extremely high sequence stability (Rodríguez-Cerezo and García-Arenal, 1989; Garcia-Arenal et al., 2001). It should be noted, that structural changes in the ToMV CP are not directly linked to the resistance breaking (Meshi et al., 1989), thus the genomic region analysed in this work could be influenced only indirectly by replication in the different tomato hosts.

Moreover, the comparison of the master sequence determined from direct PCR sequences from the initial inoculum and from leaves of tomato plants grown up to the fruit development (140 days) showed no difference (data not shown). We can hypothesize that this effect is a result of purifying selection, such that these mutations were counterselected and thus not fixed in the population.

Table 2. Analysis of the nucleotide polymorphism in the leaf samples collected from Monalbo (M), Mobaci (B) and Moperou (P) plant 21 days p.i.[a] and 48 days p.i.[b] Plants were selected from assay 2 reported in Table 1.

Sample	Number of sequences/ Number of haplotypes	Average number of nucleotide differences (k)	Haplotype diversity (Hd)	Nucleotide diversity (Pi)
M1[a]	12/5	0.833	0.576	0.00189
M2[b]	12/5	0.667	0.576	0.00152
B1	12/4	0.636	0.561	0.00145
B2	12/3	0.333	0.318	0.00076
P1	12/4	0.667	0.455	0.00152
P2	12/4	0.500	0.455	0.00114

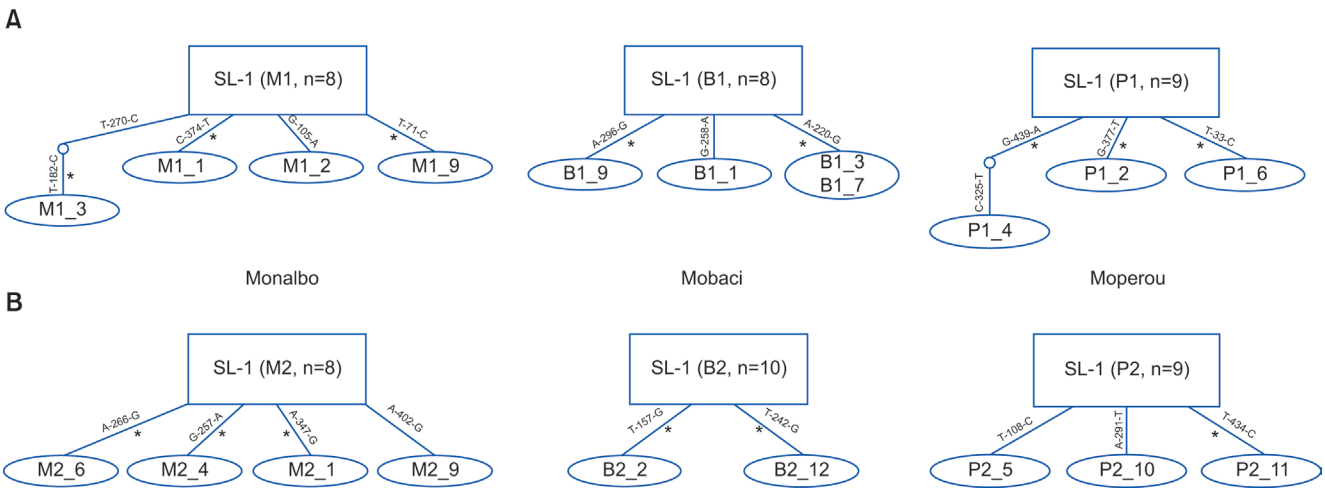

Fig. 2. Maximum parsimony network reconstructed using the TCS program for ToMV SL-1 haplotypes determined from 3 tomato genotypes (Monalbo, labelled as M, Mobaci as B and Moperou as P) in two time points (A – 21 d.p.i., B – 48 d.p.i). Each connecting line represents a single mutational step between two haplotypes, with the position of the mutated nucleotide indicated. n= number of haplotypes identical with the SL-1 sequence. Mutations leading to an aminoacid change are marked by an asterisk.

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