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SHORT COMMUNICATION

Molecular identification of tomato brown rugose fruit virus in tomato in Palestine

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Abstract

Tomato, a top cash crop, is infected by a number of viruses that cause drastic yield losses. Recently an unusual viral syndrome that resembled somewhat that induced by tobacco mosaic virus has been observed in Northern Palestine. The most affected tomatoes were of cultivars 'Ikram' and 'Azmeer'. A study aimed at revealing the cause of the disease, identified the presence of an apparently undescribed tobamovirus. The virus genome was entirely sequenced and shown to be composed of 6391 nucleotides. Sequence analysis indicated that this virus was an isolate of tomato brown rugose fruit virus (TBRFV). This is the first time TBRFV was detected in Palestine on tomatoes and the name tomato brown rugose fruit virus-Palestinian isolate (TBRFV-Ps) is suggested. Molecular tools were developed for specific detection of the virus and sanitary actions to protect tomato production from TBRFV were recommended.

Keywords Virus · Tomato · *Tobamovirus* · TBRFV · Phylogenetic analysis

Diseases induced by intracellular infectious agents (viruses, viroids, phytoplasmas) represent major threats to a wide number of crops (McGovern and Elmer 2018; Alkowni and Srouji 2009). Control is mainly based on the use of virus-free propagating material, through clonal and sanitary selection (with combination of sanitation if necessary) (Meng et al. 2017; Alkowni 2017).

Tomato (*Solanum lycopersicum* L., family Solanaceae) is a major vegetable crop that has achieved tremendous popularity over the last century and is now grown worldwide (Dorais et al. 2001; Hanssen and Lapidot 2012), with a global production that has increased fivefold in the last decade (FAO 2017). This crop, however, is susceptible to a number of pathogens among which viruses, i.e. tomato yellow leaf curl virus (TYLCV), tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), tomato spotted wilt virus (TSWV), pepino mosaic virus (PepMV), cucumber mosaic virus (CMV) and many others that can severely limit its production.

Recently, tomatoes grown in the Palestinian districts of Qalqilia, Jenin and Tubas exhibited symptoms recalling those induced by TMV, which prompted investigations aimed at identifying the putative causal agent of the disease. Field surveys were carried out in different areas of the West Bank (Qalqilia, Jayus, Tammon and Jenin), where tomatoes of cvs 'Ikram', 'Azmeer', 'Just', and 'Shekran' are commonly grown, for evaluating disease incidence and spread, as well as collecting samples for laboratory analysis.

In parallel, greenhouse-grown plants cvs 'Ikram' and 'Azmeer' from different regions of Palestine were found infected, in some cases at a rate of up to 100%. Symptomatic plants exhibited mild to severe mosaic, narrow leaves and yellow spotted fruits (Fig. 1), i.e. symptoms similar to those induced by the tobamoviruses TMV and ToMV. Samples were collected from symptomatic plants and transferred to the Biotechnology laboratory of An-Najah National University and National Agriculture Research Center (NARC) for mechanical inoculation of the following indicators: *Nicotiana benthamiana, N. tabacum, N. occidentalis, Chenopodium amaranticolor* and *C. quinoa*, and also on tomato plants of different commercial cultivars.

About one gram of leaf tissue from symptomatic plants was ground in two ml of 0.01 M sodium phosphate buffer (pH 7.0; 1:2 *w*/*v*) and the extract was rubbed on celite-dusted leaves of the indicators. Symptoms were evaluated 10 to 20 days post inoculation and the virus was maintained on systemically infected tomato plants that served as propagation host. All virus-infected plants were grown in an insect-proof growth chamber inside a glasshouse and sprayed regularly with insecticides.

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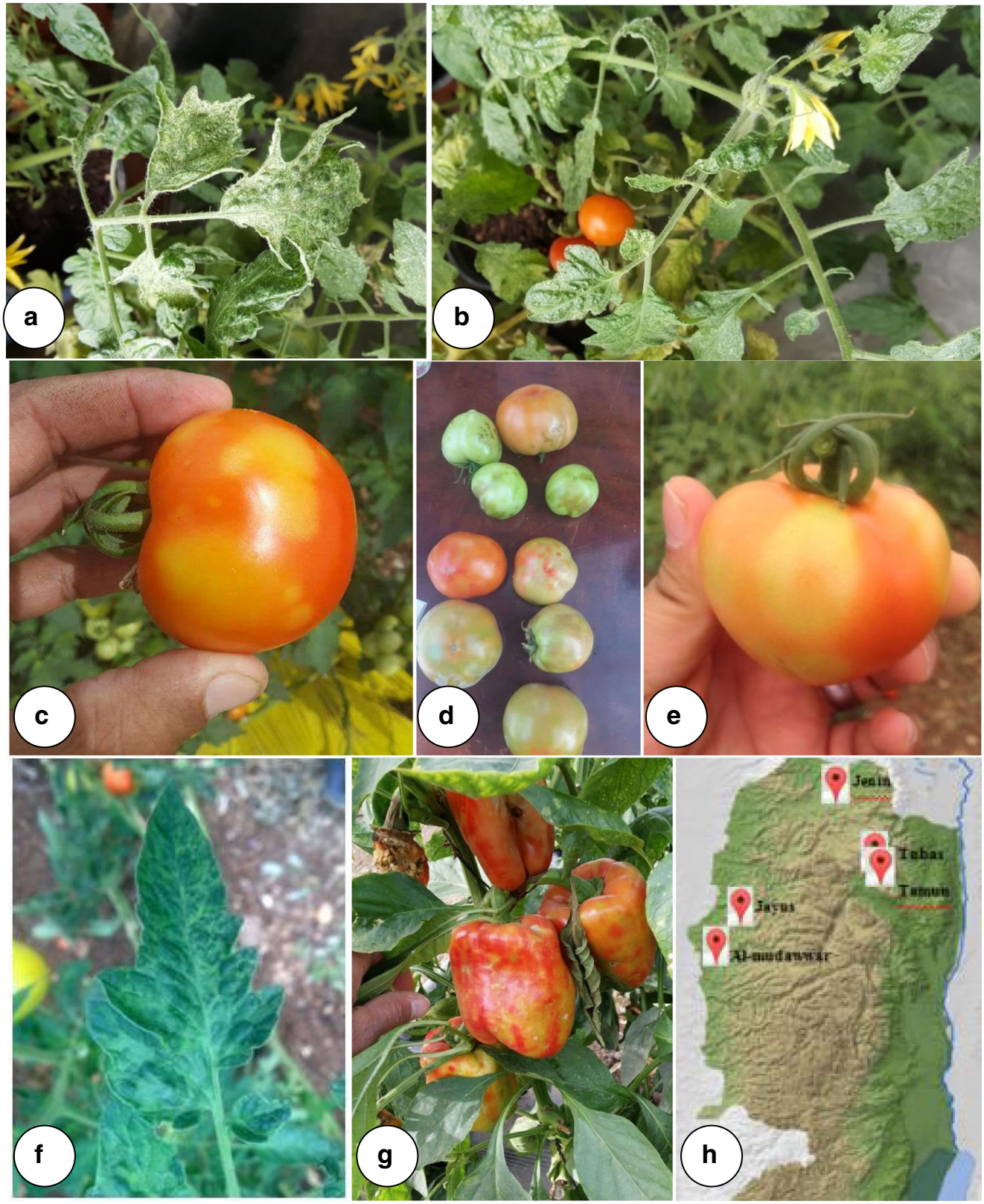

Fig. 1 Foliar symptoms of deformation and mottling (**a**, **b** and **f**) and yellow blotching on fruits (**c**, **d** and **e**) of tomato. Small yellow dots and degenerate blotches on greenhouse-grown pepper fruits (**g**). Locations in Northern Governorates of the West Bank in Palestine where tomato samples were collected (**h**)

Table 1 List of primers used for screening plants for any of known viruses

Virus	Primers (5′ ——————— > 3′)	Product size	Reference
TYLCV	TYv2337F: ACGTAGGTCTTGAC ATCTGTTGAGCTC TYc138-R: AAGTGGGTCCCACA TATTGCAAGAC	634-bp	Anfoka et al. 2005
TMV	TMV-F: CGACATCAGCCGAT GCAGC TMV-R: ACCGTTTTCGAACC GAGACT	880-bp	Kumar et al. 2011
ToMV	ToMV-F: CGAGAGGGGCAACA AACAT ToMV-R: ACCTGTCTCCATCT CTTTGG	318-bp	
TSWV	F: CACAAGGCAAAGACCTTGAG R: GCTGGAGCTAAGTATAGCAGC	620-bp	Salem et al. 2012
ToMMV	ToMMV-F: AAAAGGGCGGTCTA ATTTC ToMMV-R: TAATTTCGTCCTTT ATTAC	600-bp	Turina et al. 2016

Among the indicators that expressed symptoms, *Chenopodium quinoa* reacted with local and necrotic lesions. The field syndrome was also consistently reproduced on mechanically inoculated tomato plants of different commercial cultivars.

Total RNA was extracted from fruits and leaves of symptomatic plants, using an "ISOLATE II RNA Plant Kit" (Bioline, UK). Total RNA served as a template for cDNA synthesis using Reverse Transcriptase cDNA kit (Tetro™ cDNA Synthesis Kit, Bioline, UK). PCR for detecting the main viruses infecting tomato was performed using the primers listed in Table 1. The PCR reaction was done in a 25 µl volume, containing 0.4 mM of dNTPs, 0.2 µM of each primer, one unit of *Taq* DNA polymerase, 2.5 µl of 10X PCR buffer, 0.75 mM $MgCl_2$, and 2.5 µl of cDNA. PCR parameters were 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C, and a final extension step for 7 min at 72 °C.

Infected tomato leaf extracts were screened for the presence of TYLCV, TSWV, TMV, and ToMV but none of these viruses was detected, in agreement with what was reported for tomato mottle mosaic virus (ToMMV) in Mexico, Spain and Israel (Li et al. 2013; Ambros et al. 2016; Turina et al. 2016). However, RT-PCR using the general tobamovirus primer set F-3666 (5'-ATGGTACGAACGGCGGCAG-3′) and R-4718 (5'-CAATCCTTGATGTGTTTAGCAC-3′) amplified a 1052 bp fragment from all samples collected in Qalqilia, Jenin and Tubas governorates. These primers were designed in a conserved ORF2 region of the Israeli isolate of TBRFV (Luria et al. 2017) as well as the new Jordanian isolate of TBRFV (Salem et al. 2016).

The 1052-bp RT-PCR amplicon (referred to as F3) obtained from the replicase region of four Palestinian isolates (referred to as M, A, B, and SH1), and corresponding to the conserved ORF2 region of solanaceae-infecting tobamoviruses (Luria et al. 2017), was directly sequenced by Sanger DNA sequencing. Multiple sequence alignments and sequence relationship were produced using Clustal Omega (Goujon et al. 2010; Sievers et al. 2011). The sequences of the 1052 bp fragments showed a high similarity with the isolates TBRFV-Jo and TBRFV-IL from Jordan and Israel respectively, using BLASTN 2.8.0+ searching tools (Zhang et al. 2000; Morgulis et al. 2008; Johnson et al. 2008). Although the four Palestinian isolate sequences showed high identity scores with one another (98%), they also revealed the presence of silent point mutations.

The full-length sequence of the virus consisted of 6391 nucleotides (nt) and showed a high similarity (99%) with TBRFV-IL (GenBank accession number KX619418) from Israel and TBRFV-Jo (KT383474) from Jordan (Luria et al. 2017; Salem et al. 2016) and an almost identical genomic size using RT-PCR genome walking based on the available sequences from both isolates of the virus (-IL and –Jo). Based on the genome sequences, this virus was determined to be an isolate of TBRFV, whose sequence was deposited in GenBank as TBRFV-Ps (MK165457).

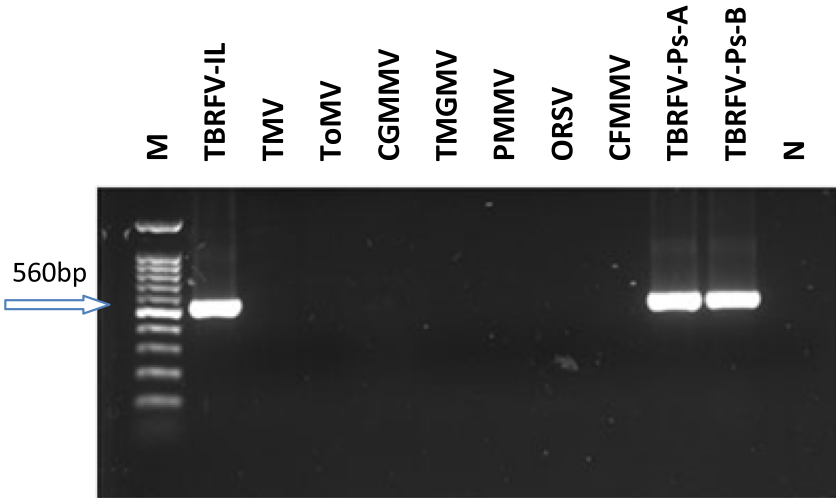

Fig. 2 TBRFV detection by RT-PCR using primers TBRFV-F and TBRFV-R in tomato plants infected with TBRFV isolates IL, Ps-A and Ps-B and seven other tobamoviruses (TMV, ToMV, CGMMV; TMGMV, PMMV, ORSV and CFMMV). The 100 bp Ladder was referred to as (M) while (N) is for negative control

Full genome sequence alignments for all tobamovirus species retrieved from GenBank were visually scanned for unique nonhomologous DNA sequence. PCR primers were developed based on these sequences in the small replicase subunit region. Thermal stability, T_m, GC content, primer dimer and hairpins were taken into consideration for primer design. Moreover, potential primer sequences were checked for the lack of sequence homology with other prokaryotic and eukaryotic DNA sequences with a BLAST search.

Primers developed based on these sequences in the small replicase subunit region were forward primer (ToBRFV-F) (5-AATGTCCATGTTTGTTACGCC-3) and reverse primer (ToBRFV-R) (5-CGAATGTGATTTAAAACTGTGAAT-3). PCR conditions were the same as above.

These primers proved to be specific for the new tobamovirus isolates. They amplified only the target sequence in the small replicase subunit region yielding a PCR product of 560 bp. However, the same primers in the same PCR conditions were unable to detect any of the other available tobamoviruses: TMV, ToMV, tobacco mild green mosaic virus (TMGMV), pepper mild mottle virus (PMMV), odontoglossum ringspot virus (ORSV) and cucumber fruit mottle mosaic virus (CFMMV), thus showing their specificity for the detection of the new tobamovirus (Fig. 2).

New emerging viruses are expected on tomato as it is planted extensively in the world (George et al. 2004; Kimura and Sinha 2008; Hanssen et al. 2010). In Palestine, tomatoes were reported to be infected by several pathogens and viruses (Amro et al. 2014), among which TYLCV was the most devastating virus causing crop losses of up 100% in some areas (George et al. 2004).

Very likely, TBRFV was introduced with imported plants, which calls for the urgent need to implement a certification program (Alkowni 2017) for which the development of molecular tools is essential for an accurate and reliable diagnosis.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest Corresponding Author has received research grants from An-Najah National University; in collaboration with co-authors from National Agricultural Research Center. Authors declare that they have no conflict of interest.

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