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Identification of Tomato mottle mosaic virus in historic seed accessions originating from France, the Netherlands and Spain, indicates a wider presence before its first description

Ruben Schoen · Pier de Koning · Carla Oplaat · Annelien Roenhorst · Marcel Westenberg · Dirk Jan van der Gaag · Ruud Barnhoorn · Harrie Koenraadt · Willem van Dooijeweert · Rik Lievers · Bert Woudt · Carla Pinto Tavares · Marleen Botermans

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Abstract Tomato mottle mosaic virus (ToMMV) is a tobamovirus found in a *Solanum lycopersicum* sample collected in Mexico in 2009. To assess the possible presence of ToMMV in Europe, accessions from a historic seed collection were tested by real-time RT-PCR and Illumina sequencing. ToMMV was identified in historical seed accessions produced in France, the Netherlands and Spain. Three different near complete genome sequences were obtained, each corresponding to the country in which the seeds had been produced. Positive samples from France and Spain could be related to the same production location and year, respectively, while the identical genome sequences from the Netherlands were obtained from samples produced in different locations and years between 1981 and 2007. The latter could be due to the fact that the Dutch seed accessions had been repacked in the past at the same location and time as accessions with a relatively high virus load from 2007. This indicates that possibly only the seeds from 2007 originated from ToMMV-infected plants, while the detection of ToMMV in the older seed accessions resulted from cross contamination. This data shows that ToMMV has been around in Europe before its first description and is possibly more widespread than currently known.

Keywords Tobamovirus · ToMMV · Real-time RT-PCR · Illumina sequencing · Seed accessions

R. Schoen (✉) · P. de Koning · C. Oplaat · A. Roenhorst · M. Westenberg · M. Botermans
Netherlands Institute for Vectors, Invasive Plants and Plant Health, Netherlands Food and Product Safety Authority, Wageningen, The Netherlands
e-mail: r.schoen@nvwa.nl

D. J. van der Gaag
Office for Risk Assessment & Research, Netherlands Food and Product Safety Authority, Utrecht, The Netherlands

R. Barnhoorn · H. Koenraadt
Naktuinbouw, Roelofarendsveen, The Netherlands

W. van Dooijeweert · R. Lievers
Centre for Genetic Resources, the Netherlands, Wageningen, The Netherlands

B. Woudt
Syngenta, Enkhuizen, The Netherlands

C. P. Tavares
Enza Zaden Seed Operations BV, Enkhuizen, The Netherlands

Tomato mottle mosaic virus (ToMMV) is a tobamovirus first detected in *Solanum lycopersicum* in Mexico in 2009 (Li et al., 2013). Since ToMMV is a relatively recently described virus there are still uncertainties about its origin and geographical distribution. In the past ToMMV may have been identified as one of the common tobamoviruses in *S. lycopersicum*, i.e. tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV), because of similarities in symptomatology and cross-reactivity in serological tests (Sui et al., 2017). ToMMV infections in *S. lycopersicum*

Table 1 Primers and probes used in a multiplex reaction for detection of ToMMV

Primers/Probes	Sequence 5'-3'
ToMMV CaTa 9-F	ATGTGGAGGAACCCTCTATGA
ToMMV CaTa 9-R	AATCTCCTCGCTCCTTGTAAAC
ToMMV CaTa 9-P	ATTO532-TCAATGGCCCGTGGTGAGTTACAA-BHQ1
ToMMV2-F	GAAACATTGGATGCCACTCG
ToMMV2-R	CTCTGGTTGTAGAAACCTGTTCC
ToMMV2-P	FAM-CGATGCTACGGTTGCGATCAGGTC-BHQ1

plants collected in Brazil in 1992, for instance, were not reported before 2018 (Nagai et al., 2018). To gain more insight into its presence in Europe, historical seed accessions of *S. lycopersicum* and *Capsicum* spp. (including wild relatives) from the collection of the Centre of Genetic Resources, the Netherlands (CGN), were tested for ToMMV.

Seeds from different accessions were bulked per host, country, production location and production year (total 800–1200 seeds per sample) and screened by a validated ToMMV-specific multiplex real-time RT-PCR test, (see Table 1 for primer and probe sequences), using bacopa chlorosis virus as an internal control (ISF, 2020).

Seeds were ground in GH + buffer, followed by RNA extraction using the Sbeadex® maxi plant kit (LGC genomics) on a KingFisher KF96 system following the manufacturer's instructions (Botermans et al., 2013). Reaction mixtures consisted of 6.25 µl UltraPlex™ 1-Step ToughMix® (4x) (Quanta Biosciences), 0.4 µM of each primer, 0.2 µM of each probe, 6 µl RNA extract, and sterile water added up to a final volume of 25 µl. Real-time RT-PCRs were carried out in 96-well plates on a Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories), cycle conditions 10 min 50 °C, 3 min 95 °C, followed by 40 cycles 10 s 95 °C and 1 min 60 °C, and results were analyzed using Bio-Rad CFX manager 2.0 software (Bio-Rad Laboratories).

From the RNA extracts that tested positive for ToMMV (Cq values ≤ 30) a selection of sixteen bulked samples was made for analysis by Illumina sequencing. The selection was based on virus load (Cq values 3–23), variation in host, country, production location and production year (Table 2). RNA extracts were rRNA-depleted using Ribo-Zero Plant Kit (Illumina, USA).

Table 2 Samples selected for analysis by Illumina sequencing, including information on host, number of accessions in bulked sample, country, location and production year(s)

Sample ID	Host (number of accessions)[1]	Country	Production location	Production year(s)
NL-41833893	Tomato (31)	Netherlands	1	1981
NL-41833906	Tomato (34)	Netherlands	1	1982
NL-41833914	Tomato (21)	Netherlands	1	2002, 2004, 2005
NL-41833922	Tomato (22)	Netherlands	2	2006
NL-41833930	Tomato (19)	Netherlands	3	2006
NL-41979261	Tomato (13)	Netherlands	4	2006
NL-41979518	Tomato (17)	Netherlands	5	2006
NL-41979454	Tomato (18)	Netherlands	4	2007
NL-41979462	Tomato (9)	Netherlands	5	2007
NL-41979470	Tomato (4)	Netherlands	6	2007
NL-41979489	Tomato (14)	Netherlands	2	2007
FR-41979446	Tomato (5)	France	7	2010
FR-41979421	Tomato (8)	France	7	2011
FR-41979438	Tomato (6)	France	7	2011
ES-41979403	Pepper (7)	Spain	8	2015, 2016
ES-41979411	Pepper (13)	Spain	8	2016

[1]Includes accessions of wild relatives of listed species

Library preparation was performed using the NEB Next Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs), followed by sequencing on a NovaSeq 6000 platform (Illumina) as 150 bp paired-end reads. The RNA-seq data were analyzed with CLC Genomics workbench v. 12.0.1 (Qiagen, Germany) by a custom workflow designed for the detection of *de-novo* assembled viral contigs as described in Hammond et al. (2021). For analysis of the consensus sequences (sequence depth > 10; size > 100nt), megaBLAST and DIAMOND (Buchfink et al., 2015) were used in combination with local downloaded versions of the NCBI nr /nt databases. BLAST results were visualized in Krona (Ondov et al., 2011). Additionally, the viral contigs were checked to make sure they were not created from different genotypes of ToMMV. ToMMV and ToMV viral sequences were further analyzed in Geneious Prime 2022.1.1 (Biomatters, New Zealand).

ToMMV was identified in all sixteen samples analyzed with Illumina sequencing, but only three different (near) complete ToMMV genome sequences with 99.5–99.6% nucleotide identity were obtained (Fig. 1). The remaining sequences were identical to one of these three genome sequences. The three unique genotypes each appeared to relate to the country where the seeds had been produced, i.e. the Netherlands (NL-41979470, tomato, 2007) (9 M mapped reads, 39% of total reads for an average coverage of 215 K; ON987482), France (FR-41979438, tomato, 2011) (25 M mapped reads, 86% of total reads for an average coverage of 566 K; ON987481), and Spain (ES-41979411, pepper, 2016) (8 M mapped reads, 6% of total reads for an average coverage of 187 K; ON987480). Sequences from the same country of origin were 100% identical. The shared sequence identities with the GenBank reference sequence of ToMMV (NC_022230) were 99.6% (NL;ON987482), 99.7% (FR; ON987481) and 99.7% (ES; ON987480). Additionally, various ToMV sequences were detected in the seed samples originating from the Netherlands (ON987475 to ON987479).

The obtained ToMMV sequence from pepper seeds from Spain showed 99.1% nucleotide identity to a sequence previously reported from tomato in that country (KU594507) (Ambrós et al., 2017). Since the genome diversity of ToMMV is relatively low, i.e. 98–100% nucleotide identity for all known ToMMV

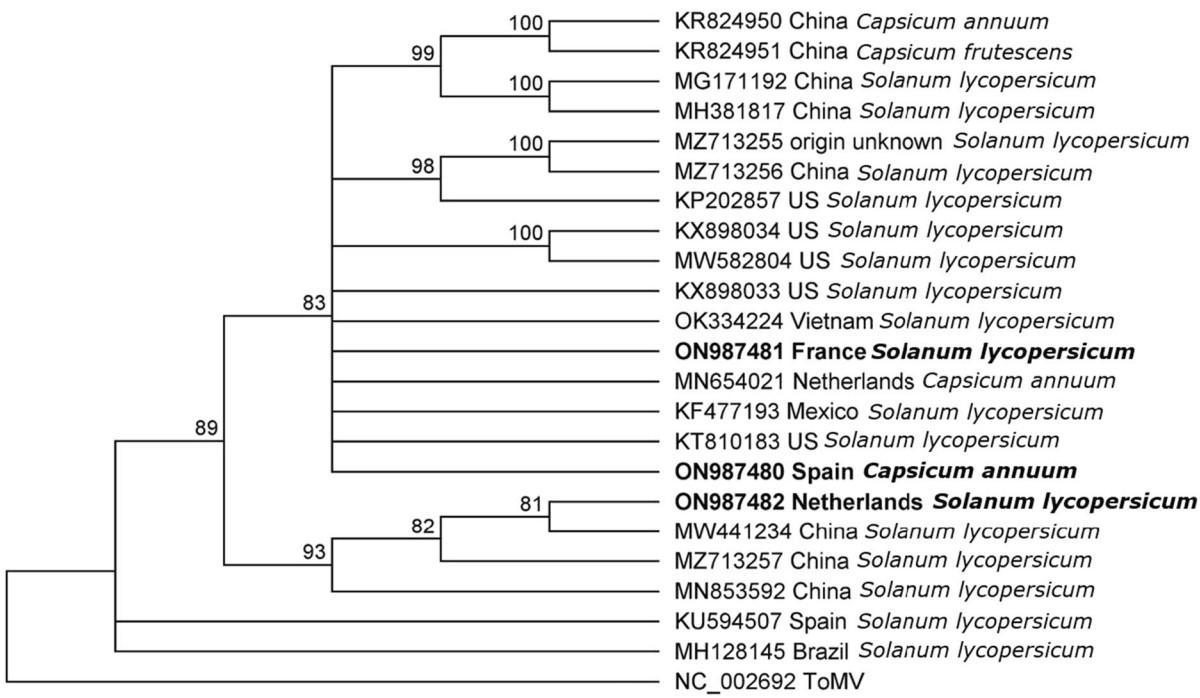

Fig. 1 Neighbor-Joining tree (100 bootstrap replicates) of complete ToMMV sequences available in GenBank and obtained in this study from France, the Netherlands and Spain (bold), ToMV as outgroup

genomes, a nucleotide identity of 99.1% suggests that the newly obtained and previously published Spanish sequence originated from different sources. Furthermore, the similarity of the genome sequences obtained from the three French tomato and two Spanish pepper samples in this study, was not unexpected because the tested accessions were from the same host and had been produced at the same location in the same years. In contrast, the similarity between the sequences from the eleven Dutch tomato samples was unexpected because the tested seeds had been produced at six different geographic locations in different years between 1981 and 2007. This raised the question whether cross contamination could have occurred between the accessions. One of the samples from 2007, NL-41979470, had an exceptional high virus load (Cq 3), in comparison to the others (Cq 13–23). Cross contamination during the current testing was not considered likely, based on the conformity of the first-line controls during RNA isolation and RT-PCR. However, cross contamination might have occurred during repacking of the seed accessions at CGN in the past. From their archives it appeared that most of the old seed accessions tested, had been repacked at the same location and time as the seeds with the high virus load from 2007. For accessions produced before 2007, it was not possible to trace back whether these seeds had become infested during production or by cross contamination during repacking. Therefore, it was concluded that at least the seeds from one or more accessions of NL-41979470 produced in 2007 originated from ToMMV-infected plants. Cross contamination after production of these seeds was considered unlikely due to the high virus load (Cq 3).

In summary, ToMMV was identified in seed accessions produced in different countries before its first description in 2013 (Li et al., 2013). ToMMV has already been reported from Spain (Ambrós et al., 2017) and the Netherlands (Fowkes et al., 2022) but to our knowledge this is the first report of ToMMV in seeds produced in France. The results of this study, together with the report on its presence in a sample misidentified as ToMV in the past (Nagai et al., 2018), indicate that ToMMV had already been present before its first description and is possibly more widespread than currently known.

Declarations

Ethics This article did not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

References

Ambrós, S., Martínez, F., Ivars, P., Hernández, C., De La Iglesia, F., & Elena, S. F. (2017). Molecular and biological characterization of an isolate of Tomato mottle mosaic virus (ToMMV) infecting tomato and other experimental hosts in eastern Spain. *European Journal of Plant Pathology, 149*, 261–268. https://doi.org/10.1007/s10658-017-1180-2

Botermans, M., Van De Vossenberg, B. T., Verhoeven, J. T. J., Roenhorst, J. W., Hooftman, M., Dekter, R., & Meekes, E. T. (2013). Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. *Journal of Virological Methods, 187*, 43–50. https://doi.org/10.1016/j.jviromet.2012.09.004

Buchfink, B., Xie, C., & Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nature Methods, 12*, 59–60. https://doi.org/10.1038/nmeth.3176

Fowkes, A. R., Botermans, M., Frew, L., De Koning, P. P. M., Buxton-Kirk, A., Westenberg, M., Ward, R., Schenk, M. F., Webster, G., Alraiss, K., Harju, V., Skelton, A., Conyers, C., Barrett, B., Adams, I. P., Mcgreig, S., Fox, A. & Vazquez-Iglesias, I. (2022). First report of Tomato mottle mosaic virus in Solanum lycopersicum seeds in The Netherlands and intercepted in seed imported from Asia. *New Disease Reports, 45,* e12067. https://doi.org/10.1002/ndr2.12067

Hammond, J., Adams, I. P., Fowkes, A. R., Mcgreig, S., Botermans, M., Oorspronk, J. J. A., Westenberg, M., Verbeek, M., Dullemans, A. M., Stijger, C. C. M. M., Blouin, A. G., Massart, S., De Jonghe, K., Heyneman, M., Walsh, J. A., & Fox, A. (2021). Sequence analysis of 43-year old samples of Plantago lanceolata show that Plantain virus X is synonymous with Actinidia virus X and is widely distributed. *Plant Pathology, 70*, 249–258. https://doi.org/10.1111/ppa.13310

ISF. (2020). Detection of Infectious Tomato brown rugose fruit virus (ToBRFV) in Tomato and Pepper Seed. https://www.worldseed.org/wp-content/uploads/2020/03/Tomato-ToBRFV_2020.03.pdf. Accessed 7 September 2022

Li, R., Gao, S., Fei, Z. & Ling, K. S. (2013). Complete genome sequence of a new tobamovirus naturally infecting tomatoes in Mexico. *Genome Announcements,* 1. https://doi.org/10.1128/genomeA.00794-13

Nagai, A., Duarte, L. M. L., Chaves, A. L. R., Alexandre, M. a. V., Ramos-Gonzalez, P. L., Chabi-Jesus, C., Harakava, R. & Dos Santos, D. (2018). First Complete Genome Sequence of an Isolate of Tomato Mottle Mosaic Virus Infecting Plants of Solanum lycopersicum in South America. *Genome Announcements,* 6. https://doi.org/10.1128/genomeA.00427-18

Ondov, B. D., Bergman, N. H., & Phillippy, A. M. (2011). Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics, 12*, 385. https://doi.org/10.1186/1471-2105-12-385

Sui, X., Zheng, Y., Li, R., Padmanabhan, C., Tian, T., Groth-Helms, D., Keinath, A. P., Fei, Z., Wu, Z., & Ling, K. S. (2017). Molecular and Biological Characterization of Tomato mottle mosaic virus and Development of RT-PCR Detection. *Plant Disease, 101*, 704–711. https://doi.org/10.1094/PDIS-10-16-1504-RE