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Tomato marchitez virus, a new plant picorna-like virus from tomato related to tomato torrado virus

M. Verbeek[1], **A. M. Dullemans**[1], **J. F. J. M. van den Heuvel**[2], **P. C. Maris**[2], **R. A. A. van der Vlugt**[1]

[1] Plant Research International BV, Wageningen, The Netherlands
[2] De Ruiter Seeds, Bergschenhoek, The Netherlands

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Summary

A new virus was isolated from a tomato plant from the state of Sinaloa in Mexico. This plant showed symptoms locally known as ‘marchitez disease’: severe leaf necrosis, beginning at the base of the leaflets, and necrotic rings on the fruits. A virus was isolated from the infected plant consisting of isometric particles with a diameter of approximately 28 nm. The viral genome consists of two (+)ssRNA molecules of 7221 (RNA1) and 4898 nts (RNA2). The viral capsid contains three coat proteins of 35, 26 and 24 kDa, respectively. The abovementioned characteristics: symptoms, morphology, number and size of coat proteins, and number of RNAs are similar to those of the previously described tomato torrado virus (ToTV). Sequence analysis of the entire viral genome shows that this new virus is related to, but distinct from, ToTV and that these members of two obviously new virus species belong to the recently proposed plant virus genus Torradovirus. For this new virus, the name tomato marchitez virus (ToMarV) is proposed.

Introduction

In 2003 a new disease was found in tomatoes grown in the fields and screenhouses in the state of Sinaloa in Mexico. Typical symptoms observed were leaf necrosis, beginning at the base of the leaflets (Fig. 1A), as well as dark necrotic rings on the fruits, already visible on the unripe green fruits (Fig. 1B). In severely infected plants, the top of the plant showed necrosis and malformation. Economic losses are substantial due to the inhibited growth of the tomato plant and to the unmarketable fruits. Locally, this disease is known as ‘marchitez’, meaning wilted or withered.

Because of the resemblance in leaf symptoms, it was initially assumed that Marchitez disease was caused by the recently described tomato torrado virus (ToTV) [15]. However, ToTV could not be detected in tomato plants showing symptoms of Marchitez disease with the aid of ToTV-specific primers in PCR [15]. In infected material, a spherical virus was found that shares several characteristics with ToTV. In this paper, we describe and characterize this new virus, related to but clearly distinct from ToTV, which we tentatively name to-

Nucleotide sequence data reported are available in the GenBank database under accession numbers EF681764 (RNA1) and EF681765 (RNA2).

Correspondence: Martin Verbeek, Plant Research International BV, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands
e-mail: martin.verbeek@wur.nl

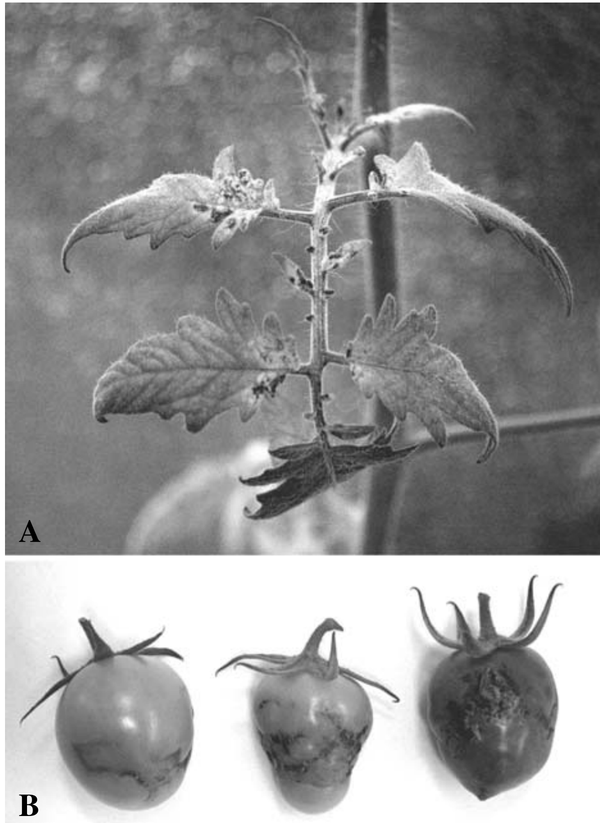

Fig. 1. Typical symptoms of tomato marchitez virus in (**A**) tomato leaves: necrosis surrounded by a yellow or bright green area, beginning at the base of the leaflets, and (**B**) tomato fruits: necrotic rings and patches

mato marchitez virus (ToMarV), a member of the newly proposed genus Torradovirus.

Material and methods

Virus transmission and propagation

ToMarV was isolated in 2005 from a tomato plant from Culiacán, Sinaloa, Mexico, that showed severe necrosis. The isolate was designated PRI-TMarV0601. Using 0.03 M sodium/potassium phosphate buffer, pH 7.7, the virus was mechanically inoculated to and maintained in *Nicotiana glutinosa* 'PRI', *Physalis floridana* or *N. benthamiana*.

Virus purification

All centrifugation steps were carried out at 6 °C. Systemically infected leaves of *N. benthamiana* were harvested approximately 14 days post-inoculation, homogenized in 5 volumes (w/v) extraction buffer (0.1 M Tris–HCl, pH 8, containing 20 mM Na_2SO_3, 10 mM Na-DIECA and 5 mM Na-EDTA) and squeezed through cheesecloth. Three volumes of a 1:1 mixture of chloroform/butanol were added and, after mixing, the suspension was centrifuged at 16,500 *g* for 10 min. Triton X-100 was added up to a concentration of 1% to the water phase and the suspension was stirred for 30 min. Then, PEG 6000 and NaCl were added to concentrations of 5% and 2.3%, respectively, and solubilized by stirring for 1 h. The suspension was left to settle for 1 h and centrifuged for 15 min at 21,500 *g*. The pellets were resuspended in a total of 80 ml extraction buffer and centrifuged for 10 min at 15,000 *g*, and the supernatant was placed on a 30% sucrose cushion. After centrifugation for 3 h at 70,500 *g*, the pellets were resuspended in a total of 2 ml Tris buffer, pH 8.0, and centrifuged for 2 min at 14,000 *g*. The virus suspension was loaded onto a 10–40% cesium sulfate gradient and centrifuged for 16 h at 126,000 *g*. Virus bands were collected and dialyzed against 0.1 M Tris–HCl, pH 8.0.

Electron microscopy

Virus suspensions were mounted on formvar-carbon coated grids, stained with 2% uranyl acetate and examined using a Philips CM12 electron microscope.

Polyacrylamide gel electrophoresis

Viral proteins were separated by subjecting purified virus particles to 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE) [6], and visualized by silver staining.

Nucleic acid isolation and evaluation

Purified virus was concentrated by centrifugation (at 115,000 *g* for 2 h). RNA was extracted from pelleted virus particles using a Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions.

RNA concentration was determined in a Beckmann DU 530 UV-spectrophotometer. Viral RNA integrity and size were checked on a 1% agarose gel using a formaldehyde/formamide/HEPES buffer system. After electrophoresis, the RNA was stained using ortho-toluidine blue.

Total RNA for RT-PCR was isolated from ToMarV-infected *Nicotiana occidentalis* 'P1' plants using an RNeasy plant mini kit (Qiagen).

RT-PCR and 5′ RACE

PCR fragments were obtained by one-tube RT-PCR (Access RT-PCR system, Promega). RT-PCRs were initiated using a universal oligo(dT) primer [14] and various primers derived from the ToTV RNA1 and RNA2 sequences (GenBank accession numbers DQ388879 and DQ388880, respectively). The 5′ regions of the ToMarV RNAs were determined by walking towards the 5′ end of the viral genome through

repeated use of a 5′ RACE kit (Roche) in combination with the Expand high-fidelity PCR system (Roche), essentially as described previously [9, 13]. cDNA primers for the 5′-RACE strategy and primer sets for additional RT-PCR reactions were based on newly obtained ToMarV sequence data.

Nucleotide sequencing and sequence analysis

All PCR products (also those resulting from the 5′ RACE) were purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced.

Sequence analysis was performed with an Applied Biosystems 3100 Genetic Analyser, using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham) and the primers that were used for amplification. For longer PCR fragments, ToMarV-specific primers were used for primer walking sequencing.

Nucleotide and amino acid sequence data were analyzed and assembled using the DNASTAR package (Lasergene).

Sequence comparisons with other viruses were performed with programs from the PHYLIP package. Multiple alignments and phylogenies were performed with the CLUSTAL X program after bootstrapping in 1000 replicates. Neighbour-joining consensus phylogenies were viewed using the NJplot program [11] and printed by using TreeView [10].

Table 1. Symptoms of tomato marchitez virus (ToMarV) and tomato torrado virus (ToTV) on experimental host plants

Tested experimental host plants	Symptoms (local/systemic)	
	ToMarV	ToTV
Chenopodium quinoa	nl/noi	−/noi
Gomphrena globosa	n.t.	−/noi
Nicotiana benthamiana	−/c, mf	−/c, mf
Nicotiana clevelandii	−/c	−/c
Nicotiana glutinosa ‘PRI’	occasionally cl/c	−/c
Nicotiana hesperis ‘67A’	nl/c, n, mf	nl/c, n, mf
Nicotiana occidentalis ‘P1’	nl/c, n, mf	nl/c, n, mf
Nicotiana rustica	−/la	−/la
Nicotiana tabacum ‘white burley’	occasionally cl/la	−/la
Physalis floridana	nl/mo	nl/c, n, mf, do

c Chlorosis; *cl* chlorotic lesions/rings; *do* die-off; *la* latent infection (verified by inoculation on indicator plants); *mf* malformation; *mo* mottle; *n* necrosis; *noi* no infection (verified by inoculation on indicator plants); *nl* necrotic lesions; *n.t.* not tested; – no symptoms.

Results and discussion

Virus characterization

ToMarV was easily transmitted to a number of indicator plants by mechanical inoculation. Table 1 gives an overview of the experimental hosts used and their reactions to ToMarV and ToTV. Most symptoms of ToMarV and ToTV in indicator plants resemble each other, except for the reactions in two host plants. In *P. floridana*, ToMarV induces a systemic mottle and only occasionally necrosis in the locally infected leaves, whereas ToTV causes severe necrosis and die-off in this plant. In *Chenopodium quinoa*, ToMarV causes necrotic pin point lesions in inoculated leaves which remain symptomless when inoculated with ToTV.

Electron microscopic examinations of leaf extracts from tomato showing marchitez symptoms and from systemically infected leaves of *Nicotiana occidentalis* ‘P1’ revealed the presence of spherical virus particles with a diameter of 28–30 nm. The particles of ToMarV clearly resembled the particles of ToTV in shape and size.

In initial attempts at ToMarV purification, the protocol designed for ToTV was used [15]. This protocol did not lead to visible virus bands in the final Cs_2SO_4 gradient. Electron microscopic analysis of gradient fractions revealed that virus particles were present in the bottom part of the gradient. However, this part of the gradient also contained plant constituents, veiling the virus bands. Therefore, another purification protocol was designed in which the separation of the virus from plant components was aided by the use of a chloroform-butanol mixture. This protocol resulted in one diffuse band in the Cs_2SO_4 gradient. This result is in contrast with that obtained for ToTV purifications, which always yielded two distinct bands following Cs_2SO_4 gradient centrifugation. The ToMarV band was collected from the gradient and examined by electron microscopy. Virions of the expected size of 28 nm were present in the collected band and were infectious when mechanically inoculated to test plants. Purified virions were also mechanically transmitted to tomato plants, which showed characteristic symptoms of marchitez disease two weeks after inoculation. The presence of ToMarV in these

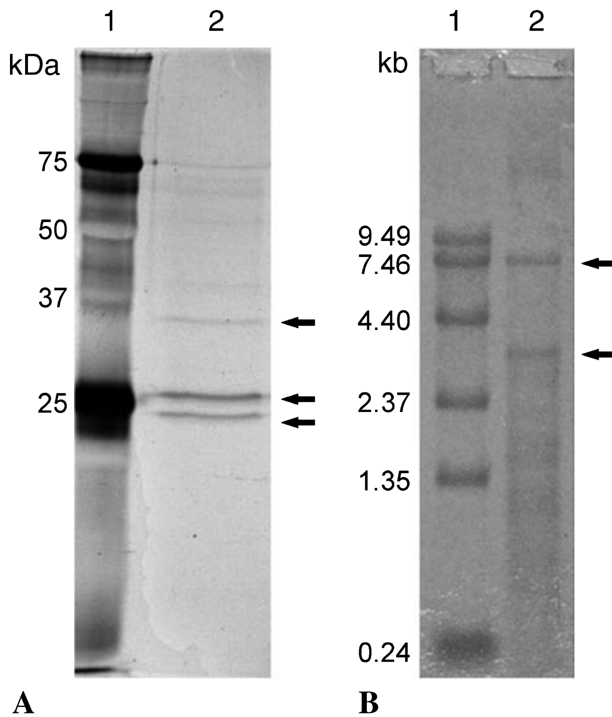

Fig. 2. (**A**) Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of ToMarV capsid proteins. Proteins were visualized by silver staining. *1* Molecular weight markers (Bio-Rad Precision Plus Protein Standards (note: the pre-stained 37-kDa and 50-kDa standards do not stain in the silver staining method used); *2* ToMarV purified virions after Cs_2SO_4 buoyant density gradient centrifugation. *Arrows* indicate positions of the coat protein bands. (**B**) Denaturing agarose gel electrophoresis of RNA extracted from ToMarV virions and stained with ortho-toluidine blue. *1* Molecular size standard (Invitrogen 0.24–9.5-kb RNA Ladder); *2* RNA purified from ToMarV virions. *Arrows* indicate positions of the RNA bands

plants was confirmed by electron microscopy and RT-PCR.

Purified virions were subjected to SDS-PAGE, and three viral proteins were detected with estimated sizes of 35, 26 and 24 kDa, named Vp35, Vp26 and Vp24, respectively (Fig. 2A). The number and estimated molecular sizes of the viral coat proteins of ToMarV are the same as previously found for ToTV [15].

When purified virus preparations of ToMarV were analyzed on a denaturing RNA gel, two RNA molecules with estimated sizes of 7.5 kb (RNA1) and 4 kb (RNA2) were visualized (Fig. 2B). The number of RNAs found is in accordance with ToTV, but their estimated sizes are smaller (ToTV RNA1 [8.5 kb] and RNA2 [5.5 kb]).

Viral RNA analysis

Based on biological and structural data like indicator plant symptoms, particle sizes and morphology, number and sizes of coat proteins, and number of RNAs obtained for ToMarV, a possible relationship with ToTV was suspected. Therefore, different upstream primers were derived from the ToTV RNA1 (DQ388879) and RNA2 (DQ388880) sequences and used in combination with a general oligo(dT) primer for RT-PCR. This resulted in a limited number of PCR fragments, indicating possible differences in RNA sequences between the two viruses. Sequence analyses of these fragments revealed low levels of similarity with the ToTV RNAs 1 and 2. Based on the obtained sequence information, new cDNA primers were generated and used to obtain additional sequence information in a 5′-RACE sequence-walking strategy.

ToMarV-specific primers derived from newly obtained sequences were used for RT-PCR to confirm sequences of both RNAs in two orientations.

RNA1

RNA1 [7221 nucleotides (nts), excluding poly(A) tail] contains one open reading frame (RNA1-ORF1) of 6453 nts encoding a predicted polyprotein of 2151 amino acids (aa) with a molecular mass of 237 kDa (Fig. 3). The first in-frame AUG is found at nt positions 141–143. The ORF has an UGA stop codon at positions 6594–6596. The putative polyprotein sequence contains several conserved regions with motifs typical for helicase and

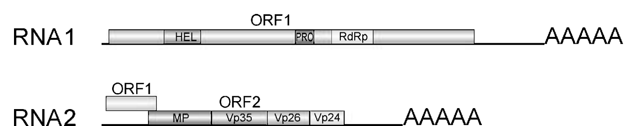

Fig. 3. Genome organization of tomato marchitez virus. Relative positions of regions containing motifs of helicase (*HEL*), protease (*Pro*), and RNA-dependent RNA polymerase (*RdRp*) on RNA1 and of the putative movement protein (*MP*) and the three coat proteins (*Vp35*, *Vp26* and *Vp24*) encoded on RNA2 are indicated

RNA-dependent RNA polymerase (RdRp). The ORF1-encoding region shows 65% overall identity with the ToTV-RNA1 (DQ388879) at both the nucleotide level (6474 nts) and the amino acid level (2158 aa).

Typical helicase motifs A (GKS), B (D), C (N) were identified at aa positions 397–399, 443 and 494 of the putative ORF1-encoded protein. The RdRp region could be identified between aa 1305 and 1553 by the presence of the typical motifs I (KDE) to VII (FLSR) [5].

The helicase region between motifs A and C in the C-terminal part of RNA1-ORF1 showed 95.6% aa identity with the corresponding region of ToTV and significantly lower levels of identity with other viruses ranging from 46.9% for maize chlorotic dwarf virus (MCDV, genus *Waikavirus*, AAV86083) to 25.4% for acute bee paralysis virus (ABPV, unassigned species in the family *Dicistroviridae*, NP_066241).

Levels of aa identity for the RdRp region, motifs I to IV in ORF1 of RNA1, were relatively high with ToTV (85%) and significantly lower with other viruses: 40% for cherry rasp leaf virus (CRLV, genus *Cheravirus*, YP_081444) to 38% for strawberry latent ringspot virus (SLRSV, genus *Sadwavirus*, NC_006764). These levels of identity indicate a close level of relationship between ToMarV and ToTV.

A BLAST search with our available data for ToMarV RNA1 revealed a remarkably high level of overall sequence identity (95%) with a partial sequence in the NCBI data base submitted under the name of tomato apex necrosis virus (ToANV, accession number EF0636641), which only became available during the course of our work. In the coding region of RNA1, the overall level of identity between ToMarV and ToANV is 96% at the nt level and 99% at the protein level with levels of aa sequence identity of 100% for both the RdRp and helicase motifs. These percentages suggest a close relationship between ToMarV and the partial sequence deposited as ToANV RNA1.

RNA2

As with ToTV, RNA2 [4898 nts, excluding poly(A) tail] of ToMarV contains two open reading frames (RNA2-ORF1 and RNA2-ORF2) encoding predicted polyproteins of 190 and 1191 amino acids (aa) with molecular masses of 21 and 131 kDa, respectively (Fig. 3). The first in-frame AUG is at position 139–141. It is likely that this start codon maps the start of ORF1 given the lack of significant sequence identity of the ToMarV sequence directly upstream of this AUG start codon with the 5′UTR of ToTV and the significant levels of aa sequence identity with ORF1 of ToTV just downstream from this AUG start. The ORF has an UAA stop codon at positions 709–711, implying a partial overlap with ORF2. The overall level of sequence identity between ORF1 of ToMarV and ToTV is around 63% at both the nt level (570 nts) and the aa level (190 aa). A BLAST search revealed no significant homologies with other viruses either at the nt or the aa level.

For the second ORF (RNA2-ORF2), the first inframe AUG is at position 709–711. A UAA stop codon is found at nt positions 4244–4246. ORF2 encodes a putative protein of 1191 aa. This ORF shows 66.1% identity with the ToTV RNA2-ORF2 (DQ388880) at the nt level (3573 nts) and 69.9% at the aa level (1191 aa). The only other significant similarity is found with the incomplete sequence of ToANV RNA2 (EF0636642). In comparable regions of ToMarV RNA2-ORF2, the nt and aa sequence identities are 78% and 90%, respectively.

By analogy with ToTV, the RNA2-ORF2-encoded protein is likely to be a polyprotein coding for a putative movement protein (MP) and three structural coat proteins (CPs). Indeed the N-terminal region of the RNA2-ORF2 polyprotein most likely codes for an MP since the motif LRVPTL, which is highly similar to the proposed movement protein consensus sequence LxxPxL [8], was found at aa position 263–268. The overall level of identity in the movement protein region (aa 1–470) between ToMarV and ToTV is 62.1%.

The relative order of the three putative coat proteins of ToMarV in the RNA2-ORF2 was derived from direct aa sequence comparisons with the ToTV CPs. The first CP (ToMarV-Vp35; aa 477–718) shows 72% identity with Vp35 of ToTV, the second CP (ToMarV-Vp26; aa 723–959) shows

86% identity with Vp26 of ToTV, and the third CP (ToMarV-Vp24; aa 975–1191) shows 71% identity with Vp23 of ToTV.

From direct aa sequence comparison of the ToMarV CP region with the corresponding region of the ToANV RNA2 it is likely that the relative order of the ToANV CPs is similar to that of ToMarV and ToTV. The ToANV CPs of 38.4, 28 and 23.8 kDa [12] show aa sequence identities of 89%, 98% and 94% with ToMarV Vp35, Vp26 and Vp24, respectively. In ToTV we identified putative cleavage regions between the different proteins encoded on ORF2 on the basis of direct protein sequencing of CPs [15]. No homologous regions were apparent in the ToMarV ORF2, and no homologies with known protease cleavage sites were found, leaving the exact polyprotein cleavage sites to be determined.

Since the RNA2 sequence 5′ of the three putative CPs of ToANV, including the putative movement protein region and the ORF1 is not available, no direct comparison with this region of ToMarV could be made.

5′- and 3′-untranslated regions (UTRs)

The 5′-UTR sizes of RNA1 and RNA2 are 140 and 138 nts, respectively, and share an overall level of identity of 58%, with the eight 5′-terminal nucleotides of RNA1 and RNA2 being identical.

The 3′-UTRs of RNA1 and RNA2 are 628 and 655 nts in length, respectively, and share an overall level of identity of 91%. The 553 most 3′-terminal nucleotides of both RNAs are almost perfectly conserved (99% identity). The nearly identical regions in the 3′-part of both 3′-UTRs are a characteristic that ToMarV shares with ToTV and the sequence of ToANV. However, direct sequence comparisons between the total 3′-UTRs of RNA1 and RNA2 of ToMarV and ToTV reveals only 49.0% and 48.9% sequence identity, respectively, between the two viruses, with a large difference in length of the 3′-UTRs of ToMarV (628 and 655 nts) and ToTV (1210 and 1092 nts). A comparison with the 3′-UTRs of ToMarV and ToANV RNA1 (630 nts) and RNA2 (650 nts), showed 88.5% and 85.6% sequence identity.

Taxonomic position of ToMarV

Tomato marchitez virus (ToMarV) showed similar host plant symptoms and shared virion characteristics and its genome organization with ToTV, but based on levels of nt and aa sequence identities, the two viruses should be considered related but distinct. Remarkably high levels of identity were observed between both RNAs of ToMarV and incomplete RNA sequences deposited for ToANV in the NCBI database. Only after submission of our paper did additional information on ToANV become available [12]. In this paper, ToANV is reported to also have three CPs of 38.4, 28 and 23.8 kDa, respectively. These correlate relatively well with the CP sizes of ToMarV (35, 26 and 24 kDa, respectively). The most distinctive symptoms for ToANV were described as necrosis of growing points, resulting in a brown brittle dieback. For ToMarV, leaf necrosis starting at the base of the leaflet was the most distinctive symptom, and necrosis of growing points was never observed upon back-inoculation on tomato plants using Cs_2SO_4 gradient purified virus. ToANV has been reported to induce mild mosaic symptoms on *N. clevelandii* and *N. tabacum* 'White Burley', while ToMarV causes systemic chlorosis in *N. clevelandii* and a latent systemic infection in *N. tabacum* 'White Burley'.

In addition, the relatively low levels of nucleotide sequence identity (less than 90%) between the two viruses in the 3′-UTRs of both RNA1 and RNA2 (88.5% and 85.6%, respectively) and in the largest putative CP (Vp35) (89%) suggest that the two viruses are not identical. They may be strains or isolates of the same virus species. Additional biological and molecular data on ToANV, especially the possible presence of ORF1 on RNA2, will be needed to determine its precise relationship to ToMarV.

Phylogenetic analysis based on the aa region between the CG protease motif [2] and the GDD RdRp active site [1] in the RNA1–ORF1 were performed to determine the relationships between ToMarV, ToTV, ToANV and other viruses from the genera *Sadwavirus*, *Cheravirus* and the families *Sequiviridae*, *Comoviridae*, *Dicistroviridae* and *Picornaviridae*. This region is proposed to be a good

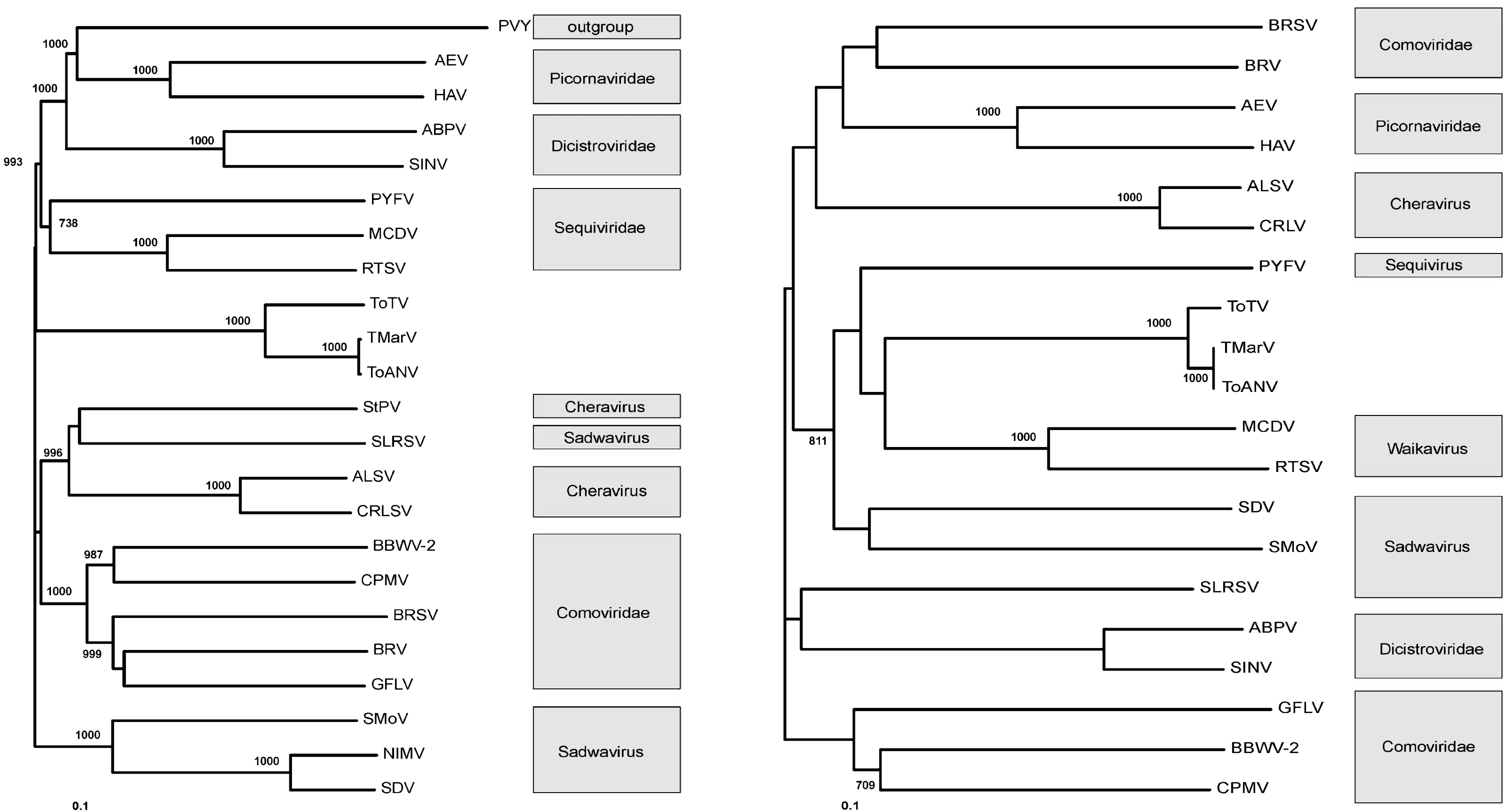

Fig. 4. Phylogenetic analysis of ToMarV and related viruses based on the alignment of (**A**) the region between the protease CG motif and the GDD RdRp motif (aa 1042–1499 of RNA1–ORF1) and (**B**) the helicase region between motifs A and C (aa 381–494 of RNA1–ORF1). Sequences included in the analysis are those of (with virus acronyms, genus and accession numbers in parentheses): acute bee paralysis virus (ABPV; unassigned species in the family *Dicistroviridae*; NP_066241), apple latent spherical virus (ALSV; *Cheravirus* NP_620568), avian encephalomyelitis virus (AEV; *Hepatovirus*; NP_653151), beet ringspot virus (BRSV; *Nepovirus*; NP_620112), blackcurrant reversion virus (BRV; *Nepovirus*; NP_612604), broad bean wilt virus-2 (BBWV-2; *Fabavirus*; AAK27841), cherry rasp leaf virus (CRLV; *Cheravirus*; YP_081444), cowpea mosaic virus (CPMV; *Comovirus*; NP_613283), grapevine fanleaf virus (GFLV; *Nepovirus*; NP_619689), hepatitis A virus (HAV; *Hepatovirus*; NP_041008), maize chlorotic dwarf virus (MCDV; *Waikavirus*; NP_619716), navel orange infectious mottling virus (NIMV; *Sadwavirus*; BAA74537), parsnip yellow fleck virus (PYFV; *Sequivirus*; NP_619734), rice tungro spherical virus (RTSV; *Waikavirus*; NP_042507), satsuma dwarf virus (SDV; *Sadwavirus*; NP_620566), Solenopsis invicta virus (SinV; unassigned species in the family *Dicistroviridae*; YP_164440), strawberry mottle virus (SMoV; *Sadwavirus*; NP_599086). strawberry latent ringspot virus (SLRSV; *Sadwavirus*; NC_006764), stocky prune virus (StPV; *Cheravirus*; DQ143874), tomato torrado virus (ToTV; ABD38934), tomato apex necrosis virus (ToANV; ABK33525). Potato virus Y (PVY; *Potyvirus*; ABA28320) was used as an outgroup sequence in the analyses. The numbers at each node are the bootstrap values for 1000 replicates (values below 700 are not shown). The scale bar represents the number of residue substitutions per site

taxonomic predictor for classifying picorna-like viruses [4]. The resulting dendrogram (Fig. 4A) shows that ToMarV virus clusters with ToTV and ToANV in a distinct branch. It also confirms the separate taxonomic position of these viruses from members of the genus *Cheravirus* [7, 15], the only other related plant virus genus whose members have 3 CPs and 2 RNAs. A similar phylogenetic analysis on the basis of the helicase region between the motifs A and C [3] (aa 397–494) confirms the separate taxonomic position of ToMarV, ToTV and ToANV (Fig. 4B).

The data we present in this paper describe ToMarV as a new picorna-like plant virus, related to but distinct from ToTV [15]. ToMarV and ToTV both share two ORFs on RNA2, which is a unique feature that clearly distinguishes them from other plant picorna-like viruses. These two viruses are likely to belong to the same new genus for which the name Torradovirus was recently proposed by the ICTV study group on plant picorna-like viruses.

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