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Characterization of a novel tymovirus on tomato plants in Brazil

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Abstract A tymovirus was isolated in Brazil from tomato plants with severe symptoms of leaf mosaic and blistering. The virus was mechanically transmissible to solanaceous indicator host species. The infected plants contained icosahedral particles and chloroplasts with membrane deformations which are typical cytopathic effects caused by tymoviruses. Its coat protein amino acid sequence shares the maximum of 64 % identity with the tymovirus Chiltepin yellow mosaic virus, which suggested that it can be considered as a distinct member of the genus *Tymovirus*. In a phylogenetic tree, this tymovirus was clustered with other solanaceous-infecting tymoviruses. It was tentatively named as Tomato blistering mosaic virus (ToBMV).

Keywords *Solanum lycopersicum* · *Tymoviridae* · Tomato blistering mosaic virus (ToBMV)

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Viruses in the genus *Tymovirus*, family *Tymoviridae,* generally replicate in high yields in plant hosts and are transmissible by flea beetles over short distances, but rarely by seeds [1]. They infect mainly dicotyledonous plants, including *Cucurbitaceae*, *Brassicaceae*, and *Solanaceae*, and occasionally cause serious diseases [1]. Most tymovirus species have natural and experimental host ranges restricted to one or very few plant families and their geographic distribution is also often restricted [2, 3]. To date, only three tymovirus species are reported in Brazil: *Passion fruit yellow mosaic virus* [4], *Eggplant mosaic virus* (EMV) [5–7], and *Petunia vein banding virus* [8].

The tymoviruses have a monopartite positive-sense single-stranded RNA genome. Virions are non-enveloped icosahedrons with a diameter of 28 nm ($T = 3$), composed of a single 20-kDa coat protein (CP) which is clustered in 20 hexameric and 12 pentameric subunits [2, 9]. Complete particles and empty protein shells coexist in the infected tissue. Typical tymoviral genomes (e.g., of *Turnip yellow mosaic virus*, TYMV) have approximately 6.3-kb long RNA with a cap structure (^{m7}GpppG) at the 5′ genomic end and with a tRNA-like structure (TLS), to which valine can be covalently linked, in the 3′ end of the virus genome [9]. The genome has a distinctive skewed C-rich, G-poor composition (39 % C, 17 % G for TYMV) [9] with three open reading frames (ORFs). ORF 1, the largest one, encodes a protein of 206 kDa with conserved motifs of a metyltransferase, a proteinase, a helicase, and an RNA-dependent RNA polymerase (RdRp). ORF 2 encodes a 69-kDa protein necessary for cell-to-cell movement, which is also involved in the suppression of RNA silencing [10, 11]. At the 3′ end of ORF 1, there is the so-called tymobox, a 16-nucleotide motif highly conserved among tymoviruses, which is thought to be the promoter for the subgenomic RNA for ORF 3 [12, 13], from which the 20-kDa CP is translated. CP amino acid

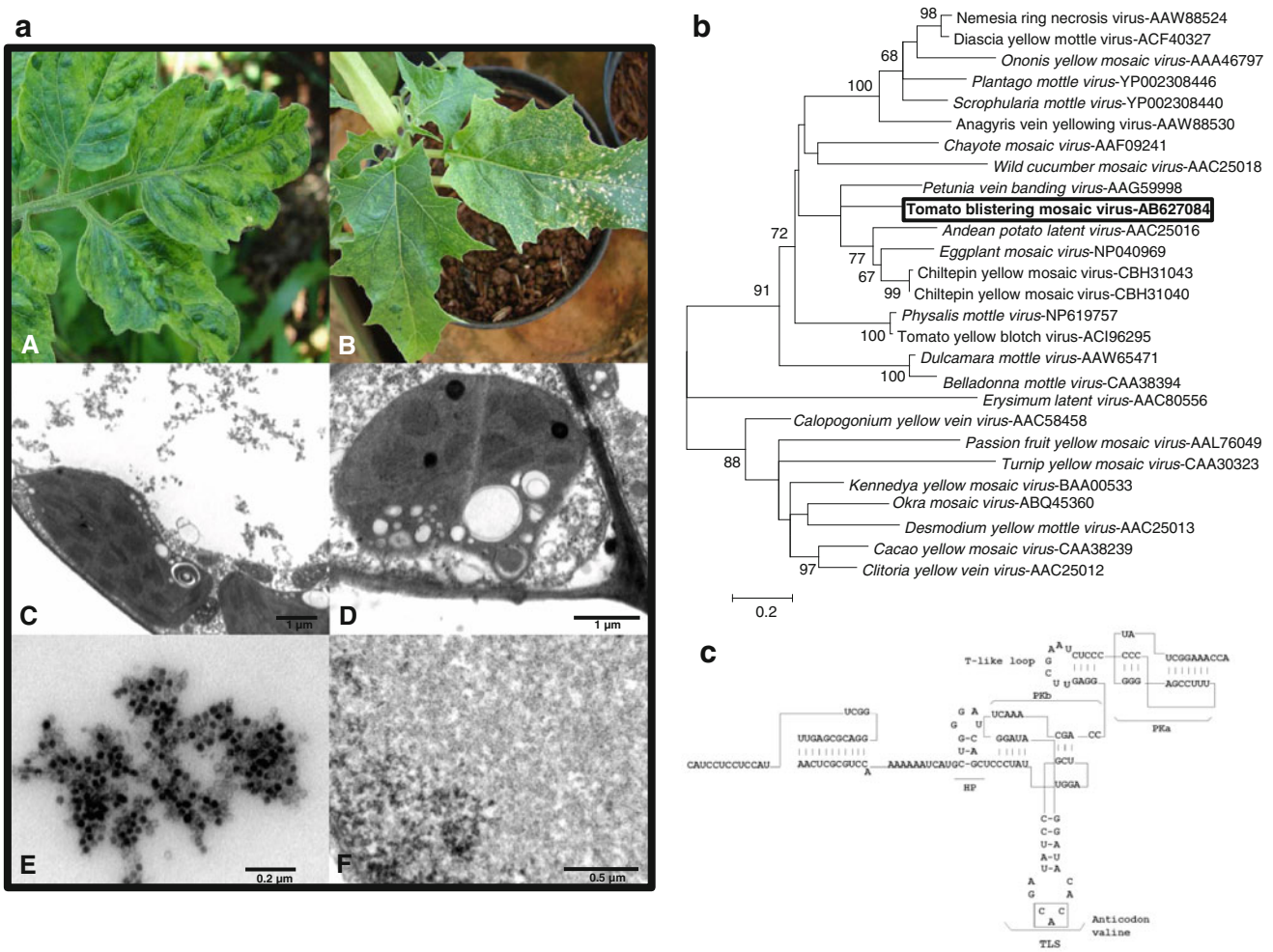

Fig. 1 **a** Plants infected by an isolate of Tomato blistering mosaic virus (ToBMV) (*A*, *B*). Symptoms of blistering and mosaic shown on a naturally infected tomato plant (*A*); and veinal necrosis, necrotic spots, and mottling on a sap inoculated *Datura stramonium* plant (*B*); transmission electron micrographs of ultra-thin sections of *Nicotiana benthamiana* leaf infected by Tomato blistering mosaic virus (ToBMV) (*C*, *D*, *E*, *F*). Part of spongy parenchyma cells showing chloroplasts with intense vesicularization at their borders (*D*). In (*C*), presumed virions are present in the vacuole, a detail of which can be seen in (*E*); (*F*) a nucleus of a palisade parenchyma cell showing a rarefied nucleoplasm, which is occupied with particles, interpreted as empty virions. **b** Phylogenetic tree based on the CP amino acid sequence of tymoviruses by the Maximum-Likelihood method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Tomato blistering mosaic virus (ToBMV) is boxed. [a]Accession numbers. **c**. Predicted secondary structure of the Tomato blistering mosaic virus (ToBMV) 3′UTR. The hairpin (HP) structure, anticodon stem-loop, T-like loop, and pseudoknots (PKa and PKb) are indicated. The anticodon for valine is shown in the box

sequences sharing less than 90 % identity is one of the species demarcation criteria in the genus [2].

Tomato plants with mosaic and blistering (Fig. 1a-A) were found in a commercial field in the State of Santa Catarina (SC) in March, 2010 (Coord. 27°47′51.3″S, 51°04′29.2″W, 1,001 m). Symptomatic plants were often found along a planting line, which strongly suggested a property of easy mechanical transmission by routine plant handlings. Less than five percent of the plants were symptomatic in three out of eight visited fields in the area. The absence of mixed infection with other tomato-infecting viruses reported in Brazil was confirmed (data not shown) by serological assays using antibodies against the potyviruses *Potato virus Y* and *Pepper yellow mosaic virus*, the tospoviruses *Tomato spotted wilt virus, Tomato chlorotic spot virus*, *Groundnut ringspot virus* and *Chrysanthemum stem necrosis virus*, the cucumovirus *Cucumber mosaic virus*, and the tobamovirus *Tomato mosaic virus*, and by PCR with universal primers for begomoviruses [14].

A biologic study of this presumed tymovirus was started with the mechanical inoculation on 12 test-plant species with sap prepared from freshly infected *D. stramonium* tissue diluted in 0.05 M phosphate buffer, pH 7.0, and 0.1 % sodium sulfite, previously dusted with carborundum.

Table 1 Symptoms caused by ToBMV on test plants after mechanical inoculation

Host plant	Symptoms
Capsicum annuum 'Ikeda'	–
C. chinenese 'PI 159236′	–
Chenopodium amaranticolor	Chlorotic spot
C. quinoa	Chlorotic spot
C. murale	Chlorotic spot
Datura metel	Necrotic spot, veinal clearing, mottling, leaf distortion
Datura stramonium	Necrotic ring spot, veinal necrosis, mosaic, blistering
Nicotiana benthamiana	Veinal clearing, mottling, leaf distortion, stuting
N. rustica	Chlorotic ring spot, veinal clearing, mottling, leaf distortion
N. tabacum TNN	–
Physalis pubescens	Veinal clearing, mottling, blistering
Solanum lycopersicum	Blistering, mosaic, mottling

The inoculated plants were maintained in a greenhouse, and the symptoms were monitored by visual observation until 30 days. The virus caused systemic chlorotic spots on *Chenopodium amaranticolor, C. quinoa*, and *C. murale* leaves. *Nicotiana rustica* showed chlorotic ring spots on the inoculated leaves and mottling in upper leaves. *Capsicum annuum*, *C. chinense*, and *N. tabacum* did not exhibit any symptoms. Severe systemic symptoms were observed on *Datura metel*, *D. stramonium* (Fig. 1a-B), *N. benthamiana*, *Physalis pubescens*, and tomato plants (Table 1). Symptoms observed on the infected tomato plants in the field in SC State were reproduced on the inoculated tomato plants. In addition to tomato plants, *D. stramonium* and *P. pubescens* were shown to be good propagation hosts.

Sections of an infected *N. benthamiana* plant were examined by transmission electron microscopy. Leaves were cut into small pieces, fixed in a mixture of 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, for 4 h, rinsed, and then post-fixed in 1 % osmium tetroxide and potassium ferrocianate at room temperature for 1 h. After dehydration on a graded acetone series, the tissues were embedded in Spurr Resin (Electron Microscopy Sciences, Tennessee, USA). Ultrathin sections were examined with the electron microscope JEM 1011 (JEOL Ltd., Tokyo, Japan). The cytopathic effects usually seen on tymovirus infected tissue were observed, such as double-membrane vesicles on the periphery of the chloroplasts; a large number of presumed isometric virions in the cytoplasm and vacuole; and empty isometric particles in the nucleus (Fig. 1a-C, D, E, F) [2, 15].

Together with electron microscopic observations, the positive reaction to EMV antibody by indirect ELISA (data not shown) suggested that the causal agent is a member of the *Tymovirus* genus, perhaps an isolate of EMV. In order to identify this virus, degenerated primers which targeted the conserved regions of the tymovirus *RdRp* gene were designed and Reverse transcription (RT)-polymerase chain reaction (PCR) was carried out. First, virus particles were purified as described by Gibbs and Mackenzie [16] and used for genomic RNA extraction using the Trizol LS reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Two primers were designed for RT-PCR: Tymo4547 For (5′-GNT CYG AYC CNG ACT GGC G-3′–N = A/G/C/T; Y = C/T) and Tymo5620 Rev (5′-ARR AGD GAA GCA ATT CAG ACT-3′–R = A/G; D = A/G/T), both targeting conserved regions of the *RdRp* gene (ORF1, close to the C-terminus of the *RdRp* region) and based on an alignment of five tymovirus sequences related to EMV, *Physalis mottle virus* (PhyMV, accession Y16104), Tomato yellow blotch virus (ToYBV, EU779803), *Okra mosaic virus* (OkMV, EF554577), *Plantago mottle virus* (PlMV, AY751779), and EMV (J04374). The RT reaction was performed using Superscript III (Invitrogen) and the primer Tymo5620 Rev followed by PCR using LongAmp *Taq* DNA polymerase (New England Biolabs Ipswich, MA, USA) according to the manufacturer's instructions. The amplicon (ca. 1,100 bp) was cloned into pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) according to manufacturer's instructions, and three clones were selected and sequenced at Macrogen Inc. (South Korea). A new forward primer TymoF3Race For (5′-TCC AAT CCA TGC CTT TTG AT-3), directed to the region close to the 3′ terminus of the obtained sequence, was designed for implementation of the 3′ RACE procedure to recover the cDNA of the entire ORF3 gene and 3′ UTR. First, a poly(A) tail was added to the 3′ hydroxyl termini of viral RNA molecules using the poly(A) polymerase and rATP (New England Biolabs) and the cDNA was synthesized with the OligodT50PacIM10 primer (5′-AAG CAG TGT TAT CAA CGC AGA TTA ATT AAT_{50}) using Superscript III RT (Invitrogen). Then, the viral genomic region of the 3′ cDNA end was amplified by PCR using the specific TymoF3Race For and anchor (M10: 5′-AAG CAG TGT TAT CAA CGC AGA-3′) primers, producing an amplicon of approximately 780 bp. These amplified cDNA fragments were directly sequenced or cloned and four independent clones were sequenced as described above.

Contigs were assembled by the Staden package program version 5.1 [17]. The sequence was 1,782 nucleotides (nts)-long, corresponding to 1,083 nts of the 3′ end of ORF 1 (*RdRp* region), an intergenic region of 4 nts, the complete *CP* gene of 573 nts, and an UTR of 122 nts. The strategy of polyadenylation of the genomic RNA was carried out to enable the cloning of the 3′ terminal end, therefore it was not possible to confirm precisely the presence of the

adenosine residue at the upmost terminal end. The 3′ end genomic nucleotide sequence of this tymovirus was deposited in the DDBJ/EMBL/GenBank database under accession number AB627084. Hereafter, we tentatively refer to as Tomato blistering mosaic virus (ToBMV).

The ToBMV genome contains the typical tymobox sequence (Supplementary Fig. S1) present 19 nts upstream the initiation codon of the *CP* gene. This tymobox was identical to the ones of related tymoviruses, except for a single nucleotide substitution at the sixth position. While other tymoviruses have only one initiation box—CAAU—upstream the stop codon of the *RdRp* gene, ToBMV genome seemed to have a duplicated one. For the confirmation of the tymobox sequence, the amplified products were directly sequenced.

Nucleotide and deduced amino acid sequence distance matrices were calculated for phylogenetic studies by MEGA program version 5.03 [18]. A phylogenetic tree was constructed with the Maximum-Likelihood algorithm with 1,000 bootstrap replications. Twenty-six CP amino acid sequence of tymoviruses deposited in public databases were used for alignment, which are from the following species: *Anagyris vein yellowing virus*, *Andean potato latent virus*, *Belladonna mottle virus*, *Cacao yellow mosaic virus*, *Calopogonium yellow vein virus*, *Chayote mosaic virus,* Chiltepin yellow mosaic virus, *Clitoria yellow vein virus*, *Desmodium yellow mottle virus*, Diascia yellow mottle virus, *Dulcamara mottle virus*, *Eggplant mosaic virus*, *Erysimum latent virus*, *Kennedya yellow mosaic virus*, *Nemesia ring necrosis virus*, *Okra mosaic virus*, *Ononis yellow mosaic virus*, *Passion fruit yellow mosaic virus*, *Petunia vein banding virus*, *Plantago mottle virus*, *Physalis mottle virus*, *Scrophularia mottle virus*, Tomato yellow blotch virus, *Turnip yellow mosaic virus*, and *Wild cucumber mosaic virus*.

The phylogenetic tree (Fig. 1b) consists of two major clusters. All tymoviruses infecting solanaceous plants were grouped in the first cluster, suggesting a possible common ancestor. The ToBMV was clearly distinct from other tymoviruses, such as *Petunia vein banding virus*, *Andean potato latent virus* (APLV), EMV, and Chiltepin yellow mosaic virus (ChiYMV) (Fig. 1b). The CP amino acid sequence of ToBMV shares the maximum identity of 64 % with that of ChiYMV and 63 % with EMV and APLV. The 3′-UTR portion (122 nts, downstream of the *CP* gene) contained a sequence capable of forming a TLS (Fig. 1c). This structure was predicted by the STAR program [19] and unlike most of tymoviruses is essentially similar to the TLS of tobamoviruses [20–22]. The secondary structure (Fig. 1c) comprised one T-like loop, two pseudoknot structures (PKa and PKb), and an anticodon arm (valine-CAC). Furthermore, the ToBMV TLS had a shorter five-base paired stem of the valine anticodon arm when compared to other tymovirus TLS [22–24].

This report presents a description of a new tymovirus infecting tomato plants in Brazil though it is not the first report of a tomato-infecting tymovirus in this country. A tymovirus tentatively named Tomato white necrosis virus (TWNV) was first described by Chagas et al. [6] in tomato leaf samples showing white necrosis, and later identified as an isolate of EMV [5]. Owing to the lack of molecular data regarding this virus isolate, the real relationship between this virus and our ToBMV isolate is still unknown.

As observed for other tymoviruses, ToBMV was readily transmitted by mechanical inoculation and infected some solanaceous species. According to the host range studies, this virus is possibly more adapted to solanaceous plants since most tymoviruses have a narrow experimental host range [2, 3] although it systemically infected *Chenopodium* species.

Abundant virus particles were observed in the cytoplasm and vacuoles of infected leaves (Fig. 1a-E, F) and chloroplasts showed distinctive modifications (Fig. 1a-C, D). It is believed that TYMV, the best studied tymovirus, replicates its RNA genome inside the vesicles of the outer and inner membranes of the chloroplasts [25]. It is suggested that virus particles are assembled at the necks of the peripheral vesicles from an emerging genomic strand of RNA combining with pentamers and hexamers of CP lying in an oriented fashion within the lipid bilayer of the chloroplast membrane [25]. Later studies demonstrated the association of TYMV-derived proteins with these vesicles [26, 27].

The tymobox, a 16-nts conserved sequence upstream of the initiation site of the subgenomic (sg) RNA of tymoviral RNAs [12] was found at the end of the *RdRp* gene (Supplementary Fig. S1). When compared to other tymoviruses, the tymobox of ToBMV had one nucleotide substitution, U to G, at the sixth position. The importance of the tymobox sequence and its adjacent region on the control of sgRNA production was evaluated by Schirawski et al. [28], who constructed mutants with different nucleotide exchanges within the tymobox and/or the initiation box of the TYMV cDNA constructs. Schirawski et al. [14] concluded that the preservation of the original sequence was essential since substitutions inhibited the transcription activity in TYMV. The initiation box (CAAU) of ToBMV is duplicated, which is peculiar within the tymoviruses. In most tymoviruses, this initiation box occurs downstream of a 7 or 8 nts CU-rich region next to the tymobox. The first CAAU of ToBMV was just one nt downstream the tymobox. This duplicated initiation box may have an enhancing effect on the transcription efficiency, and this hypothesis should be tested in a future study.

At the 3′ genome terminus (Fig. 1c), a TLS was predicted to have one hairpin (HP), an anticodon stem-loop, a T-like loop, and two pseudoknot structures (PKa e PKb), a structure found on tobamoviruses rather than on

tymoviruses [13, 22–24, 29–32]. This type of TLS is also found in the tymovirus *Nemesia ring necrosis virus* [22]. It is thought that the TLS provides a major contribution to the ability of the 3′ UTR to enhance the translation in protoplasts of reporter mRNAs [9] and to serve as a translational enhancer in a synergistic interaction with the 5′ genomic region [33].

The International Committee on Taxonomy of Viruses lists five species demarcation criteria for the genus *Tymovirus*: (1) overall sequence identity of the genomic RNAs of less than 80 %; (2) CP sequences less than 90 % identity; (3) differential host range; (4) differences in the 3′ terminal structure; and (5) serological specificity. Here, we demonstrated that the virus causing mosaic and blistering symptoms in tomato plants is a tymovirus, and based on the criteria (2) and (4), this virus should be classified as an isolate of a new species. Although it shared a serological relationship with EMV, the low identity in the CP amino acid sequence of ToBMV and EMV suggests that they are distinct viruses. The name Tomato blistering mosaic virus (acronym: ToBMV) is proposed due to the strong symptom of mosaic and blistering it causes on tomato plants.

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