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Biological and molecular properties of *Tomato rugose mosaic virus* (ToRMV), a new tomato-infecting begomovirus from Brazil

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A viral complex causing golden mosaic and leaf distortion (rugosity) in tomato plants was obtained from viruliferous whiteflies, and named TGV-Ub1. This complex was sap-transmitted from tomato to *Nicotiana benthamiana*. PCR amplification using universal begomovirus primers yielded two distinct fragments for DNA-A, suggesting that the TGV-Ub1 complex comprised at least two distinct viruses. Clones corresponding to full-length viral genomes were obtained from tomato plants infected with TGV-Ub1. Comparisons of the complete sequences of clones pUb1-49 (DNA-A), pUb1-62 and pUb1-81 (both DNA-B) indicated that they constitute novel western hemisphere begomoviruses. Clones pUb1-49 and pUB1-81 have identical common regions, thus representing the cognate DNA-A and -B of a novel begomovirus, named *Tomato rugose mosaic virus* (ToRMV). Clone pUb1-62 has a distinct common region from ToRMV and all other geminiviruses. A cognate DNA-A for pUb1-62 was not found. Clones containing 1·8 copies of the genomic components were constructed. Infectivity assays of these clones in tomato and *N. benthamiana* demonstrated that the clones corresponding to ToRMV systemically infected both hosts. Symptoms were analogous to those observed when using the pure isolates obtained in this study. The combination of pUb1-49 and -62 did not result in systemic infection, indicating that these components do not form a viable virus. ToRMV was sap-transmitted from *N. benthamiana* to *N. benthamiana*, and by grafting to *Solanum tuberosum* and *Datura stramonium*. ToRMV-A and ToRMV-B were detected in plants of *Nicandra physaloides* and *Phaseolus vulgaris*, respectively, growing in nearby tomato fields, in association with distinct DNA components.

Keywords: geminivirus, *Bemisia*, whitefly, genetic diversity

Introduction

Viruses in the family *Geminiviridae* are characterized by a circular, single-stranded DNA genome encapsidated in twinned icosahedral particles. The family is divided into four genera according to genome organization, type of insect vector and host range. The genus *Begomovirus* includes species with one or two genomic components, transmitted by whiteflies (*Bemisia tabaci*) and infecting mostly dicotyledonous plants (Stanley *et al*., 2005).

In Brazil, Costa *et al*. (1975) described six begomoviruses inducing golden mosaic in tomatoes (*Lycopersicon esculentum*) in São Paulo state. One of these viruses was purified and named *Tomato golden mosaic virus* (TGMV) (Matyis *et al*., 1975). At the time, the incidence of tomato golden mosaic was attributed to the migration of the whitefly vector from soybean and beans and to the existence of natural reservoirs of the virus, with a higher incidence in processing tomatoes during the autumn months (from April until June) (Costa, 1976).

In the mid-1990s, the B biotype of *B. tabaci* was introduced in Brazil. As opposed to the previously existent A biotype, which normally does not colonize tomatoes, the B biotype heavily infests this host (Lourenção & Nagai, 1994; França *et al*., 1996). The B biotype was quickly disseminated throughout the country, and the widespread incidence of the insect in tomato fields led to several reports of begomovirus infection in this crop (Ribeiro *et al*., 2003).

In the state of Minas Gerais, in 1996, the occurrence of begomoviruses in tomatoes was reported in the cities of Uberlândia and Igarapé, located in distinct geographical

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regions more than 500 km apart (Rezende *et al*., 1996; Zerbini *et al*., 1996). The isolate collected at Igarapé was shown to comprise a new species named *Tomato chlorotic mottle virus* (ToCMoV) (Ambrozevicius *et al*., 2002; Ribeiro *et al*., 2003). The analysis of tomato samples collected at Uberlândia by single-stranded conformational patterns in low ionic strength buffer (LIS-SSCP) suggested the existence of mixed infections, with more than one variant begomovirus in the same plant (Rezende *et al*., 1997). A partial DNA-A sequence of one of these viruses was analysed by Ribeiro *et al*. (2003) during a nationwide survey of tomato-infecting begomoviruses.

Considering the high level of incidence and severity of tomato begomoviruses in Brazil, and the limited characterization of the aetiological agents, the present work was carried out in order to characterize new begomoviruses infecting tomatoes at Uberlândia, Minas Gerais state, Brazil.

Materials and methods

Viral isolate

The isolate named TGV-Ub1 (*Tomato geminivirus*-Uberlândia 1) was obtained by collecting whiteflies from tomato plants showing yellow mosaic and severe leaf distortion (rugosity), grown under glasshouse conditions at Uberlândia, Minas Gerais state, Brazil (Fig. 1a). The insects were transferred to young plants of the tomato cv. Ângela Hiper. The presence of a begomovirus was tested by PCR-based amplification of fragments of the viral genome, using the general begomovirus primers PAL1v, 1978/PAR1c496 (DNA-A) and PCRc1/PBL1v 2040 (DNA-B) (Rojas *et al*., 1993). DNA extraction was carried out according to Dellaporta *et al*. (1983), using 5 mm leaf disks taken from young, noninoculated leaves. PCR was carried out essentially as described by Rojas *et al*. (1993).

Due to the impossibility of adequate whitefly containment in the glasshouse, the isolate was propagated by periodical grafting into tomato plants. Grafted plants were kept in a screenhouse. Sap inoculation was carried out from tomato to *Nicotiana benthamiana* using 0·1 m sodium phosphate buffer, pH 8·0, plus 1 g L^{-1} sodium sulphite. Inoculated plants were kept in a screenhouse until they developed symptoms. Viral infection was confirmed by PCR.

Host range of the TGV-Ub1 isolate

Plants of the families Solanaceae, Cucurbitaceae, Malvaceae, Fabaceae, Chenopodiaceae and Amaranthaceae (Table 1) were sap- and graft-inoculated as described above. The sap-inoculation experiment was repeated four times and the graft-inoculation experiment was repeated twice. In each replication, plants were inoculated three times to prevent escapes. In every experiment, two mock-inoculated plants of each species/cultivar were used as negative controls. Plants were kept in a screenhouse and observed for the development of symptoms for up to 45 days after inoculation. Viral infection was confirmed by PCR.

Biological purification and cloning of a begomovirus from the TGV-Ub1 isolate

Sap-inoculated *N. benthamiana* plants from the host range experiment, in which a single PCR fragment had been amplified, were selected for graft inoculation of cv. Miller Early tomato plants. Grafted tomato plants were kept in a screenhouse until symptom development, and viral infection was confirmed by PCR.

The replicative form (RF) of the viral DNA was purified from the graft-inoculated cv. Miller Early tomato plants, using the method described by Gilbertson *et al*. (1991). The RF was linearized, ligated to the pBLUESCRIPT KS+ phagemid vector (Stratagene) and introduced into *E. coli* DH5α using standard techniques (Sambrook *et al*., 1989). Recombinant clones corresponding to complete DNA-A and DNA-B were identified by hybridization under low stringency conditions using probes TGV-BZ-A-*Eco*RI,

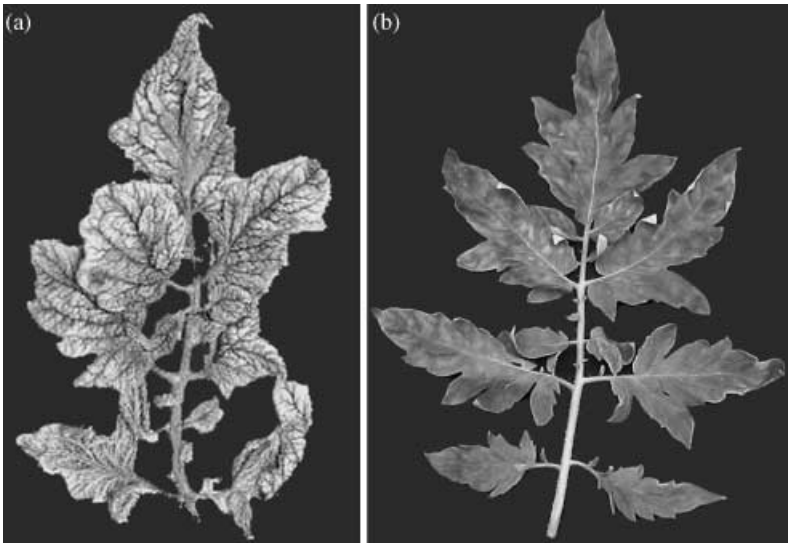

Figure 1 Symptoms in tomato plants. (a) Yellow mosaic and severe rugosity in the original field sample infected with the TGV-Ub1 viral complex isolate. (b) Yellow mosaic and mild rugosity in a plant biolistically inoculated with *Tomato rugose mosaic virus* (ToRMV).

Table 1 Host range of the TGV-Ub1 viral complex isolate and of *Tomato rugose mosaic virus* (ToRMV) after sap or graft inoculation. Infection by TGV-Ub1 was evaluated by visual observation of symptoms at 31 and 45 dpi, and confirmed by PCR amplification of a DNA-A fragment using general begomovirus primers. Infection by ToRMV was determined by Southern hybridization of the PCR-amplified DNA-A fragment, using digoxigenin-labelled probe RFUb49-total at high stringency

	TGV-Ub1					
	Sap transmission		Graft transmission		ToRMV	
Species tested	Symptoms[a]	PCR	Symptoms	PCR	Sap	Graft
Capsicum annuum Casca Dura Ikeda	ND	ND	–	–	ND	ND
Datura stramonium	ND	ND	ep, gm, r, s	+	ND	ND
Lycopersicon esculentum						
Angela Hipper	ep, gm, r, s, vc	+	ep, gm, r, s, vc	+	ND	ND
Miller Early	ep, ym, r, s, vc	+	ND	ND	ND	ND
Rutgers	ep, ym, r, s, vc	+	ep, gm, r, s, vc	+	0 (1)[b]	1 (1)
Santa Clara	ep, gm, r, s, vc	+	ep, gm, r, s, vc	+	ND	2 (2)
Nicotiana benthamiana	ep, r, vc, ym	+	ND	ND	17 (19)	ND
N. clevelandii	r, ym	+	ND	ND	4 (6)	ND
N. glutinosa	ep, r, vc, ys	+	ND	ND	10 (18)	ND
N. rustica	–	+	m, r, s	+	8 (12)	2(2)
N. tabacum						
Havana 425	–	+	r, s, ym	+	1 (1)	5 (5)
Sansum	–	+	m, r	+	2 (2)	5 (5)
TNN	–	+	r, s, ym	+	1 (1)	8 (8)
Turkish	–	+	m, r	+	1 (1)	4 (4)
White Burley	–	+	r, s, ym	+	2 (2)	8 (8)
Xanthi	–	+	m, r	+	2 (2)	6 (6)
Nicandra physaloides	ep, gm, r	+	ND	ND	ND	ND
Physalis floridana	–	–	–	–	ND	ND
Solanum gilo Verde Claro Comprido	ND	ND	–	–	ND	ND
Solanum melongena Embu	ND	ND	–	–	ND	ND
Solanum nigrum	–	–	–	–	ND	ND
Solanum tuberosum	ND	ND	r, s, ys	+	ND	ND
Chenopodium quinoa	–	–	ND	ND	ND	ND
Cucurbita pepo Caserta	–	–	ND	ND	ND	ND
Gossipium hirsutum DeltaPine Acala 90	–	–	ND	ND	ND	ND
Phaseolus vulgaris						
Carioca 80	–	–	ND	ND	ND	ND
Emgopa Ouro 201	–	–	ND	ND	ND	ND
Preto 153	–	–	ND	ND	ND	ND
Santana	–	–	ND	ND	ND	ND
Sida rhombifolia	–	–	–	–	ND	ND

[a]Symptoms: ep, epinasty; gm, golden mosaic; m, mosaic; r, rugosity; s, stunting; vc, vein clearing; ym, yellow mosaic; ys, yellow spots; –, no symptoms; ND, not done.

[b]Outside parenthesis, number of positive plants; within parenthesis, total number of plants tested.

Ub49 (A1 + 496), pTG1·4B and Ub62 (*Nco*I-*Hind*III) (Table 2). Probes were labelled with the DIG DNA Labelling and Detection kit (Roche Applied Sciences) according to the manufacturer's instructions. Both genomic components were completely sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and an ABI 310 automated sequencer (Applied Biosystems).

Sequence analysis

Nucleotide and deduced amino acid sequences were compared with those of other begomoviruses deposited in the GenBank. Phylogenetic trees were prepared using MEGA 3·1 (Kumar *et al*., 2004), from multiple alignments obtained with Clustal W (www.ebi.ac.uk/clustalw).

Cloning of viral genomes as 1·8mers

The DNA-A cloned in pUb1-49 was linearized with *Eco*RI, separated by electrophoresis and the 2600 nt fragment was gel purified. The subclone pUb1-49-s16, consisting of a 2200 nt insert flanked by *Cla*I and *Eco*RI sites, was cut with *Eco*RI and dephosphorylated. The 2600 nt fragment from pUb1-49 was then ligated to the *Eco*RI site of pUb1-49-s16, generating clone pUb1-G1, containing 1·8 copies of the DNA-A from pUb1-49. Clones corresponding to partial repeats of the DNA-B cloned in pUb1-81 (pUb1-G4) and pUb1-62 (pUb1-G7) were obtained in a similar fashion. Approximately 10 μg of each 1·8mer (G1 + G4 or G7) were biolistically inoculated (Aragão *et al*., 1996) into *N. benthamiana* and tomato plants (cv. Santa Clara),

Table 2 Description of probes used in molecular hybridization assays

Probe	Origin	Corresponding region of the viral genome[a]	Hybridization conditions
TGV-BZ-A-*Eco*RI	*Eco*RI digestion of the PCR fragment amplified from field samples using universal primers PAL1v1978 and PAR1c496 (Rojas *et al.*, 1993)	ToRMV-A, nucleotides 2278 to 484	58°C/2×SSC
RFUb49-total	Cloned viral genome	ToRMV-A, full-length	68°C/0.5×SSC (high) 58°C/2×SSC (low)
Ub49 (A1 + 496)	PCR amplification from the DNA-A component in pUb1-49 using primers Ub-A1-v (5′-GGTAGTTATGGTAGCTC-3′) and PAR1c496 (Rojas *et al.*, 1993)	ToRMV-A, nucleotides 64 to 484	68°C/0·5×SSC
pTG1·4B	Cloned viral genome (Hamilton *et al.*, 1984)	TGMV-B, full-length	58°C/2×SSC
Ub62 (*Nco*I-*Hin*dIII)	*Nco*I-*Hin*dIII digestion of the DNA-B component in pUb1-62	DNA-B in pUb1-62, nucleotides 2371 to 319	68°C/0·5×SSC

[a]Nucleotide positions refer to the sequences deposited in GenBank.

at the two- to four-leaf stage. Recombinant plasmids containing partial repeats of TGMV DNA-A and -B (Hamilton *et al.*, 1984) and of *Tomato yellow spot virus* (ToYSV, GenBank accession number DQ336350) were used as controls for the inoculation. The plants were transferred to a glasshouse 24 h after inoculation and observed for the appearance of symptoms until 21 days postinoculation (dpi). PCR-RFLP and hybridization using probe RFUb49-total (Table 2) were used to confirm that the viral infection was actually caused by the inoculated virus.

Sap transmission of ToRMV

Infected leaves of *N. benthamiana* plants biolistically inoculated with ToRMV (clones pUb1-G1 and pUb1-G4) were used as inoculum source for sap transmission of the virus to healthy *N. benthamiana* plants. Inoculated plants were maintained in a screenhouse until 30 dpi, and viral infection was confirmed by PCR.

Host range of ToRMV

The host range of ToRMV was inferred by hybridization of DNA fragments amplified from plants infected with the isolate TGV-Ub1 for the host range assay of this isolate, using probe RFUb49-total (Table 2) under high stringency conditions. Also, the top portions of tomato plants biolistically infected with ToRMV were grafted onto five plants of *Solanum tuberosum* cv. Bintje and six plants of *Datura stramonium*. After grafting, the plants were kept in a screenhouse and observed for the development of symptoms until 35 dpi. Viral infection was confirmed by PCR.

Detection of the ToRMV genomic components in wild hosts

Leaf samples of wild bean (*Phaseolus vulgaris*) and *Nicandra physaloides* plants with begomovirus-like symptoms were collected near a tomato field in Araguari, MG, approximately 30 km from Uberlândia. DNA fragments were PCR-amplified from these plants using primers PAL1v 1978/PAR1c496 (DNA-A) and PCRc1/PBL1v 2040 (DNA-B) (Rojas *et al.*, 1993), cloned and sequenced in order to identify the virus.

Results

Transmission of the TGV-Ub1 isolate

Graft-inoculated tomato plants (cv. Santa Clara) developed golden mosaic and severe rugosity, the same symptoms displayed by plants inoculated with the whitefly vector. The isolate TGV-Ub1 was sap-transmitted to *N. benthamiana* and from this host to tomato. However, the isolate was not transmitted from tomato to tomato. PCR amplification from *N. benthamiana* and tomato plants infected with TGV-Ub1 yielded two DNA-A fragments with approximately 1200 nt, but slightly different mobilities in the gel. This suggested the presence of at least two distinct begomoviruses in these plants.

Host range of the TGV-Ub1 isolate

The symptoms observed in sap- and graft-inoculated plants are presented in Table 1. Viral infection was confirmed by PCR in all plants with, and in some cases without, symptoms.

Only solanaceous plants were infected by TGV-Ub1. Symptoms in sap-inoculated *N. glutinosa* became progressively more severe during the course of the four experiments. The opposite was observed in *N. clevelandii* plants (data not shown). Symptoms in *Nicandra physaloides*, although not observed in every inoculated plant, were always very severe. Sap-inoculated *N. rustica* and all *N. tabacum* cultivars had latent infections. All graft-inoculated *N. tabacum* cultivars displayed mild symptoms.

Biological purification and cloning of a begomovirus from the TGV-Ub1 isolate

Sap-inoculated *N. benthamiana* plants could be divided into two groups displaying distinct symptoms. A low

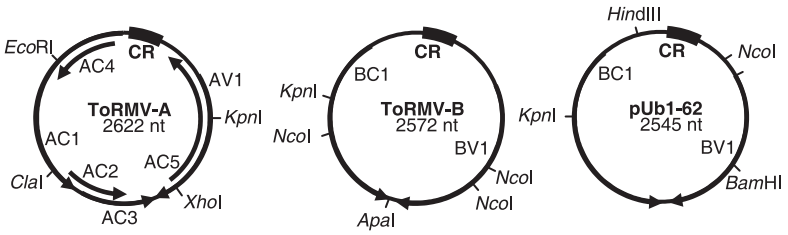

Figure 2 Schematic representation of *Tomato rugose mosaic virus* (ToRMV) -A and -B, and of the DNA-B cloned in pUb1-62. All open reading frames (ORFs) encoding for proteins > 10 kDa are indicated: AC1, replication-associated protein; AC2, *trans*-activating protein; AC3, replication-enhancer protein; AC5, unknown function; AV1, capsid protein; BC1, movement protein; BV1, nuclear shuttle protein. CR, common region. Some endonuclease restriction sites are also indicated.

percentage of inoculated plants displayed mosaic, mild rugosity and stunting. Most plants displayed severe yellow mosaic, epinasty, rugosity and stunting. PCR amplification using general begomovirus primers yielded a single DNA-A fragment for plants displaying mild symptoms, and two fragments for plants displaying severe symptoms.

Two tomato plants (cv. Miller Early, labelled E11 and E13), graft-inoculated with the top portions of *N. benthamiana* displaying mild symptoms, developed vein clearing symptoms followed by yellow mosaic, rugosity and epinasty. In general, these symptoms were less severe than those induced by the TGV-Ub1 isolate. PCR amplification using general begomovirus primers yielded a single DNA-A fragment with approximately 1200 nt for both plants. However, the fragment amplified from E11 migrated slightly slower in agarose gels, compared with the fragment amplified from E13. The sizes of these fragments corresponded to those amplified from plants infected with the TGV-Ub1 isolate (data not shown). The plant labelled E13 was selected for further experiments.

The replicative form of begomovirus DNA was extracted from tomato plants graft-inoculated with the top portion of plant E13, linearized and ligated to pKS+. Selection of clones with an approximately 2600 nt insert resulted in 96 candidate clones. Sequence of one of the ends of these inserts from randomly selected plasmids indicated that all clones had significant sequence identity with begomoviruses (data not shown). Clones pUb1-49 (DNA-A), pUB1-62 and pUb1-81 (both DNA-B) were selected for complete sequencing.

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                 Rep binding site        TATA box
BGYMV-A     GGA-G-ACTGGAGTA-CAATATATACTAGTACCCTCAATCTCGTGAATTATC
BGYMV-B     GGA-G-ACTGGAGTA-CAATATATACTAGTACCCTCAATCTCGTGAATTATC
SiGMV-A     GGA-GTATTAGAGTATCCATATATACTACTAGTCTCAATCTTGG-TT-----
SiGMV-B     GGA-GTATTAGAGTA-CCATATATACTACTTGTGTTTATCTTGG-TT-----
SiMoV-A     GGA-GTAT-GGAGTA-CAATATATAGTAGGAGTTCC--TAAGGGG-------
SiYMV-A     GGG-G-ACTGGGGTC-TTATATATAGCAGA-CTCCATATAA----TTTTT--
ToCMoV-A    GGG-G-ACTGGGGTC-CTATATATACTAGAACCCTCTATAGAACTTTCAATC
ToCMoV-B    GGG-G-ACTGGGGTC-CTATATATACTAGAACCCTCTATAGAACTTTCAATC
BGMV-A      GGT-GTAATGGTGCC--AATATATAGTAAGAAGTTCTTTAAGGATC-TG---
BGMV-B      GGT-GTAATGGTGCC--AATATATAGTATGAAGTTCTTTAAGGATC-TG---
TGMV-A      GGTAGTAA-GGTAGC-TCTTATATATTAGAAGTTCC--TAAGGGG-------
TGMV-B      GGTAGTAA-GGTAGC-TCTTATATATTAGAAGTTCC--TAAGGGG-------
ToSRV-A     GGTAGTAATGGTAGC-TCTTATATAGTAGAAGTTCCTTTAAGGAGA-TT---
ToRMV-A     GGTAGTTATGGTAGC-TCTTATATACTAGAAGTTCCTTTAAGGAGA-TT---
ToRMV-B     GGTAGTAATGGTAGC-TCTTATATAGTAGAAGTTCCTTTAAGGAGA-TT---
pUb1-62-B   GGG-G-AATGGGTCTC-AATATATAGTA-GAGACCAT-TATAGAATATAA--
            **  *     *        ******  *          *

                    G box      Stem      Loop      Stem
BGYMV-A     AGATTCACACACGTGGC-GGCCATCCGATATAATATTACCGGATGGCCGCCC
BGYMV-B     AGATTCACACACGTGGC-GGCCATCCGATATAATATTACCGGATGGCCGCGC
SiGMV-A     ---TTGATACACGTGGC-GGCCATCCG-TATAATATTACCGGATGGCCGCGC
SiGMV-B     ---TTGGTACACGTGGC-GGCCATCCG-TATAATATTACCGGATGGCCGCGC
SiMoV-A     ---------CACGTGGC-GGCCATCCG-TCTAATATTACCGGATGGCCGCGC
SiYMV-A     -GAT-GG-ACACGTGGC-GGCCATCCGCTATAATATTACCGGATGGCCGGCG
ToCMoV-A    TCGTT--CACACGTGGC-GGCCATCCGTTATAATATTACCGGATGGCCGCGG
ToCMoV-B    TCGTT--CACACGTGGC-GGCCATCCGTTATAATATTACCGGATGGCCGCGG
BGMV-A      -----TAGACACGTGGC-GGCCATCCGCTATAATATTACCGGATGGCCGCGC
BGMV-B      -----GAGACACGTGGC-GGCCATCCGTTATAATATTACCGGATGGCCGCGC
TGMV-A      ---------CACGTGGC-GGCCATCCGTT-TAATATTACCGGATGGCCGCGC
TGMV-B      ---------CACGTGGC-GGCCATCCGTTTTAATATTACCGGATGGCCGCGC
ToSRV-A     -----GCTACACGTGGC-GGCCATCCGTTATAATATTACCGGATGGCCGCGC
ToRMV-A     -----GCTACACGTGGCCGGCCATCCGATATAATATTACCGGATGGCCGCGC
ToRMV-B     -----GCTACACGTGGCCGGCCATCCGTTATAATATTACCGGATGGCCGCCC
pUb1-62-B   -----TTGCCACGTGGC-GGCCATCCGCTATAATATTACCGGATGGCCGCGC
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Figure 3 Sequence alignment of part of the common region of *Tomato rugose mosaic virus* (ToRMV)-A and -B, the DNA-B cloned in pUb1-62, and other begomoviruses. The conserved *cis*-acting elements (iterons) relevant for DNA replication are indicated above the alignment.

Sequence analysis

The complete sequences of clones pUb1-49, pUb1-62 and pUb1-81 were deposited in GenBank under accession numbers AF291705, DQ336352 and AF291706, respectively.

Sequence analysis indicated that the genomic components cloned in pUb1-49, pUb1-62 and pUb1-81 have a genetic organization typical of DNA-A (pUb1-49) and -B (pUb1-62 and pUb1-81) of western hemisphere, bipartite begomoviruses (Fig. 2). Six ORFs were identified in DNA-A, and two ORFs in DNA-B. Analysis of common region sequences (Fig. 3) indicated that the genomic components cloned in pUb1-49 and pUb1-81 constitute cognate DNA components of a single virus. The nucleotide sequence of the DNA-A cloned in pUB1-49 has < 89% identity with any other begomovirus sequenced so far. Therefore, and in accordance with current taxonomic criteria for the *Geminiviridae* (Fauquet *et al*., 2003; Stanley *et al*., 2005), the genomic components cloned in pUb1-49 and pUb1-81 comprise the genome of a new species of begomovirus, named *Tomato rugose mosaic virus* (ToRMV). Henceforth, the DNA-A cloned in pUb1-49 will be referred to as ToRMV-A, and the DNA-B cloned in pUb1-81 will be referred to as ToRMV-B. The DNA-B cloned in pUb1-62 has 70, 67 and 65% identity with ToRMV-B, BGMV-B and TGMV-B, respectively.

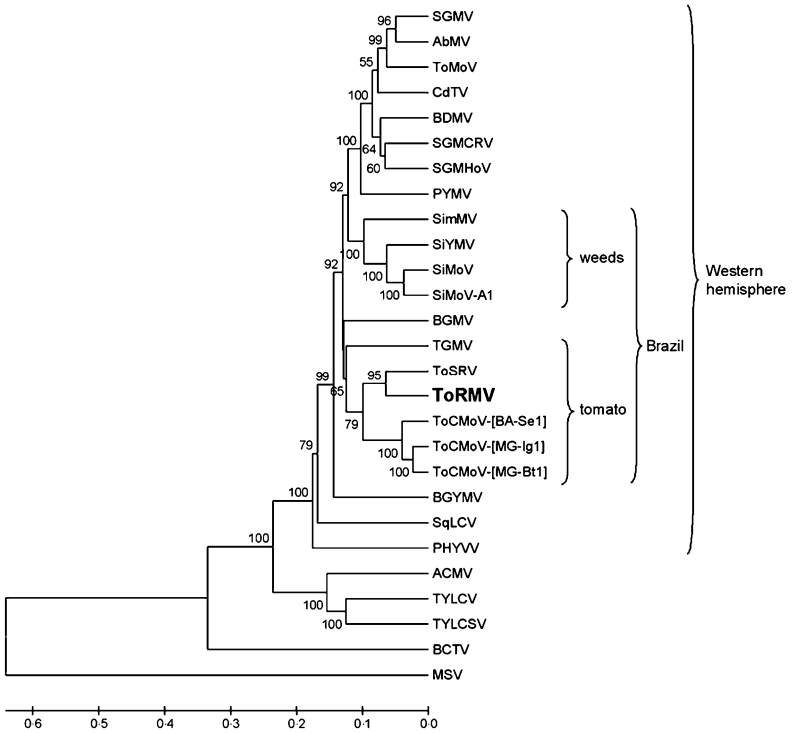

Figure 4 Phylogenetic tree based on a multiple sequence alignment of full-length DNA-A sequences using the UPGMA method. Branches were bootstrapped with 2000 replications. AbMV, *Abutilon mosaic virus* (GenBank accession number NC_001928); ACMV, *African cassava mosaic virus* (NC_001467); BCTV, *Beet curly top virus* (NC_001412); BDMV, *Bean dwarf mosaic virus* (NC_001931); BGMV, *Bean golden mosaic virus* (NC_004042); BGYMV, *Bean golden yellow mosaic virus* (NC_001439); CdTV, *Chino del tomate virus* (NC_003830); MSV, *Maize streak virus* (NC_001346); PHYVV, *Pepper huasteco yellow vein virus* (NC_001359); PYMV, *Potato yellow mosaic virus* (NC_001934); SGMV, *Sida golden mosaic virus* (NC_002046); SGMCRV, *Sida golden mosaic Costa Rica virus* (NC_004657); SGMHoV, *Sida golden mosaic Honduras virus* (NC_004659); SiMoV, *Sida mottle virus* (NC_004637; isolate A1, AJ557450); SiYMV, *Sida yellow mosaic virus* (NC_004639); SimMV, *Sida micrantha mosaic virus* (NC_005330); SqLCV, *Squash leaf curl virus* (NC_001936); TGMV, *Tomato golden mosaic virus* (NC_001507); ToCMoV, *Tomato chlorotic mottle virus* (BA-Se1, NC_003664; MG-Bt1, AY090557; MG-Ig1, DQ336353); ToSRV, *Tomato severe rugose virus* (AY029750); ToRMV, *Tomato rugose mosaic virus* (AF291705 = NC_002555); ToMoV, *Tomato mottle virus* (NC_001938); TYLCV, *Tomato yellow leaf curl virus* (NC_004005); TYLCSV, *Tomato yellow leaf curl Sardinia virus* (NC_003828).

Phylogenetic analysis

A phylogenetic tree based on the complete nucleotide sequence of DNA-A is presented in Fig. 4. Begomoviruses are clearly separated in terms of geographical origin, with a monophyletic cluster including those from the western hemisphere. Viruses reported from Brazil are split into two clusters. One cluster includes begomoviruses originally detected in crop plants (bean and tomato). Viruses originally detected in weed species form a second cluster. ToRMV, so far the only Brazilian virus found in both weeds (Jovel *et al*., 2004) and crop species, was positioned among Brazilian begomoviruses from crop species. Phylogenetic trees based on the complete nucleotide sequence of DNA-B and on the deduced amino acid sequences of Rep, Trap, Ren, CP, MP and NS yielded equivalent results (data not shown).

Infectivity of the cloned virus

Nicotiana benthamiana and tomato plants biolistically inoculated with clones pUb1-G1 + pUb1-G4 (ToRMV) displayed symptoms of yellow mosaic and mild rugosity approximately 3 weeks after inoculation (Fig. 1B). Control plants inoculated with TGMV displayed symptoms of yellow spots, leaf curling and stunting at 2 weeks post-inoculation. Plants inoculated with clones pUb1-G1 + pUb1-G7 did not show any symptoms at 6 weeks post-inoculation.

PCR-based analysis of plants inoculated with ToRMV and with TGMV resulted in the amplification of an approximately 1200 nt fragment, only from plants with symptoms. For ToRMV, 25% of the inoculated plants were confirmed to be infected by PCR. Amplification also occurred from 11·7% of the plants inoculated with pUb1-G1 + pUb1-G7, but only when DNA was extracted from inoculated leaves.

Hybridization with probe RFUb49-total at 68°C and 0·5 × SSC (high stringency) detected PCR-amplified DNA-A fragments from noninoculated leaves of tomato and *N. benthamiana* plants inoculated with ToRMV (Fig. 5), and from inoculated leaves of plants inoculated with pUb1-G1 + pUb1-G7. Under these hybridization conditions, no signal was observed with DNA amplified from plants infected with other begomoviruses (Fig. 5).

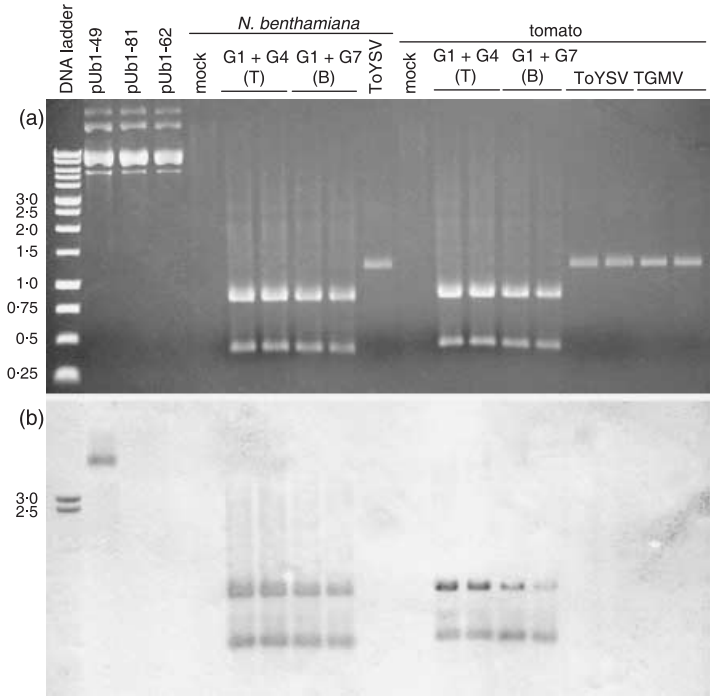

Figure 5 Detection of *Tomato rugose mosaic virus* (ToRMV) in tomato and *Nicotiana benthamiana* plants biolistically inoculated with cloned DNA components. (a) Ethidium bromide-stained agarose gel showing PCR-amplified DNA-A fragments using general begomovirus primers from plants inoculated with clones G1 + G4 (1·8mers of ToRMV-A and -B), G1 + G7 (1·8mers of ToRMV-A and the DNA-B cloned in pUb1-62), *Tomato yellow spot virus* (ToYSV)-A and -B, or *Tomato golden mosaic virus* (TGMV)-A and -B. PCR fragments were digested with *Sst*I prior to electrophoresis. This enzyme cleaves the 1·2 kb DNA fragment amplified from ToRMV, generating two fragments with approximately 0·8 and 0·4 kb. The fragments amplified from ToYSV and TGMV are not cleaved by the enzyme. T, DNA extracted from the top portion of the plant (noninoculated); B, DNA extracted from the inoculated leaf. Controls include plasmid DNA preparations from clones pUb1-49 (ToRMV-A), pUb1-81 (ToRMV-B) and pUb1-62. (b) Hybridization with digoxigenin-labelled RFUb49-total probe at 68°C and 0·5 × SSC (high stringency). The probe hybridizes with the DNA-A fragments amplified from ToRMV, but not from ToYSV or TGMV.

Sap transmission of ToRMV

Approximately 2 weeks after inoculation, 10–12·5% of *N. benthamiana* sap-inoculated with ToRMV showed systemic symptoms of viral infection. PCR-based detection indicated that 37·5–40% of the plants were infected. At 36 dpi, all infected plants had symptoms of yellowing, vein banding, mild rugosity and stunting. These symptoms were milder than those observed in plants inoculated with the isolate TGV-Ub1.

Host range of ToRMV

The DNA-A fragment from ToRMV was detected by specific hybridization in *N. benthamiana*, *N. glutinosa*, *N. clevelandii*, *N. rustica* and *N. tabacum* cvs Sansum, TNN, Havana 425, Xanthi, White Burley and Turkish sap-inoculated with the isolate TGV-Ub1 (Table 1). The virus was not detected in some plants of *N. benthamiana*, *N. glutinosa*, *N. rustica*, *N. clevelandii* and tomato cv. Rutgers. PCR amplification of a DNA fragment using universal begomovirus primers indicated that these plants were infected by a distinct begomovirus also present in the TGV-Ub1 isolate.

The DNA-A fragment amplified from ToRMV was detected in *N. rustica*, *N. tabacum* cvs Sansum, TNN, Havana 425, Xanthi, White Burley and Turkish, and tomato cvs Santa Clara and Rutgers graft-inoculated with TGV-Ub1 (Table 1). All five potato plants graft-inoculated with ToRMV developed systemic symptoms of yellow spots, mild rugosity and stunting. PCR-based detection resulted in the amplification of a DNA-A fragment from these plants, corresponding in size to the fragment amplified for ToRMV. *D. stramonium* plants grafted with ToRMV-infected cv. Santa Clara tomato did not show any symptoms of viral infection. However, PCR-based detection resulted in the amplification of a DNA-A fragment corresponding in size to the fragment amplified for ToRMV. No fragment was amplified from mock-inoculated plants.

Detection of the ToRMV genomic components in alternative hosts

PCR-based analysis of *Nicandra physaloides* and *Phaseolus vulgaris* plants using universal begomovirus primers resulted in the amplification of DNA-A and -B fragments with approximately 1200 and 400 nt, respectively. Amplified fragments were cloned and partially sequenced. The DNA-A fragment from *N. physaloides* was 99·8% identical to ToRMV-A, while the DNA-B fragment amplified from this plant was only 71·3% identical to the ToRMV-B (data not shown). The opposite was verified with the fragments amplified from *P. vulgaris*. The DNA-A and -B fragments were 72·2 and 96·9% identical to ToRMV-A and -B, respectively.

Discussion

Differences in symptom severity among tomato and *N. benthamiana* plants graft-inoculated with the TGV-Ub1 isolate, as well as the PCR amplification of two distinct DNA fragments (based on their sizes), were strong indications that this viral isolate comprised more than one begomovirus species or strain. The PCR amplification of a single DNA fragment from plants showing attenuated symptoms in relation to the original isolate suggested

that one of these species/strains had been biologically purified.

Successful sap transmission of the TGV-Ub1 isolate was accomplished in nine *Nicotiana* species and cultivars. However, symptoms of viral infection were only observed in *N. benthamiana*, *N. glutinosa* and *N. clevelandii*. In the remaining species and cultivars, the infection was confirmed by PCR and, in general, infectivity was low. *Nicotiana glutinosa* and *N. rustica* were an exception, with 90 and 100% infectivity, respectively, although all *N. rustica* plants remained symptomless. The absence of symptoms in most of these species and cultivars may be due to infection by only one of the viruses that comprise the isolate. In support of this hypothesis, ToRMV was not detected by hybridization in some plants in which PCR amplification with universal begomovirus primers was successful. Mixed infections by begomoviruses have been shown to increase symptom severity in tomato (Umaharan *et al*., 1998), cassava (Pita *et al*., 2001; Vanitharani *et al*., 2004) and several weed species (Umaharan *et al*., 1998; Saunders *et al*., 2001). The low infectivity of the TGV-Ub1 isolate for *N. tabacum* cultivars may be due to the low adaptability of the viruses present in TGV-Ub1 to these hosts. Morra & Petty (2000) showed that begomoviruses may not be able to colonize mesophyll cells systemically in hosts to which they are poorly adapted. It is significant that TGV-Ub1 was able to systemically infect *Nicandra physaloides* and *Datura stramonium*, two common weeds in tomato fields in Brazil. This result suggests that these species could act as virus reservoirs and inoculum sources in the field.

Sequence analysis of the begomovirus components cloned from TGV-Ub1, namely pUb1-49 (DNA-A), pUb1-62 and pUb1-81 (both DNA-B), showed that they have a genomic organization typical of western hemisphere, bipartite begomoviruses. The sequence identity of pUb1-49 is below 89% with all previously characterized begomoviruses. Therefore, based on the current criteria for begomovirus taxonomy (Fauquet *et al*., 2003; Stanley *et al*., 2005), this clone represents a novel species of this genus, named *Tomato rugose mosaic virus* (ToRMV). The common region of clone pUb1-81 is highly homologous, and its iterons are identical to those from clone pUb1-49. These clones were infectious when inoculated in combination into tomato and *N. benthamiana*. They comprise the complete genome of ToRMV. The common region of clone pUb1-62 has a low sequence identity with ToRMV and with other begomoviruses, and the combination of clones pUb1-49 and pUb1-62 was not infectious to tomato or *N. benthamiana*. Thus, clone pUb1-62 most likely represents a second new species, with an unidentified cognate DNA-A. These results, coupled with the observation of attenuated symptoms in tomato and *N. benthamiana* plants infected with ToRMV in relation to TGV-Ub1, are in agreement with the hypothesis that TGV-Ub1 comprises at least two begomovirus species or strains. Whether the cognate DNA-A of clone pUb1-62 represents the second species in TGV-Ub1 remains to be demonstrated.

The host range of ToRMV was inferred based on the hybridization of probe RFUb49-total at high stringency with PCR-amplified DNA fragments from plants infected with TGV-Ub1. ToRMV was detected in all 41 plants graft-inoculated with TGV-Ub1, mostly *N. tabacum* cultivars. The virus was also detected in all species and cultivars of plants sap-inoculated with TGV-Ub1, but not in all plants of *N. benthamiana*, *N. clevelandii* and *N. glutinosa*. This result demonstrates that, under these hybridization conditions, probe RFUb49-total was specific for ToRMV-A, and reinforces the hypothesis of the presence of a viral complex in TGV-Ub1.

Sequence analysis of the DNA-A and DNA-B fragments amplified from *N. physaloides* and *P. vulgaris* plants collected near tomato fields confirmed the presence of a begomovirus in these plants. These results demonstrated that the DNA-A fragment from *N. physaloides* and the DNA-B fragment from *P. vulgaris* are highly similar (> 96% sequence identity) to ToRMV-A and -B, respectively, suggesting that ToRMV might be able to infect these hosts in the field. However, during the course of this work, bean plants were never infected by ToRMV or by TGV-Ub1, either biolistically or by sap inoculation. It is possible that *P. vulgaris* could be infected by ToRMV using whiteflies. Alternatively, these results could suggest that the genomic components of ToRMV can be associated with distinct, unidentified begomovirus components. This is in agreement with the hypothesis that begomoviruses currently infecting tomatoes in Brazil were previously present in the native flora and have been introduced, disseminated and maintained in tomato plants by the biotype B of *B. tabaci*. Begomovirus isolates infecting tomato, other crops and weeds were also detected in Trinidad and Tobago (Umaharan *et al*., 1998), in Mexico and in the USA (Torres-Pacheco *et al*., 1996). The presence of at least three distinct begomoviruses, including ToRMV, was demonstrated in *Sida micrantha* samples originally collected in Brazil in the 1970s and maintained by vegatative propagation in Germany for over 25 years (Jovel *et al*., 2004). The mixing of indigenous viruses previously confined to distinct plant species allows the occurrence of recombination and pseudorecombination events, giving rise to new species which are better adapted to the new host. This phenomenon may be the driving force in geminivirus evolution and diversity under current agricultural practices (Pita *et al*., 2001; Monci *et al*., 2002; Mansoor *et al*., 2003; Preiss & Jeske, 2003).

Recent survey results have indicated a high incidence of ToRMV infection in tomato plants in the major tomato-producing states of São Paulo, Minas Gerais and Goiás (Inoue-Nagata *et al*., 2004; S. S. Ferreira, E. C. Andrade, A. T. L. Lima & F. M. Zerbini, unpublished results). However, other begomoviruses are also present at high incidence, particularly *Tomato chlorotic mottle virus* (ToCMoV), *Tomato severe rugose virus* (ToRSV) and *Tomato yellow vein streak virus* (TYVSV). Control efforts, particularly those based on host resistance, should take into consideration the presence of multiple begomovirus species in the field. It is also important to monitor whether ToRMV, ToCMoV, ToSRV and TYVSV will remain as the prevalent species over the years, or whether new species

will continue to emerge. The high degree of genetic diversity among tomato-infecting begomoviruses in Brazil (Ambrozevicius *et al*., 2002; Ribeiro *et al*., 2003), coupled with initial reports of recombination and pseudorecombination among these viruses (Galvão *et al*., 2003), indicate the potential to generate a large number of novel viruses, some of which might be well adapted to the tomato plant and could therefore become established in this crop.

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