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Detection and Identification of TMV Infecting Tomato Under Protected Cultivation in Paraná State

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ABSTRACT

During an inspection in plastic houses in Sapopema, Paraná, 90% of tomato plants showed leaf abnormalities, probably associated with herbicide toxity. However, virus like symptoms developed in selected hosts after mechanical inoculatation. RT-PCR reactions using primers for an internal region within the movement protein gene of TMV and ToMV resulted in the amplification of a 409 bp cDNA fragment only by TMV primers. Deduced amino acids showed 100% identity when compared to TMV movement protein and 94% with ToMV. The RT-PCR protocol was efficient for quick and conclusive determination of virus species. The virus was purified and a polyclonal antiserum was raised for future surveys in tomato crops of Paraná. The partial genomic sequence obtained for TMV-Sapopema has been deposited under the accession number DQ173945, which is the first partial genomic sequence of an isolate of TMV from Brazil in the GenBank, and the first tomato virus isolate from Paraná to have some of its biological and molecular properties determined.

Key words: TMV, P30 protein, plasticulture

INTRODUCTION

Crop production based on plasticulture has allowed substantial gains in yields for many horticultural crops. However, this practice may increase the impact of some biotic and abiotic diseases. Among the viruses reported to cause significant losses in tomato (*Lycopersicum esculentum* Mill.), the commonly found are *Cucumovirus*, *Begomovirus*, *Luteovirus*, *Potexvirus*, *Potyvirus*, *Tobamovirus*, and *Tospovirus* (Eiras et al., 2002). Fukuda et al., (1981) classified the species of *Tobamovirus* into two subgroups according to the location of their origin of assembly. The subgroup 1 viruses, *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), *Pepper mild mottle virus* (PMMoV), *Tobacco mild green mosaic virus* (TMGMV) and *Odontoglossum ringspot virus* (ORSV) mainly infect solanaceous plants except ORSV, which is a prevalent virus in orchids (Park et al., 1990; Paul, 1975). TMV is the type member of the genus *Tobamovirus*. Its virion is a rigid rod (18 nm x 300 nm) consisting of about 2130 identical coat protein subunits stacked in a helix around a single strand of positive sense RNA,

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6395 nucleotides in length. The genome of TMV encodes at least four proteins (Goelet et al., 1982). The 3' proximal genes encode a 30-kDa cell-to-cell movement protein, also called P30 protein, and a 17.5-kDa coat protein (Meshi et al., 1987, Citovsky et al., 1992). There are reports about *Tobamovirus* infection in tomato, mainly from São Paulo, Minas Gerais, and Federal District (Resende and Cupertino, 1996). The host range of some isolates include, *Nicotiana tabacum* L., *Petunia* sp, tomato and species of *Zinnia* for TMV, and Tomato for ToMV; (Caner et al., 1990; Bastos et al., 1999; Maritan and Gaspar, 2001; Moreira et al., 2001; Eiras et al., 2002). In Sapopema, tomato plants growing under plastic houses, showing narrowing and leaf distortion in 90% of the plants, suggested a toxity caused by herbicide residues. However, after mechanical inoculation, virus similar symptoms developed in selected hosts. The objective of this work was to identify at species, a tomato virus found in Sapopema, determine some of its biological and molecular properties, and to produce an antiserum for future surveys in tomato producing areas of Paraná State.

MATERIAL AND METHODS

Source and maintenance of the virus

Leaves of tomato 'Santa Clara' showing narrowing, light and dark green mottled areas, producing fruits reduced in size and number with uneven ripening, cultivated in plastic houses in Sapopema-PR, were collected for host range, serology, electron microscopy and RT-PCR analysis. The virus isolate was purified by local lesion transfers on *Nicotiana tabacum* L. 'Debney', before systemic propagation in 'Santa Clara'. Four hundred grams of leaves were collected from infected plants and cut in 1 cm^2 pieces, placed in glass bottles, filled with 300 ml of phosphate buffer (pH 7.0, 0.02M), plus the same volume of glycerol and stored at -20^0C. Fresh material collected from symptomatic plants or the stored material was used for virus propagation in 'Santa Clara' tomato to conduct this work.

Host range

One gram of symptomatic leaves of 'Santa Clara' tissue was ground in 2 ml of 0.01M sodium phosphate buffer (pH 7.0; 1:2 w/v). The extract was rubbed onto leaves of several botanical species, previously dusted with Carborundum® 300 mesh. Symptoms were evaluated at 10 and 20 days after inoculation. The presence of the virus in asymptomatic plants was verified by back inoculation to 'Santa Clara' the same species in which the virus was propagated.

Virus purification

Infected leaves of tomato were harvested approximately 30 days after inoculation. The virus particles were purified by adding 20% of polietileneglicol (PEG) and 5M NaCl according to Gooding and Hebert (1967). Further, the semi-purified preparation was submitted to a linear density sucrose gradient (10-40%), and ultra centrifuged at 80,000g for 2.5h. To determine the yields, dilutions of small aliquots of the preparation were measured at the absorbance of 260 and 280 nm. The yield was estimated assuming an extinction coefficient of three for tobamovirus according to Gibbs and Harrison (1976).

Antiserum production and serological analysis

Antiserum was produced by mixing equal volumes of purified viral solution (containing approximately 5 mg/ml) with Freund's complete adjuvant (Difco) and intramuscular injection of rabbit. Second, third, and fourth injections were prepared with incomplete adjuvant, containing the same amount of virus and made at 10-day intervals. Bleedings began two weeks after the first injection and continued for six weeks. Double diffusion tests were performed according to Purcifull and Bachelor (1977).

Electron microscopy

Small pieces of leaves from infected plants were fixed in a modified Kanovsky fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.05M cacodylate buffer, pH 7.2), post-fixed with 1% OsO_4, dehydrated in acetone and embedded in Spurr's low viscosity resin. Sections were stained with uranyl acetate and Reynold's led citrate and examined in a Zeiss EM 900 transmission electron microscope. Viral particles were also observed in leaf dip preparations negatively stained with uranyl acetate (Kitajima, 1965).

Total nucleic acid extraction and reverse transcription

Total RNA was extracted from 100 mg of fresh leaf tissue ground in liquid nitrogen according to

the RNeasy® Plant Kit protocol (Qiagen®, Chatsworth, CA). The cDNA was synthesized using reverse transcriptase (Gibco BRL) and buffers according to the manufacturer in a final volume of 30µl. From the total RNA extraction solution, 10 ml were taken and mixed with 2µl of 10 µM reverse primer TMV-2 (5'-GAA AGC GGA CAGAAA CCC GCT G-3') or 2µl of reverse primer ToMV-5 (5'-CTC CAT CGT TCA CAC TCG TTA CT-3') (Jacobi et al, 1998). Afterwards, the samples were incubated at 70 ^{0}C for 10 min and quenched on ice for at least 1 min. The other reaction components were added in the following order: 3µl of 10X PCR buffer (200 mM Tris-HCl, pH 8,4), 500 mM KCl, 2,5 mM $MgCl_2$ (2µl), 10 mM dNTPmix (1 ml), 0.1M dithiothreitol (2µl) and 1µl (20 U) of M-MLV reverse transcriptase (Gibco BRL), and DEPC treated water to give a reaction volume of 30µl. The tubes were incubated for 1h at 42 ^{0}C on an Eppendorf thermocycler model mastercycler personal followed by 10 min of heating at 65 ^{0}C to denature the enzyme.

Amplification by PCR

Reverse primer TMV2 and forward primer TMV1 (5'-GAC CTG ACA AAAATG GAG AAG ATC T-3') are expected to amplify a 422 bp PCR product within the viral movement protein gene of TMV, while primer ToMV-5 and forward primer ToMV-6 (5'-GAT CTG TCA AAG TCT GAG AAA CTT C-3') amplify a product of 508 bp within the same gene of ToMV (Jacobi et al., 1998). PCR reactions were performed in a 40µl volume, containing 10mM each of dATP, dCTP, dGTP and dTTP, 2µl of each primer (10 µM), 2.5 U of *Taq* DNA Polymerase (Gibco BRL), 4µl of 10X PCR buffer, 25mM MgCl, and 4µl of the first strand synthesis product. Thirty-five reaction cycles were performed as follows: template denaturation at 94 ^{0}C for 30s, primer annealing at 62 ^{0}C for 45s, and elongation at 72^{0}C for 1 min. A final 5 min elongation step at 72^{0}C was performed at the end of the 35 cycles.

Sequence comparison

The RT-PCR product was direct sequenced by the dideoxy chain termination method, using a 377 DNA sequencer (ABI, USA). Nucleotide sequence was compared with those available in GenBank (http://www.ncbi.nih.gov/gorf/gorf. html), and the amino acid sequence was estimated through the translation tool from Expasy Software (http://us.expasy.org/tools/). Multiple sequence alignments and sequence relationship were produced using CLUSTAL W (Thompson et al., 1994), and calculation of percentage of identity was performed by analysis on http:// bioinformatics.org/ sms/ ident_sim.html.

RESULTS AND DISCUSSION

Tomato production of Paraná accounts for approximately 3.4% of the total country production and the north region, where Sapopema is located, concentrates 60% of this (Seab/Deral, 2006). TMV-Sapopema could not infect *Petunia hybrida* described as a host for TMV and ToMV (Moreira et al., 2003; Hollings and Huttinga, 1976). However, severe mosaic symptoms developed in tomato 'Santa Clara' and 'Avansus' with strong leaf narrowing, while the cultivar Carmem developed only mild symptoms. Misinterpretation of host range symptoms for species determination in *Tobamovirus* is very common. There are reports of isolates capable to overcome the resistance of some tomato varieties expressing, for example, the resistance factor 'Tm-1' that provides resistance for ToMV, and the resistance factors 'Tm-2' and 'Tm-2^2' for TMV (Moreira et al., 2003). Additionally few indicator plants distinguish reliably between TMV and ToMV (Jacobi et al. 1998). In this work, electron microscopy examinations of ultratin sections revealed rod shaped particles, and combined with host range, allowed to conclude that a *Tobamovirus* was associated with the diseased tomatoes from Sapopema (Table 1 and Fig.1). The method for virus purification yielded an average of 5 mg/ml. The antiserum produced reacted to leaf extracts from symptomatic plants in homologous serological reactions and not with healthy plants. According to some authors, serological cross-reaction between ToMV and TMV limits the efficiency of serological differentiation of these two viruses (Jacobi et al., 1998). In this regard, low cross-reaction was observed by Duarte et al., (2002) for a monoclonal antiserum that showed strong specificity for ToMV, but problems for raising this kind of antibodies limited their immediate use. In this work, definitive determination of virus species was possible after RT-PCR assays that reliably discriminated these two viruses. The TMV-1/-2 primer pair was very

effective to amplify a 409 bp product located in the TMV movement protein gene (Fig.2).

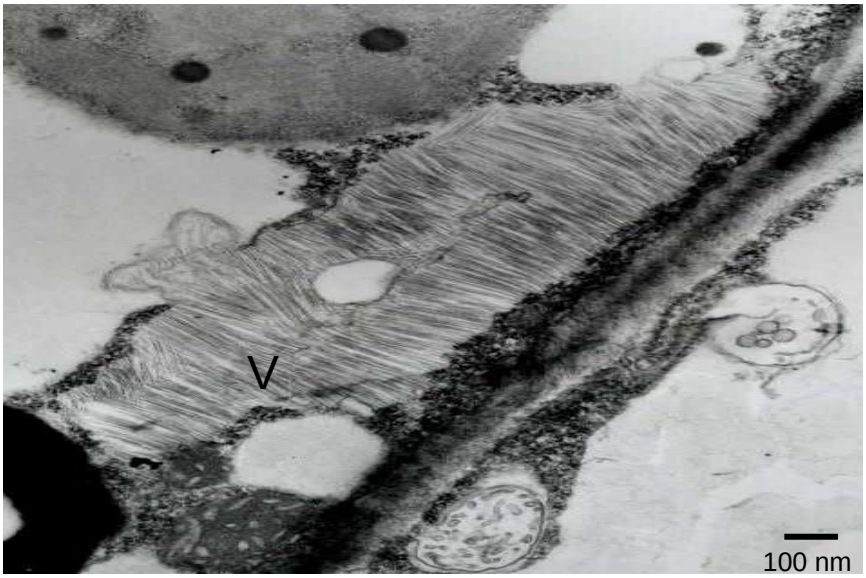

Figure 1 - Transmission electron micrograph of ultratin sections of *Lycopersicum esculentum* 'Santa Clara' leaves, showing masses of presumable virus aggregates (V) in the cell cytoplasm.

Table 1 - Predominant symptoms induced in the host range of the TMV isolate from Sapopema-PR.

Family	Species	Local Symptom*	Systemic Symptom*
Chenopodiaceae	*Chenopodium amaranticolor* Coste and Reyn	LL	-
Solanaceae	*Capsicum annum L.*	LD	LD, MO
	Datura stramonium	LL	-
	Lycopersicum esculentum 'Santa Clara'	LL, LD	LD, MO, LN, SN
	'Avansus'	LL, LD	LD, MO, LN
	'Carmem'	LL	MO
	'Santa Cruz Kada'	LL	LD, MO, LN
	Nicandra Physaloides	LL	-
	Nicotiana tabacum 'Turkish NN'	LL	-
	'Samsum'	LL	-
	Nictotiana ocidentalis	LL	MO
	N. glutinosa L.	LL	MO
	Petunia hybrida	-	-
Leguminosae	*Glycine max (L.) Merr.*	NS	
	Phaseolus vulgaris L.	NS	
Compositae	*Gonphrena globosa*	NS	
Cucurbitaceae	*Cucumis sativus*	NS	

*Abbreviations: NS= No symptom, LL = Local lesion, LD = Leaf distortion, LN = Leaf narrowing, MO = mosaic, SN = Systemic necrosis

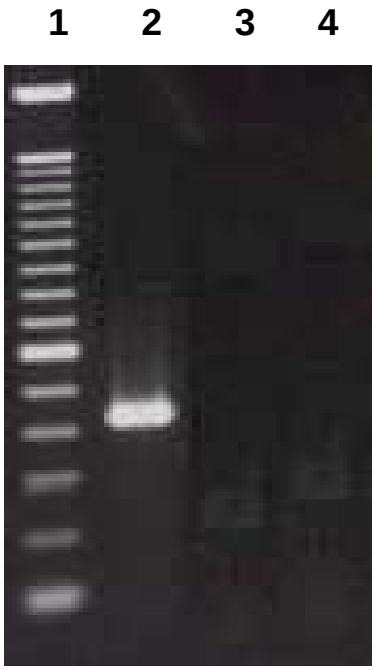

Figure 2 - RT-PCR analysis of total RNA extracted from tomato samples collected in Sapopema, PR. Lane 2 – 409 bp fragment amplified by primer pair TMV-1/-2. Lane 3 – No amplification by primer pair ToMV- 5/-6. Lane 4 – Healthy plant extract tested with TMV-1/-2 and ToMV-5/-6 primers in a multiplex RT-PCR. Lane 1- 100 bp DNA ladder.

Table 2 - Percentage of identities of the deduced amino acid sequence of the 409 bp RT-PCR product located in the movement protein gene of TMV from Sapopema-PR, compared to sequences from GenBank.

Virus isolate*	**TMV-Sapopema**	**GenBank accession** *DQ173945*
TMV-U1- China	100	AAP59459
TMV-OM - Japan	100	P03582
ToMV - Australia	94	AAK17990
ToMV - Canada	94	AAB97422
PMMV-KR – South Korea	88	BAD90600
ORSV- Singapore	84	Q84135
ORSV- Japan	84	P22590
TMGMV - Japan	76	BAB83988

(TMV – *Tobacco mosaic virus*, ToMV – *Tomato mosaic virus*, PMMoV – *Pepper mild mottle virus*, ORSV – *Odontoglossum mosaic virus*, TMGMV – *Tomato mild green mosaic virus*)

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atggagaagatcttaccgtcgatgtttacccctgtaaagagtgtcatgtgttccaaagtt
 M  E  K  I  L  P  S  M  F  T  P  V  K  S  V  M  C  S  K  V     38
Gataaaataatggttcatgagaatgaatcattgtcagaggtaaaccttcttaaaggagtt
 D  K  I  M  V  H  E  N  E  S  L  S  E  V  N  L  L  K  G  V .   58
aagcttattgatagtggatacgtctgtttagccggtctggtcgtcacgggcgagtggaac
 K  L  I  D  S  G  Y  V  C  L  A  G  L  V  V  T  G  E  W  N     78
ttgcccgacaattgcagaggaggtgtgagcgtgtgtctggtggacaaaaggatggaaaga
 L  P  D  N  C  R  G  G  V  S  V  C  L  V  D  K  R  M  E  R     98
gccgacgaggccactctcggatcttactacacagcagctgcaaagaaaagatttcagttc
 A  D  E  A  T  L  G  S  Y  Y  T  A  A  A  K  K  R  F  Q  F    118
aaggtcgttcccaattatgcaataaccacccaagacgcgatgaaaaacgtctggcaagtt
 K  V  V  P  N  Y  A  I  T  T  Q  D  A  M  K  N  V  W  Q  V    138
ttagtcaatattagaaatgtgaagatgtcagcgggtttctgtccgcttt
 L  V  N  I  R  N  V  K  M  S  A  G  F  C  P  L     .  154
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Figure 3 - Nucleotide and deduced amino acid sequence located in the internal region of P30 movement protein gene of TMV isolate from Sapopema. Amino acids are numbered from the 5' end according to their actual position in the P30 sequence. Amino acid residues 65 to 86 (underlined) represent the domain implicated in protein folding. Residues 112 to 154 represent part of binding domain A, responsible for single strand nucleic acid interaction. The sequence access number in the GenBank for TMV-Sapopema is DQ173945.

Sequence analysis of deduced amino acids showed 100% identity with part of the P30 protein of TMV isolates from China and Japan, 94% for ToMV isolates from Australia and Canada and less than 88% with other tobamoviruses (Table2). This sequence revealed two domains of TMV P30 movement protein responsible for the virus cell-to-cell spread through intercellular connections, as described by Citovsky et al., (1992) (Fig. 3). After many attempts, amplification of this genomic region by primer pair ToMV-5/-6 was not effective, demonstrating the specificity of this primer set for its own virus species (Fig.2). The antiserum produced would facilitate future surveys of TMV and ToMV in combination with RT-PCR, allowing quick and precise species differentiation of this two *Tobamoviruses.* To our knowledge, this work detailed for the first time, some biological and molecular properties of a TMV isolate from Paraná, responsible for production decrease in two plasticulture locations in Sapopema.

RESUMO

Durante uma inspeção em cultivos protegidos de tomate em Sapopema, Paraná, foram observadas anormalidades foliares em 90% das plantas, indicando possivelmente a existência de um problema de fitotoxidade causada por herbicidas. Todavia, os sintomas manifestados nas hospedeiras após os ensaios de inoculação mecânica revelaram que os sintomas estariam relacionados a uma infecção por *Tobamovirus.* As reações de RT-PCR com oligonucleotídeos específicos para uma região interna da proteína de movimento de dois vírus comuns em tomate, TMV e ToMV, resultaram na amplificação de um fragmento de 409 pares de bases, apenas com os oligonucleotídeos específicos para o TMV. Após o sequenciamento, os aminoácidos deduzidos apresentaram identidade de 100% quando comparados com as seqüências das proteínas de movimento de outros isolados do TMV, e 94% de identidade com seqüências do ToMV. A RT-PCR demonstrou ser um método eficiente para a rápida e conclusiva determinação da espécie viral envolvida na infecção do tomateiro. A seqüência parcial do genoma do isolado de TMV de Sapopema, está depositada no GenBank sob o número de acesso DQ173945, sendo esta a primeira seqüência genômica parcial de um isolado de TMV do Brasil, e conforme o nosso conhecimento, o primeiro isolado de TMV do Paraná a ter algumas de suas propriedades biológicas e moleculares determinadas. Um anti-soro policlonal foi produzido, o que permitirá futuros levantamentos da ocorrência de *Tobamovirus* nas principais áreas de cultivo de tomate do Paraná.

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