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SHORT COMMUNICATION / *COMUNICAÇÃO*

Genetic structure of a Brazilian population of the begomovirus *Tomato severe rugose virus* (ToSRV)

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ABSTRACT

Begomoviruses are whitefly-transmitted single-stranded DNA viruses of great economic importance in the tropics and subtropics. Several begomovirus species have been reported in tomatoes in Brazil, but only a few predominate in the field, for unknown reasons. In this study begomovirus-infected tomato samples were collected in Viçosa, State of Minas Gerais, in Nov/2009 and Dec/2010. Viral genomes were amplified, cloned and sequenced. A total of 36 DNA-A components were obtained. Sequence comparisons indicated the presence of a single begomovirus, *Tomato severe rugose virus* (ToSRV), with pairwise identities between isolates ranging from 97.3 to 100%. Subdivision tests indicated the existence of a single population. The analysis of variability descriptors indicated that the ToSRV population has a genetic variability similar to other begomovirus populations described in Brazil infecting tomato. Neutrality tests suggested the occurrence of purifying selection acting upon the population. Recombination analysis identified recombination events with begomoviruses from the weed species *Sida micrantha*. The wide distribution of ToSRV in the field and the detection of recombination indicate that continuous monitoring of viral populations in the field will be required to enable an efficient resistance-based control strategy for begomoviruses.

Key words*:* geminivirus, genetic variability, recombination.

Viruses belonging to the family *Geminiviridae* have a genome comprised of circular ssDNA molecules encapsidated in a twinned icosahedral capsid (Rojas et al., 2005). The family is divided into four genera (*Mastrevirus*, *Curtovirus*, *Topocovirus* and *Begomovirus*) according to the type of insect vector, host range, genome organization and phylogeny (Brown et al., 2012). Begomoviruses are transmitted by whiteflies (*Bemisia tabaci* - Homoptera: Aleyrodidae) and infect dicotyledonous plants. In Brazil, most begomoviruses are bipartite with two DNA components, DNA-A and DNA-B. For identification of a begomovirus isolate, the analysis of the full DNA-A sequence is essential.

In Brazil as well as in other countries in Latin America, the incidence and severity of the diseases caused by begomoviruses has greatly increased since the 1980's, due to the dissemination of aggressive biotypes of the whitefly vector (Lourenção & Nagai, 1994; Villas-Bôas et al., 2002). As a result of the introduction and dissemination of *B. tabaci* biotype B in Brazil, a number of new begomovirus species have been described infecting tomatoes (Ribeiro et al., 2003; Castillo-Urquiza et al., 2008; Fernandes et al., 2008). However, and in spite of the large number of begomovirus species described, only a few seem to predominate in the field, being detected in >90% of the samples analyzed in field surveys (Cotrim et al., 2007; Castillo-Urquiza et al., 2008; Fernandes et al., 2008). *Tomato severe rugose virus* (ToSRV) is one of these "field-prevalent" begomoviruses (Colariccio et al., 2006; Fernandes et al., 2008; Barbosa et al., 2011; Rocha, 2011). In addition to tomato it also naturally infects pepper, potato and tobacco, and under experimental conditions it is capable of infecting at least nine weed species (Souza-Dias et al., 2008; Barbosa et al., 2009; Nozaki et al., 2010; Barbosa et al., 2011). This broad host range may have important implications for disease management and development of resistant cultivars to ToSRV at the field level.

The study of geminivirus populations has assisted in the understanding of the evolution of these pathogens under field conditions (Prasanna et al., 2010; Ramos-Sobrinho et al., 2010; Rocha, 2011; Silva et al., 2011a, b). Such information is useful in the development of management strategies based on natural or engineered resistance, as it allows for a greater understanding of the evolutionary forces acting upon the pathogen (Prasanna et al., 2010).

The aim of this study was to determine the genetic make up of a population of ToSRV obtained from tomato

The sequences described in this study have been deposited in GenBank under the accession numbers JX865615-JX865650.

samples collected over two years (2009 and 2010) in Viçosa, State of Minas Gerais.

Tomato samples showing typical symptoms of begomovirus infection were collected in November/2009 and December/2010 at an experimental field of Universidade Federal de Viçosa (UFV) (20º45'14"S, 42º52'53''W, 648 m elevation). DNA extraction was carried out from fresh leaves according to Dellaporta et al. (1983). The presence of begomoviruses was confirmed by non-radioactive molecular hybridization according to specifications of the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences). Full length viral genomes were amplified from positive samples by rolling-circle amplification (RCA) (Inoue-Nagata et al., 2004), cloned in pBLUESCRIPT KS+ (Stratagene) after monomerization with the restriction enzymes *Bam*H I, *Cla* I and *Kpn* I and sequenced commercially (Macrogen, Seoul, South Korea).

DNA-A nucleotide sequences were initially submitted to a BLAST search for preliminary species assignment based on the 89% threshold level established by the *Geminiviridae* Study Group of the International Committee on Taxonomy of Viruses (Brown et al., 2011). Additional pairwise nucleotide sequence comparisons were made with DNAMan version 6.0 (Lynnon Biosoft Corporation) using the Optimal Alignment option with the following parameters: Ktuple = 2, Gap penalty = 7, Gap open = 10, Gap extension = 5. Nucleotide sequences of begomoviruses used in the recombination and phylogenetic analyses (Table 1) were aligned using the Muscle module in MEGA 5.0 (Tamura et al., 2011). A phylogenetic tree based on the DNA-A sequence alignment was constructed with MEGA 5.0 using the Neighbour-Joining method. Bootstrap analysis (10,000 replications) was carried out to verify the significance of each tree branch.

The program DnaSP version 5 (Librado & Rojas, 2009) was used to determine the extent of genetic differentiation or level of gene flow between the two putative subpopulations (samples collected in Nov/2009

TABLE 1 - Begomoviruses used for pairwise sequence comparisons, phylogenetic and recombination analysis

Species	Acronym	GenBank access number
Brazil		
Bean golden mosaic virus	BGMV	M88686
Blainvillea yellow spot virus	BlYSV	EU710756
Sida common mosaic virus	SiCmMV	EU710751
Sida micrantha mosaic virus	SiMMV	FJ686693
Sida mosaic Brazil virus	SiMBV	FN436001
Sida mottle virus	SiMoV	AY090555
Sida yellow leaf curl virus	SiYLCV	EU710750
Sida yellow mosaic virus	SiYMV	AY090558
Tomato chlorotic mottle virus	ToCMoV	AF490004
Tomato common mosaic virus	ToCmMV	EU710754
Tomato leaf distortion virus	ToLDV	EU710749
Tomato mild mosaic virus	ToMIMV	EU710752
Tomato rugose mosaic virus	ToRMV	AF291705
Tomato severe rugose virus	ToSRV	DQ207749
Tomato severe rugose virus	ToSRV	HQ606467
Tomato severe rugose virus	ToSRV	FJ824808
Tomato yellow spot virus	ToYSV	DQ336350
Tomato yellow vein streak virus	ToYVSV	EF417915
Others countries of the Americas		
Sida golden yellow vein virus	SiGYVV	AJ577395
Sida yellow mosaic Yucatan virus	SiYMYuV	DQ875872
Sida yellow vein virus	SiYVV	Y11099
Tomato Chino La Paz virus	ToChLPV	AY339618
Tomato golden motlle virus	ToGMoV	AF132852
Tomato mosaic Havana virus	ToMHV	EF088197
Tomato mottle Taino virus	ToMoTV	AF012300
Tomato mottle virus	ToMoV	AY965900
Tomato severe leaf curl virus	ToSLCV	AF130415
Tomato leaf curl Sinaloa virus	ToLCSV	AJ608286
Tomato leaf curl Cuba virus	ToLCCUV	AM050143
Tomato yellow leaf distortion virus	ToYLDV	FJ174698
Old World		
Tomato leaf curl New Delhi virus	ToLCNDV	U15015

and in Dec/2010) using Wright's *Fst* value (Wright, 1951). Wright's *Fst* is a measure of the proportion of total genetic variation contained in a subpopulation relative to the total genetic variation. Values can range between 0 and 1 and *Fst* values > 0.05 (5%) suggest a degree of differentiation between subpopulations.

The main descriptors of genetic variability were quantified: number of polymorphic sites (S), total number of mutations (Eta), average number of nucleotide differences (k), nucleotide diversity (π), mutation frequency, number of haplotypes (H), haplotype diversity (Hd), Watterson's estimate of the population mutation rate based on the total number of segregating sites (θw-S) and on the total number of mutations (θ-Eta). The analysis was performed using DnaSP version 5. The sequences of each ORF in the DNA-A (CP, capsid protein; Rep, replication-associated protein; Trap, trans-activating protein; Ren, replication-enhancer protein; and AC4) were aligned using the Muscle module in MEGA 5.0. Tajima's *D* (Tajima, 1989) and Fu and Li's *D** and *F** (Fu & Li, 1993) were computed for each ORF using DnaSP version 5.

Phylogenetic network analysis for evidence of recombination was performed among ToSRV isolates (including the ones obtained in Viçosa) with the Neighbor-Net method implemented in the program SplitsTree4 (Huson & Bryant, 2006). Analysis of potential recombination events was carried out using the Recombination Detection Program (RDP) version 3.0 (Martin et al., 2010) using default parameters. To omit unreliable signals, only recombination events supported by at least four different methods were considered. The dataset included some of the isolates obtained in this study and other begomoviruses previously described. Some of the isolates of ToSRV were excluded of the dataset after analysis of the minimum genetic distance threshold recommended in the RDP manual.

The percentage of tomato and weed plants displaying symptoms of begomovirus infection at the collection site was very high on both years, as well as the level of whitefly infestation. This site has been used for screening material from UFV's tomato breeding program for begomovirus resistance (Xavier et al., 2011). A total of 65 tomato samples were collected: 28 in Nov/2009 and 37 in Dec/2010. All samples were positive for the presence of begomoviruses based on non-radioactive molecular hybridization (data not shown). A total of 36 full-length DNA-A components were cloned. Clones were named according to the recommendations of the *Geminiviridae* Study Group of the ICTV (Fauquet et al., 2008): country code:isolate reference:year of collection (*eg*, BR:Vic1:09).

BLAST analysis and pairwise sequence comparisons of the DNA-A clones indicated that they all corresponded to isolates of the species *Tomato severe rugose virus* (ToSRV), with nucleotide sequence identities between isolates ranging from 97.3 to 100%. These results were confirmed by phylogenetic analysis, in which all isolates clustered in a single branch with previously sequenced ToSRV isolates (GenBank accession numbers DQ207749, FJ824808 and HQ606467), supported by a bootstrap value of 100% (data not shown). Moreover, all ToSRV isolates, including the ones described here, formed a group with begomoviruses previously reported in Brazil, which diverged from another group of begomoviruses described from other American countries (data not shown). The detection of a single begomovirus may be related to the fact that all clones were obtained with the same enzyme (*Bam*H I). Although most of the begomoviruses described infecting tomatoes in Brazil possess a unique *BamH* I site either in the DNA-A or in the DNA-B, one particular virus which has been commonly detected in the region, *Tomato common mosaic virus* (ToCmMV), does not. Thus, it is possible that the true diversity of begomoviruses present in that field was not represented, and it is not possible to claim that ToSRV is the only begomovirus present at the sampled site. Additional clones should be obtained using different enzymes in order to verify (or rule out) the presence of other begomoviruses.

The ToSRV isolates were initially divided into two putative subpopulations according to the date of collection (Nov/2009 or Dec/2010). However, the value obtained for the *Fst* test (0.036) indicated that these two putative subpopulations were actually not structured. Therefore they were treated as a single population comprised of 36 DNA-A clones.

The ToSRV population obtained here, when compared with two other ToSRV populations also obtained from tomatoes in Minas Gerais (municipalities of Florestal and Carandaí) (Rocha, 2011), has shown a similar genetic variability. This could be related to the proximity of the collecting sites (*ca*. 100 km between Viçosa and Carandaí, 230 km between Viçosa and Florestal, and 150 km between Florestal and Carandaí). Nevertheless, there was a large time span between sample collection: samples from Carandaí and Florestal were collected in Jul/2008, 17 and 29 months before the samples from Viçosa. When compared with populations of ToCmMV and *Tomato yellow vein streak virus* (ToYVSV) from tomato (Castillo-Urquiza, 2008), *Bean golden mosaic virus* (BGMV) from lima bean (Ramos-Sobrinho et al., 2010), *Cleome* leaf crumple virus (ClLCrV) from *Cleome affinis* (Silva et al., 2011a) and *Macroptilium* yellow spot virus (MaYSV) from *Macroptilium* spp. (Silva et al., 2011b), the genetic variability was lower for the ToSRV population. The ClLCrV and MaYSV population showed greater genetic variability (Table 2).

The genetic variability of the ToSRV population from this work was similar to those from two other ToSRV populations obtained from tomatoes in Minas Gerais (Rocha, 2011). Conversely, the variability of these ToSRV populations is much lower than those from begomovirus populations infecting lima bean or weeds (Ramos-Sobrinho et al., 2010; Silva et al., 2011a, b). A low degree of genetic variability could be a feature of ToSRV populations. However, we favor a hypothesis based on all ToSRV populations being sampled from the same host (tomato),

TABLE 2 - Genetic structure of the population of *Tomato severe rugose virus* (ToSRV) collected from tomato in Viçosa, State of Minas Gerais, Brazil, compared with populations of *Tomato severe rugose virus* (ToSRV), *Tomato yellow vein streak virus* (ToYVSV) and *Tomato common mosaic virus* (ToCmMV) from tomato in the Southeastern Brazil and *Bean golden mosaic virus* (BGMV) from *Phaseolus lunatus* (lima bean) in Northeastern Brazil. Analysis based on the DNA-A sequence

Population		Genome length (nt)	No. of sequences	S[a]	Eta[b]	k[c]	π[d]	Mutation frequency[e]	H[f]	Hd[g]	θw-S[h]	θ-Eta[i]
Virus	Location											
ToSRV	Viçosa (Minas Gerais)	2593	36	144	149	11.528	0.0045	1.6×10^{-3}	34	0.997	0.0134	0.0139
	Carandaí (Minas Gerais)[1]	2589	19	73	74	10.474	0.0040	1.8×10^{-3}	18	0.994	0.0080	0.0081
	Florestal (Minas Gerais)[1]	2592	5	37	37	19.000	0.0073	3.5×10^{-3}	5	1.000	0.0068	0.0068
BGMV	(Alagoas)[2]	2616	20	251	265	40.321	0.0154	5.0×10^{-3}	18	0.989	0.0271	0.0295
ToCmMV	Paty de Alferes (Rio de Janeiro)[3]	2560	10	11	11	2.200	0.0009	4.3×10^{-4}	8	0.933	0.0015	0.0015
ToCmMV	Coimbra (Minas Gerais)[3]	2560	12	91	92	26.258	0.0103	3.0×10^{-3}	11	0.985	0.0118	0.0119
ToYVSV	Paty de Alferes (Rio de Janeiro)[3]	2562	26	49	49	5.381	0.0021	7.4×10^{-4}	25	0.997	0.0050	0.0050

[a] Number of polymorphic sites; [b] Total number of mutations; [c] Average number of nucleotide differences; [d] Nucleotide diversity; [e] Total number of mutations / Total number of sequenced nucleotides; [f] Number of haplotypes; [g] Haplotype diversity; [h] Watterson's estimate of the population mutation rate based on the total number of segregating sites; [i] Watterson's estimate of the population mutation rate based on the total number of mutation; [1] Data from Rocha (2011); [2] Samples were collected at multiple locations throughout the state; Data from Ramos-Sobrinho et al. (2010); [3] Data from Castillo-Urquiza (2008).

which also has a narrow genetic basis. In other words, the genetic variability of begomovirus populations could be modulated by the genetic variability of the host. Since ToSRV is known to infect multiple hosts, including crops such as peppers, and weeds such *Nicandra physaloides* and *Solanum nigrum* (Cotrim et al., 2007; Barbosa et al., 2009; Nozaki et al., 2010; Barbosa et al., 2011), which supposedly have distinct genetic bases, this hypothesis could be tested by analyzing ToSRV populations obtained from these different hosts.

Neutrality tests were applied to assess whether there was evidence of selection on genomic regions encoding the CP, Rep, Ren, Trap and AC4 ORFs. Values for Tajima's *D* and Fu and Li's *D** and *F** tests were negative for all ORFs, but were not significant for Trap and AC4 (except for Fu and Li's *F** for AC4; Table 3). These results indicate that purifying selection is acting upon all ORFs in the DNA-A of the ToSRV population from tomato, coinciding with the results obtained by Rocha (2011). During the viral infection cycle, several factors may impose purifying selection on the population. The biology of the vector and its feeding habits create bottlenecks for the maintenance of structural and functional characteristics of the viral proteins. Interaction with host factors may also impose purifying selection (García-Arenal et al., 2001).

Phylogenetic relationships inferred by neighbor-net analysis based on a data set consisting of the three previously reported ToRSV isolates (DQ207749, FJ824808 and HQ606467) plus the ToSRV population described here, revealed evidence of multiple recombination events (Figure 1). To investigate these putative recombination signals in greater detail, a data set including additional sequences of Brazilian begomoviruses was analyzed using the RDP3 package. This analysis identified many unique recombination signals (Table 4), including two recombination events for the ToSRV isolates described here. The first event was observed for all isolates, encompassing nucleotides 1,910 to 2,135 (within the Rep and AC4 ORFs), with *Sida micrantha mosaic virus* (SiMMV) (AJ557451) and other unknown begomovirus as putative parents. The second event was detected for isolate BR:Vic30:10, encompassing nucleotides 1,910 to 2,166 (also within the Rep and AC4 ORFs) with the same putative parents of the other event (Table 4). Additionally, the isolate BR:Vic20:10 and *Bean golden mosaic virus* (BGMV) (M88686) were identified as putative parents in a recombination event involving *Tomato rugose mosaic virus* (ToRMV) (AF291705) with breakpoints located at nucleotides 1,520 and 2,596 (Rep ORF and common region) (Table 4; Figure 2). Lefeuvre et al. (2007a)

TABLE 3 - Neutrality tests based on variation in the CP, Rep, Ren, Trap and AC4 ORFs of the isolates of *Tomato severe rugose virus* (ToSRV) collected from tomato samples in Viçosa, State of Minas Gerais, Brazil

Tests/ORFs	CP	Rep	Ren	Trap	AC4
Tajima's *D*	-4.56*	-3.16*	-3.33*	-0.46^{NS}	-1.78^{NS}
Fu and Li's *D**	-4.62*	-3.39*	-3.35*	-0.86^{NS}	-2.17^{NS}
Fu and Li's *F**	-2.61**	-2.29**	-2.37**	-1.45^{NS}	-1.96*

* Significant values, $P<0.05$
** Significant values, $P<0.01$
^{NS} Non significant values

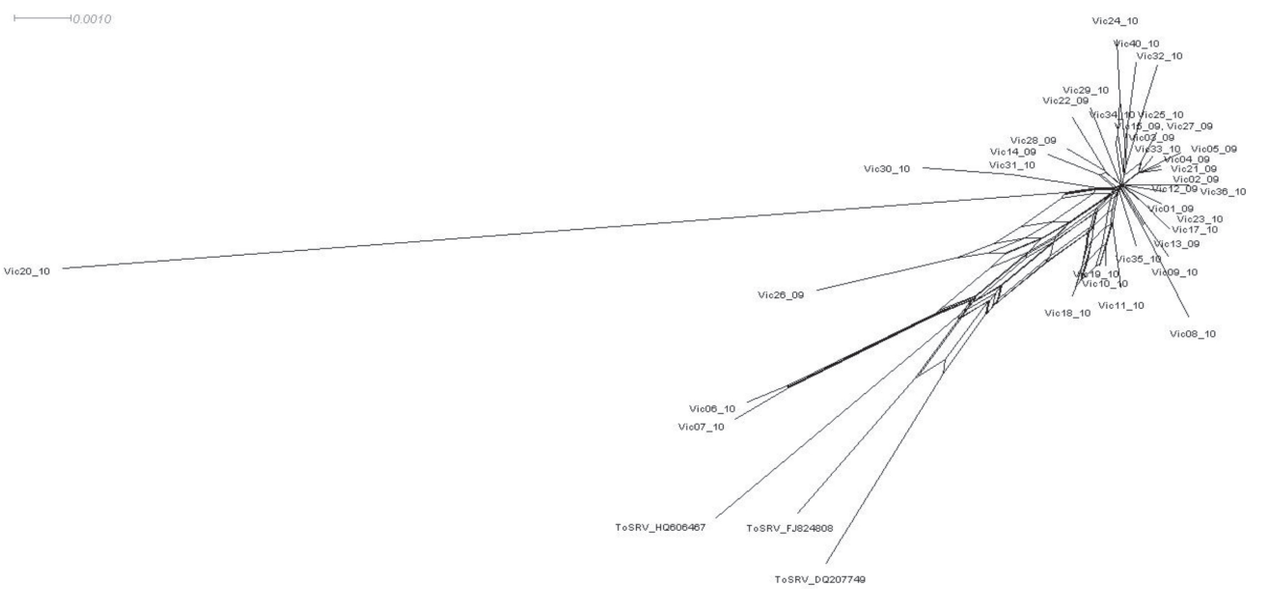

FIGURE 1 - Phylogenetic evidence for recombination among ToSRV (*Tomato severe rugose virus*) isolates, including the ones obtained in Viçosa, State of Minas Gerais, Brazil. Neighbor-net analysis was performed using the program SplitsTree4. Formation of a reticular network instead of a single forked tree is suggestive of recombination.

TABLE 4 - Recombination events detected between begomoviruses previously described in Brazil and the isolates of *Tomato severe rugose virus* (ToSRV) from Viçosa, State of Minas Gerais, Brazil

Recombinants	Breakpoints		Parents		Programs[1]	*P*-value
	Initial	Final	Major	Minor		
BR:Vic01:09, BR:Vic06:10, BR:Vic20:10	1,910	2,135	Unknown	SiMMV(AJ557451)	RGBMCS	5.2×10^{-16}
BR:Vic30:10	1,910	2,166	Unknown	SiMMV(AJ557451)	RGBMCS	5.2×10^{-16}
ToRMV (AF291705)	1,520	2,596	BGMV(M88686)	BR:Vic20:10	RGBMCS3	1.9×10^{-32}
ToSRV (DQ207749)	1,910	2,135	Unknown	SiMMV(AJ557451)	RGBMCS	5.2×10^{-16}
ToSRV (FJ824808)	1,910	2,135	Unknown	SiMMV(AJ557451)	RGBMCS	5.2×10^{-16}
ToSRV (HQ606467)	1,910	2,135	Unknown	SiMMV(AJ557451)	RGBMCS	5.2×10^{-16}

[1]Programs that detected recombination events: R=RDP; G=GeneConv; B=Bootscan; M=MaxChi; C=CHIMAERA; S=SisScan; 3=3SEQ. The program underlined yielded the lowest *P*-value.

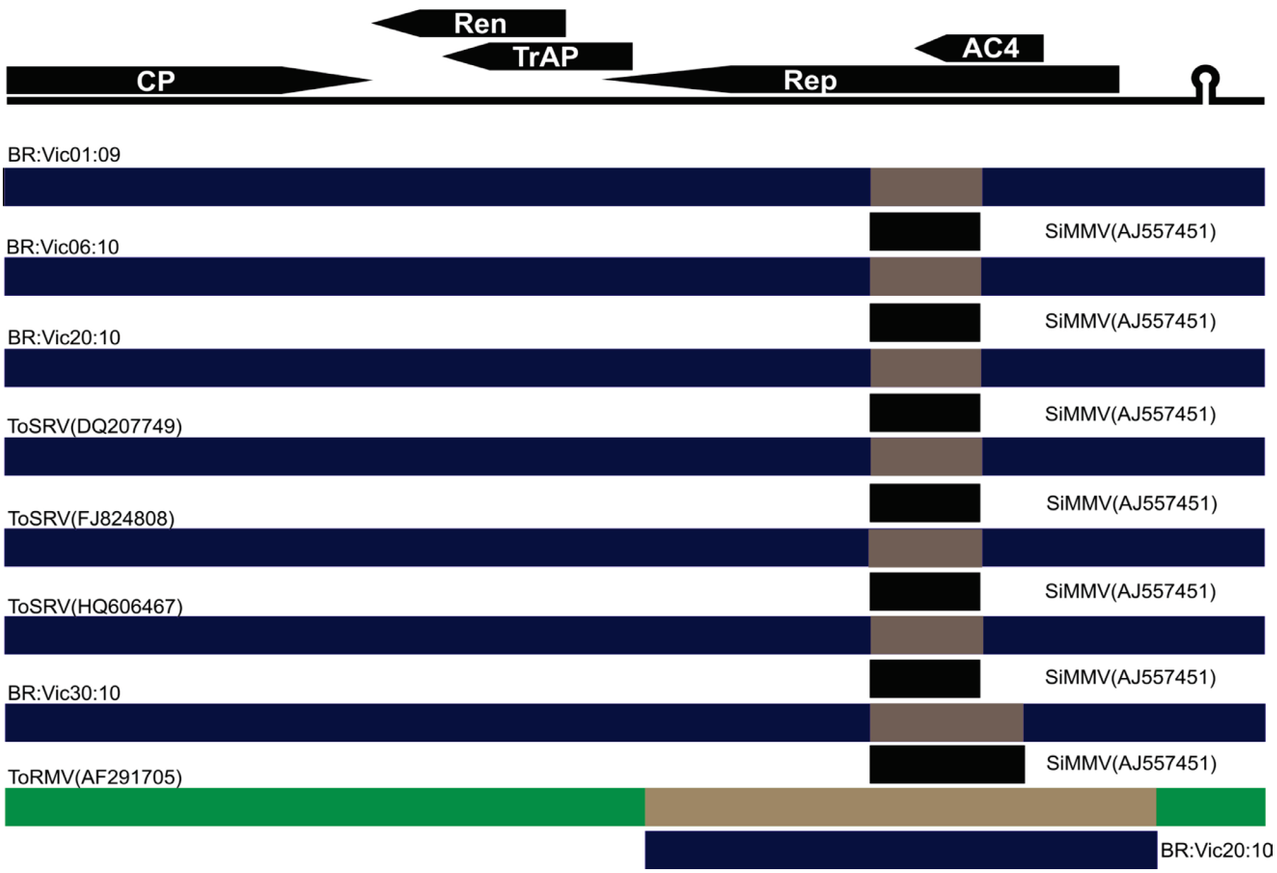

FIGURE 2 - Evidence of recombination among ToSRV isolates. Blue boxes represent the DNA-A sequences of the ToSRV isolates and black or brown boxes represent the regions of sequence with a potentially recombinant origin. Black boxes indicate recombinant region from minor parent detected with a high probability ($P < 0.05$) by at least four methods.

suggested that coding regions are generally less susceptible to recombination due to structural bottlenecks. However, the regions encoding the Rep and CP of begomoviruses have been shown to be hotspots of recombination (García-Andrés et al., 2007; Lefeuvre et al., 2007b), coinciding with the results obtained in our study.

Several studies have shown that recombination is a common source of genetic variability for begomoviruses in Brazil (Galvão et al., 2003; Inoue-Nagata et al., 2006) and worldwide (Pita et al., 2001; Monci et al., 2002; García-Andrés et al., 2007). Recombination events detected in this work showed that viruses detected mostly in weeds (SiMMV) have recombined with others viruses, possibly giving rise to isolates which are better adapted to tomato.

The wide distribution of ToSRV in the State of Minas Gerais and its low degree of genetic variability have

important implications for the development of disease management strategies based on genetic resistance. The evolutionary processes on these populations were little influenced by environmental factors such as geographical distance, temperature or altitude. A low degree of variability in the viral population could facilitate the obtainment of durable resistance, as long as there are no natural reservoirs where more diverse populations could be hiding.

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