Eur J Plant Pathol (2011) 129:1–7
DOI 10.1007/s10658-010-9699-5

Tomato leaf deformation virus, a novel begomovirus associated with a severe disease of tomato in Peru

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Accepted: 7 October 2010 / Published online: 22 October 2010

Abstract Begomovirus infection was suspected in tomato plants exhibiting symptoms of curling and deformation of leaves observed in a survey conducted in northern and central Peru. Rolling circle amplification and restriction fragment length polymorphism analyses suggested that a begomovirus was present in symptomatic plants. The full-length sequence of a begomovirus DNA component was determined, comprising 2591 nucleotides. Based on its genome organization, we suggest it corresponds to the DNA-A of a New World begomovirus. Less than 89% nucleotide sequence identity to known begomoviruses was found, indicating that it corresponds to an isolate of a distinct begomovirus species for which the name tomato leaf deformation virus (ToLDeV) is proposed. Different stretches of the genomic component have the highest sequence identity with different viruses compatible with a recombinant origin. Sequence segments shared common ancestors with isolates of either soybean blistering mosaic virus, tomato yellow spot virus, or tomato chino La Paz virus. Partial sequence analysis of begomovirus isolates present in symptomatic tomato samples collected in northern and central Peru suggested widespread occurrence of this new begomovirus. This is the first confirmation of a begomovirus infection in tomatoes in Peru.

Keywords *Begomovirus* · *Geminiviridae* · Recombinant · *Solanum lycopersicum* · Tomato leaf deformation virus

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Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) are plant-infecting viruses with circular single-stranded DNA (ssDNA) genomes encapsidated in twinned icosahedral virions (Fauquet et al. 2008). These viruses have emerged to become one of the largest and most economically important group of plant-infecting viruses, being a major threat to vegetable production in warm and temperate regions worldwide (Fargette et al. 2006; Mansoor et al. 2006; Moriones and Navas-Castillo 2000; Seal et al. 2006; Varma and Malathi 2003). The global spread of

begomoviruses is associated with the pantropical dissemination of the whitefly (*Hemiptera*: *Aleyrodidae*) vector *Bemisia tabaci* Gennadius, including Latin America (Morales and Anderson 2001). New World begomoviruses have a bipartite genome composed of two ~2.6 kb DNA components, referred to as DNA-A and DNA-B (Stanley et al. 2005). There are coding regions in both the virion (V) and complementary (C) sense strands, separated by intergenic (IR) non coding regions. The DNA-A and DNA-B components share an approximately 200-nucleotide sequence referred to as the common region (CR), which contains the viral origin of replication and regulatory sequences (Jeske 2009). The DNA-A components encode five or six genes. These genes are *AV1* (CP), *AV2* (precoat gene, not for New World bipartite begomoviruses), *AC1* (Rep), *AC2* (TrAP), *AC3* (Ren), or *AC4*. The DNA-B components encode two genes, *BV1* and *BC1* (Rojas et al. 2005; Stanley et al. 2005). Recently, rolling-circle amplification (RCA) using the bacteriophage ϕ29 DNA polymerase has become a powerful tool for the detection, diagnosis and genetic characterization of begomoviruses (Haible et al. 2006; Jeske 2007; Inoue-Nagata et al. 2004).

Epidemics of a disease associated with infestations of the whitefly *B. tabaci* are causing severe damage to tomato (*Solanum lycopersicum* L.) crops of Peru along the Pacific Ocean coast since the early 2000s. Symptoms consisting of curling and deformation of leaves and stunted growth of plants (Fig. 1) occurred at incidences up to 100% causing significant yield losses. These symptoms are typical of begomovirus infections. The presence of *B. tabaci* infestations associated with geminivirus-like symptoms in tomato in the coastal Valley of Ica has been reported previously (Morales and Anderson 2001). However, as far as we know, no characterization of the possible begomovirus(es) involved has been reported to date. Therefore, samples were collected from symptomatic plants at several locations in the major fresh market tomato-growing regions of the Pacific Ocean coastal lowlands in northern (Reque and Batán Grande areas) and central (Carabayllo and Huaral areas) Peru, during 2003 and 2008 (Table 1), and analyzed for begomovirus infection. Total DNA was isolated from 10 mg samples of dried leaf material using a CTAB-based purification method (Haible et al. 2006). Samples were tested for presence of begomovirus DNAs by PCR using three pairs of degenerate primers, PAL1v1978/PAR1c496 and PCRc1/PBL1v2040 for DNA-A and DNA-B, respectively (Rojas et al. 1993), and prBV1855/prBC656 for DNA-B (Idris and Brown 1998). The expected ~1.1 kbp DNA fragment was amplified from all samples with the DNA-A specific degenerate primer pair, whereas no amplification product was obtained from any samples with degenerate primers specific for DNA-B.

Sequences of the IR obtained by direct sequencing using primer PAR1c496 (Macrogen Inc., Seoul, South Korea) of PCR products amplified from a number of

Fig. 1 Symptoms of upward curling of leaflet margins (panel **a**) and leaflet deformation (panel **b**) characteristic of diseased tomato plants

Table 1 Tomato samples collected during 2003 and 2008 in commercial fresh market tomato fields of Carabayllo and Huaral areas in the central and Reque and Batán Grande areas in the northern regions of the Pacific Ocean coastal lowlands of Peru

Year of collection	Location of collection	Isolate[a]	GenBank accession numbers for IR[b]
2003	Carabayllo	PT1:2003	GQ334473
	Carabayllo	PT2:2003	
	Carabayllo	PT3:2003	GQ334474
	Carabayllo	PT4:2003	GQ334475
	Huaral	PT5:2003	
	Huaral	PT6:2003	GQ334476
2008	Reque	PT7:2008	
	Reque	PT8:2008	
	Reque	PT9:2008	GQ334477
	Reque	PT10:2008	
	Batán Grande	PT11:2008	GQ334478
	Batán Grande	PT12:2008	

[a] In 2003 isolates were collected from commercial tomato fields growing tomato cultivars with no begomovirus resistance and in 2008 from fields of tomato cv. 'Dominator' (Seminis, Saint Louis, USA) with tolerance to tomato yellow leaf curl disease

[b] Intergenic region (IR) nucleotide sequences of begomovirus DNA-A were obtained for a number of samples by direct sequencing of the DNA product derived from PCR amplification using the degenerate primer pair PAL1v1978/PAR1c496 for begomovirus DNA-A (Rojas et al. 1993). The GenBank accession numbers are given for the sequences obtained

samples revealed 99 to 100% identity between them (GenBank accession numbers provided in Table 1), suggesting that all plants were infected with isolates of the same begomovirus species. Amplification of begomovirus-associated circular DNA was performed from total DNA extracts using the RCA methodology (TempliPhi kit, GE Healthcare, Little Chalfont, UK) essentially as described by Inoue-Nagata et al. (2004), but initially heating extracts for 3 min at 95°C to improve the amplification from dried leaf material (Shepherd et al. 2008). Restriction fragment length polymorphism (RFLP) analysis of the RCA products using the restriction enzymes *Hpa*II and *Sau*3AI resulted in identical RFLP profile in all cases (exemplified in Fig. 2 for *Hpa*II), again suggesting the same begomovirus infecting those plants. Therefore, the isolate from sample PT1:2003 was selected for further genetic characterization of the begomovirus involved in infection. A ~2.7 kbp fragment corresponding to a full-length genome component was obtained from this sample by digestion of the RCA product with *Kpn*I and was cloned in pBluescriptII SK+ (Stratagene, La Jolla, USA) to generate pPT1K7-1.0 which was completely sequenced by primer walking (Macrogen Inc.). Sequence data were analyzed using SeqMan and SeqBuilder softwares included in the DNASTAR package (DNASTAR Inc., Madison, USA) and the pairwise percent of nucleotide sequence identity was calculated using MegAlign based on the multiple sequence alignment Clustal V algorithm (DNASTAR package) with default parameters. The fragment cloned from isolate PT1:2003 consisted of 2591 nucleotides (GenBank accession number GQ334472) and represented a complete component with a genome organization typical of the DNA-A of a New World bipartite begomovirus (Stanley et al. 2005), with five genes, one on the viral strand corresponding to *AV1* and four on the complementary-sense strand corresponding to *AC1*, *AC2*, *AC3*, and *AC4* (schematically represented in Fig. 3b). Viral and complementary sense genes were separated by an IR that exhibited features of members of the genus *Begomovirus*, with hallmarks such as the TATA box for Rep and CP promoters and the putative stem loop structure with the conserved nonanucleotide TAATATT↓AC in the loop, including the nicking site (shown by the arrow) for the initiation of viral strand replication (Jeske 2007). Moreover, three repeated DNA motifs (iterons) which are predicted to function as species-specific primary high-affinity

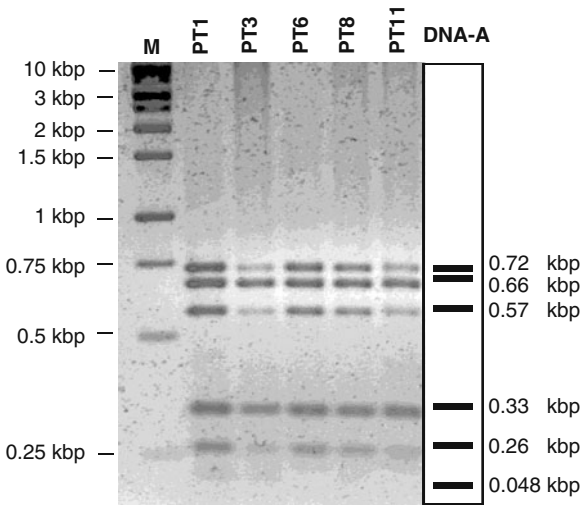

Fig. 2 Rolling circle amplification products obtained from a number of symptomatic tomato samples digested with *Hpa*II and separated on 2% agarose gel. The *Hpa*II restriction products obtained in silico (NEBcutter v2.0 tool, New England BioLabs, Ipswich, USA) for the complete DNA-A sequence of the virus characterized in this work (GenBank accession number GQ334472) is shown on the right side of the gel. M, molecular marker. Lengths of DNA fragments are indicated at the left and right sides of the gel

Rep binding motifs (Argüello-Astorga and Ruiz-Medrano 2001), were also identified. Efforts to detect and clone the putative cognate DNA-B component from isolate PT1:2003 or the other collected isolates were unsuccessful. Although the genome organization of the component cloned here is homologous to the DNA-A component of New World bipartite begomoviruses (with no *AV2*-precoat-gene present), the analyses conducted here might suggest that the virus is monopartite. Specifically, PCR with DNA-B primers did not produce amplification products, and RFLP analysis conducted on RCA product appears to show the presence of only a single circular DNA. Also, cloning and sequencing of putative DNA-B fragments corresponding to bands revealed after hybridization at low stringency conditions of Southern blots of RCA digestion products, using a probe corresponding to a fragment of the DNA-B of a begomovirus recently described in Cuba (Fiallo-Olivé et al. 2009), did not yield DNA fragments homologous to DNA-B. The presence of a monopartite begomovirus would be a significant finding for the New World since, with the exception of the introduced tomato yellow leaf curl virus (Duffy and Holmes 2007), no other monopartite begomovirus has been reported there. However, we cannot exclude the possibility that the DNA-B exists but was missed because it is present at very low amounts, as similar difficulties in detecting and isolating the DNA-B components have been reported for other putative bipartite begomoviruses (Inoue-Nagata et al. 2004). Therefore, this is an aspect that will require further study including infectivity analysis of the molecule cloned here.

Analysis of the complete nucleotide sequence of the DNA-A obtained from isolate PT1:2003 showed that it was less than 89% identical to that of any previously characterized begomovirus, with ~75% nucleotide sequence identity with the closest related begomovirus, an isolate of soybean blistering mosaic virus, SbBMV (GenBank EF016486). The current species demarcation criteria for begomoviruses are based on complete DNA-A nucleotide sequence identity, establishing 89% as threshold level (Fauquet and Stanley 2005; Fauquet et al. 2008). Therefore, the DNA-A characterized here corresponds to an isolate of a novel begomovirus species for which the name tomato leaf deformation virus (ToLDeV) is proposed. The SWeBLAST tool (Fourment et al. 2008) was used to locate the most closely related begomoviruses using the BLAST facilities of GenBank, allowing at the same time a check for possible recombination features in the molecule avoiding the significant problem to have first to decide which sequences to compare. Interestingly, comparisons performed in successive stretches of the nucleotide sequence revealed that the relationships varied depending on the sequence position, suggesting a recombination origin for ToLDeV DNA-A. Three begomoviruses were found to be closely related to ToLDeV DNA-A: an isolate of SbBMV (see above), an isolate of tomato yellow spot virus (ToYSV) (GenBank DQ336350) and an isolate of tomato chino La Paz virus (ToChLPV) (GenBank DQ347948). A diagrammatic representation of the results for recombination analyis showing the genetic relationships of the different segments of the ToLDeV DNA-A based on the sequence identities of the most closely related viruses is shown in Fig. 3. It should be noted that in general nucleotide sequence identities below 90% were detected (Fig. 3a), suggesting common but not recent ances-

a

Begomovirus	GenBank n°	DNA-A	Nucleotides									
			1-260	261-520	521-780	781-1040	1041-1300	1301-1560	1561-1820	1821-2080	2081-2340	2341-2591
SbBMV	EF016486	75.1	53.8	76.6	80.5	81.9	80.0	74.6	76.9	79.2	82.3	64.9
ToYSV	DQ336350	72.4	48.8	80.4	82.5	83.1	77.3	65.8	78.5	76.9	81.2	54.3
ToChLPV	DQ347948	72.2	46.2	74.2	75.8	78.5	81.5	75.9	85.4	70.0	84.6	57.8

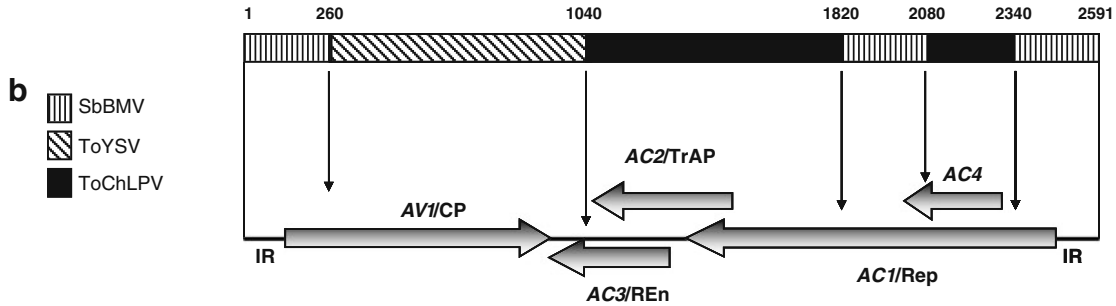

Fig. 3 Begomovirus sequences related to that determined in this study (named tomato leaf deformation virus, ToLDeV) deduced by using SWeBLAST (Fourment et al. 2008). The pairwise percent of nucleotide sequence identity of ToLDeV with the most closely related begomoviruses calculated based on Clustal V alignment for the complete DNA-A and for successive stretches of the nucleotide sequence is shown; nucleotide positions refer to the nucleotide sequence of ToLDeV (GenBank accession number GQ334472) (panel **a**). Based on the sequence identities found, a proposal is made for the genetic relationships of the different segments of the ToLDeV DNA-A taking into account the most closely related virus in each sequence segment and nucleotide positions of ToLDeV sequence (panel **b**); a schematic representation of the virus DNA-A is shown at the bottom of this panel showing the intergenic region (IR) and genes *AV1* (that encodes the coat protein, CP), *AC1* (encoding the replication associated protein, Rep), *AC2* (encoding the transcriptional activator protein, TrAP), *AC3* (encoding the replication enhancer protein, Ren), and *AC4*. Viruses included in comparisons are isolates of soybean blistering mosaic virus (SbBMV), tomato yellow spot virus (ToYSV), and tomato chino La Paz virus (ToChLPV) (GenBank accession numbers EF016486, DQ336350, and DQ347948, respectively)

tors shared with DNA-A of ToChLPV, ToYSV and ToChLPV. The analysis shows that both non-coding and coding sequences have been exchanged among the ancestors of ToLDeV resulting in chimeric genes (Fig. 3b). The complex cis interactions within the genomes (genomic components) of begomoviruses suggest that the survival and spread of this recombinant must have been the result of strong positive selection resulting in improved fitness (Martin et al. 2005a). Using another approach to detect recombination, the Recombination Detection Program (RDP3 Beta 27 software, using a multiple comparison corrected P-value cut-off of 0.05 and default settings) (Martin et al. 2005b), only one significant recombination event was supported by several algorithms, involving ToYSV in a region between nucleotides 470 and 1,040, in which a relatively high sequence identity to this virus was detected (Fig. 3a). Therefore, the analysis demonstrates the usefulness of SWeBLAST to trace the evolutionary origin of a newly reported virus. Altogether, our data suggest that the begomovirus genome component characterized here represents a novel begomovirus with a complex history of recombination, which provides further evidence for the importance of recombination in begomovirus evolution and emergence (Galvao et al. 2003; Moriones et al. 2007; Padidam et al. 1999; Seal et al. 2006; van der Walt et al. 2009; Zhou et al. 2001).

In summary, the results shown here indicate that the begomovirus characterized is widespread in tomato crops in Peru. This study confirms earlier preliminary findings about the existence of a begomovirus infections in tomatoes in Peru (Murayama et al. 2005), and identifies the associated virus as a new begomovirus species. This information is relevant because Peru is a centre of origin of cultivated tomato with the presence of wild *Solanum* section *Lycopersicum* reservoirs (Rick 1976) that might be adversely affected by the spread of this begomovirus.

Acknowledgements This work was supported through grant GEN2006-27770-C2-2 (Ministerio de Educación y Ciencia, Spain, co-financed by FEDER) in the frame of the ERA-NET Plant Genomics project ERA-PG 040B "RCA-GENOMICS" of the European Commission 6th Framework Programme for research and the Trilateral Cooperation GABI-GENOPLANTE-MEC. B. Márquez contract was financed by this same project. E. Fiallo-Olivé was supported by a MAEC-AECID fellowship from Ministerio de Asuntos Exteriores y de Cooperación, Spain. We thank S. García-Andrés for preliminar analyses, and M. V. Martín, R Tovar, and R. Campos for technical assistance.

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