Accepted Manuscript

Title: Fulfilling Koch's postulates confirms the monopartite nature of tomato leaf deformation virus, a begomovirus native to the New World

Authors: S. Sánchez-Campos, A. Martínez-Ayala, B. Márquez-Martín, L. Aragón-Caballero, J. Navas-Castillo, E. Moriones

PII: S0168-1702(13)00051-8
DOI: doi:10.1016/j.virusres.2013.02.002
Reference: VIRUS 95929

To appear in: *Virus Research*

Received date: 1-12-2012
Revised date: 25-1-2013
Accepted date: 5-2-2013

Please cite this article as: Sánchez-Campos, S., Martínez-Ayala, A., Márquez-Martín, B., Aragón-Caballero, L., Navas-Castillo, J., Moriones, E., Fulfilling Koch's postulates confirms the monopartite nature of tomato leaf deformation virus, a begomovirus native to the New World, *Virus Research* (2010), doi:10.1016/j.virusres.2013.02.002

Highlights

- Koch's postulates are fulfilled for ToLDeV as a monopartite begomovirus native of the New World
- ToLDeV is found throughout the major tomato growing regions of Peru
- Three major genetic types are found present in the ToLDeV population
- Local evolution is suggested for the ToLDeV population present in Peru
- Geographic segregation was observed for the population of ToLDeV.

Fulfilling Koch's postulates confirms the monopartite nature of tomato leaf deformation virus, a begomovirus native to the New World

Sánchez-Campos, S.[1§:], Martínez-Ayala, A.[1§:], Márquez-Martín, B.[1], Aragón-Caballero, L.[2], Navas-Castillo, J.[1], Moriones, E.[1*]

[1] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora" (IHSM-UMA-CSIC), Consejo Superior de Investigaciones Científicas, Estación Experimental "La Mayora", 29750 Algarrobo-Costa, Málaga, Spain.

[2] Facultad de Agronomía, Universidad Nacional Agraria La Molina, Av. La Molina s/n, La Molina, Lima, Perú.

§: These authors contributed equally to this work.

[*]**Corresponding Author:** Enrique Moriones, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora" (IHSM-UMA-CSIC), Consejo Superior de Investigaciones Científicas, Estación Experimental "La Mayora", 29750 Algarrobo-Costa, Málaga, Spain. Phone number: (+34) 952548990: Fax: (+34)952552677; e-mail address: moriones@eelm.csic.es.

Summary

The monopartite nature of the begomovirus tomato leaf deformation virus (ToLDeV) reported in Peru is demonstrated here. The DNA molecule cloned from an infected plant was shown to be fully infectious in tomatoes inducing leaf curling and stunted growth similar to that observed in field-infected plants. The viral DNA was reisolated from systemically infected tissues of inoculated plants, thus fulfilling Koch's postulates. ToLDeV was demonstrated, therefore, as the causal agent of the disease syndrome widespread in tomato crops in Peru. This virus was shown to be present throughout the major tomato-growing regions of this country, both in tomatoes and wild plants. Analyses of the sequences of 51 ToLDeV isolates revealed a significant genetic diversity with three major genetic types co-circulating in the population. A geographical segregation was observed which should be taken into account for virus control. Constraints to genetic divergence found for the C4 gene of ToLDeV isolates suggest a relevant function for this protein. The results obtained confirm ToLDeV as a monopartite begomovirus native to the New World, which is a significant finding for this region.

Key words: Begomovirus, *Geminiviridae*, *Solanum lycopersicum*, tomato, Tomato leaf deformation virus, Genetic diversity.

1. Introduction

Begomoviruses (genus *Begomovirus*) are small plant viruses with twinned, quasi-isometric virions encapsidating genomes of circular single-stranded DNA (ssDNA) characteristic of members of the family *Geminiviridae* (Brown et al., 2012). Begomoviruses are an emerging threat worldwide (Varma et al., 2012) and are

transmitted in nature by the whitefly *Bemisia tabaci* Genn. (*Hemiptera*: *Aleyrodidae*). The genus *Begomovirus* consists of viruses with either monopartite or bipartite genomes. Globally, begomoviruses are grouped into two major phylogenetic clades named Old World (OW) and New World (NW) (Briddon et al., 2010). Most begomoviruses have bipartite genomes with components designated DNA-A and DNA-B, each of which is 2.5–2.8 kilobases. Both components are required for infectivity (Stanley, 1983). Demonstration of the infectivity of a single component was done for the first time for the begomovirus tomato yellow leaf curl virus (TYLCV), thus showing that begomoviruses with a single genomic component exist (Navot et al., 1991). Remarkably, no monopartite begomovirus native to the NW has been identified to date, although recently the OW monopartite begomovirus TYLCV was inadvertently introduced and has spread in the NW (Duffy and Holmes, 2007; Polston et al., 1999). Another exception is monopartite begomoviruses that infect sweet potato [*Ipomoea batatas* (L.) Lam], which probably are spreading worldwide through the exchange of infected propagating material (tubers). These are known as sweepoviruses and cluster in a monophyletic clade separated from OW and NW begomoviruses (Albuquerque et al., 2012).

Coding sequences are present in both the virion (V) and complementary (C) sense strands of genome components of monopartite and bipartite begomoviruses, separated by the an intergenic non coding region (IR). In bipartite begomoviruses, the DNA-A component has five or six genes. These are AV1 (which encodes the coat protein, CP), AV2 (precoat protein), AC1 (replication associated protein, Rep), AC2 (transcriptional activator protein, TrAP), AC3 (replication enhancer protein, Ren), and AC4. Interestingly, DNA-As of NW begomoviruses are characterized for the absence of AV2. The DNA-B component has two genes that encode proteins directly involved in

movement, BV1 (which encodes the nuclear shuttle protein, NSP), and BC1 (movement protein, MP), on the V-sense and C-sense strands respectively (Brown et al., 2012). The genomes of monopartite begomoviruses have a similar organization and genes homologous to DNA-A of bipartite begomoviruses (Brown et al., 2012). These genes include V1 (CP protein), V2 (precoat), C1 (Rep), C2 (TrAP),C3 (Ren), and C4. Different gene requirements exist for monopartite and bipartite begomoviruses. Thus, monopartite begomoviruses absolutely require a functional CP for systemic infection and C4 as a pathogenicity factor, whereas both proteins can be dispensable in bipartite begomoviruses; also, V2 is essential for virus-host interaction and pathogenicity, whereas it is absent in NW bitpartite begomoviruses (Luna et al., 2012; Pooma et al., 1996; Pooma and Petty, 1996; Rojas et al., 2001; Wartig et al., 1997). The IR contains the promoters for transcription of the V- and C- sense genes (Hanley-Bowdoin et al., 1999), the stem-loop structure with the nonanucleotide sequence conserved in geminiviruses, TAATATTAC (Jeske 2007; Lazarowitz et al., 1992), and virus-specific repeated sequences (iterons) where the Rep binds to initiate replication (Argüello-Astorga et al., 1994).

The spread of begomoviruses causing severe damage to important food crops in tropical and subtropical regions in the last two decades is associated with the spread of their insect vector *B. tabaci* and the global movement of plant materials (Navas-Castillo et al., 2011; Seal et al., 2006; Varma et al., 2011). Emergence of these viruses is especially important in Latin America in regions where reproduction of *B. tabaci* is favored by high temperatures (Morales, 2010; Morales and Jones 2004; Navas-Castillo et al., 2011; Rojas and Gilbertson, 2008). Control of begomoviruses is mainly based on intensive insecticide treatment programs to control vector transmission, with limited success to reduce virus spread (Nauen and Denholm, 2005). The use of host genetic

resistance if available, therefore, is the best control option, such as for the *Ty-1* gene widely used commercially to control damage caused by TYLCV (Michelson et al., 1994). Durable control based on genetic resistance, however, requires knowledge about the genetic diversity of the virus population (García-Arenal and McDonald, 2003).

A new begomovirus named tomato leaf deformation virus (ToLDeV) has been recently described infecting tomato (*Solanum lycopersicum* L.) crops in Peru (Márquez-Martín et al., 2011). Tomato plants infected with ToLDeV exhibited a disease syndrome consisting of upward curling of leaflet margins, leaflet deformation and growth stunting with dramatic yield losses when infections occur in early growth stages (Márquez-Martín et al., 2011). The complete nucleotide sequence was obtained for a circular ssDNA molecule associated with symptomatic plants (GenBank accession number GQ334472), showing a genome organization typical of DNA-A of NW begomoviruses (with no AV-2 gene present) and phylogenetic relationships with this group of viruses (Márquez-Martín et al., 2011). No DNA-B could be isolated from symptomatic plants suggesting that ToLDeV might be a monopartite begomovirus (Márquez-Martín et al., 2011). Demonstration of the infectivity of the cloned DNA, however, was lacking as was fulfilling of Koch's postulates for this virus as the causal agent of the disease observed in tomato. The presence of a monopartite begomovirus would be a significant finding for the New World. Also, no information was available about the genetic diversity of the ToLDeV population in Peru, which is essential to implement durable control strategies.

In the present study, we demonstrated the monopartite nature of ToLDeV and that it is the causal agent of the disease spreading in tomato crops in Peru. Isolates of this virus were found throughout this country. The genetic diversity of ToLDeV was characterized suggesting the presence of a locally evolved population with three major

131 genotypes. Also, infection of ToLDeV was shown in wild hosts and a geographical
132 segregation of the population is suggested. This demonstrates ToLDeV as a monopartite
133 begomovirus native to the New World.
134 .

135 **2. Materials and methods**

136 2.1. Virus isolates

137 A total of 250 samples were collected from cultivated and wild host plants
138 exhibiting begomovirus-like symptoms throughout vegetable growing regions of Peru
139 (departments of Lambayeque, La Libertad, Lima, Ica and Arequipa) during 2003 and
140 2008 to 2010. Surveys comprised plants from cultivated hosts such as tomato, pepper
141 (*Capsicum annuum* L., *Capsicum chinense* Jack, *Capsicum bacatum* cv. *Pendulum* (Willd.)
142 Eshbaugh), bean (*Phaseolus vulgaris* L., *Phaseolus lunatus* L.), and cucurbit (*Citrullus lanatus*
143 (Thunb.) Matsum. & Nakai), wild relatives of tomato indigenous to Peru, and weeds (Table
144 1). A sample from each plant consisted of apical young leaves that were stored dried at
145 4ºC until used. After confirming ToLDeV presence by dot-blot hybridizaion (see
146 below), 48 isolates from tomato representing all growing areas of Peru along with the
147 three additional isolates detected in wild hosts were included in the analysis (Table 1).
148

149 2.3 Sample analysis

150 Field samples and agroinoculated plants were analyzed for the presence of
151 ToLDeV by squash blot or by dot-blot hybridization using 1! l of total DNA (see
152 below), on positively charged nylon membranes (Roche Diagnostics, Mannheim,
153 Germany). For hybridization, a digoxigenin (DIG)-labeled DNA probe, specific to the
154 ToLDeV IR region was used. The probe was prepared by PCR according to the DIG-
155 labeling detection kit (Roche Diagnostics) and as described by Navas-Castillo et al.

(1999), using the primer pair MA1644 (5′-CTTAAAGGCCTTAGGTGGGGGC-3′, nucleotides 2436 to 2457) and MA1645 (5′-CGCCATGGGGCATCCCGCTTWGG-3′, nucleotides 201 to 179) designed on the sequence reported for ToLDeV DNA (GenBank accession number GQ334472). Hybridization was carried out under high stringency conditions (washing steps at 65°C in 0.1× SSC [15 mM NaCl and 1.5 mM sodium citrate] and 0.1% sodium dodecyl sulfate) following standard procedures.

2.3 Total DNA extraction, Southern-blot hybridization and amplification

Total DNA was extracted from 10 mg of dried leaf tissue using a CTAB-based purification method (Haible et al., 2006). Different viral DNA forms were visualized in Southern-blot hybridizations of total DNA extracts performed as reported by Noris et al. (1998) using the DIG-labeled DNA probe indicated above. Begomovirus DNA was amplified from total DNA extracts by using the degenerate primers MA55 (5′-GCCCACATYGTCTTYCCNGT-3′) and MA56 (5′-GGCTTYCTRTACATRGG-3′), based on primers PAL1v1978 and PAR1c476, respectively, universal for begomoviral DNA-A and designed by Rojas et al. (1993) (Fiallo-Olivé et al., 2012). For DNA-B amplification, primers designed by Rojas et al. (1993) and Idris and Brown (1998) were used. Rolling-circle amplification (RCA) with ! 29 DNA polymerase was used for full-length genome amplification as described by Inoue-Nagata et al. (2004) using a TempliPhi DNA Amplification Kit (GE Healthcare, Little Chalfont, UK).

2.4 Sequence analysis

The genetic diversity study was conducted by using a sequence of 921 nucleotides (nucleotides 1846 to 175 of ToLDeV sequence, GenBank GQ334472) deduced from the PCR products amplified from the 51 ToLDeV isolates detected in the

survey (Supplementary Table 1). This sequence comprises the IR and the 5' terminal two-thirds of the Rep gene that includes the overlapping C4 gene (Fig. 1). Nucleotide sequences were obtained through the commercial service offered by Macrogen Inc. (Seoul, South Korea). EditSeq, SeqBuilder and SeqMan softwares (available in "Lasergene", DNAStar Inc., Madison, WI, USA) were used for assembly and analysis of the sequences. Genetic distances for synonymous (d_S) and nonsynonymous substitutions (d_{NS}) were estimated using the Pamilo-Bianchi-Li method in *MEGA* version 4 (Pamilo and Bianchi 1993; Tamura et al., 2007). *MEGA* version 5 (Tamura et al., 2011) was used for phylogenetic inference by the Maximum-Likelihood method. The nucleotide sequences reported in this work were deposited in the GenBank database under the accession numbers KC237066 to KC237115, as indicated in Supplementary Table 1.

2.4 Construction of an infectious clone of ToLDeV and infectivity assays

A head-to-tail dimer construct for the infectivity of clone pPT1K7-1.0 obtained from isolate PT1:2003 (Márquez-Martín et al., 2011) was produced. For this, a 1-mer fragment was excised from pPT1K7-1.0 by digestion with the restriction enzyme *Kpn*I (Roche Diagnostics GmbH, Mannheim, Germany) and religated to obtain the 1-mer circular genome. Then, RCA amplification was performed and the product obtained was partially digested with *Hind*III (Roche Diagnostics) to recover a 2-mer fragment that was cloned into the unique *Hind*III site of the pCAMBIA 0380 vector (Cambia, Canberra, Australia) to produce pPT1/03K7-2.0 which was transformed into *Agrobacterium tumefaciens* strain LBA 4404.

For plant inoculation, liquid cultures of *A. tumefaciens* containing pPT1/03K7-2.0 were produced, adjusted to an OD of 1.0 at 600 nm, and used to inoculate test plants

by stem puncture inoculation (Monci et al., 2005). Inoculated plants were maintained in an insect-free growth chamber (26 °C during the day and 18 °C at night, 70% relative humidity, with a 16-h photoperiod at 250 µmol s–1 m–2 photosynthetically active radiation) until analyzed.

3. Results

3.1. Widespread occurrence of ToLDeV in Peru.

An extensive field survey was conducted throughout the major tomato growing regions of Peru between 2003 and 2010. Analysis of samples collected showed a widespread occurrence of ToLDeV. A total of 51 out of 250 plant samples analyzed resulted in positive reaction for the presence of ToLDeV (Table 1). Among cultivated host plants sampled, the presence of ToLDeV was only detected in tomatoes, with a similar prevalence observed in plants of susceptible and resistant cultivars, the latter based on the *Ty-1* resistance gene (Table 1). Very mild or no symptoms, however, were observed in plants of resistant cultivars in contrast to the striking symptoms of begomovirus infection observed in those of susceptible cultivars. Interestingly, ToLDeV was also detected infecting weeds sampled close to tomato crops, such as in *Anoda cristata* (L.) Schltdl or *Tanacetum parthenium* L. The presence of ToLDeV in wild relatives of tomato was only identified in a single *Solanum penelli* Correll plant located close to an infected tomato crop (Table 1).

3.2. Genetic diversity and structure of the ToLDeV population in Peru

Sequence analysis of a 921-nucleotide (nt) region (Fig. 1) from the genome of 51 virus isolates revealed a significant genetic diversity in the ToLDeV population present in Peru. Nucleotide sequences of isolates were grouped in three phylogenetically

separated clades (Groups 1 to 3, Fig. 2). High percentages of nucleotide sequence identity were observed among sequences of single clades (98.3 to 100.0% in Group 1, 96.9 to 100.0% in Group 2, and 92.4 to 99.8% in Group 3) (Supplementary Fig. 1).. Percentages of nucleotide sequence identity among sequences from different clades were 91.7 to 93.8% between Groups 1 and 2, 84.4 to 90.4% between Groups 2 and 3, and 85.5 to 92.5% between Groups 1 and 3 (Supplementary Fig. 1). Group 3 contained the most divergent sequences (especially those of isolates PE7:2010_To_Ica and PE12:2010_To_Ica) (Fig. 2A). Even within a single clade, slight nucleotide sequence variation was observed suggesting genetic variability in the population (Fig. 2A). Interestingly, a geographical segregation of the population was observed. Thus, isolates of Group 1 were detected only in the Departments of Lambayeque, La Libertad and Lima in central-north Peru, whereas isolates of Group 3 and most of those of Group 2 (except isolates P22:2009 and P110:2009) were detected in Ica and Arequipa (south Peru) (Fig.2). Virus isolates collected from wild hosts belonged to Group 1 (isolate P17:2009 Ac) and Group 2 (isolates P110:2009 Tp and P182:2009 Sp).

Repeated sequences (iterons) were localized in the intergenic region of ToLDeV as described previously (Márquez-Martín et al., 2011). These iterons were located upstream from of the TATA box and presented three different patterns correlated with the three groups of isolates observed in the phylogenetic analysis of the 921-nt region sequenced (Supplementary Fig. 2). Thus, the first pattern ACACC / GGTGT / GGAGT corresponded to isolates from Group 1 (Fig. 2) that includes the isolate PT1:2003 for which the complete genome sequence is available. The other two patterns, (ACCCC / GGGGT / GGGGT and TTACC / GGTAG / GGTAA) corresponded to isolates from Group 2 and Group 3, respectively (Supplementary Fig. 2).

Nucleotide diversities were calculated for sequences obtained in this study at synonymous (d_S) and nonsynonymous (d_{NS}) positions of the open reading frames (ORFs) contained in the sequenced 92-nt fragment that includes 5´-terminal two-thirds of the C1 ORF and the C4 ORF completely overlapped in the C1 ORF. As summarized in Table 2, d_{NS}/d_S ratios lower than 1 were obtained, suggesting a positive selection. For the C1 ORF, the d_{NS}/d_S ratio was about three times lower (0.201) than the one obtained for C4 (0.581) suggesting stronger constraints to variation. The d_{NS}/d_S values of C1 were of the same order in overlapping and nonoverlapping regions (0.184 vs. 0.207 respectively).

3.3. ToLDeV is a monopartite begomovirus

As for samples collected in years 2003 and 2008 included in a previous analysis (Márquez-Martín et al., 2011), efforts to detect and clone a putative cognate DNA-B component from isolates collected during years 2009 and 2010 were unsuccessful. Specifically, PCR with primers designed for DNA-B amplification did not produce any products, and RFLP analysis conducted on RCA products appear to show the presence of only a single circular DNA for all analyzed samples. As the presence of a monopartite begomovirus would be a significant finding for the NW, further studies were conducted. In this sense, the infectivity of the DNA molecule cloned from sample PT1:2003 (Márquez-Martín et al., 2011) was studied. For this, a head-to-tail dimer clone was obtained and inoculated in test plants via *A. tumefaciens*. Twenty-five tomato and five *Nicotiana benthamiana* plants were inoculated resulting in all of them infected systemically with ToLDeV based on tissue-blot hybridization (Fig. 3E). Moreover, Southern-blot analysis of DNA extracted from young noninoculated leaves of agroinoculated tomato and *N. benthamiana* plants clearly exhibited the presence of

replication intermediate forms of the DNA genome of the ToLDeV infectious clone (Fig. 3F). *N. benthamiana* plants exhibited yellowing, curling of apical leaves and significant plant-growth stunting (compare ToLDeV-infected plant with mock-inoculated plant in right and left positions, respetively, of Fig. 3A). Tomato plants developed symptoms similar to those observed in field-infected plants consisting of curling and deformation of apical leaves, and yellowing (Figs. 3B and 3D -mock inoculated on the right-) and stunted growth (Fig. 3C -mock-inoculated on the right). Sequencing of DNA fragments amplified from young noninoculated tissues of infected tomato and *N. benthamiana* test plants (two plants analyzed per host species) using primer pair MA55 / MA56 confirmed 100% nucleotide sequence identity with the corresponding sequence of isolate PT1:2003 inoculated. We conclude, therefore, that the DNA molecule cloned from PT1:2003 is fully infectious and biologically active in the absence of DNA-B both in tomato and *N. benthamiana.* Also, in tomato it was able to reproduce the disease symptoms observed in field-infected plants. Thus, Koch's postulates were fulfilled for this molecule as the causal agent of the disease syndrome observed in field-infected tomato plants.

4. Discussion

The curling and leaf deformation syndrome causing damage to commercial tomatoes grown along the Pacific Ocean coast of Peru since the early 2000s, was demonstrated to be caused by ToLDeV. A previous report suggested an association of this virus with symptomatic plants (Márquez-Martín et al., 2011). Only one circular ssDNA molecule, however, similar to DNA-A of NW begomoviruses (with no AV2-precoat gene present) could be isolated from symptomatic plants. Efforts to detect and clone the putative cognate DNA-B component characteristic of NW begomoviruses were unsuccessful,

suggesting that this virus could be a monopartite begomovirus. Here, we demonstrated that the DNA-A-like molecule was infectious and causes the disease syndrome observed in field tomatoes, thus, demonstrating that ToLDeV is a monopartite begomovirus. The high genetic diversity found in the ToLDeV population present in Peru is not consistent with a genetic bottleneck typical of a founder effect due to the introduction of a small number of individuals of a virus in a new niche as observed in other cases (Delatte et al., 2007; García-Arenal et al., 2001; Sánchez-Campos et al., 2002). In contrast, the presence of a locally evolved population is more plausible based on the results obtained. Three major groups of virus variants related phylogenetically were detected among ToLDeV isolates collected throughout Peru's tomato-growing regions. Such a population structure might reflect independent evolution due to genetic isolation. These data, together with phylogenetic clustering of ToLDeV with NW begomoviruses (Márquez-Martin et al., 2011) support this virus as a monopartite begomovirus native to the NW. Based on the surveys conducted here, a narrow host range was found in nature for ToLDeV which was almost confined to tomatoes. No infection was found in any sample collected from other cultivated hosts and a limited presence was detected in wild hosts. Nevertheless, more intensive samplings would be needed to investigate this aspect. The availability of an infectious clone would help to perform controlled inoculations under experimental conditions that will inform about the host range of this virus. This information is essential to establish control strategies in affected areas (Cooper and Jones, 2006).

The demonstration of the presence of a native monopartite begomovirus in the NW is a significant finding. Monopartite begomoviruses have been reported so far only in the OW, where they cause diseases in the absence of DNA-B. OW monopartite begomoviruses have been shown to associate also with ssDNA satellites known as

330 betasatellites to effectively infect plants and induce disease (Amin et al., 2011; Briddon
331 and Stanley, 2006). Mutagenesis studies with the OW monopartite begomovirus
332 TYLCV have shown that the CP, V2 and C4 proteins mediate movement in plants
333 including nuclear shuttling (CP) and cell-to-cell movement (V2, C4), respectively, the
334 functions carried out by the DNA-B-encoded proteins of bipartite viruses (Rojas et al.,
335 2001; Wartig et al., 1997). It has been speculated, moreover, that the lack of
336 monopartite begomoviruses in the NW might be related to the absence of the V2 gene
337 (Briddon et al., 2010). In this sudy we showed, however, that the single DNA
338 component characterized for ToLDeV is fully infectious and causes disease in tomatoes
339 in the absence of V2. The V2 protein is involved in virus-host interactions in OW
340 monopartite begomoviruses, being a strong suppressor of gene silencing that might
341 determine host range (Luna et al., 2012) and essential for virus movement (Rojas et al.,
342 2001). The results obtained here, however, demonstrated that the presence of V2 is not
343 essential for fully biological activity of this NW monopartite begomovirus. ToLDeV
344 has evolved in such a way that the genes present in its genome component can
345 complement functions provided by DNA-B-encoded genes of bipartite begomoviruses
346 and by the V2 gene of OW monopartite begomoviruses. It is tempting to speculate if
347 ToLDeV has evolved from the DNA-A molecule of a bipartite begomovirus which
348 would give some clues about begomovirus evolution. The requirement of C4 and/or CP
349 for virus movement and pathogenicity for this NW monopartite begomovirus as for OW
350 monopartite begomoviruses and in contrast to most NW bipartite begomoviruses
351 (Pooma and Petty, 1996; Rojas et al., 2001) needs also to be studied. These are aspects
352 that merit further research to help to shed light into the evolutionary aspects of
353 begomoviruses and the need for monopartite or bipartite genomes to survive in nature
354 (Briddon et al., 2010). Mutagenesis analyses of genes encoded by ToLDeV and the

subcellular localization of coded proteins would provide further insight into this aspect as shown in other cases (Rojas et al., 2001; Wartig et al., 1997).

The ratio of nucleotide diversity values at nonsynonymous to synonymous sites (d_{NS}/d_S) can be used to estimate the degree of selective constraint on a coding region (Fraile et al., 1996; Hall 2006). Here, we found that the genomic regions of ToLDeV analyzed (partial C1 and complete C4 genes) are under negative selection, stronger in the case of C1 with a d_{NS}/d_S ratio almost three times smaller than that of C4 (Table 2). The d_{NS}/d_S values obtained for the C1 region (overlapping region = 0.184 and nonoverlapping = 0.207) are similar to those obtained for both overlapping and nonoverlapping regions of the C1 gene of other begomoviruses such as cotton leaf curl-like (CLCu-like) viruses (0.233 and 0.168, respectively) (Sanz et al., 1999) or East African cassava mosaic virus (EACMV) (0.180) (Duffy and Holmes, 2009). Great differences, however, were found for d_{NS}/d_S values of the C4 ORF of ToLDeV and those reported for other OW monopartite begomoviruses, such as for CLCu-like or tobacco leaf curl-like (TbLC-like) viruses (Sanz et al., 1999; Yahara et al., 1998). A d_{NS}/d_S value of 0.581 for ToLDeV C4 supports functional constraints to genetic divergence. In contrast, d_{NS}/d_S values > 1 were reported for C4s of CLCu-like or TbLC-like begomoviruses and explained by their overlap nature with C1 and that constrictions imposed upon C1 prevail upon those acting on C4 (Sanz et al., 1999; Yahara et al., 1998). Also, similar d_{NS}/d_S values > 1 are found for C4s of NW bipartite begomoviruses, based on complete genome sequences available in databases [e.g., *d*NS/*d*S = 1.433 for Macroptilium yellow spot virus (n = 56 sequences), *d*NS/*d*S=1.444 for Bean golden mosaic virus (n = 11), or *d*NS/*d*S = 2.154 for Sida golden yellow vein virus (n = 6)]. It could be speculated that the C4 protein of ToLDeV is assuming roles of the V2 protein of monopartite begomoviruses and/or proteins present in DNA-B of

bipartite begomoviruses making it less permissive to variation. This is an aspect that merits further research.

The tomato is an economically and socially important vegetable crop in Peru, with about 225,000 tons produced in 6,000 ha in 2010 (Anonymous, 2010). Therefore, the widespread occurrence of ToLDeV throughout Peru in the tomato-growing regions is a major concern. As suggested for similar monopartite begomovirus epidemics, the use of host genetic resistance would be the best alternative to limit damage (Polston and Lapidot, 2007). A wide genetic diversity was found for the ToLDeV population, which is a challenge when resistance genes are deployed. Thus, for epidemics of tomato yellow leaf curl disease caused by monopartite begomoviruses (Moriones and Navas-Castillo, 2000), not every resistance gene is effective against every virus variant (Picó et al., 2000). Deployment of resistance genes, moreover, in regions in which virus genetic diversity occurs can result into population structure changes that can limit durability of the resistance (García-Andrés et al., 2009). Broad-spectrum resistance genes would be better introduced for durable resistance (Seal et al., 2006). The occurrence of three major groups of ToLDeV isolates has been shown here with some geographical segregation of the population. Therefore, breeding for resistance and deployment of resistance genes should take into account this genetic diversity. The best approach to breed for wide-range resistance would be to obtain infectious clones of representative isolates of each ToLDeV genetic group and challenge begomovirus resistance genes available in tomatoes (Polston and Lapidot, 2007). Pyramidation of effective genes could help to produce broad-spectrum resistance (Vidavski et al., 2008). If no useful resistance gene was found, new sources of resistance should be explored (Picó et al. 2000). Results obtained here suggest that the *Ty-1* gene widely used commercially to

404 control TYLCV (Michelson et al., 1994) might provide ToLDeV-tolerance, although
405 virus accumulation occurs in infected plants.
406
407 **Acknowledgements**
408 S. S.-C. was a recipient of a JAE-DOC postdoctoral contract from the CSIC (Spain) co-
409 financed by FSE. A. M.-A. was a recipient. of an AECID (Spain) fellowship. The work
410 was partially supported by grant GEN2006-27770-C2-2 (Ministerio de Educación y
411 Ciencia, Spain, cofinanced by FEDER) in the frame of the ERA-NET Plant Genomics
412 project ERA-PG 040B RCA-Genomics (6th EU Framework Programme for Research
413 and Technological Development, FP6) and network 111RT0433 from Fundación
414 CYTED. J. N.-C. and E. M. are members of the Research Group AGR-214, partially
415 funded by the Consejería de Economía, Innovación y Ciencia, Junta de Andalucía,
416 Spain (cofinanced by FEDER-FSE). We thank M.V. Martín and R. Tovar for technical
417 assistance and B. Jaffee for scientific English editing of the manuscript.
418
419 **References**
420 Albuquerque, L., Inoue-Nagata, A., Pinheiro, B., Resende, R., Moriones, E., Navas-
421 Castillo, J. 2012. Genetic diversity and recombination analysis of sweepoviruses
422 from Brazil. Virology J. 9, 241.
423 Amin, I., Hussain, K., Akbergenov, R., Yadav, J.S., Qazi, J., Mansoor, S., Hohn, T.,
424 Fauquet, C.M., Briddon, R. W. 2011. Suppressors of RNA Silencing Encoded
425 by the Components of the Cotton Leaf Curl Begomovirus-BetaSatellite
426 Complex. Mol. Plant Microbe Interact. 24, 973-983.
427 Anonymous, 2010. Producción Hortofrutícola 2010, Oficina de Estudios
428 Estadísticos y Económicos. Ministerio de Agricultura, Perú.

Argüello-Astorga, G.R., Guevara-Gónzalez, R.G., Herrera-Estrella, L.R., Rivera-Bustamante, R.F., 1994. Geminivirus replication origins have a group-specific organization of iterative elements: a model for replication. Virology 203, 90-100.

Briddon, R., Patil, B., Bagewadi, B., Nawaz-ul-Rehman, M., Fauquet, C., 2010. Distinct evolutionary histories of the DNA-A and DNA-B components of bipartite begomoviruses. BMC Evol Biol 10, 97.

Briddon, R.W., Stanley J., 2006. Sub-viral agents associated with plant-infecting single-stranded DNA viruses. Virology 2006, 344, 198-210

Brown, J.K, Fauquet, C.M., Briddon, R.W., Zerbini, M., Moriones, E., Navas-Castillo, J., 2012. *Geminiviridae*, in: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., (Eds.) Virus Taxonomy. Ninth Report of the ICTV. Elsevier/Academic Press, London, pp 351–373.

Cooper, I., Jones, R.A.C., 2006. Wild plants and viruses: under-investigated ecosystems. Adv. Virus Res. 67: 1-47.

Delatte, H., Holota, H., Moury, B., Reynaud, B., Lett, J.M., Peterschmitt, M., 2007. Evidence for a founder effect after introduction of Tomato yellow leaf curl virus-Mild in an insular environment. J. Mol. Evol. 65, 112-118.

Duffy, S., Holmes, E.C., 2007. Multiple introductions of the Old World begomovirus tomato yellow leaf curl virus into the New World. Appl. Environ. Microbiol. 73, 7114-7117.

Duffy, S., Holmes, E.C., 2009. Validation of high rates of nucleotide substitution in geminiviruses: phylogenetic evidence from East African cassava mosaic viruses. J. Gen. Virol. 90, 1539-1547.

453 Fiallo-Olivé, E., Martínez-Zubiaur, Y., Moriones, E., Navas-Castillo, J., 2012. A
454 novel class of DNA satellites associated with New World begomoviruses.
455 Virology 426, 1-6.
456 Fraile, A., Malpica, J. M., Aranda, M. A., Rodríguez-Cerezo, E., García-Arenal, F.,
457 1996. Genetic diversity in tobacco mild green mosaic tobamovirus infecting the
458 wild plant Nicotiana glauca. Virology 223, 148-155.
459 García-Andrés, S., Tomás, D.M., Navas-Castillo, J., Moriones, E., 2009. Resistance-
460 driven selection of begomoviruses associated with the tomato yellow leaf curl
461 disease. Virus Res. 146, 66-72.
462 García-Arenal, F., McDonald, B.A., 2003. An analysis of the durability of resistance
463 to plant viruses. Phytopathology 93, 941-952.
464 García-Arenal, F., Fraile, A., Malpica, J.M., 2001. Variability and genetic structure
465 of plant virus populations. Annu. Rev. Phytopathol. 39, 157-186.
466 Haible, D., Kober, S., Jeske, H., 2006. Rolling circle amplification revolutionizes
467 diagnosis and genomics of geminiviruses. J. Virol. Methods 135, 9-16.
468 Hall, G., 2006. Selective constraint and genetic differentiation in geographically
469 distant barley yellow dwarf virus populations. J. Gen. Virol. 87, 3067-3075.
470 Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S., Robertson, D., 1999.
471 Geminiviruses: models for plant DNA replication, transcription, and cell cycle
472 regulation. Crit. Rev. Plant Sci. 18, 71-106.
473 Idris, A.M., Brown, J.K., 1998. Sinaloa tomato leaf curl geminivirus: Biological and
474 molecular evidence for a new subgroup III virus. Phytopathology 88, 648-657.
475 Inoue-Nagata, A.K., Albuquerque, L.C., Rocha, W.B., Nagata, T., 2004. A simple
476 method for cloning the complete begomovirus genome using the bacteriophage
477 phi 29 DNA polymerase. J. Virol. Methods 116, 209-211.

Jeske, H., 2007. Replication of geminiviruses and the use of rolling circle amplification for their diagnosis, in: Czosnek, H. (Ed.), Tomato yellow leaf curl virus disease management, molecular biology, breeding for resistance. Springer, pp. 141-156.

Lazarowitz, S.G., Wu, L.C., Rogers, S.G., Elmer, J.S., 1992. Sequence-specific interaction with the viral Al1 protein identifies a geminivirus DNA-Replication Origin. Plant Cell 4, 799-809.

Luna, A.P., Morilla, G., Voinnet, O., Bejarano, E.R., 2012. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. Mol. Plant Microbe Interact. 25, 1294-1306.

Márquez-Martín, B., Aragón-Caballero, L., Fiallo-Olivé, E., Navas-Castillo, J., Moriones, E., 2011. Tomato leaf deformation virus, a novel begomovirus associated with a severe disease of tomato in Peru. Eur. J. Plant Pathol. 129, 1-7.

Michelson, I., Zamir, D., Czosnek, H., 1994. Accumulation and translocation of tomato yellow leaf curl virus (TYLCV) in a *Lycopersicon esculentum* breeding line containing the *L. chilense* TYLCV tolerance gene *Ty-1*. Phytopathology 84, 928-933.

Monci, F., García-Andrés, S., Maldonado, J.A., Moriones, E., 2005. Resistance to monopartite begomoviruses associated with the bean leaf crumple disease in *Phaseolus vulgaris* controlled by a single dominant gene. Phytopathology 95, 819-826.

Morales, F.J., Jones, P.G., 2004. The ecology and epidemiology of whitefly-transmitted viruses in Latin America. Virus Res. 100, 57-65.

Morales, F.J., 2010. Distribution and dissemination of begomoviruses in Latin America and the Caribbean, in: Stansley PA, Naranjo SE (Eds.) Bemisia:

503 bionomics and management of a global pest. Springer, Dordrecht, Heidelberg,
504 London, New York, pp 283–318.
505 Moriones, E., Navas-Castillo, J., 2000. Tomato yellow leaf curl virus, an emerging
506 virus complex causing epidemics worldwide. Virus Res. 71, 123-134.
507 Nauen, R., Denholm, I., 2005. Resistance of insect pests to neonicotinoid
508 insecticides: current status and future prospects. Arch. Insect. Biochem. Physiol.
509 58, 200-215.
510 Navas-Castillo, J., Fiallo-Olivé, E., Sánchez-Campos, S., 2011. Emerging virus
511 diseases transmitted by whiteflies. Annu. Rev. Phytopathol. 49, 219-248.
512 Navas-Castillo, J., Sánchez-Campos, S., Díaz, J.A., Sáez-Alonso, E., Moriones, E.,
513 1999. Tomato yellow leaf curl virus-Is causes a novel disease of common bean
514 and severe epidemics in tomato in Spain. Plant Dis. 83, 29-32.
515 Navot, N., Pichersky, E., Zeidan, M., Zamir, D., Czosnek, H. 1991. Tomato yellow
516 leaf curl virus - a whitefly-transmitted geminivirus with a single genomic
517 component. Virology 185, 151-161.
518 Noris, E., Vaira, A.M., Caciagli, P., Masenga, V., Gronenborn, B., Accotto, G.P.,
519 1998. Amino acids in the capsid protein of tomato yellow leaf curl virus that are
520 crucial for systemic infection, particle formation, and insect transmission. J,
521 Virol. 72, 10050-10057.
522 Pamilo, P., Bianchi, N.O., 1993. Evolution of the Zfx and Zfy genes: rates and
523 interdependence between the genes. Mol. Biol. Evol. 10, 271-281.
524 Picó, B., Sifres, A., Elia, M., Díez, M.J., Nuez, F., 2000. Searching for new
525 resistance sources to tomato yellow leaf curl virus within a highly variable wild
526 *Lycopersicon* genetic pool. Acta Physiologiae Plantarum 22: 344-350.

527 Polston, J., McGovern, R., Brown, L., 1999. Introduction of tomato yellow leaf curl
528 virus in Florida and implications for the spread of this and other geminiviruses
529 of tomato. Plant Dis. 83, 984-988.
530 Polston, J., Lapidot, M., 2007. Management of tomato yellow leaf curl virus: US
531 and Israel Perspectives, in: Czosnek, Henryk (Ed.), Tomato yellow leaf curl
532 virus disease. Springer Netherlands, pp. 251-262.
533 Pooma, W., Gillette, W.K., Jeffrey, J.L. Petty, I.T.D., 1996. Host and viral factors
534 determine the dispensability of coat protein for bipartite geminivirus systemic
535 movement. Virology 218, 264-268.
536 Pooma, W., Petty, I.T.D., 1996. Tomato golden mosaic virus open reading frame
537 AL4 is genetically distinct from its C4 analogue in monopartite geminiviruses. J.
538 Gen. Virol. 77, 1947-1951.
539 Rojas, M.R., Gilbertson, R.J., Rusell, D.R., Maxwell, D.P., 1993. Use of degenerate
540 primers in the polymerase chain-reaction to detect whitefly-transmitted
541 geminiviruses. Plant Dis. 77, 340-347.
542 Rojas, M.R., Gilbertson, R.L., 2008. Emerging plant viruses: a diversity of
543 mechanisms and opportunities, in: Roossinck, M.J. (Ed.), Plant Virus Evolution.
544 Springer-Verlar, Berlin, Heidelberg, pp. 27–51.
545 Rojas, M.R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M.R., Lucas,
546 W.J., Gilbertson, R.L., 2001. Functional analysis of proteins involved in
547 movement of the monopartite begomovirus, tomato yellow leaf curl virus.
548 Virology 291, 110-125.
549 Sánchez-Campos, S., Díaz, J.A., Monci, F., Bejarano, E.R., Reina, J., Navas-Castillo,
550 J., Aranda, M.A., Moriones, E., 2002. High genetic stability of the begomovirus

Tomato yellow leaf curl Sardinia virus in southern Spain over an 8-year period. Phytopathology 92, 842-849.

Sanz, A.I., Fraile, A., Gallego, J.M., Malpica, J.M., García-Arenal, F., 1999. Genetic variability of natural populations of cotton leaf curl geminivirus, a single-stranded DNA virus. J. Mol. Evol. 49, 672-681.

Seal, S.E., vandenBosch, F., Jeger, M.J., 2006. Factors influencing begomovirus evolution and their increasing global significance: Implications for sustainable control. C. R. Plant Sci. 25, 23-46.

Stanley, J., 1983. Infectivity of the cloned geminivirus genome requires sequences from both DNAs. Nature 305: 643-645.

Tamura, K., Dudley, J., Nei, M., Kumar, S,. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.

Varma, A., Mandal, B., Singh, M.K, 2011. Global emergence and spread of whitefly *Bemisia tabaci* transmitted geminiviruses, in: Thompson, W.M.O. (Ed.) The Whitefly, *Bemisia tabaci* (*Homoptera: Aleyrodidae*) Interaction with Geminivirus-Infected Host Plants. Springer Netherlands, pp. 205-292.

Vidavski, F., Czosnek, H., Gazit, S., Levy, D., Lapidot, M., 2008. Pyramiding of genes conferring resistance to Tomato yellow leaf curl virus from different wild tomato species. Plant Breeding 127, 625-631.

575 Wartig, L., Kheyr-Pour, A., Noris, E., De Kouchkovsky, F., Jouanneau, F.,
576 Gronenborn, B., Jupin, I., 1997. Genetic analysis of the monopartite tomato
577 yellow leaf curl geminivirus: Roles of V1, V2, and C2 ORFs in viral
578 pathogenesis. Virology 228, 132-140.
579 Yahara, T., Ooi, K., Oshita, S., Ishii, I., Ikegami, M., 1998. Molecular evolution of a
580 host-range gene in geminiviruses infecting asexual populations of *Eupatorium*
581 *makinoi*. Genes Genet. Syst. 73, 137-141.
582

582

583 **Table 1.** Presence of tomato leaf deformation virus (ToLDeV) in samples exhibiting begomovirus-
584 like symptoms collected in Peru during a survey conducted between 2003 and 2010.

Host plants	Species	No. ToLDeV infected samples / Total No. of plants analyzed (% infected) [a]
CULTIVATED HOSTS [b]		
Tomato Resistant cv.	*Solanum lycopersicum* L.	30/71 (42.2)
Tomato Susceptible cv.	*Solanum lycopersicum* L.	18/44 (40.9)
Sweet pepper	*Capsicum annuum* L.	0/10 (0)
Habanero pepper	*Capsicum chinense* Jack	0/3 (0)
Red Peruvian chile	*Capsicum bacatum* cv. *Pendulum* (Willd.) Eshbaugh	0/4 (0)
Watermelon	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	0/3 (0)
Common bean	*Phaseolus vulgaris* L.	0/5 (0)
Pallar bean	*Phaseolus lunatus* L.	0/5 (0)
WILD HOSTS		
Weeds	21 species	2/60 (3.3) [c]
Wild relatives of tomato	4 species	1/45 (2.2) [d]
TOTAL		51/250 (20.4)

585 [a] Samples were analyzed by dot-blot hybridization using a digoxigenin-labeled DNA probe specific
586 to the tomato leaf deformation virus.

587 [b] Tomato resistant cvs. are commercial tomato cultivars with tolerance to tomato yellow leaf curl
588 disease (TYLCD), the most widespread: 'Dominator' (Seminis, Saint Louis, USA) and 'Tyson'
589 (Hazera Genetics, Shikmim, Israel). Tomato susceptible cvs. are several commercial tomato
590 cultivars not tolerant to TYLCD.

591 [c] Samples positive to ToLDeV were from a plant of the species *Anoda cristata* (L.) Schltdl. (family
592 *Malvaceae*) and from a plant of *Tanacetum parthenium* L. (family *Compositae*).

593 [d] The sample from a plant of a wild relative of tomato infected with ToLDeV corresponds to the
594 species *Solanum pennellii* Correll.

595

595 **Table 2.** Nucleotide diversities for the genomic regions included in the
596 ToLDeV sequence fragment analyzed in this study [a].

ToLDeV genomic region	d_{NS}	d_S	d_{NS}/d_S
C1 5'-2/3 ORF (610 nt)	0.042	0.209	0.201
C1 ORF nonoverlapping regions (353 nt)	0.047	0.227	0.207
C1–C4 overlap (257 nt)			
C1 ORF	0.035	0.190	0.184
C4 ORF	0.061	0.105	0.581

597 [a] Nucleotide diversity was computed separately for nonsynonymous (d_{NS}) and
598 for synonymous (d_S) sites by using the Pamilo-Bianchi-Li method (Pamilo and
599 Bianchi, 1993).
600

Figure legends

Figure 1. Schematic representation of the genome of tomato leaf deformation virus composed of a circular single stranded DNA molecule of ~2.6 kb in length. Arrows indicate open reading frames that putatively encode the coat protein (CP) in the virus sense strand, and the replication-associated protein (Rep), protein C4, the transcriptional activator protein (TrAP), and the replication enhancer protein (REn) in the complementary sense strand. The intergenic region (IR) that contains the stem-loop structure is shown. The black box represents the 921-nt fragment sequenced for the genetic diversity study.

Figure 2. A. Phylogenetic tree based upon an alignment of the sequences of a 921-nt fragment (nucleotides 1846 to 175, numbers based on the sequence of ToLDeV isolate PT1:2003, GenBank accession number GQ334472) obtained in this work for 51 tomato leaf deformation virus (ToLDeV) isolates collected in Peru during a survey conducted between 2003 and 2010. The fragment comprises the intergenic region and 5´ terminal two-thirds of the C1 coding region, including the overlapping the C4 gene**.** The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood is shown. Bootstrap (1,000 replicates) values are shown as percentage values, and only the nodes with values greater than 50% are labeled. The isolate of ToLDeV described by Márquez-Martín et al. (2011), PT1:2003, was shown (black shadow). Also, the sequences of nine additional ToLDeV isolates available in databases were included in the analyses (boxed). The DNA A component of tomato chino La Paz begomovirus (ToChLPV) (GenBank accession number DQ347948), was used as an outgroup. Whereas horizontal bars represent genetic distances as indicated by the scale bar, vertical distances are

arbitrary. The bar below the tree indicates 0.05 nucleotide substitutions per site. For isolates from this study, the name of each isolate indicates the isolate code number, the year of collection, the plant species [To: tomato, Ac: *Anoda cristata* (L.) Schltdl; Tp: *Tanacetum parthenium* L.; Sp: Solanum pennellii Correll.] and the geographic Department of origin. The GenBank accession number of the nucleotide sequence is indicated in each case. **B**. Map of Peru showing the location of the Departments surveyed. Blue, red and green circles on the map indicate the presence of isolates corresponding to Group 1, Group 2 or Group 3, respectively, as shown in the phylogenetic tree of Figure 2A.

Figure 3. A to **D**. Leaf symptoms caused by tomato leaf deformation virus (ToLDeV) isolate PT1:2003 on agroinfected *Nicotiana benthamiana* (**A**) and tomato 'Moneymaker' (**B** to **D**) plants. Mock-inoculated controls are shown in panels A (left), and C–D (right). **E**. Analysis of ToLDeV-agroinoculated plants at 30 days postinoculation (dpi) for virus presence by hybridization of tissue blots of petiole cross sections of newly emerged young leaves performed on positively charged nylon membranes (two prints per sample were performed in each individual square); the results for ten tomato and ten *N. benthamiana* plants are shown in the lines A and B, respectively; tissue prints of mock-inoculated *N. benthamiana* (C1 and C2) and tomato (C3 and C4) plants, and dot-blots of DNA extracts obtained from ToLDeV-infected tomato plants (C9 and C10) were included as negative and positive controls, respectively. **F**. Southern-blot analysis of DNA extracted (at 30 dpi) from young noninoculated leaves of *N. benthamiana* (lines lines 1 to 3) and tomato (lines 4 to 6) plants mock-inoculated (lines 1 and 4) or agroinoculated with the infectious clone of ToLDeV (lines 2-3 and 5-6); DNA extracts from field-infected PT1:2003 and P92:2009

650 tomato plants were included (lines 7 and 8, respectively); positions are indicated for the
651 single-stranded genomic DNA (SS) and for the open circular (OC), linear (LIN), and
652 supercoiled (SC) double-stranded DNA forms.
653

Figure 1 Sanchez-Campos et al.pptx

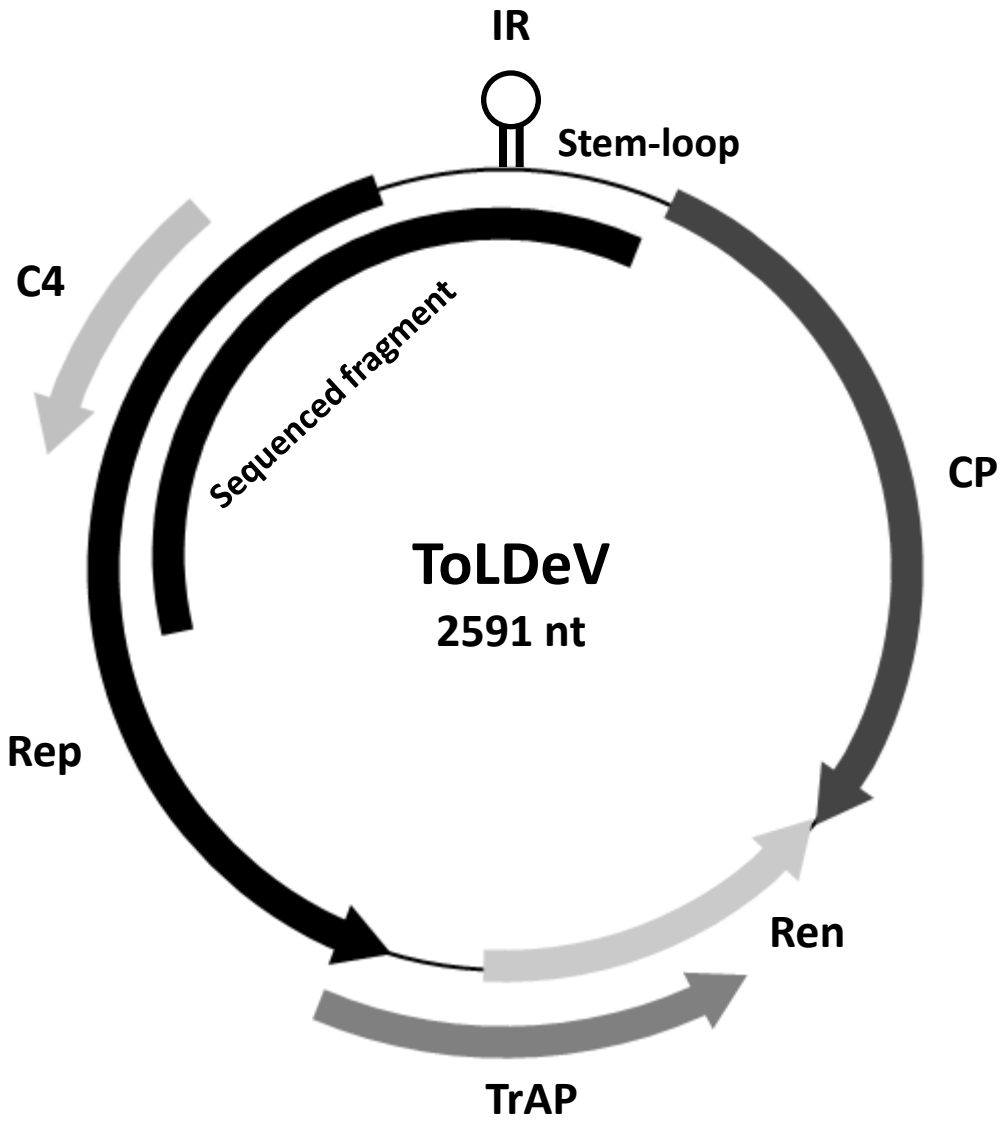

Figure 1, Sánchez-Campos et al., *Virus Research*

Figure 2 Sanchez-Campos et al.pptx

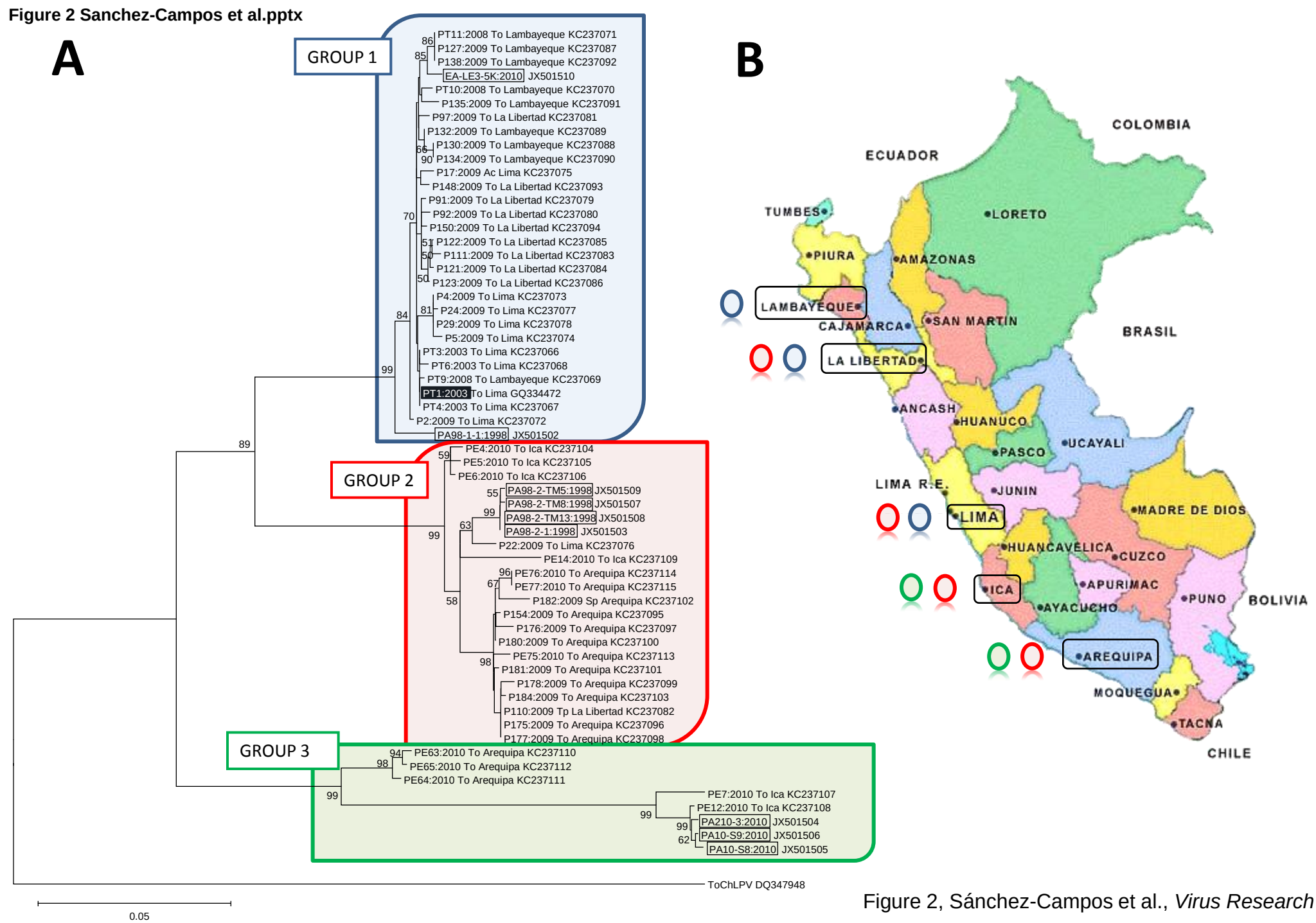

Figure 2, Sánchez-Campos et al., *Virus Research*

Figure 3 Sánchez-Campos et al.ppt

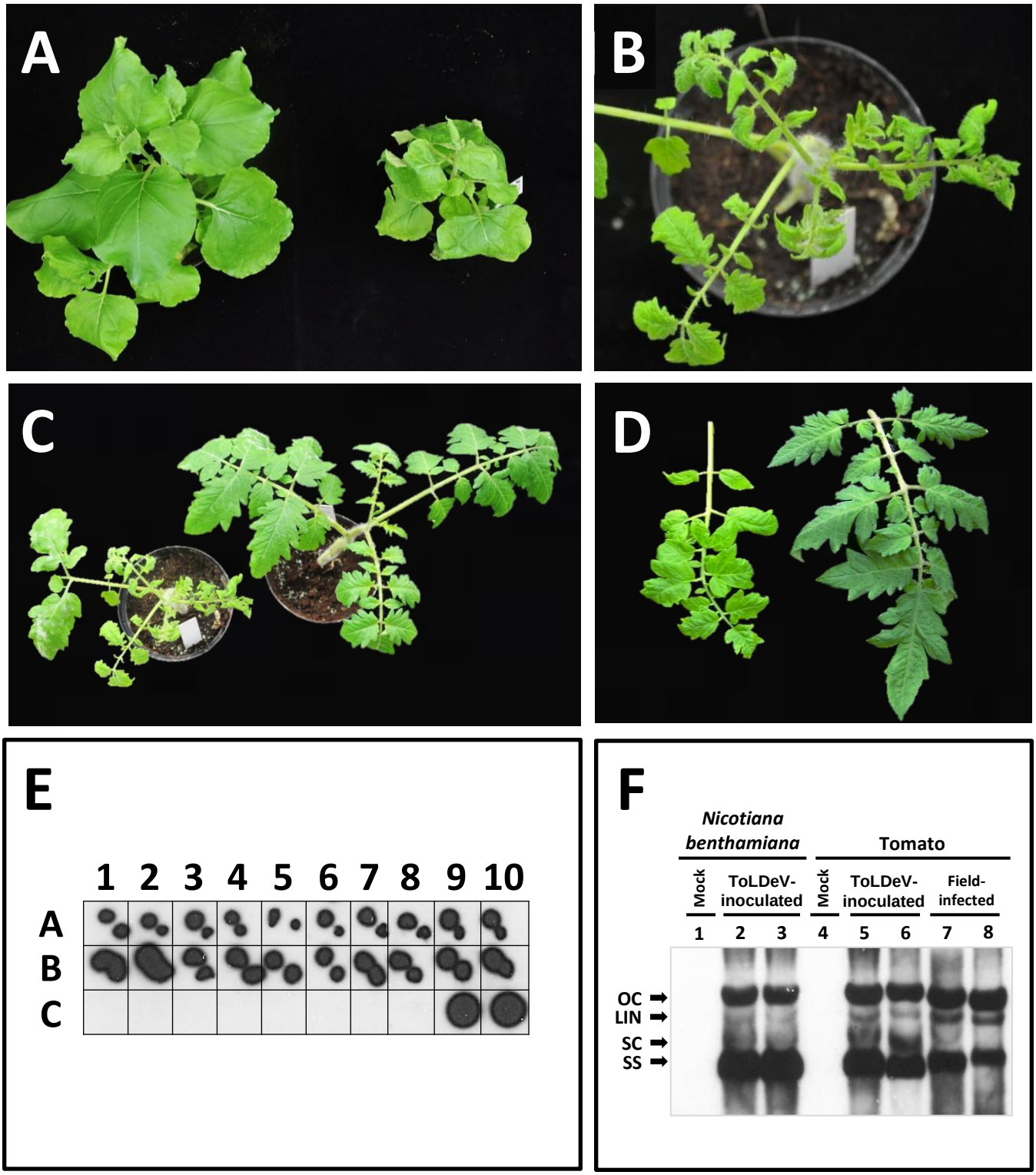

Figure 3, Sánchez-Campos et al., *Virus Research*