Pepper (*Capsicum annuum*) Is a Dead-End Host for *Tomato yellow leaf curl virus*

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ABSTRACT

Morilla, G., Janssen, D., García-Andrés, S., Moriones, E., Cuadrado, I. M., and Bejarano, E. R. 2005. Pepper (*Capsicum annuum*) is a dead-end host for *Tomato yellow leaf curl virus*. *Phytopathology* 95:1089-1097.

Tomato yellow leaf curl (TYLC) is one of the most devastating pathogens affecting tomato (*Lycopersicon esculentum*) worldwide. The disease is caused by a complex of begomovirus species, two of which, *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato yellow leaf curl virus* (TYLCV), are responsible for epidemics in Southern Spain. TYLCV also has been reported to cause severe damage to common bean (*Phaseolus vulgaris*) crops. Pepper (*Capsicum annuum*) plants collected from commercial crops were found to be infected by isolates of two TYLCV strains: TYLCV-Mld[ES01/99], an isolate of the mild strain

similar to other TYLCVs isolated from tomato crops in Spain, and TYLCV-[Alm], an isolate of the more virulent TYLCV type strain, not previously reported in the Iberian Peninsula. In this work, pepper, *Nicotiana benthamiana*, common bean, and tomato were tested for susceptibility to TYLCV-Mld[ES01/99] and TYLCV-[Alm] by *Agrobacterium tumefaciens* infiltration, biolistic bombardment, or *Bemisia tabaci* inoculation. Results indicate that both strains are able to infect plants of these species, including pepper. This is the first time that infection of pepper plants with TYLCV clones has been shown. Implications of pepper infection for the epidemiology of TYLCV are discussed.

Additional keywords: geminivirus.

Tomato yellow leaf curl disease (TYLCD) is one of the most important diseases affecting tomato crops in many tropical and subtropical regions of the world (16). The disease, caused by several species of the genus *Begomovirus*, was first described in Israel in 1931 and now occurs widely in Asia, Africa, Europe, and America. With the exception of *Tomato yellow leaf curl Thailand virus* (27), all begomovirus species that cause the disease have monopartite single-stranded DNA genomes of about 2.8 kb. In the Mediterranean Basin, two species cause TYLCD: *Tomato yellow leaf curl Sardinia virus* (TYLCSV), previously known as TYLCV-Sar, and *Tomato yellow leaf curl virus* (TYLCV), previously known as TYLCV-Is. TYLCSV and TYLCV were originally isolated in Italy and Israel, respectively (1,9), and share 74% nucleotide sequence identity.

The initial TYLCD epidemics during 1992 in Southern Spain were caused by the ES strain of TYLCSV (TYLCSV-ES) (21). In 1995, the Mld strain of TYLCV (TYLCV-Mld) was detected in the south of Portugal (12), and in 1997 an isolate of this TYLCV strain also was recovered from tomato in Southeastern Spain (hereafter referred to as TYLCV-Mld[ES72/97]) (18). TYLCV-Mld, but not TYLCSV-ES, was found to be the causal agent of a similar disease in common bean (17). Although TYLCV-Mld has become prevalent in Spain (33), both TYLCV-Mld and TYLCSV-ES coexist in the field, and have recombined to generate viruses with new biological properties (13).

TYLCV remains by far the most widely distributed and economically important TYLCD-causing virus. Infections of *Capsicum* crops, *C. annuum* and *C. chilensis*, by TYLCV species have

been reported in different parts of the world, including Southern Spain (26), Dominican Republic (30), Cuba (25), Jamaica (29), and Tanzania (8). In spite of the commercial importance of pepper, there is little information available on infection of pepper crop by TYLCV. In this article we provide definitive evidence, based on biological and molecular data, demonstrating that *C. annuum* plants can be infected by two strains of TYLCV with different biological properties.

MATERIALS AND METHODS

Microorganisms and general methods. Manipulations of nucleic acids and bacteria were carried out following standard procedures (31). For Southern blot hybridization, total DNA from plant leaves was isolated as described (3), separated in 0.8% Tris-borate-EDTA agarose gels (2 µg for *Lycopersicon esculentum* L. and 8 to 12 µg for *C. annuum*), and transferred to Hybond N+ nylon membranes (Amersham Biosciences Europe GmbH, Freiberg, Germany). Blots were hybridized under high stringency conditions with probes specific to either TYLCSV or TYLCV (15), or with the entire genome of TYLCV-Mld[ES72/97] (17). Fragments were labeled with [α^{32} P]-dCTP by random priming (Megaprime DNA Labeling Systems, Amersham Biosciences). Hybridization signal was detected with FUJIX Film and a FUJIX BAS1000 detector (Fujix). Hybridization images were acquired and processed with the Image Science 2003 Software (Fuji Photo Film Co., Tokyo). For restriction fragment length polymorphism (RFLP) analysis, 1 µg of total DNA extracted from *C. annuum* plants was used as template for polymerase chain reaction (PCR) using the primers OTYA3 (5'-GGGTCGACGTCATCAATGACG-3') and OTYA6 (5'-CTACATGAGAATGGGGAAC-3'). These primers were designed to amplify a 649-bp fragment of all known TYLCV/TYLCSV strains. PCR conditions used were 1 min at

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95°C, 25 cycles of 30 s at 95°C/30 s at 55°C/30 s at 72°C, and a final cycle of 7 min at 72°C.

Cloning and sequencing of the viral genomes. Pepper plant samples were obtained from surveys conducted in Almería on commercial pepper crops during 1999 and 2000. Total DNA was extracted from pepper leaves and used as template for PCR with primers OTYA3 and OTYA6. Amplified fragments from sample Ma398/99 (for TYLCV-like isolate) and Ma01/99 (for TYLCV-Mld-like isolate) were sequenced and used to design primers OTYANco1 (5'-TAAATACCATGGCCGCGCAGCGGAAGACACGACGTTTC-3') and OTYANco2 (5'-TATAATCCATGGAGACCTAATAGTATTGTCATTGAGGGTGA-3') to amplify the whole virus genome using the overlapping primer method (23). Long PCRs were performed using *Pfu* DNA polymerase (Promega Corporation, Madison, WI). The resulting double-stranded DNA fragments of approximately 2.8 kb were separated in 0.8% agarose gels, excised and purified (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences), and cloned into the *EcoRV* site of pBluescriptII SK+ (Stratagene, La Jolla, CA). Plasmids containing full-length genome clones from sample Ma01/99 (pTYPB1) and Ma398/99 (pTYCZ10) were obtained, and one clone of each sample was fully sequenced. Virus genomes, cloned in pTYPB1 and pTYCZ10, were named TYLCV-Mld[ES01/99] (GenBank accession no. AJ519441) and TYLCV-[Alm] (GenBank accession no. AJ489258), respectively.

Construction of infectious clones of isolates TYLCV-Mld[ES01/99] and TYLCV-[Alm]. An infectious clone of TYLCV-Mld[ES01/99] was obtained by constructing a 1.5-mer clone of the insert of pTYPB1. First, a plasmid (pTYPB20) containing 0.5-mer clone was obtained by deleting a *Clal* fragment of pTYPB1. Then, a 2.8-kb *NcoI* fragment of pTYPB1, containing the full-length genome, was inserted into the unique *NcoI* site of pTYPB20, yielding pTYPB30. The 1.5-mer clone of TYLCV-Mld[ES01/99] was then excised with *KpnI* and *SacI* from pTYPB30 and ligated into the binary vector pGA482 digested with *KpnI* and *SacI* yielding pGTYPB30. A similar strategy was used to obtain a 1.5-mer clone of TYLCV-[Alm]. A 0.5-mer clone (pTYCZ20) was produced by deleting a *Clal* fragment of pTYCZ10. Then, a full-length genome fragment of TYLCV-[Alm] was excised with *NcoI* from pTYCZ10 and inserted into the unique *NcoI* site of pTYCZ20 generating pTYCZ30. Half of the genome containing the intergenic region (IR) was excised from pTYCZ30 with *SphI* and cloned into the unique *SphI* site of pTYCZ10, producing the 1.5-mer clone pTYCZ40. The *BamHI-Sall* fragment from pTYCZ40 was cloned in a *BamHI-Sall*-digested binary vector pBIN-PLUS yielding pBICZ40.

Phylogenetic analysis. Phylogenetic analyses were performed with the neighbor-joining method using CLUSTAL W (34). For sequence comparison we used the genomic sequences of the following viruses (GenBank accession numbers are indicated): TYLCV (X15656), TYLCV-Mld (X76319), TYLCSV (X61153), TYLCSV-Sic (Z28390; TYLCSV isolate from Sicily), TYLCSV-ES[1] and TYLCSV-ES[2] (Z25751 and L27708, respectively, TYLCSV isolates from Spain), TYLCV-Mld[ES72/97]) and TYLCV-Mld[PT] (AF071228 and AF105975; TYLCV-Mld isolates from Spain and Portugal, respectively), TYLCMaV (AF271234; a natural recombinant between TYLCV-Mld and TYLCSV-ES), and two TYLCV isolates from Central America, TYLCV-[CU] (AJ223505) and TYLCV-[DO] (AF024715). *Tomato leaf curl virus* (S53251) was used as the outgroup. Distances were calculated using the Tamura-Nei method. Gaps were distributed proportionally.

Plant material. *Nicotiana benthamiana* Domin, *L. esculentum* cv. Marmande, *Phaseolus vulgaris* cv. Donna (Nunhems, Almería, Spain), and *C. annuum* cv. Cadia (Lamuyo type, Enza-Zaden Seeds, Almería, Spain) and Italiano type cv. Dulce de España (Vilmorin-Oxadis, La Verpilliere, France) were grown in

an insect-free growth chamber, at 24°C for a 16-h photoperiod with 250 $\mu\text{mol s}^{-1}\text{m}^{-2}$ photosynthetically active radiation.

Agroinfection. Plant agroinoculation was carried out using *Agrobacterium tumefaciens* as described (10). *A. tumefaciens* strains LBA4404 (6) and EHA105 (7) were transformed with recombinant plasmids pGTYPB20 and pBICZ40, respectively. Plants were inoculated with cultures of *A. tumefaciens* transformed with plasmids containing infectious clones: pGTYA2 (for TYLCSV-ES[2] infections) (15), pGTYILES (a binary vector containing a dimer of TYLCV-Mld[ES72/97]) (17), pGTYPB20 (for TYLCV-Mld[ES01/99] infections), and pBICZ40 (for TYLCV-[Alm] infections). Negative control plants were inoculated with *A. tumefaciens* harboring the empty binary vector pGA482 or pBINPLUS. *N. benthamiana*, pepper, tomato, and common bean plants were inoculated at the five- to six-leaf growth stage. After agroinoculation, plants were maintained in a growth chamber (conditions indicated previously) for 21 to 28 days and the youngest newly emerged leaves were analyzed by hybridization of tissue blots from petiole cross sections using specific DNA probes (13,15).

Biolistic transmission. Bombardment was performed using 1 μm gold particles coated with undigested DNA of the 1.5-mer infectious clones of TYLCV-[Alm] (pTYCZ40) and TYLCV-Mld[ES01/99] (pGTYPB20), the 1.7-mer clone of TYLCV-Mld[ES72/97] (p1.7SP72/97) (17), and the 2.0-mer clone of TYLCSV-ES[2] (pTYA55) (15). Particles were delivered using a helium pressure-based apparatus (Helios Biolistic PDS-1000; Bio-Rad, Hercules, CA). Bombardment was performed at a pressure of 650 psi. After bombardment, plants were kept in a humid environment at 24°C for 1 day and then maintained in a growth chamber (conditions indicated previously) for 15 days. Presence of viral DNA in young newly emerged leaf tissues was confirmed by Southern blot analysis, and PCR amplification of a 1.6-kb fragment using primers OTYA7 and OTYA8 (14).

Bemisia tabaci transmission. Whitefly transmission experiments were performed using *B. tabaci* biotype Q nonviruliferous individuals obtained from a virus-free colony reared on melon (*Cucumis melo*). Brief and extended inoculation access period (IAP) experiments were done. The brief IAP (48 h) experiments were as described (13). For extended IAP experiments, viruliferous whiteflies were given 30 days free range within a wooden cage covered with a whitefly-proof net. Within the cage, whiteflies were free to move between test plants and virus-infected source. After the IAP, insects were eliminated by insecticide treatment. Presence of virus in young leaf tissues of inoculated plants was analyzed by Southern blot and PCR analysis, as indicated previously. Tomato and pepper control plants were obtained following the same procedure, but using *B. tabaci* adults exposed only to uninfected source plants. Transmission experiments were performed in a growth chamber (conditions indicated previously).

Field survey. A survey was conducted on commercial pepper crops grown under plastic at the major growing areas of Southern Spain to determine incidence of TYLCV/TYLCSV. Samples were collected at random from 18 greenhouses (two independent samples per plant, 45 plants per greenhouse) located in different growing areas of the Almería Province during September 2002, November 2002, and January 2003. Each sample was tested for infection with TYLCV and TYLCSV by tissue blot hybridizations using specific probes and conditions described previously. Two independent tissue prints were made per sample.

RESULTS

Pepper crops in Spain are infected by at least two TYLCV strains. During a routine survey conducted in June 1999, samples of pepper plants (cv. Aristocrata, Enza Zaden) from a commercial greenhouse in La Mojonera, Almería (Southern Spain) were col-

Fig. 1. A, Genome map of *Tomato yellow leaf curl virus* (TYLCV)-Mld[ES72/97] showing the position of open reading frames (arrows), the position of selected restriction endonuclease cleavage sites, and the position of a DNA fragment amplified by polymerase chain reaction with the primers OTYA6 and OTYA3. **B**, Southern blot analysis of field-grown infected plants collected in Almeria: one tomato (2 µg per lane) and four pepper plants (8 µg per lane) (samples Ma1 [1], Ma15 [15], Ma [398], and Ma463 [463]). DNA extracted from young tissues of each plant, either undigested (nd) or digested with the restriction enzyme *NcoI* are shown. **C**, Southern blot analysis from one of the infected pepper plants (sample Ma15). Total DNA undigested (nd) or digested with *EcoRI*, *Sall*, *BamHI*, or *HindIII* was loaded in each lane (12 µg per lane). The positions of *EcoRI* 2.71-kb fragment (EI) and open-circular (oc), single-stranded (ssc), linear (lin), and supercoiled (sc) DNA forms are indicated. **D**, Restriction fragment length polymorphism analysis: primers OTYA6 and OTYA3 were used to amplify a fragment from six infected pepper plants (samples Ma1 [1], Ma15 [15], Ma394 [394], Ma [398], Ma405 [405], and Ma463 [463]). DNA was either undigested (nd) or digested with *HinfI* or *ClaI*. DNA from a TYLCV-infected tomato plant (C+) was used as positive control; DNA from a noninfected pepper plant was used as negative control (C-). The positions of DNA size markers (kb) in lane M are indicated. The *HinfI*-*ClaI* restriction enzyme maps of the OTYA6/OTYA3 fragments amplified from TYLCV-Mld and TYLCV genomes are shown. For Southern blot analyses, a TYLCV-specific probe (13,15) that recognizes either TYLCV or TYLCV-Mld was used.

Fig. 2. A, Genome maps of two fully sequenced genomes of *Tomato yellow leaf curl virus* (TYLCV) isolates obtained from field-infected pepper plants (TYLCV-Mld[ES01/99] and TYLCV-[Alm]) showing the positions of the open reading frames and the position of selected restriction endonuclease cleavage sites. **B,** Neighbor-joining phylogenetic trees depicting topology and distance relationships among the genomes of the two TYLCV isolates (shaded) obtained in this work and other TYLCV or *Tomato yellow leaf curl Sardinia virus* (TYLCSV) isolates. The significance of the nodes in a bootstrap analysis with 1,000 replicates is shown. GenBank accession numbers are indicated in each case. *Tomato leaf curl virus* (ToLCV) was used for rooting the tree. Scale bar represents the horizontal distance equivalent to a phylogenetic distance of 0.1 replacements per position. Horizontal distances are proportional to sequence distances; vertical lengths are arbitrary.

lected. Among these samples, a number of plants tested positive when hybridized with a probe for TYLCV-like begomoviruses, whereas no signal was detected when a TYLCSV-specific probe was used (data not shown). To further characterize the viruses present in each sample, we extracted DNA from leaves of six plants giving TYLCV-positive hybridization signals (Ma1, Ma15, Ma394, Ma398, Ma405, and Ma463) and analyzed the samples by Southern blot. The results confirmed that pepper plants were infected with TYLCV; characteristic begomoviral DNA forms were detected after hybridization with a TYLCV-specific probe (13,15) (Fig. 1B shows four representative samples). This probe hybridized with all TYLCV strains. When these DNA samples were digested with *Nco*I, a 2.8-kb fragment was obtained (all known TYLCV genomes contain a unique site for this enzyme). Interestingly, viral DNA concentration in pepper plants was considerably lower than that typical of infected tomato plants.

To determine which TYLCV viral strain was present, we digested DNA samples with several restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI, and *Sal*I), which generate characteristic restriction patterns for each known TYLCV strain. The restriction pattern obtained for some of the samples, e.g., Ma15 (Fig. 1C), did not match that expected for TYLCV-Mld[ES72/97], the strain previously reported in Spain (Fig. 1A). Therefore, we designed primers (OTYA3/OTYA6) to amplify a 650-bp fragment from any known TYLCV/TYLCSV isolate and performed a more accurate RFLP analysis of the samples. A single fragment with the expected size was amplified from all samples (Fig. 1D). However, when these fragments were digested with *Hinf*I or *Cla*I (Fig. 1D), three different patterns were obtained. While the pattern obtained with sample Ma1 corresponded to that expected for the TYLCV-Mld strain, those obtained for samples Ma394 and Ma398 corresponded to that expected for the TYLCV type strain (20). Sample Ma15 displayed a mixed-pattern of bands in the RFLP analysis suggesting infection with both the Mld and type strains of TYLCV. Similar results of mixed-infection were obtained with Ma405 and Ma463. Taken together, the results suggest that pepper plants were infected by two different TYLCV strains. To confirm this hypothesis we cloned and sequenced the OTYA3/OTYA6 PCR fragments from Ma1 and Ma398. The DNA sequence from sample Ma1 clearly matched with the sequence of TYLCV-Mld[ES72/97], while that from Ma398 was similar to that of the TYLCV type strain (20).

TYLCV-[Alm], an isolate of a new TYLCV strain in Southern Spain. Using the partial TYLCV sequences obtained from pepper samples, we designed a pair of primers (OTYANco1 and OTYANco2) to amplify the complete genome of both TYLCV strains present in Ma1 and Ma398. These primers partially overlap and were designed based on the sequence of *Rep* containing the unique *Nco*I restriction site. DNA fragments of approximately 2.7 kb were amplified from extracts of both samples, cloned into pBluescriptII SK+, and fully sequenced. The genome sequence

obtained from sample Ma1 (2,790 nucleotides) and Ma398 (2,781 nucleotides) represented the isolates named TYLCV-Mld[ES01/99] and TYLCV-[Alm] (GenBank accession nos. AJ519441 and AJ489258, respectively). The genome organization of both TYLCV isolates was similar to that reported for other TYLCVs, with two open reading frames in the virion-sense and four in the complementary-sense strand, separated by the IR which contains the putative stem-loop structure and the TAATATTAC nona-nucleotide found in all geminiviruses (Fig. 2A).

Alignment of the complete genome sequences of TYLCV-Mld[ES01/99] and TYLCV-[Alm] with other TYLCV and TYLCSV isolates reported previously, including all fully sequenced isolates from the Iberian Peninsula, confirmed our previous results. Thus, as shown in Figure 2B, TYLCV-Mld[ES01/99] is related to other TYLCV-Mld isolates including the TYLCVs previously reported from the Iberian Peninsula (TYLCV-Mld[ES72/97] and TYLCV-Mld[PT]), while TYLCV-[Alm] is more similar to isolates of the TYLCV type strain described in Israel (TYLCV), Cuba (TYLCV-[CU]), and Dominican Republic (TYLCV-[DO]). Sequence comparison between TYLCVs isolated from pepper plants (TYLCV-Mld[ES01/99] and TYLCV-[Alm]) with TYLCV-Mld[ES72/97] and the type strain of TYLCV are shown in Table 1.

Tomato, common bean, and *N. benthamiana*, but not pepper, can be agroinfected with TYLCV-Mld[ES01/99] and TYLCV-[Alm]. A 1.5-mer DNA copy of the TYLCV-Mld[ES01/99] and TYLCV-[Alm] genomes were cloned into *Agrobacterium* binary vectors to produce pGTYPB30 and pBICZ40, respectively. *A. tumefaciens* strain LBA4404 cultures transformed with these plasmids were then used for agroinoculation experiments. As agroinoculation controls, infectious clones of TYLCV-Mld[ES72/97] and TYLCSV-ES[2] were used. *N. benthamiana*, common bean, pepper, and tomato plants were inoculated (Table 2). All TYLCV clones were able to infect

TABLE 2. Agroinoculation of pepper, *Nicotiana benthamiana*, tomato, and common bean plants with *Tomato yellow leaf curl Sardinia virus* (TYLCSV)-ES[2], *Tomato yellow leaf curl virus* (TYLCV)-Mld[ES72/97], TYLCV-Mld[ES01/99], and TYLCV-[Alm]^a

Host plant	No. of plants infected/no. of plants inoculated			
	TYLCSV-ES[2]	TYLCV-Mld[ES72/97]	TYLCV-Mld[ES01/99]	TYLCV-[Alm]
Pepper	0/40	0/40	0/40	0/40
<i>N. benthamiana</i>	10/10	10/10	10/10	10/10
Tomato	10/10	10/10	10/10	10/10
Common bean ^b	0/4	3/4	4/4	4/4

^a Data shown are pooled from two independent experiments with 5 (*N. benthamiana* and tomato) or 20 (pepper) plants per virus. Plants were analyzed 28 days after inoculation by hybridization of tissue blots. Pepper plants also were analyzed by polymerase chain reaction.

^b Only one experiment with four plants per virus species was performed.

TABLE 1. Comparisons of nucleotide and amino acid sequences deduced from the genomes of *Tomato yellow leaf curl virus* (TYLCV) isolates described in this work (TYLCV-Mld[ES01/99] and TYLCV-[Alm]), and that of TYLCV-Mld[ES72/97] and the type strain of TYLCV

DNA	TYLCV-[ES01/99] ^a			TYLCV-[Alm] ^a	
	TYLCV-[Alm]	TYLCV-Mld[ES72/97]	TYLCV	TYLCV-Mld[ES72/97]	TYLCV
Genome	91.9	98.1	90.2	91.0	98.1
IR	75.4	95.9	75.1	78.9	95.5
Proteins					
V2	100.0	98.3	100.0	98.3	100.0
CP	99.2	98.1	96.9	98.1	96.9
REn	99.3	96.3	97.8	97.0	98.5
Trap	100.0	95.5	99.3	95.5	99.3
Rep	88.8	98.6	87.7	89.9	98.0
C4	46.4	98.0	47.4	45.4	96.9

^a GenBank accession numbers of genome sequences of TYLCV-Mld[ES72/97] and the type strain of TYLCV are AF01228 and X15656, respectively. Percentage of identity between DNA sequences and amino acid sequences are shown. The table shows the percentage of identity of the nucleotide sequence (DNA) of whole genome or intergenic region (IR), as well as that of the amino acids (proteins).

tomato, *N. benthamiana*, and common bean, whereas TYLCV-ES[2] infected tomato and *N. benthamiana*, but was unable to infect common bean, as previously described (17). Therefore, pTYPB30 and pTYCZ40 contained infectious copies of TYLCV-Mld[ES01/99] and TYLCV-[Alm], respectively. None of the inoculated pepper plants were infected with TYLCSV or any of the TYLCV clones used. Equivalent results were obtained when the agroinoculation experiments were repeated using the more virulent *A. tumefaciens* strain EHA105 (data not shown).

All plants infected with TYLCV or TYLCSV clones developed symptoms characteristic of infection; however, those induced by TYLCV-[Alm] were more severe in the three plant species assayed (shown for TYLCVs in Fig. 3). Leaves from TYLCV-[Alm]-infected plants exhibited stronger leaf curling and dwarfing and a more intense internode reduction than those infected with TYLCV-Mld[ES72/97] or TYLCV-Mld[ES01/99]. We also noticed that *N. benthamiana* leaves infected with TYLCV-[Alm] cupped downwards as occurs with other TYLCV strains, whereas upward curling was characteristic of TYLCSV infections, as reported (15).

TYLCV-Mld[ES01/99] and TYLCV-[Alm] infect pepper and *N. benthamiana* plants by genegun bombardment. Pepper plants are known to be recalcitrant to transformation by *A. tumefaciens* (11). Since this could explain failure to agroinfect pepper test plants, we delivered viral DNA inocula via biolistic

bombardment, as previously reported for other pepper-infecting geminiviruses (4). Replicative forms of viral DNA were detected in most of the pepper plants inoculated with TYLCV-Mld[ES01/99] or TYLCV-[Alm] (Table 3; Fig. 4A and B), but not in the plants inoculated with TYLCSV-ES[2] (Table 3; Fig. 4C). Interestingly, we only detected viral replicative forms in the inoculated leaves, but not in young tissues. None of the biolistically inoculated pepper plants developed symptoms (plants were observed up to 10 weeks postinoculation). Tomato and

TABLE 3. Inoculation by biolistic bombardment of pepper, *Nicotiana benthamiana*, and tomato plants with *Tomato yellow leaf curl Sardinia virus* (TYLCSV)-ES[2], *Tomato yellow leaf curl virus* (TYLCV)-[ES01/99], and TYLCV-[Alm]^a

Host plant	No. of plants infected/no. of plants inoculated with		
	TYLCSV-ES[2]	TYLCV-Mld[ES01/99]	TYLCV-[Alm]
Pepper	0/7	5/7	4/7
<i>N. benthamiana</i>	2/4	3/4	3/4
Tomato	3/4	2/4	2/4

^a Plants were analyzed 28 days after inoculation by hybridization of tissue blots. Inoculated pepper plants also were analyzed by Southern blot. For each inoculated pepper plant the most apical and one or two bombarded leaves were collected, and total DNA was extracted and analyzed by Southern blot.

Fig. 3. Representative symptoms observed at **A** and **C**, 4 weeks postinoculation or **B**, 8 weeks postinoculation in young leaves of **A**, *Nicotiana benthamiana*, **B**, tomato, or **C**, common bean plants mock-inoculated or inoculated with *Tomato yellow leaf curl virus* (TYLCV)-Mld[ES72/97], TYLCV-Mld[ES01/99], or TYLCV-[Alm].

N. benthamiana were successfully infected with either virus following bombardment (Table 3) and developed characteristic symptoms of infection (data not shown).

TYLCV-[Alm] is transmitted by *B. tabaci* to tomato, but inefficiently to pepper. The progeny of the infectious clone of TYLCV-[Alm] (pTYCZ40) was tested for *B. tabaci* transmission. Brief IAP for whiteflies fed on infected tomato source plants resulted in efficient transmission of TYLCV-[Alm] to tomato, whereas none of the pepper plants became infected (Table 4). However, no TYLCV-[Alm] transmission was obtained either to tomato or to pepper when whiteflies were fed on biolistically inoculated pepper source plants (bombarded and young leaves) during short IAP experiments (Table 4).

Fig. 4. Southern blot analysis from pepper plants (lanes 1 to 7) inoculated by biolistic bombardment with *Tomato yellow leaf curl virus* (TYLCV)-Mid[ES01/99], TYLCV-[Alm], or *Tomato yellow leaf curl Sardinia virus* (TYLCSV)-ES[2]. Total DNA was extracted from each plant from apical leaves (A) and from bombarded leaves (B), and 10 µg of undigested DNA was loaded in each lane. The positions of DNA size markers (kb) are indicated in lane M. The main band present in infected samples corresponds to single-stranded and double-stranded supercoiled form of the viral genome. Blots were hybridized with the probes specific to TYLCV or to TYLCSV.

TABLE 4. *Bemisia tabaci* transmission of *Tomato yellow leaf curl virus* (TYLCV)-[Alm] to tomato or pepper test plants after acquisition on infected pepper or tomato plants^a

Inoculated plant	No. of plants infected as virus acquisition source			
	Tomato		Pepper	
	48 h IAP ^b	30 days IAP ^c	48 h IAP ^b	30 days IAP ^c
Pepper	0/15	2/15	0/17	0/15
Tomato	5/5	5/5	0/15	0/15

^a Young leaves from inoculated test plants were analyzed 28 days after inoculation (for 48 h inoculation access period [IAP] experiment) or 7 days after the whiteflies were removed (for 30 days IAP experiment) by hybridization of tissue blot (tomato) or by Southern blot and polymerase chain reaction (pepper).

^b Healthy plants (tomato and pepper) were exposed for a 48 h IAP to *B. tabaci* adult individuals (50 adults per plant) that were given a 48 h acquisition access period on virus source plants infected with TYLCV-[Alm] 3 weeks earlier by bombardment.

^c Healthy plants (tomato and pepper) were exposed for a 30 day IAP within a wooden cage to *B. tabaci* adults (50 adults per plant) that were given a 48 h acquisition access period on TYLCV-[Alm]-infected source plants, in the presence of tomato or pepper plant infected by bombardment with TYLCV-[Alm] 3 weeks earlier.

In an effort to mimic natural transmission conditions, a long IAP system was assayed. As before, when using tomato as virus source, all tomato test plants were infected. However, in these experimental conditions successful transmission to pepper test plants also was obtained, as viral DNA was detected in 2 out of 15 pepper plants (Table 4). In contrast to the biolistic experiments, viral DNA accumulation also was detected in young leaves, although titer was lower compared with older leaves (Fig. 5). Identical results were obtained by Southern blot (data not shown). None of the infected plants developed symptoms. When similar experiments were carried out using pepper plants as viral source, neither tomato nor pepper plants were infected (Table 4).

TYLCV is detected in pepper plants from commercial greenhouse crops. To evaluate the incidence of TYLCV in greenhouse-grown pepper crops, samples were collected during a survey conducted between September 2002 and January 2003 at the main pepper production area of Almería Province (Fig. 6). Tomato greenhouses were included in the survey as a control for TYLCV presence.

TYLCV was found widely dispersed throughout pepper crops of Almería, although the percentage of infected plants was much lower than that observed in the tomato crops present in the same area (Table 5). At the beginning of the growing season, 21% of the tomato plants analyzed contained TYLCV DNA while only

Fig. 5. Polymerase chain reaction amplification of extracts from pepper plants inoculated with *Tomato yellow leaf curl virus* (TYLCV)-[Alm] by whitefly transmission. Primers OTYA7 and OTYA8 were used to amplify a fragment of the virus genome. Three of the fourteen plants inoculated are shown; two of them contained viral DNA (lanes 2 and 3), whereas the other did not (lane 1). For every plant, DNA was extracted from leaves already present during the whitefly inoculation (B) and from those newly developed (A). DNA extracted from mock-inoculated pepper plants was used as negative control (C-). Positive (C+) control consisted of 10 ng of pTYXZ40 (a plasmid containing a dimer of TYLCV-[Alm] genome) added to the extract from mock-inoculated plants. The sizes of the DNA marker bands in lane M are indicated in kilobases.

Fig. 6. Localization of the Almería Province (shadowed in the map of Spain) and the greenhouses (black dots) where the field surveys for *Tomato yellow leaf curl virus* (TYLCV) isolates were conducted between 1999 and 2003.

1% of the analyzed pepper plants did. As the growing season progressed, the percentage of infected plants increased, reaching a peak during the November/January samplings, when TYLCV was detected in 6% of the pepper plants and in about 50% of the tomato plants tested. No correlation was observed between TYLCV detection in pepper plants and the presence of characteristic disease symptoms in contrast to that normally observed in tomato or common bean plants.

DISCUSSION

TYLC-like viruses constitute a complex of begomoviruses causing serious disease of tomato and/or common bean. In this work we demonstrate that some viruses belonging to this complex also infect pepper. Although there have been previous reports of pepper infection by TYLCV (25,26,29,30), this is the first time that infectious clones have been isolated from TYLCV-infected field-grown pepper plants, and that viral DNA has been subsequently delivered into pepper plants through either gene bombardment or via its natural vector *B. tabaci*, to reproduce the infection experimentally.

Representatives of two different TYLCV strains have been isolated and characterized from infected pepper plants collected from commercial greenhouses of Almería (Spain). Interestingly, in addition to the presence of the TYLCV-Mld strain, already reported in Spain (19), we detected the TYLCV type strain (20) in pepper. Infectious clones have been obtained for isolates of both TYLCV type strain and TYLCV-Mld recovered from pepper, allowing us to study their biological characteristics. Although both isolates display a similar host range, they differ in the severity of the symptoms produced in symptomatic species such as tomato, common bean, or *N. benthamiana*. None of these isolates induced noticeable symptoms in pepper plants infected experimentally, and no correlation was found between TYLCV presence and symptom development in the field survey undertaken during autumn-winter 2002/2003. Therefore, we concluded that although TYLCD-like viruses are able to replicate in pepper cells and invade plants, they do not cause significant damage to pepper crops in contrast to that observed in tomato or common bean. We speculate that absence of symptoms in pepper may correlate with limited viral DNA accumulation in pepper tissues, which is consistently lower than that observed in symptomatic hosts. Whether limited viral titer in pepper plants is due to a restraint in virus replication, movement, or both cannot be discerned from our data. Results from the bombardment inoculation experiments suggest that movement of the virus was impaired in pepper plants, since several weeks after inoculation the amount of virus in bombarded leaves was clearly higher than in young newly developed leaves. However, this conclusion is not supported by the results from *B. tabaci* inoculation experiments. This apparent discrepancy could be explained from the differences in the inoculation method. TYLCV is a phloem-restricted virus, at least in tomato and *N. benthamiana* (15,28). We speculate that since white-

flies inoculate the virus directly into the phloem, virus could be translocated directly to other parts of the plant. However, when viral DNA is inoculated biolistically, it must replicate and move in nonphloem cells before entering into the phloem. Nevertheless, limitations to TYLCV replication and/or movement should exist, since the amount of virus accumulated systemically in *B. tabaci*-inoculated pepper plants is manifestly lower to that found in tomato and *N. benthamiana* plants.

We have been unable to transmit TYLCV from infected pepper plants, most likely because of the low viral titers present. This suggests that pepper is a dead end in the epidemiological cycle of this virus. However, we cannot completely rule out that pepper may serve as an inefficient reservoir when vector populations are extremely high.

Under the experimental conditions used, we have not been able to systemically infect pepper plants with TYLCSV or detect replicative viral forms in inoculated tissues after bombardment. This confirms a previous report of Crespi et al. (2). However, during our field survey, hybridization with a TYLCSV-specific probe was detected for some pepper samples (data not shown). Therefore, we cannot completely rule out that this virus species also may infect pepper.

Mixed viral infections are quite common under field conditions, and may result in antagonistic, additive, or even synergistic effects. Mixed infections also provide an opportunity for genetic recombination, a demonstrated property of geminiviruses (5,22). The risk of new recombinant viruses appearing in an area depends on the coexistence of different viral strains or species in the region. TYLC-like viruses have become widely dispersed during the last 10 years (24). In the Iberian Peninsula, a genetically stable population of TYLCSV was originally present (32). However, spread of the Mld strain of TYLCV produced a dramatic change in the population structure, resulting in displacement of TYLCSV by TYLCV (33) and emergence of recombinant viruses with new biological characteristics (13). Therefore, confirmation of spread of the TYLCV type strain in Southern Spain is of particular interest. The introduction of a more aggressive strain of TYLCV, such as TYLCV-[Alm], may result in novel changes in the virus population structure with unknown epidemiological consequences.

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TABLE 5. Analysis of pepper and tomato samples randomly collected in a survey conducted during 2002 and 2003 in commercial crops of the Almería Province

Date	Plant	Number of samples collected (number of greenhouses visited)	Number (percentage) of samples infected with TYLCV ^a
September 2002	Pepper	764 (18)	12 (1.6%)
	Tomato	114 (2)	24 (21%)
November 2002	Pepper	765 (18)	43 (6%)
	Tomato	180 (2)	85 (47%)
January 2003	Pepper	765 (18)	45 (6%)
	Tomato	180 (2)	78 (43%)

^a Plants were analyzed by hybridization of tissue blots with *Tomato yellow leaf curl virus* (TYLCV)-specific probes.

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