Tomato Yellow Leaf Curl Virus-Is Causes a Novel Disease of Common Bean and Severe Epidemics in Tomato in Spain

Jesús Navas-Castillo, Sonia Sánchez-Campos, and Juan Antonio Díaz, Estación Experimental "La Mayora," Consejo Superior de Investigaciones Científicas, 29750 Algarrobo-Costa, Málaga, Spain; Elisa Sáez-Alonso, Departamento de Sanidad Vegetal, Consejería de Agricultura y Pesca, Almería, Spain; and Enrique Moriones, Estación Experimental "La Mayora," Consejo Superior de Investigaciones Científicas, 29750 Algarrobo-Costa, Málaga, Spain

ABSTRACT

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Field surveys were conducted in the autumn of 1997 in the main tomato (*Lycopersicon esculentum*)-growing regions of southern Spain following a severe tomato yellow leaf curl epidemic in tomato. Tomato yellow leaf curl virus (TYLCV)-Is was found to have spread to all regions and to coexist with TYLCV-Sr, which has been present since 1992. TYLCV-Is was also shown to be the causal agent of bean leaf crumple, a novel disease that has caused severe economic losses in fresh-market common bean (*Phaseolus vulgaris*) crops of southern Spain since September 1997. The disease was reproduced by infecting beans with cloned TYLCV-Is obtained from infected tomato plants collected in Almería. This is the first report of bean leaf crumple disease and the first report of a geminivirus in bean from Spain.

Additional keywords: geminivirus, *Lycopersicon esculentum*, *Phaseolus vulgaris*, TYLCV

Tomato yellow leaf curl disease (TYLC) causes devastating annual damage in tomato (*Lycopersicon esculentum*) crops of many tropical, subtropical, and temperate regions of the world (9). The disease is caused by several whitefly (*Bemisia tabaci*)-transmitted geminiviruses, all of which are referred to as tomato yellow leaf curl virus (TYLCV). TYLCV isolates reported so far have been grouped in several species in the "subgroup III Geminivirus" (proposed to be named *Begomovirus*) genus of the family Geminiviridae (4). All, except for a TYLCV from Thailand (27), have a monopartite genome. Monopartite TYLCVs have a similar genome organization consisting of two virion sense (pre-coat, V1; and coat, V2) and four complementary sense (C1, C2, C3, and C4) genes separated by a short intergenic region (IR; 2,8,12,19,21).

TYLC has threatened tomato crops of southern Spain since 1992 (17). Two iso-

lates of TYLCV from the first epidemic outbreak, named TYLCV-M and TYLCV-Alm, have been cloned and sequenced (21,26). These isolates are almost identical (99% nucleotide sequence identity) and belong to the TYLCV-Sr species of the *Begomovirus* genus. In June 1997, a severe outbreak of TYLC disease was reported in tomato crops of Almería. This outbreak was associated with infections caused by isolates of the TYLCV-Is species (18) which had not been previously reported in Spain. In this paper we report the widespread occurrence of TYLCV-Is in the main tomato-growing regions of southern Spain and evidence for its involvement in a novel disease of fresh-market bean (*Phaseolus vulgaris*). This is the first report of a geminivirus infection in common bean from Spain.

MATERIALS AND METHODS

Virus sources. Tomato (*L. esculentum* cv. Daniela) plants exhibiting an abnormally strong upward curling of leaflet margins (Fig. 1A) relative to symptoms normally observed in TYLC-affected plants in Spain (17; Fig. 1B) were collected in June 1997 from a plastic house in Almería (region III, Fig. 2). Following previously reported analyses (18), sample SP72/97 was demonstrated to be infected only by TYLCV-Is and was selected for further characterization.

Construction of an infectious clone of SP72/97-TYLCV-Is. A putative full-length fragment of SP72/97-TYLCV-Is

was detected, excised, and extracted from low-melting-temperature agarose gels after electrophoresis of total nucleic acids from sample SP72/97 digested with *Bam*HI. An aliquot was cloned into pBluescript SK+ (Stratagene, La Jolla, CA) and a clone (pSP72/97) containing a potentially full-length insert was selected for further analysis. A 1.7-mer clone of the insert of pSP72/97 was constructed by adding the full-length fragment into the unique *Bam*HI site of a 0.7-mer clone previously obtained by a *Sma*I-*Sph*I deletion in pSP72/97. The 1.7-mer fragment was subcloned into the binary vector pBin19 (3). The recombinant clone (p1.7SP72/97) has proved to be infectious, and transmission of progeny virus by *Bemisia tabaci* has been demonstrated on tomato (*unpublished*). DNA manipulation and cloning were carried out following standard methods (29) or methods described by Noris et al. (21). For hybridization, a digoxigenin-labeled DNA probe covering the IR was prepared from the clone pPORT2 of an isolate of TYLCV-Is from Portugal (14). The probe was prepared by polymerase chain reaction (PCR) with primers MA-30 (5'-GAGCACTTAGGATATGTGAGG-3', nucleotides 2,566 to 2,586) and MA-31 (5'-AGTGGATCCACATATTGC-3', nucleotides 170 to 152) based on the sequence of the clone pPORT2 (*unpublished*).

Etiology of a novel disease of common bean. Unusual virus-like disease symptoms were observed in September 1997 in plastic house-grown (Almería, region III, Fig. 2) and field-grown (Málaga, region I, Fig. 2) staked fresh market bean (*P. vulgaris* cv. Donna and Garrafal Oro, respectively) heavily infested with *B. tabaci*. Samples from 40 symptomatic and 15 asymptomatic plants were collected and analyzed for the presence of viruses reported in common bean in Spain, including potyviruses, cucumber mosaic cucumovirus (CMV), and tomato spotted wilt tospovirus (TSWV; 5,6; E. Sáez-Alonso, *unpublished*). Analyses were done by direct double-antibody sandwich enzyme-linked-immunosorbent assay (DAS-ELISA; 7). Antibodies were against a broad-spectrum of potyviruses (anti-Potyvirus group; Agdia, Inc., Elkhart, IN), CMV, or TSWV (Loewe Biochemica GmbH, Otterfing,

Corresponding author: E. Moriones
E-mail: moriones@eelm.csic.es

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Fig. 1. Symptoms of tomato yellow leaf curl virus (TYLCV) infection in Spain. (A) Severe and (B) normal upward curling of leaflet margins in tomato (*Lycopersicon esculentum*) caused by TYLCV-Is and TYLCV-Sr species, respectively; and (C) leaf crumple, thickening, and upward curling and (D) abnormal apical shoot proliferation in common bean (*Phaseolus vulgaris*) caused by TYLCV-Is.

Fig. 2. Location of regions I to IV where field surveys for occurrence of tomato yellow leaf curl virus-Is in fresh market tomato (*Lycopersicon esculentum*) were made during autumn 1997. The main production areas are shadowed.

Germany), and were used as recommended by the suppliers. The same samples were also analyzed by hybridization of petiole cross-sections printed on nylon⁺ membranes (Boehringer Mannheim, Mannheim, Germany; hereafter referred to as tissue blotting) with probes specific to TYLCV-Sr and TYLCV-Is. As the IR is the least conserved region among geminivirus genomes (1), probes specific to TYLCV-Sr and -Is were prepared by PCR to IR of

TYLCV-Sr isolate M and TYLCV-Is isolate SP72/97, respectively. PCR amplification reactions were performed with the primer pair MA-14/MA-15 (MA-14: 5'-TGCATTATTTGAAAACG-3', nucleotides 2,587 to 2,604; MA-15: 5'-AAAG-GATCCCACATATTG-3', nucleotides 162 to 145), based on the sequence of TYLCV-M (21) for TYLCV-Sr and MA-30/MA-31 for TYLCV-Is. Total nucleic acids were also extracted from the collected samples

and specific fragments of the VI and C2 genes were amplified as reported (18) for single-strand conformation polymorphism (SSCP) analysis (13) and direct sequencing. Cultures of *Agrobacterium tumefaciens* strain LBA4404 transformed with the recombinant plasmid p1.7SP72/97 (11) were used to inoculate (hereafter referred to as agroinoculation) bean (*P. vulgaris* cv. Donna) plants at the first trifoliate leaf growth stage. After 20 days, the 36 plants inoculated were analyzed for TYLCV infection by tissue blotting, and young tissues were extracted for SSCP analyses of both pre-coat (VI) and C2 fragments. Symptoms were recorded 30 days after inoculation.

Field surveys. A survey was undertaken during autumn 1997 in the main tomato-growing regions of southern Spain, covering a transect of about 500 km along the Mediterranean coast (regions I to IV, Fig. 2) to determine the occurrence of TYLCV-Is. Samples from TYLC-affected tomato plants were collected from fields and plastic houses. Also, to ascertain if the unusually severe TYLC symptoms observed in tomato crops were associated to infections caused by TYLCV-Is, several tomato samples exhibiting the severe (Fig. 1A) or normal (Fig. 1B) TYLC symptoms were collected from a plastic-house crop grown in Almería (region III, Fig. 2) where plants with both kind of symptoms coexisted. Samples were analyzed by tissue blotting with probes specific to TYLCV-Sr or TYLCV-Is as described above.

Fig. 3. Results of analysis of common bean (*Phaseolus vulgaris*) plants collected from crops affected by the bean leaf crumple disease or agroinoculated with an infectious clone obtained for the SP72/97-tomato yellow leaf curl virus (TYLCV)-Is isolate collected during the first epidemic outbreak of this geminivirus species in Spain. (A) Petiole cross section prints from 12 symptomatic plants collected from plastic-house (row a)- or field (row b)-grown crops. Row c contains prints from asymptomatic plants collected in the same survey. (B) Petiole cross-section prints from 36 agro-inoculated plants (rows a to f). Controls in row d (A) and g (B) are healthy, SP72/97-TYLCV-Is- or M-TYLCV-Sr-infected tomato (*Lycopersicon esculentum*) plants, respectively. Two prints per tested plant were made. Prints were hybridized with probes specific to TYLCV-Is.

RESULTS

Etiology of a novel disease of common bean. A novel virus-like disease was observed in field- (Málaga, region I, Fig. 2) and plastic-house-grown (Almería, region III, Fig. 2) staked fresh market bean crops in autumn 1997. Incidences of symptomatic plants was up to 80% in some fields. Symptoms consisted of thickening, epinasty, crumpling, and blade reduction of leaves; upward curling of leaves was also observed at times (Fig. 1C). A characteristic abnormal shoot proliferation and internode reduction occurred in apical parts of affected plants; the very small leaves formed resulted in a bushy appearance (Fig. 1D). Symptoms were noticed especially in new shoots emerging from the basal part of the plants. When infected early, plants showed dramatic stunting and abortion of new inflorescences, and production was entirely lost. This disease, named bean leaf crumple, was observed in crops heavily infested with *B. tabaci*. A

Fig. 4. Single-strand conformation polymorphism patterns of the pre-coat (V1) and C2 polymerase chain reaction fragments amplified from a SP72/97-tomato yellow leaf curl virus (TYLCV)-Is agro-infected bean (*Phaseolus vulgaris*) plant and from a field bean plant affected by the bean leaf crumple disease (lanes 5 and 7, respectively). Patterns from SP72/97-TYLCV-Is agroinfected and *Bemisia tabaci*-mediated-infected tomato (*Lycopersicon esculentum*) plants are shown in lanes 4 and 6, respectively. Controls are from healthy and M-TYLCV-Sr- or SP72/97-TYLCV-Is field-infected tomato plants (lanes 1, 2, and 3, respectively). The 1 kilobase-ladder DNA molecular marker is included in lanes 8. Electrophoresis was performed in 12% non-denaturing polyacrylamide gels (110 by 125 by 1 mm) at 200 V for 5 h at 4°C, and gels were stained with silver nitrate.

possible implication of TYLCV in bean leaf crumple disease was suspected, because TYLCV-affected tomato crops were present nearby. Analysis of the bean samples showed that none of the symptomatic plants reacted in ELISA for potyviruses, CMV, or TSWV. Tissue-blotting assays for TYLCV-Sr were negative (results not shown), whereas a clear hybridization signal was obtained with probes for TYLCV-Is (Fig. 3A). None of the samples collected from the asymptomatic plants reacted in ELISA or tissue blotting. SSCP patterns for V1 and C2 fragments amplified from positive-reacting samples were identical to those of SP72/97-TYLCV-Is (Fig. 4). The V1 and C2 amplified fragments were directly sequenced for one of the samples, and a 100% nucleotide sequence identity with the sequence of the corresponding fragments of SP72/97-TYLCV-Is was found. To ascertain if TYLCV-Is was the etiological agent for the bean leaf crumple syndrome, healthy bean plants were agro-inoculated with the infectious clone of SP72/97-TYLCV-Is previously obtained. Of 36 inoculated plants, 33 were infected systemically by TYLCV-Is (Fig. 3B) and developed symptoms similar to those observed in the field-infected beans. SSCP patterns for V1 and C2 fragments amplified from these plants were identical to those of SP72/97-TYLCV-Is (Fig. 4).

Occurrence of TYLCV-Is. TYLCV-Is was found widely dispersed throughout the main tomato-growing regions of southern Spain by autumn 1997. Analysis of TYLCV-symptomatic tomato samples collected from Málaga, Granada, Almería, or Murcia provinces (regions I to IV, respectively; Fig. 2) showed that TYLCV-Is occurred frequently in all regions either alone or as mixed infections with TYLCV-Sr (Table 1). A close relationship was found between symptoms exhibited in tomato plants and the TYLCV species present. A total of 19 tomato plants from a plastic-house crop

grown in Almería (where plants with different symptoms were present) were analyzed. Results consistently associated the TYLCV-Is infection with the unusually severe TYLC symptoms. All 10 plants with severe symptoms analyzed were found to be infected by TYLCV-Is, either alone or together with TYLCV-Sr; whereas, with one exception (a plant with a mixed infection of TYLCV-Is and -Sr), the 9 plants showing normal symptoms were infected only by TYLCV-Sr.

DISCUSSION

Two important results described in this paper demonstrate the widespread occurrence of TYLCV-Is in southern Spain and its association with a new disease in common bean. The presence of TYLCV-Is in Spain is of concern because it is consistently associated with more severe effects in tomato than the previously existing TYLCV-Sr species. Presence of TYLCV-Is may require changes in breeding programs for virus resistance in Spain (22) because differences in the behavior of breeding lines against different TYLCVs have been reported (10). The simultaneous occurrence of both species within a single crop and even in a single plant is important because recombinants might arise with unpredictable consequences on pathogenicity. Such recombination has been suggested as a powerful factor in the evolution of whitefly-transmitted geminiviruses, not only in the long term but also in the immediate future (28). Natural genetic recombination between geminiviruses regarded as separate species has been demonstrated for cassava mosaic geminiviruses, and caused a severe form of cassava mosaic disease in Uganda which has devastated crops since 1988 (30).

In addition to being a severe pathogen of tomato, TYLCV-Is is the causal agent of the novel bean leaf crumple disease that has recently threatened common bean

Table 1. Occurrence of tomato yellow leaf curl virus (TYLCV)-Is in fresh-market tomato (*Lycopersicon esculentum*) crops in the main tomato growing regions of southern Spain during autumn 1997

Region	No. fields surveyed	No. samples tested	No. samples infected by TYLCV ^a		
			Is	Is + Sr	Sr
I. Málaga	12	130	80	36	14
II. Granada	4	21	9	9	3
III. Almería	13	94	16	21	57
IV. Murcia	2	24	0	9	15

^a Petiole cross-sections of samples collected were printed on nylon⁺ membranes and hybridized with probes specific to TYLCV-Sr and TYLCV-Is.

crops in Spain. This is the first reported geminivirus in bean crops from Spain. There is a report of TYLCV naturally infecting beans in Israel (20); however, no information on symptoms or characterization of the virus involved were presented.

In recent years, TYLCV-Is has been reported to cause epidemics in countries from both eastern and western hemispheres (9,14–16,24,25). The origin and spread pathways of this virus, especially between distant countries, is a matter of discussion. It is possible that TYLCV-Is was spread by movement of infected plant material or by plants harboring viruliferous whiteflies between distant countries. TYLCV-Is is the only known geminivirus introduced from the eastern to the western hemisphere (23). It appears that TYLCV-Is was unknowingly introduced via infected tomato transplants to the Dominican Republic (23,24) and then expanded to other Caribbean countries (15,16,25). A similar situation may have occurred in the western Mediterranean basin, where TYLCV-Is was first reported in Portugal in 1995 (14) and a year later in Spain (18).

The present report of TYLCV-Is infecting bean in Spain is important not only because of the economic importance of this crop but because bean is often used as an intercrop between tomato crops. Furthermore, beans are grown as an alternative to tomatoes in areas severely affected by TYLCV epidemics. Therefore, maintenance of high TYLCV infection levels is ensured year-round alternately in tomato and bean crops, and reduces the possibilities for managing TYLCV epidemics. In the absence of effective control methods against TYLCV infections, consequences for the local growers' economy can be dramatic. The need for efforts to develop TYLCV-resistant cultivars has clearly increased.

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