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A Natural Recombinant between the Geminiviruses *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* Exhibits a Novel Pathogenic Phenotype and Is Becoming Prevalent in Spanish Populations

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This work provides evidence of the significant contribution of recombination to the genetic diversification of emerging begomovirus populations. In southern Spain, *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato yellow leaf curl virus* (TYLCV) are distinct geminivirus species that coexist in the field and contribute to the tomato yellow leaf curl disease epidemic. A natural recombinant between TYLCSV and TYLCV has been detected and an infectious clone of a recombinant isolate (ES421/99) was obtained and characterized. Analysis of its genome showed that the recombination sites are located in the intergenic region in which a conserved stem-loop structure occurs and at the 3′-end of the replication enhancer protein open reading frame. ES421/99 exhibited a novel pathogenic phenotype that might provide it with a selective advantage over the parental genotypes. This agrees with results from field studies which revealed that the recombinant strain is becoming prevalent in the region in which it was detected.

Key Words: begomovirus; *Bemisia tabaci* transmission; field survey; geminivirus; host range; *Lycopersicon esculentum*; *Phaseolus vulgaris*; natural recombination; tomato yellow leaf curl disease.

INTRODUCTION

The geminiviruses (family *Geminiviridae*) are a group of emerging plant viruses that have circular single-stranded (ss) DNA genomes encapsidated in twinned icosahedral virions. Members of this group cause severe diseases to a wide variety of plant species, some of them of great agricultural importance (Rybicki *et al.,* 2000). The yellow leaf curl disease of tomato (TYLCD) is one of these diseases that is causing increasing damage to tomato (*Lycopersicon esculentum* Mill.) crops in many warm and temperate regions of the world. A complex of geminivirus species in the genus *Begomovirus*, that are naturally transmitted by the whitefly *Bemisia tabaci* Genn. (*Hemiptera*: *Aleyrodidae*), has been associated with TYLCD (Moriones and Navas-Castillo, 2000). All these species, except *Tomato yellow leaf curl Thailand virus* (Rochester *et al.,* 1994; Fauquet *et al.,* 2000), have monopartite ssDNA genomes of about 2.8 kb that contain six partially overlapping open reading frames (ORFs), two partially overlapping ORFs (V2 and CP) on the virion-sense, and four partially overlapping ORFs (Rep, REn, TrAP, and C4) on the complementary-sense strand, separated by an intergenic region (IR) of about 300 nucleotides. The IR includes a stem-loop structure that is characteristic of all geminiviruses, with a conserved nonanucleotide sequence 5′-TAATATT↓AC-3′ in the loop in which the breaking and joining site (↓) for rolling-circle replication occurs (Hanley-Bowdoin *et al.,* 2000). The Rep proteins encoded by geminiviruses show specificity for the replication of their cognate genomes, conferred by recognition of a high-affinity binding site present in the IR by the N-terminal domain of the Rep protein (Gutierrez, 1999).

Epidemics of TYLCD have caused devastating damage to field and greenhouse tomato crops in Spain since 1992, when the ES strain of the species *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES, hereafter referred to as TYLCSV) was reported as the causal agent (Noris *et al.,* 1994; Fauquet *et al.,* 2000). In 1997, another begomovirus species, the Mld strain of *Tomato yellow leaf curl virus* (TYLCV-Mld, hereafter referred to as TYLCV) was also found associated with TYLCSV in the TYLCD epidemics (Navas-Castillo *et al.,* 1997, 2000). Both virus species are frequently found in mixed infections in single tomato plants (Sánchez-Campos *et al.,* 1999). TYLCV was also demonstrated to be the causal agent of bean leaf crumple disease (BLCD) of common bean (*Phaseolus vulgaris* L.) (Navas-Castillo *et al.,* 1999), a plant species that TYLCSV is unable to infect (Sánchez-Campos *et al.,* 1999).

The generation of genetic diversity in viral populations provides novel opportunities for adaptation to new hosts and changing environmental conditions. Three major forces drive the evolution of viruses: mutation, recombi-

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nation, and reassortment (for review, see Roossinck, 1997). Recombination occurs in both RNA and DNA genomes and plays a major evolutionary role in animal and plant viruses (Domingo and Holland, 1997; García-Arenal *et al.,* 2001). By recombination, viruses can acquire novel genetic information from other viruses or even from the host, as shown for the RNA virus *Potato leaf roll virus* (Mayo and Jolly, 1991), facilitating their rapid evolution (Angenent *et al.,* 1986; Simon and Bujarski, 1994; Padidam *et al.,* 1999). Viruses also use recombination as a mechanism to rescue deleterious mutations by genetic exchange with wild-type genomes (Makino *et al.,* 1986; Rao and Hall, 1993), thus overcoming the loss of fitness derived from genetic drift and Muller's ratchet (Chao, 1990). Therefore, recombination has a crucial role in virus evolution (Makino *et al.,* 1986; Nagy and Bujarski, 1992; Simon and Bujarski, 1994).

Recombination contributes to the genetic diversification of geminivirus populations (Umaharan *et al.,* 1998; Moffat, 1999; Padidam *et al.,* 1999; Sanz *et al.,* 1999, 2000) and has been related to the emergence of some serious diseases (Zhou *et al.,* 1997; Rybicki and Pietersen, 1999). In begomoviruses, recombination occurs at the strain (Hou and Gilbertson, 1996; Kirthi *et al.,* 2002), species (Zhou *et al.,* 1997; Fondong *et al.,* 2000; Navas-Castillo *et al.,* 2000; Sanz *et al.,* 2000; Martin *et al.,* 2001; Saunders *et al.,* 2002), genus (Briddon *et al.,* 1996; Klute *et al.,* 1996), and family (Saunders and Stanley, 1999) levels. Despite the evident importance of recombination in the begomoviruses, scarce work is available to document its contribution to the evolution of natural populations. During 1999, Monci *et al.* (2001) reported the occurrence in southern Spain of a natural recombinant derived from a genetic exchange between TYLCSV and TYLCV. This provided us the opportunity to analyze the consequences of recombination in the population. Here, we report the characterization of an infectious clone of the recombinant strain. Data are presented which suggest that it exhibits novel biological characteristics that could provide it with a selective advantage in nature as substantiated by its increasing frequency in the population. Thus, direct evidence is presented for the contribution of recombination to the evolution of the emerging begomovirus population present in southern Spain.

RESULTS

Isolate ES421/99 is a recombinant between the genomes of TYLCSV and TYLCV

A 1.4-mer DNA copy of the ES421/99 genome was cloned into the agrobacterium binary vector pBin19 to produce p1.4ES421/99. This clone was infectious after agroinoculation of common bean test plants (four plants infected of four plants tested). Indeed, inoculated plants reproduced the BLCD symptoms observed in the field sample and virus progeny was transmissible by *B. tabaci* to healthy common bean plants (two of four plants inoculated were infected and reproduced the BLCD symptoms). Therefore, the DNA fragment cloned in pES421/99 represented a fully biologically active copy of the genome of ES421/99. In addition, as for the field sample, in all plants infected by agroinoculation or *B. tabaci* transmission, a DNA fragment representative of the IR could only be amplified by PCR when using the primers MA116 and MA117 (see below).

The complete genome sequence of isolate ES421/99 was determined for clone pES421/99. The sequence comprised 2782 nucleotides and has been deposited in the EMBL/GenBank Nucleotide Sequence Database under Accession No. AF271234. The genome organization of ES421/99 was similar to that reported for other begomoviruses, with two ORFs in the virion-sense and four in the complementary-sense strand separated by the IR (Fig. 1). The IR contains a putative stem-loop structure and the TAATATTAC nonanucleotide found in all geminiviruses. Comparison of the nucleotide sequence with available sequences showed that the genome of ES421/99 was the result of a recombination event between the genomes of TYLCV and TYLCSV (Fig. 1). The recombination sites were located in a part of the IR in which the stem-loop structure occurs and at the 3′-end of the REn ORF. However, the precise nucleotides at which the recombination occurred could not be identified as the regions involved are conserved between TYLCV and TYLCSV (Fig. 1). As a result of this recombination, cognate Rep protein and *cis*-acting motifs in the IR required for replication were maintained in ES421/99, ensuring a viable replicative unit (Gutiérrez, 1999; Hanley-Bowdoin *et al.,* 2000). The TYLCV and TYLCSV genome sequences present in ES421/99 were closely related to those of the isolates reported from Spain. Thus, the TYLCSV-like region of ES421/99 was 99% identical to the corresponding part of TYLCSV-ES[1] and the TYLCV-like region was 99% identical to the corresponding part of TYLCV-[ES72/97]. At the amino acid level, only two nonconservative changes were detected, one in the TrAP ORF (L_{43} of ES421/99 replaces Q_{43} in TYLCV-[ES72/97]), and one in the C4 ORF (M_{47} of ES421/99 replaces T_{47} in TYLCV-[ES72/97]). A more comprehensive phylogenetic analysis with other TYLCD-causing begomoviruses remained consistent with a recombination event between TYLCV and TYLCSV isolates from Spain (Fig. 2).

The recombinant ES421/99 has a wider host range than TYLCV and TYLCSV present in Spain

The host range of ES421/99 was compared with that of TYLCV and TYLCSV isolates reported from Spain. Infectious clones of ES421/99, TYLCSV-ES[1], and TYLCV-[ES72/97] were inoculated into a panel of plant species known to be hosts of TYLCSV and/or TYLCV. As shown in Table 1, TYLCSV was able to infect tomato, *Solanum*

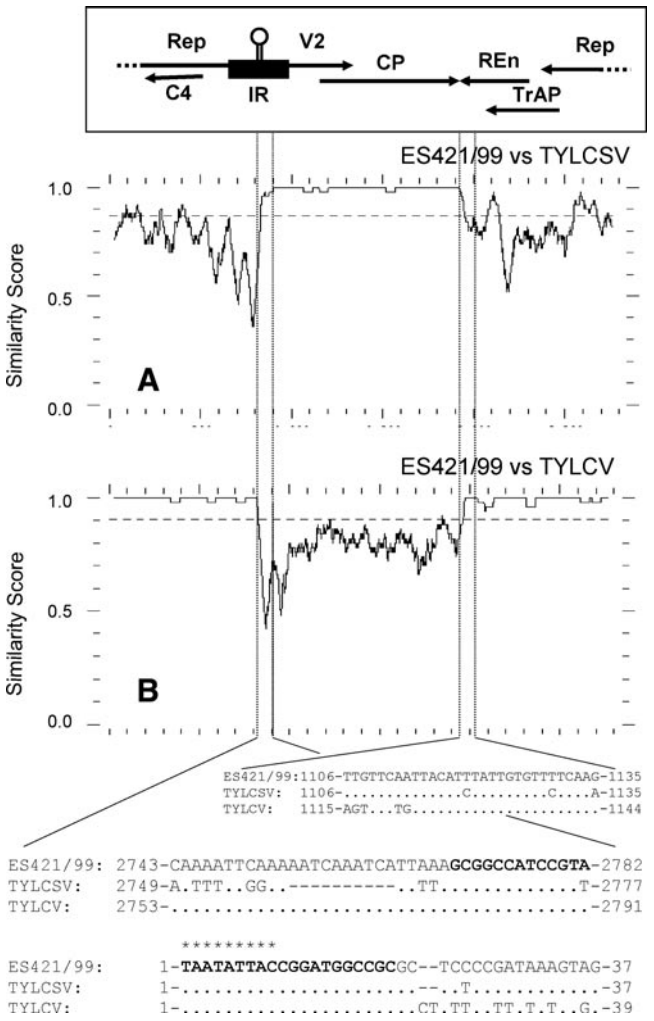

FIG. 1. Plotsimilarity diagrams (scanning window = 50 nt) comparing the nucleotide sequences of ES421/99 (this study) with either TYLCSV-ES[1] (GenBank Accession No. Z25751) (A) or TYLCV-[ES72/97] (GenBank Accession No. AF071228) (B) isolates. The putative regions involved in the recombination event are indicated by vertical lines. The nucleotide sequences of each virus in those regions are shown at the bottom of the figure. Bold letters indicate the nucleotides involved in the stem-loop structure; asterisks indicate the conserved nonanucleotide sequence present in the loop, and dashes indicate nucleotide deletions within the TYLCSV and recombinant sequences. Numbers refer to nucleotide positions in the sequence of each virus. Positions of the open reading frames and the intergenic region (IR) are indicated at the top of the figure. Horizontal broken lines indicate the mean similarity between the sequences compared.

nigrum, and *S. luteum* but not common bean, and TYLCV was able to infect tomato and common bean but not *S. nigrum* and *S. luteum*. These results agreed with field studies done in Spain (this work and unpublished data; Sánchez-Campos *et al.,* 1999, 2000), in which TYLCSV was never detected in common bean and TYLCV was never detected in *S. nigrum* and *S. luteum*. Interestingly, ES421/99 was capable of infecting tomato, common bean, *S. nigrum*, and *S. luteum*. In common bean, similar BLCD symptoms were induced by TYLCV-[ES72/97] and ES421/99. However, in the susceptible tomato tested (Moneymaker), ES421/99 induced much milder TYLCD symptoms than either TYLCV-[ES72/97] or TYLCSV-ES[1]. In the tolerant tomato Anastasia, none of these viruses induced symptoms, although they were able to infect this host species systemically. It should be noted that our results suggested that ES421/99 infected Anastasia plants more efficiently than TYLCV-[ES72/97] and TYLCSV-ES[1] (Table 1). In every case, only mild hybridization signals were detected for infected Anastasia plants in contrast to the strong signals obtained for infected Moneymaker plants. Therefore, as already demonstrated for TYLCV (Michelson *et al.,* 1994), the tolerance gene *Ty-1* also seems to limit the systemic infection of TYLCSV and ES421/99 in tomato. In *S. nigrum* and *S. luteum*, ES421/99 seemed to be less infectious than TYLCSV (Table 1), and as in the susceptible tomato, milder symptoms were induced. The identity of the virus present in systemically infected tissues with that inoculated was confirmed in every case by PCR analysis using primers based on the IR and the REn ORF (see below).

The recombinant ES421/99 is efficiently transmitted by *B. tabaci*

Differences have been reported between TYLCV and TYLCSV isolates from Spain in their ability to be transmitted by *B. tabaci*, and this was related to their differential fitness in nature (Sánchez-Campos *et al.,* 1999). As whitefly transmission is an essential step in the epidemics of geminiviruses, we compared *B. tabaci* transmission of ES421/99 with TYLCSV and TYLCV isolates reported from Spain. The results (Table 2) revealed that despite ES421/99 accumulating to ~14% of the level of TYLCV-[ES72/97] in source plants, the recombinant was transmitted as efficiently as this virus, and slightly more efficiently than TYLCSV-ES[1], which accumulated to ~19% of the level of TYLCV-[ES72/97]. Therefore, these results suggest that ES421/99 is at least as well adapted to transmission by the Q biotype of *B. tabaci* as the TYLCV isolate used.

Recombinants resembling ES421/99 are becoming prevalent in nature

Field studies can provide information regarding the fitness of ES421/99 in nature. Therefore, we studied the frequency of occurrence of isolates resembling ES421/99 among the samples collected during a field survey conducted between 1998 and 2000 in commercial bean and tomato crops of southern and southeastern Spain. A hybridization/PCR analysis system was developed which allowed us to screen samples using probes specific to the IR of either TYLCV or TYLCSV, and PCR primers that are specific for either TYLCV, TYLCSV, or ES421/99-like variants, designed on sequences around the crossover sites found in ES421/99. This PCR-based methodology

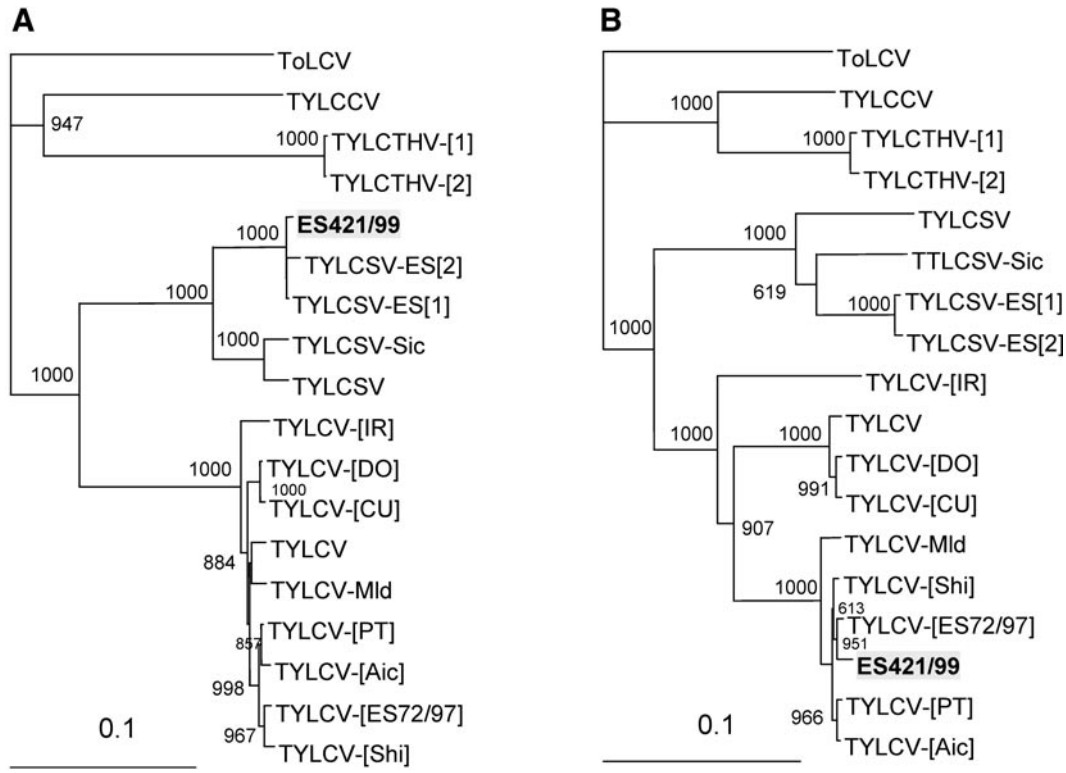

FIG. 2. Neighbor-joining phylogenetic trees depicting topology and distance relationships of nucleotide sequences in regions of ES421/99 (shadowed) resembling those in *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (A) and *Tomato yellow leaf curl virus* (TYLCV) (B) and equivalent regions of other begomoviruses causing tomato yellow leaf curl disease. The trees were rooted using *Tomato leaf curl virus* (ToLCV) as outgroup. Accession numbers for the full-length genomic sequences not determined in this study are as follows: ToLCV (AF165098), TYLCCV (AF311734), TYLCTHV-[1] (X63015), TYLCTHV-[2] (AF141922), TYLCSV-ES[1] (Z25751), TYLCSV-ES[2] (L27708), TYLCSV-Sic (Z28390), TYLCSV (X61153), TYLCV-[IR] (AJ132711), TYLCV (X15656), TYLCV-[DO] (AF024715), TYLCV-[CU] (AJ223505), TYLCV-Mld (X76319), TYLCV-[PT] (AF105975), TYLCV-[Aic] (AB014347), TYLCV-[Shi] (AB014346), TYLCV-[ES72/97] (AF071228). The significance of the nodes in a bootstrap analysis with 1000 replicates is shown. Vertical distances are arbitrary, while horizontal distances reflect genetic distance between branch nodes. Scale bars indicate the horizontal distance equivalent to 0.1 replacements per position.

allowed us to detect recombinant-like isolates from infected samples even at ratios of 1:10,000 in mixed infections with TYLCV or TYLCSV (not shown). Thus, the presence of recombinants resembling ES421/99 in single infections or mixed infected with TYLCV and/or TYLCSV could be ascertained almost unequivocally in field samples. Therefore, this methodology was used to analyze the samples collected during the survey. It should be noted that for those samples tested only by hybridization (i.e., those that reacted only with the probe for TYLCSV or TYLCV and did not give a product in the PCR reaction for a recombinant REn ORF, see below) the procedure was less sensitive because a maximum ratio of 1:100 could be resolved in mixed infections (not shown).

The analysis of the samples randomly collected from plants showing symptoms of TYLCD (in tomato) or BLCD (in common bean) revealed that in common bean ES421/99-like isolates are becoming prevalent in the Almería province. In this province in 1999 and 2000, in contrast to 1998, a predominance of ES421/99-like infections was detected in common bean (Table 3). Although few samples were available from 1998, all were collected from the same region sampled during 1999 (Berja region). None of the samples was infected with ES421/99-like isolates in 1998, while 96.5% of the samples were infected with this variant in 1999. In Málaga, no ES421/99-like variant infections were detected in common bean. In tomato, although TYLCV infections are predominant in Almería, a significant number of samples collected in 2000 contained ES421/99-like isolates (Table 4). It should be noted that in this province in 1998, ES421/99-like variant was already present in four tomato samples, in mixed infections with TYLCV and TYLCSV. In Málaga, Granada, and Murcia, ES421/99-like isolates in tomato were very rare and were only detected in two samples from Málaga, one in 1999 and another in 2000, both also containing TYLCV and TYLCSV (Table 4). A variable proportion of TYLCV and TYLCSV, and/or mixed infections of these viruses, was detected in Granada and Murcia, whereas TYLCV infections predominated in Málaga. Interestingly, both in common bean (Almería 2000; Table 3) and in tomato (Málaga 2000; Table 4), several samples gave hybridization and PCR results that differed from

TABLE 1

Host Ranges of *Tomato yellow leaf curl virus* Isolate [ES72/97] (TYLCV), *Tomato yellow leaf curl Sardinia virus* Isolate ES[1] (TYLCSV), and the Recombinant ES421/99[a]

Host plant	Number of plants infected/inoculated[b]		
	TYLCV	TYLCSV	ES421/99
Tomato			
cv. Moneymaker	10/10	10/10	10/10
cv. Anastasia	4/10	2/10	7/10
Common bean			
cv. Donna[c]	3/5	0/5	4/5
cv. Kwinthus	10/10	0/10	10/10
GG5	9/10	0/10	10/10
Solanum nigrum	0/10	10/10	4/10
Solanum luteum	0/10	9/10	3/10

[a] Plants were agroinoculated using infectious clones of TYLCV-[ES72/97] (Navas-Castillo *et al.,* 1999), TYLCSV-ES[1] (Noris *et al.,* 1994), and ES421/99 (this work).

[b] Results from two repeated experiments with five plants per virus and host species; plants were analyzed 30 days after inoculation by hybridization of tissue blots from petiole cross sections using probes prepared to the IR of either TYLCV or TYLCSV (Navas-Castillo *et al.,* 1999).

[c] Only one experiment with five plants per virus species was done.

those expected for infections with ES421/99, TYLCSC, TYLCV, or combinations of these viruses. This could be due to point mutations that inhibit primer recognition during PCR or to the presence of novel virus variants. Additional cloning and sequence analysis is needed to determine the nature of the variation associated with these samples.

During the host range studies, we noticed that ES421/99 induced very mild symptoms in tomato. We suspected that the actual frequency of ES421/99-like isolates in nature could have been underestimated in tomato during the field survey because it was based on symptomatic plants. Therefore, during autumn 2000 we conducted a new survey in commercial tomato crops of Almería, the province in which ES421/99-like isolates seemed to be more frequent. On this occasion, symptomatic and asymptomatic plants were sampled at random. The results from hybridization/PCR analyses of the samples collected are summarized in Table 5. As suspected, a lower frequency of ES421/99-like infections occurred in symptomatic samples (8.3%) compared with asymptomatic samples (26.7%) and the entire sample collection (16.7%). Hence, the frequency of ES421/99-like variant infections deduced for TYLCD symptomatic tomato plants (Table 4) probably underestimates the actual frequency of this variant in nature. As before, in this autumn survey, several samples contained unidentified virus (Table 5). These samples will require further analysis to determine the nature of the virus variant involved in the infection.

DISCUSSION

The analysis of the nucleotide sequence of one isolate (ES421/99) of the recombinant begomovirus strain recently detected in Spain (Monci *et al.,* 2001) revealed that the putative progenitors were closely related to TYLCSV and TYLCV isolates from Spain (Noris *et al.,* 1994; Navas-Castillo *et al.,* 2000). The recombination involved a region at the 3′-end of the REn ORF and part of the IR close to the nick site for initiation of rolling circle replication present in the stem-loop (Hanley-Bowdoin *et al.,* 2000; Gutierrez, 1999), which is a hot spot for recombination in geminiviruses (Stenger *et al.,* 1991; Stanley, 1995; Sanz *et al.,* 1999; Navas-Castillo *et al.,* 2000). The biological characterization of ES421/99 showed that it exhibited a novel pathogenic phenotype. It is well adapted to *B. tabaci* transmission and has a wider host range than either TYLCV or TYLCSV, which indicates a selective advantage over the putative parental genotypes. The expansion of the host range shown by this recombinant may have important epidemiologic consequences because, as observed for the geminivirus *Maize streak virus*, an increased host range may result in a wider spectrum of potential reservoir hosts, providing greater opportunities to infect crops (Martin *et al.,* 2001). With the exception of common bean, ES421/99 induced mild symptoms in susceptible plants, in contrast to the severe symptoms induced by TYLCV and TYLCSV. A similar alteration of symptom severity resulting from recombination has also been observed in other geminiviruses (Stenger *et al.,* 1994; Saunders *et al.,* 2001; Schnippenkoetter *et al.,* 2001), and such reduced aggression may be important in nature. Thus, the increased survival of mildly symptomatic plants might result in a more efficient maintenance of virus reservoirs. This together with a less efficient *B. tabaci* transmission from severely symptomatic plants,

TABLE 2

B. tabaci Transmission to Tomato Plants after Virus Acquisition from Tomato Plants Infected with Either *Tomato yellow leaf curl Sardinia virus* (TYLCSV)-ES[1], *Tomato yellow leaf curl virus* (TYLCV)-[ES72/97], or the Recombinant ES421/99[a]

	Number of plants infected/inoculated using as acquisition source plants infected with[b]:		
	TYLCV	TYLCSV	ES421/99
Experiment 1	13/25	5/25	10/25
Experiment 2	13/25	9/25	11/25

[a] Healthy tomato (cv. Moneymaker) test plants were exposed for 48 h to *B. tabaci* adults (five adults per plant) that had acquired the virus for 24 h from a tomato (cv. Moneymaker) plant agroinoculated 3 weeks before.

[b] Young leaves of inoculated plants were analyzed 30 days after inoculation by hybridization of tissue blots from petiole cross sections using probes prepared to the IR of TYLCV or TYLCSV (Navas-Castillo *et al.,* 1999).

TABLE 3

Analysis of Common Bean Samples Randomly Collected in a Survey Conducted during 1998, 1999, and 2000 in Commercial Crops of the Almería and Málaga Provinces from Plants Showing Symptoms of Bean Leaf Crumple Disease

Province	Year	Number of infected samples collected[a] (number of fields visited)	Number (%) of samples infected with[b]			
			TYLCV	Rec	Rec + TYLCV	Other[c]
Almería	1998	10 (3)	10 (100%)	—	—	—
	1999	28 (9)	1 (3.5%)	22 (78.6%)	5 (17.9%)	—
	2000	84 (13)	11 (13.1%)	64 (76.2%)	5 (5.9%)	4 (4.8%)
Málaga	1999	20 (11)	20 (100%)	—	—	—
	2000	60 (12)	60 (100%)	—	—	—

[a] Samples that gave positive results in the hybridization/PCR analyses.
[b] Suggested infection type based on the hybridization/PCR analyses; "Rec" refers to ES421/99-like recombinant.
[c] Samples for which hybridization/PCR results differ from those expected for infections with TYLCV, TYLCSV, and ES421/99-like variant described in southern Spain.

as shown by Lapidot *et al.* (2001) in tomato, might increase the chances of transmission of the recombinant. A further significant pathogenic difference exhibited by ES421/99 is that it repeatedly infected tomato with the tolerance gene *Ty-1* (Michelson *et al.,* 1994) more efficiently than TYLCV and TYLCSV, although none of these viruses induced TYLCD symptoms. As the *Ty-1* gene is increasingly used in the commercial tomato cultivars grown in Spain, further experimental and field studies are needed to analyze the consistency and possible epidemiologic implications of this result.

Once a recombination event has occurred, the long-term survival and establishment of a recombinant in nature will depend on it having a selective advantage. The novel pathogenic characteristics observed for ES421/99 may indicate some natural selective advantage for this variant. An improved fitness of ES421/99 is also suggested from the field survey, which indicates that ES421/99-like isolates are becoming prevalent in nature. Thus, in Almería, a rapid displacement of TYLCV by ES421/99-like isolates has occurred in common bean, and the occurrence of these isolates may also be increasing in tomato. An increase in the relative fitness of a recombinant plant virus has been documented (e.g., Fernández-Cuartero *et al.,* 1994), and it has been shown that the appearance of more fit recombinants may dramatically affect the genetic makeup in populations of RNA (Revers *et al.,* 1996; Moonan *et al.,* 2000) and DNA (Zhou *et al.,* 1997; Pita *et al.,* 2001) plant viruses. Interestingly, although mixed infections of TYLCV and TYLCSV are frequent in Málaga, Granada, and Murcia, recombinants have only been detected in two samples from Málaga (Tables 3 and 4). Our results indicate that recombinant genomes rapidly appear during mixed infection of tomato with TYLCV and TYLCSV (our unpublished data), similar to the findings of Schnippenkoetter

TABLE 4

Analysis of Tomato Samples Randomly Collected in a Survey Conducted during 1998 to 2000 in Commercial Crops of the Almería, Málaga, Granada, and Murcia Provinces from Plants Showing Symptoms of Tomato Yellow Leaf Curl Disease

Province	Year	Number of infected samples collected[a] (number of fields visited)	Number (%) of samples infected with[b]						
			TYLCV	TYLCSV	TYLCV + TYLCSV	Rec	Rec + TYLCV	Rec + TYLCV + TYLCSV	Other[c]
Almería	1998	35 (9)	21 (60.0%)	—	10 (28.6%)	—	—	4 (11.4%)	—
	1999	22 (4)	19 (86.4%)	—	3 (13.6%)	—	—	—	—
	2000	94 (15)	68 (72.3%)	—	8 (8.5%)	2 (2.1%)	13 (13.8%)	3 (3.2%)	—
Málaga	1999	79 (16)	70 (88.6%)	2 (2.5%)	6 (7.6%)	—	—	1 (1.3%)	—
	2000	113 (15)	93 (82.3%)	2 (1.8%)	16 (14.2%)	—	—	1 (0.9%)	1 (0.9%)
Granada	1999	26 (4)	12 (46.2%)	14 (53.8%)	—	—	—	—	—
	2000	19 (4)	13 (68.4%)	3 (15.8%)	3 (15.8%)	—	—	—	—
Murcia	1999	29 (7)	11 (37.9%)	11 (37.9%)	7 (24.1%)	—	—	—	—
	2000	36 (7)	23 (63.9%)	6 (16.7%)	7 (19.4%)	—	—	—	—

[a] Samples that gave positive results in the hybridization/PCR analyses.
[b] Suggested infection type based on the hybridization/PCR analyses; "Rec" refers to ES421/99-like recombinant.
[c] Samples for which hybridization/PCR results differ from those expected for infections with TYLCV, TYLCSV, and ES421/99-like variant described in southern Spain.

TABLE 5

Analysis of Tomato Samples Randomly Collected in a Survey Conducted during Autumn 2000 in Eight Commercial Fields of the Almería Province

Sample type[a]	Number of infected samples collected[b]	Number (%) of samples infected with[c]						
		TYLCV	TYLCSV	TYLCV + TYLCSV	Rec	Rec + TYLCV	Rec + TYLCV + TYLCSV	Other[d]
Asymptomatic	71	38 (53.5%)	—	13 (18.3%)	15 (21.1%)	3 (4.2%)	1 (1.4%)	1 (1.4%)
Symptomatic	85	59 (69.4%)	6 (7.1%)	9 (10.6%)	6 (7.1%)	—	1 (1.2%)	4 (4.7%)
Total	156	97 (62.2%)	6 (3.8%)	22 (14.1%)	21 (13.5%)	3 (1.9%)	2 (1.3%)	5 (3.2%)

[a] After random collection, samples were classified as asymptomatic and symptomatic, based on the absence or presence of yellow leaf curl symptoms, respectively.

[b] Samples that showed positive results in the hybridization/PCR analyses.

[c] Suggested infection type based on the hybridization/PCR analyses; "Rec" refers to ES421/99-like recombinant.

[d] Samples for which hybridization/PCR results differ from those expected for infections with the TYLCV, TYLCSV, and ES421/99-like variant described in southern Spain.

et al. (2001) for the geminivirus *Maize streak virus*. Therefore, although mixed infections are a prerequisite for recombination to occur, other ecological factors will contribute to the establishment and spread of a recombinant variant. However, from our data we cannot exclude that other recombinants, different from ES421/99, might be present in the population and were not detected during our analysis. In fact, hybridization/PCR results for several samples cannot be explained if only TYLCV, TYLCSV, and ES421/99-like variants are present (Tables 3 to 5). Therefore, unknown virus variants may already be present in the population and it is possible that some of these are uncharacterized recombinants. These samples are currently under study to determine the nature of the virus variant(s) involved in the infection and to investigate if the genetic makeup of the begomovirus population present in southern Spain is more complex than presumed.

We have demonstrated for TYLCSV that the population of an individual begomovirus strain can be very stable genetically over an extended period of time (Sánchez-Campos *et al.,* 2002). However, as observed in southern Spain, the incorporation of a second begomovirus species in the epidemic might result in a dramatic alteration of the population structure (Navas-Castillo *et al.,* 1997, 1999; Sánchez-Campos *et al.,* 1999). Indeed, here we have demonstrated that recombination provides additional sources for variation, selection, and potentially novel interactions. Thus, we conclude that begomovirus populations are extremely dynamic, and this should be taken into account when developing control strategies, especially when breeding for resistance. Knowledge on the diversity of the virus population is essential to obtaining durable resistance, and our work contributes to understanding the genetic diversification and evolution of the complex begomovirus pathosystem that is emerging in southern and southeastern Spain.

MATERIALS AND METHODS

Virus sources

During routine surveys, field samples from common bean plants exhibiting symptoms of the BLCD syndrome were collected in June 1999 from a commercial crop (cv. Donna, Nunhems Zaden, Haelen, The Netherlands) grown in a plastic-house in Berja, Almería (southeastern Spain). Analysis of these samples showed that several of them hybridized with probes specific to either TYLCV or TYLCSV, prepared from the intergenic region (IR), as described by Navas-Castillo *et al.* (1999). Preliminary analysis of one of these samples (ES421/99) suggested that it was infected with a virus variant that has a chimeric IR with part of the sequence from TYLCV and part from TYLCSV (Monci *et al.,* 2001). Therefore, this sample was considered for further characterization in this study.

Plants infected with TYLCSV and TYLCV were obtained by *Agrobacterium tumefaciens* mediated inoculation (hereafter referred to as agroinoculation) as described by Kheyr-Pour *et al.* (1991) using the infectious clones of the Spanish isolates TYLCSV-ES[1] (Noris *et al.,* 1994; Fauquet *et al.,* 2000) and TYLCV-[ES72/97] (Navas-Castillo *et al.,* 1999), respectively.

Cloning, sequencing, and construction of an infectious clone of isolate ES421/99

Total nucleic acids were extracted from leaf tissue of sample ES421/99, digested with *Bam*HI, and used for Southern blot analysis according to Noris *et al.* (1994), using probes specific to TYLCSV (see above) for hybridization. A putative full-length double-stranded DNA of approximately 2.8 kb was detected, excised, and extracted from 1% agarose gels using the Ultrafree-DA kit (Millipore Corp., Bedford, MA). The DNA fragment was cloned into the *Bam*HI site of pBluescript SK+ (Stratagene, La Jolla, CA), a virus-specific clone (pES421/99) containing an insert of about 2.8 kb was selected, and the sequence of both strands was determined using an

ABI PRISM DNA sequencer 377 (Perkin–Elmer, Foster City, CA) with pUC/M13 universal primers and internal primers designed from partial sequences. Sequence comparisons were done using the Wisconsin GCG software package (Devereux *et al.,* 1984). Nucleotide sequence comparisons were also carried out using the Plotsimilarity program of this software package; the proportion of identical nucleotides between pairs of sequences in a running window of a defined size were plotted to obtain Plotsimilarity diagrams. The genomic sequences of isolates TYLCSV-ES[1] and TYLCV-[ES72/97] (GenBank Accession Nos. Z25751 and AF071228, respectively) were used for comparisons. Phylogenetic analyses were performed using the neighbor-joining method of the CLUSTAL W (Thompson *et al.,* 1994) software package.

An infectious clone of ES421/99 was obtained by constructing a 1.4-mer clone of the insert of pES421/99 by inserting the full-length fragment into the unique *Bam*HI site of a 0.4-mer clone previously obtained by deletion of an *Eco*RI fragment in pES421/99. The 1.4-mer fragment obtained was excised with *Xho*I and *Sac*I and ligated into the binary vector pBin19 (Bevan, 1984) digested with *Sal*I and *Sac*I. *A. tumefaciens* strain LBA 4404 (Höfgen and Willmitzer, 1988) was transformed with the recombinant plasmid (p1.4ES421/99) and infectivity was tested by agroinoculation of common bean (cv. Donna) plants.

B. tabaci transmission

B. tabaci transmission experiments were performed using the Q biotype which is predominant in southern Spain (Moya *et al.,* 2001). Nonviruliferous *B. tabaci* were obtained from a colony reared on melon (*Cucumis melo* L. cv. ANC42, La Mayora germplasm collection). Viruliferous whiteflies were obtained by giving *B. tabaci* adults a 24-h acquisition access period (AAP) on systemically infected young leaves of common bean (cv. Donna) or tomato (cv. Moneymaker, La Mayora germplasm collection) plants agroinoculated 3 weeks before with TYLCSV-ES[1], TYLCV-[ES72/97], or ES421/99. When needed, viral DNA levels present in the acquisition source were estimated according to Sánchez-Campos *et al.* (1999). After the AAP, whiteflies were transferred to healthy test plants for a 48-h inoculation access period (IAP). For transmission to common bean, viruliferous whiteflies (10 whiteflies per test plant) were given an IAP using clip-on cages on cv. Donna seedlings in which the first trifoliate leaves were emerging. For transmission to tomato, viruliferous whiteflies (five adults per test plant) were given a free IAP on cv. Moneymaker seedlings at the three-leaf growth stage, within a wooden cage covered with a whitefly-proof net. After the IAP, insects were removed from plants, which were treated with insecticide and maintained until analyzed 30 days later by hybridization of tissue blots of petiole cross sections from young leaves using probes prepared to the IR of TYLCV or TYLCSV (see above). Controls were tomato or common bean plants obtained following the same scheme, with *B. tabaci* adults initially exposed to healthy source plants. Transmission experiments were performed in a growth chamber (27°C and 70% relative humidity, with a 16 h photoperiod at 12,000 lux) or an insect-proof glasshouse (approximately 16-h day length at 24°C and 18°C at night, with light supplementation when needed).

Determination of ES421/99 host range

The infectious clone of ES421/99 was agroinoculated into the stems of common bean (cv. Donna; cv. Kwinthus; Nickerson-Zwaan, Barendrecht, The Netherlands; line GG5; Seminis Vegetable Seeds Iberica, Almería, Spain) plants at the one trifoliate leaf growth stage, and tomato (cv. Moneymaker), *S. nigrum*, and *S. luteum* plants at the three-leaf growth stage. Plants of a partially resistant tomato (cv. Anastasia; Bruinsma Seeds, Enkhuizen, The Netherlands), which carries the *Ty-1* tolerance gene for TYLCV, were also agroinoculated. TYLCV-[ES72/97] and TYLCSV-ES[1] were used as controls in the host range studies. Liquid cultures of *A. tumefaciens* containing virus infectious clones were adjusted to an OD of 1.0 at 600 nm before agroinoculation. After agroinoculation, plants were maintained in a growth chamber (conditions described above) for 30 days, and then the youngest newly emerged leaves were analyzed by hybridization of tissue blots from petiole cross sections using probes prepared to the IR of either TYLCV or TYLCSV (see above). Two replicated experiments with five plants per virus and host species were done.

Study of the frequency of recombinants resembling ES421/99 in nature

The occurrence of the recombinant was analyzed in tomato and common bean samples collected in a survey conducted during 1998, 1999, and 2000. In this survey, commercial crops grown in the field and under plastic were randomly sampled in the main tomato and common bean growing regions of southern and southeastern Spain. A transect of about 500 km along the Mediterranean coastal area of the (from west to east) Málaga, Granada, Almería, and Murcia provinces was covered. Samples were randomly collected from TYLCD symptomatic plants present in tomato crops from all four provinces, and from BLCD symptomatic plants present in common bean crops from Málaga and Almería. During autumn 2000, tomato samples were also randomly collected from asymptomatic and symptomatic plants of commercial tomato crops from Almería. Samples consisted of one young leaf per plant that was stored in a plastic bag at 4°C until analyzed.

Each sample was analyzed for TYLCV and TYLCSV by hybridization of tissue blots from petiole cross sections

using probes specific to each viral species (see above). Recombination events at the 3′-end of the REn ORF, similar to that observed in recombinant ES421/99, were analyzed in each sample. This was done by PCR on nucleic acid extracts using the primers MA99 (5′ AAGGAGCAGTGTCTGTTG 3′), specific to TYLCSV and designed on the sequence of the ES[1] isolate (identical to nt 890 to 908), and MA100 (5′ GCCAGAGGCCATTTCTAATG 3′), specific to TYLCV and designed on the sequence of the [ES72/97] isolate (complementary to nt 1389 to 1369), which amplified a fragment of 490 bp from ES421/99 but not from TYLCSV or TYLCV. The type of IR present was analyzed by PCR of samples that gave a product in the reaction and in all the samples that reacted with both TYLCV and TYLCSV probes. For IR amplification, primers were designed on the sequences of TYLCV and TYLCSV reported from Spain. Primers MA115 (5′ GAAAGTACCCCATTCAAGAAC 3′) (identical to nt 2271 to 2291) and MA116 (5′ GTAGGGCCCACTACTTTATC 3′) (complementary to nt 47 to 27) are specific to TYLCSV, based on the ES[1] isolate. Primers MA117 (5′ TAAGGAGCACTTAGGATATG 3′) (identical to nt 2559 to 2579) and MA118 (5′ GATCCCACATATTGCAAGAC 3′) (complementary to nt 164 to 144) are specific to TYLCV, based on the [ES72/97] isolate. The combination MA115/MA116 is specific to TYLCSV and amplifies a fragment of 553 bp from TYLCSV-ES[1]; MA116/MA117 is specific to ES421/99 and amplifies a fragment of 279 bp, and MA117/MA118 is specific to TYLCV and amplifies a fragment of 396 bp from TYLCV-[ES72/97]. PCR reactions were carried out in 50 μl final volume of PCR reaction mix containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 200 μM of each dNTP, 300 nM of each primer, and 1.5 U AmpliTaq polymerase (Perkin–Elmer). Thermal cycling was undertaken in a GeneAmp PCR System 9700 (Perkin–Elmer) using 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The first denaturation step was carried out for 2 min and the last elongation step was carried out for 6 min.

Hybridizations of dot-blots of nucleic acid extracts and tissue blots made on positively charged nylon (nylon$^+$) membranes (Roche Diagnostics, Mannheim, Germany) were performed according to Accotto *et al.* (2000). Healthy tomato, *S. nigrum*, *S. luteum*, and common bean plants were used as negative controls and tomato plants were experimentally infected with either TYLCSV-ES[1] or TYLCV-[ES72/97] as positive controls.

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