Begomovirus genetic diversity in the native plant reservoir *Solanum nigrum*: Evidence for the presence of a new virus species of recombinant nature

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Abstract

We examined the native plant host *Solanum nigrum* as reservoir of genetic diversity of begomoviruses that cause the tomato yellow leaf curl disease (TYLCD) emerging in southern Spain. Presence of isolates of all the species and strains found associated with TYLCD in this area was demonstrated. Mixed infections were common, which is a prerequisite for recombination to occur. In fact, presence of a novel recombinant begomovirus was demonstrated. Analysis of an infectious clone showed that it resulted from a genetic exchange between isolates of the ES strain of *Tomato yellow leaf curl Sardinia virus* and of the type strain of *Tomato yellow leaf curl virus*. The novel biological properties suggested that it is a step forward in the ecological adaptation to the invaded area. This recombinant represents an isolate of a new begomovirus species for which the name *Tomato yellow leaf curl Axarquia virus* is proposed. Spread into commercial tomatoes is shown.
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Introduction

Emergence of viral diseases can cause considerable damage (Chua et al., 2000; Hahn et al., 2000; Rybicki and Pietersen, 1999; Schrag and Wiener, 1995). Despite this, the attributes responsible for the establishment and spread of specific invaders are often difficult to pinpoint or are unknown. Ecological studies have provided a body of information concerning factors that are important for invasive parasites (Kolar and Lodge, 2001; Schrag and Wiener, 1995). However, genetic approaches have received far less attention, even though genetic variation may determine the success of invaders. For successful emergence, introduced parasites need to evolve rapidly to circumvent loss of genetic variation normally associated with founder effects, and adapt to the novel environmental conditions. Key forces driving evolution are mutation, recombination, genetic drift, natural selection, and migration (Charlesworth and Charlesworth, 2003). Gene flow provided by sex and/or recombination is exploited by parasites to increase their evolutionary potential and to enhance local adaptation (Bürger, 1999), viruses are not an exception (García-Arenal et al., 2001; Michalakis and Roze, 2004; Moya et al., 2004; Roossinck, 1997).

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) constitute a group of plant viruses that exploit gene flow provided by recombination (Chatchawankanphanich and Maxwell, 2002; Monci et al., 2002; Padidam et al., 1999; Pita et al., 2001; Preiss and Jeske, 2003; Sanz et al., 2000; Zhou et al., 1997). They are transmitted in nature by the whitefly (*Hemiptera:Aleyrodidae*) *Bemisia tabaci* and have small twinned (geminate) icosahedral virions that encapsidate circular single-stranded (ss) DNA genomes (Stanley et al., 2005). Most begomoviruses have bipartite genomes comprising two DNA components (DNAs A and B). The DNA A encodes a replication-associated protein (Rep), the coat protein (CP), and proteins such as replication enhancer protein (REn), and transcription activator protein (TrAP), that participate in the

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control of replication and gene expression. The DNA B encodes proteins that are required for virus movement in plants. Open reading frames (ORFs) are organized bi-directionally in both genome components, separated by an intergenic region (IR) that contains key elements for the replication and transcription of the viral genome, including the origin of replication (reviewed in Hanley-Bowdoin et al., 2000). Several begomoviruses have been reported that consist of a single genomic component that resembles DNA A, among them most of the viruses associated with tomato yellow leaf curl disease (TYLCD) (Moriones and Navas-Castillo, 2000).

During the last two decades, begomoviruses have emerged worldwide as a result of the spread of their insect vector *B. tabaci* (Rybicki and Pietersen, 1999), causing diseases to a wide variety of plant species, some of them of great agricultural importance (Chatchawankanphanich and Maxwell, 2002; Czosnek and Laterrot, 1997; Ribeiro et al., 2003; Stanley et al., 2005; Stonor et al., 2003). Recent introduction of begomoviruses into new areas provides an ideal model to analyze aspects of genetic adaptation and evolution of an invading virus. The spread of begomoviruses in Spain is well documented (Sánchez-Campos et al., 1999, 2002). The first reports of infections were of the early 1990s, associated with the presence of *Tomato yellow leaf curl Sardinia virus* (TYLCSV). Subsequent introductions of *Tomato yellow leaf curl virus* (TYLCV) strains were reported, which provided the substrate for interactions, and spread to new host species (Monci et al., 2002; Morilla et al., 2003, 2005; Navas-Castillo et al., 1999).

Native species, acting as reservoirs can play an important role in the emergence of plant virus epidemics (Hull, 2002). For begomoviruses, studies are available, for example, in the *Eupatorium makinoi*–begomovirus system, that have described the effects of virus infections in natural plant communities (reviewed by Funayama-Noguchi, 2001). However, studies to understand the genetic structure and dynamics of begomovirus populations in wild reservoirs and possible effects on epidemics of cultivated species are scarce and less detailed (Frischmuth et al., 1997; Jovel et al., 2004; Ooi et al., 1997; Roye et al., 1997, 1999; Sanz et al., 2000). In this report, we provide information about a wild reservoir of TYLCD-associated viruses, that might help to get an insight into the bases of begomovirus epidemics and evolution. We examine the begomovirus population present in *Solanum nigrum* L., a wild host species (Bedford et al., 1998; Salati et al., 2002; Sánchez-Campos et al., 2000) widely distributed in the Mediterranean region. Data are presented that support the contribution of *S. nigrum* as reservoir of genetic diversity for TYLCD-associated viruses. Also, we provide evidence of the presence of a novel begomovirus of recombinant nature for which putative parents, spatial (geographical area, host), and temporal origin are suggested. This recombinant is a step forward in the ecological adaptation of begomoviruses emerging in the invaded area. Novel genetic and pathogenic characteristics made the International Committee on Taxonomy of viruses to consider it as a representative of a new begomovirus species, for which the name *Tomato yellow leaf curl Axarquia virus* was proposed.

Results

S. nigrum is a reservoir of genetic diversity for TYLCD epidemics

A total of 53 samples were collected between 2000 and 2003 in Málaga (southern Spain) from *S. nigrum* plants exhibiting symptoms of TYLCD-associated virus infection. All reacted positively in a hybridization analysis for detection of TYLCD-associated viruses. To identify the genetic diversity of the begomovirus population present in this host species, the nucleotide sequence of the IR was determined for a number of virus-related DNA fragments amplified from these samples. The 35 sequences thus obtained, have been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Database under accession numbers DQ121456 to DQ121490. Phylogenetic reconstruction showed that sequences corresponding to all the TYLCD-associated begomoviruses species and strains reported to date in Spain were present in *S. nigrum*: the type and Mld strains of TYLCV, the ES strain of TYLCSV, and the type strain of *Tomato yellow leaf curl Málaga virus* (TYLCMalV) (Stanley et al., 2005) (Fig. 1). Similar phylogenetic relationships were deduced using either maximum-likelihood, parsimony, or neighbor-joining methods (data not shown). Therefore, these results suggested the relevance of this wild host as reservoir of viral genetic diversity for TYLCD epidemics. Interestingly, the presence of mixed infections in single *S. nigrum* plants was apparent, as observed for example, in sample Sn8:00, in which TYLCV-like and TYLCSV-like sequences were detected (ES: Sn8-1:00, ES:Sn8-2:00, and ES:Sn8-3:00, in Fig. 1). The pattern observed in the phylogenetic tree shows radial divergence of clades from an ancestral state. Also, phylogenetic reconstruction demonstrated that 34% of the sequences obtained from *S. nigrum* samples consistently grouped in a clade that could not be associated with any previously reported begomovirus. Furthermore, abnormally severe infection symptoms were observed in the field samples from which those sequences were derived. Therefore, an isolate representative of this group was further studied.

Isolate ES:Alg:00 represents a previously undescribed begomovirus

The complete genome sequence of an isolate representative of the clade discussed above (isolate named ES:Alg:00) was deduced from one full-length recombinant clone derived from sample Sn8:00. The sequence consisted of 2,772 nucleotides and has been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Database under accession number AY227892. The genome organization is typical of monopartite begomoviruses, containing in the IR the stem–loop structure conserved in the geminiviruses that includes the Rep nick site (TAATAT-T↓AC) where rolling circle replication is initiated (Laufs et al., 1995). Detailed analysis of the genome of ES:Alg:00 revealed that it shares less than 89% nucleotide sequence identity relative to any previously characterized begomovirus; moreover, comparison of individual ORFs suggested that Rep and C4
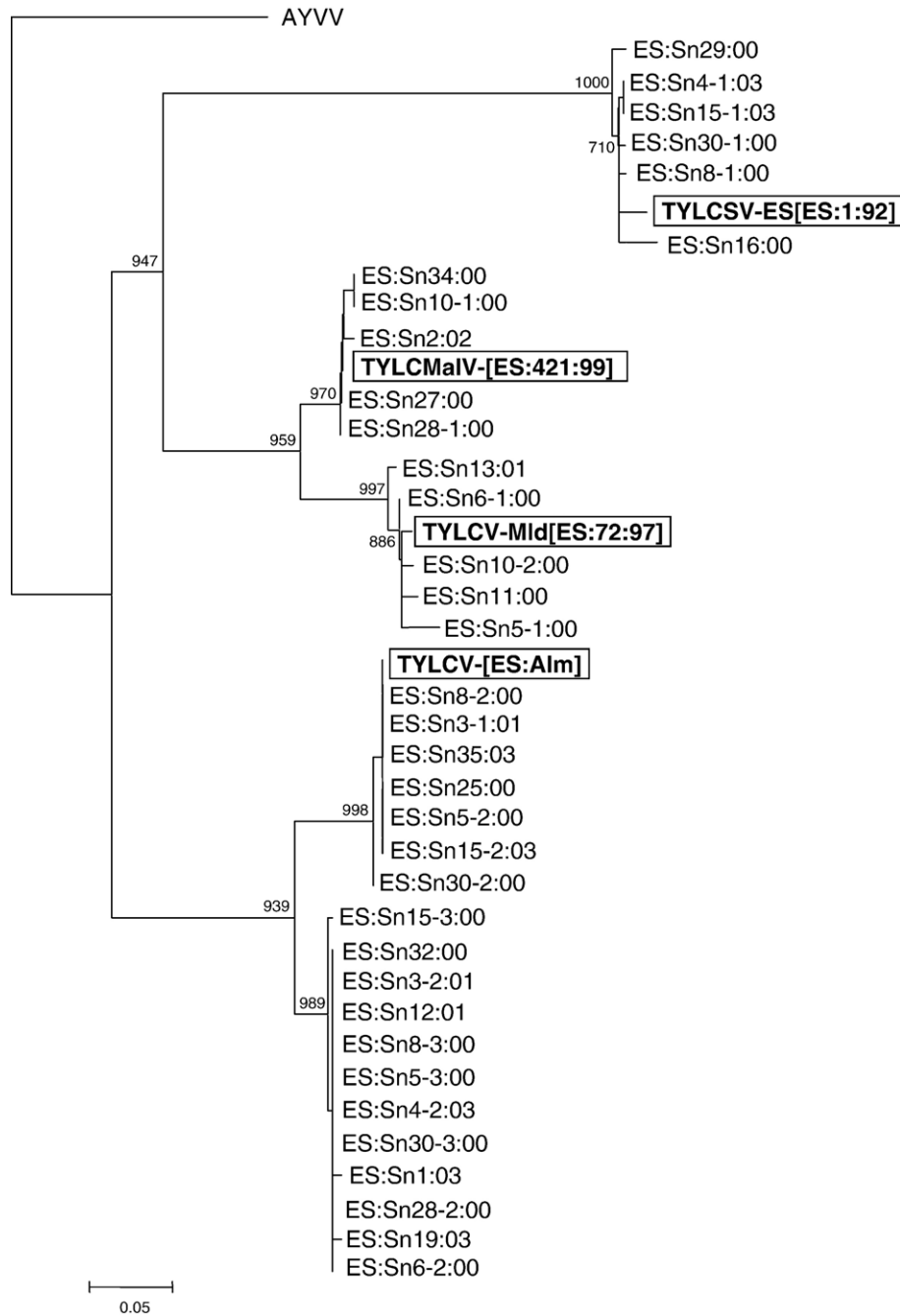
Fig. 1. Phylogenetic relationships among begomoviruses detected in plants of the population of the native reservoir *Solanum nigrum* present in Málaga (southern Spain). Relationships were inferred by neighbor-joining analysis of sequences covering the intergenic region (about 300 nucleotides). Support for nodes in a bootstrap analysis with 1000 replicates is shown for figures over 700 and most branches not supported collapsed. Vertical distances are arbitrary and branch lengths are drawn to scale; the bar indicates 0.05 nucleotides substitutions per site. Begomovirus isolates are named according to indications of the *Geminiviridae* Study Group of the International Committee on Taxonomy of Viruses, indicating the code of the country of origin, sample name, and year of collection (e.g., "ES:Sn15-1:03", in which "ES" refers to Spain, "Sn15-1", to isolate 1 derived from the sample number 15 of *S. nigrum*, and "03" to year 2003). Representative isolates are included of begomovirus species associated with tomato yellow leaf curl disease in the Mediterranean area, the ES strain of *Tomato yellow leaf curl Sardinia virus* (TYLCSV), the type and Mld strains of *Tomato yellow leaf curl virus* (TYLCV), and *Tomato yellow leaf curl Málaga virus* (TYLCMalV) (GenBank accession numbers Z25751, AJ489258, AF071228, and AF271234, respectively) (bold letters and boxed). As outgroup, an isolate of *Ageratum yellow vein virus* (AYVV) was used (GenBank X74516).

genes have an evolutionary history different to that of Ren, TrAp, CP, and V2 genes (Table 1), and that a recombination between TYLCV- and TYLCSV-like isolates might have occurred. In fact, thorough nucleotide sequence comparisons showed that ES:Alg:00 seems to have derived from a genetic exchange between isolates of the type strain of TYLCV and of the ES strain of TYLCSV (Fig. 2). The recombination sites were located in a part of the IR in which the stem–loop structure occurs and in the 3′-end of the Rep ORF. However, the precise nucleotides at which the recombination occurred could not be

Table 1

Comparisons of ES:Alg:00 with isolates of tomato yellow leaf curl disease-associated begomoviruses reported from the Mediterranean region [a]

Virus isolates	Percentage of nucleotide identity (%)						
	Complete genome	V2	CP	C4	Rep	TrAP	REn
TYLCSV-[IT:Sar]	83	95	93	74	79	93	90
TYLCSV-Sic[IT:Sic]	82	95	93	70	78	91	88
TYLCSV-ES[ES:1:92]	88	100	99	72	80	99	98
TYLCV-[IL]	84	82	80	98	95	77	77
TYLCV-[ES:Alm]	84	82	81	100	96	77	77
TYLCV-Mld[ES:72:97]	78	81	81	78	83	76	77
TYLCMalV-[ES:421:99]	87	99	99	78	83	77	80

[a] Percentage of nucleotide identity was calculated for the complete genome or for open reading frames of the different putative proteins V2, CP, C4, Rep, TrAP, and REn. For comparison the sequences of isolates of the type strain of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (GenBank accession number X61153) and of the Sic and ES strains of TYLCSV (GenBank Z28390 and Z25751, respectively), the type strain of *Tomato yellow leaf curl virus* (TYLCV) (GenBank X15656 and AJ489258 for [IL] and [ES:Alm] isolates, respectively), the Mld strain of TYLCV (GenBank AF071228), and the *Tomato yellow leaf curl Málaga virus* (TYLCMalV) (GenBank AF271234), were used.

identified as the sequences involved are conserved between TYLCSV-ES and TYLCV (data not shown). As a result of this recombination, cognate Rep protein and *cis*-acting motifs in the IR required for replication were maintained ensuring a viable replicative unit (Gutiérrez, 1999; Hanley-Bowdoin et al., 2000). ES:Alg:00 seemed to have resulted from a recent recombination event because over 99% nucleotide sequence identity existed with the corresponding genomic regions of Spanish isolates (TYLCV-[ES:Alm] and TYLCSV-ES[ES:1:92], Fig. 2) of the putative parental viruses. At the amino acid level, six non-conservative changes were detected, two in each Rep (I_{213} replaces N_{213}, and A_{320} replaces T_{320}), TrAP (T_{95} replaces P_{95}, and Q_{103} replaces P_{103}), and REn (L_{45} replaces F_{45}, and N_{54} replaces H_{54}) ORFs.

Isolate ES:Alg:00 exhibits novel pathogenic characteristics

An infectious construct of the clone of ES:Alg:00 sequenced was produced. This construct readily infected *S. nigrum* plants by agroinoculation; moreover, infected plants exhibited symptoms resembling those observed in the field sample from which this isolate was derived. Also, the progeny virus present in agroinoculated plants was readily transmissible by *B. tabaci* to healthy *S. nigrum* plants (6 out of 6 plants inoculated were infected and reproduced symptoms). Therefore, the DNA fragment cloned represented a fully biologically active copy of the genome of the begomovirus isolate ES:Alg:00 present in sample Sn8:00. When tested on a panel of plant species reported as natural hosts of TYLCD-associated begomoviruses (tomato, common bean, and *S. nigrum*), ES:Alg:00 infected all plant
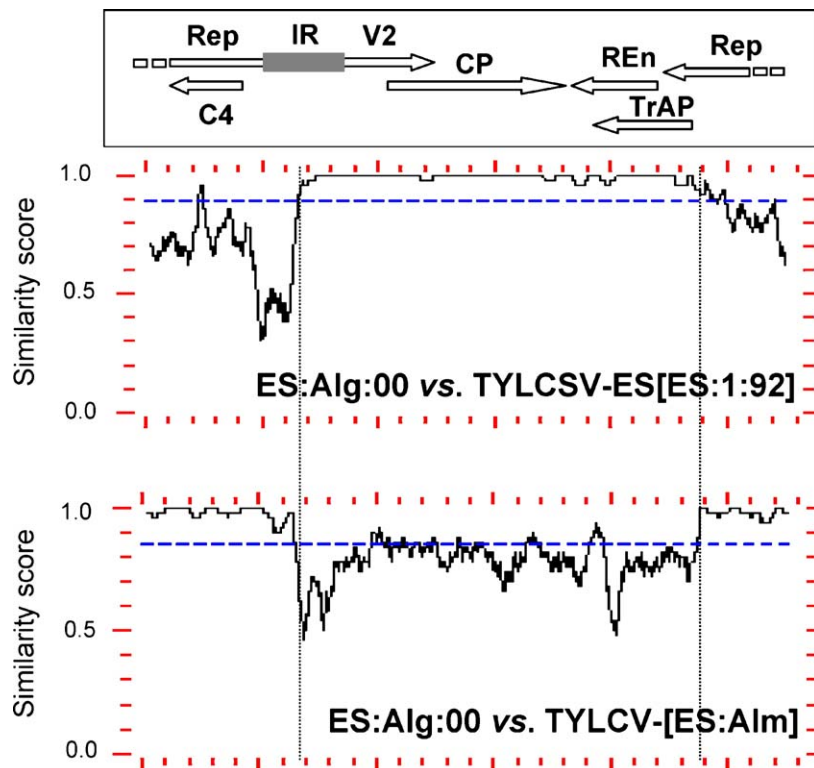

Fig. 2. Comparison of the genome of the recombinant begomovirus ES:Alg:00 with that of representative isolates of the putative parental viruses. Plotsimilarity diagrams (scanning window = 50 nt) comparing complete genome sequence of ES:Alg:00 with that of either TYLCSV-ES[ES:1:92] (GenBank accession number Z25751) or TYLCV-[ES:Alm] (GenBank AJ489258) isolates. Putative regions involved in the recombination event are indicated by vertical dotted lines. Positions of the putative open reading frames (V2, CP, Rep, TrAP, REn, and C4) and of the intergenic region (IR) are indicated at the top of the figure. Horizontal broken lines indicate the mean similarity between the sequences compared.

Table 2
Host range of the recombinant ES:Alg:00 compared to that of isolates of putative donor parents, the type strain of *Tomato yellow leaf curl virus* (TYLCV) and the ES strain of *Tomato yellow leaf curl Sardinia virus* (TYLCSV)

Plant species	Number of plants infected / number of plants inoculated [a]		
	TYLCSV-ES[ES:1:92]	TYLCV-[ES:Alm]	ES:Alg:00
Solanum nigrum	10/10	0/10	10/10
	10/10	0/10	10/10
Tomato	10/10	10/10	10/10
	5/5	5/5	5/5
Common bean	0/8	8/9	4/9
	0/10	6/10	4/10

[a] Two independent agroinoculation experiments were performed with each plant species using infectious clones of TYLCSV-ES[ES:1:92] (Noris et al., 1994), TYLCV-[ES:Alm] (kindly supplied by E. R. Bejarano, Málaga University, Spain), or ES:Alg:00 (this work). After agroinoculation, plants were maintained in a growth chamber and analyzed 30 days after inoculation by hybridization of tissue blots from petiole cross-sections of young non-inoculated leaves, using a mixture of probes specific to TYLCV and TYLCSV (Navas-Castillo et al., 1999).

species (Table 2). In contrast, the putative parental virus TYLCSV-ES was only detected in tomato and *S. nigrum* plants, and TYLCV was only detected in tomato and common bean plants. In common bean, similarly to TYLCV-[ES:Alm], escapes were observed for ES:Alg:00 with the procedure used. As TYLCV was reported that can infect *S. nigrum* when analyzed by PCR (Salati et al., 2002), plants inoculated with TYLCV, that tested negative by hybridization (Table 2), were further tested by PCR. Using this technique, TYLCV was detected in non-inoculated leaves of *S. nigrum* test plants, although lower amounts of amplification product were obtained compared to those obtained from plants inoculated with TYLCSV-ES or ES:Alg:00 (Fig. 3). Therefore, infection of TYLCV in this host species seems to be impaired. Indeed, attempts to transmit TYLCV-[ES:Alm] from the PCR-positive *S. nigrum* plants to healthy tomato test plants via *B. tabaci* were unsuccessful (0 plants infected out of 10 plants inoculated), while ES:Alg:00 or TYLCSV-ES[ES:1:92] were readily transmitted (10 plants infected out of 10 plants inoculated). TYLCSV could not be detected in non-inoculated leaves of common bean test plants by PCR amplification (Fig. 3), thus demonstrating that this virus is unable to infect this host species, confirming previous reports (Monci et al., 2002; Sánchez-Campos et al., 1999). It should be noted that as shown in Fig. 4, ES:Alg:00 induced abnormally severe symptoms in infected tomato and *S. nigrum* plants, whereas in common bean, milder
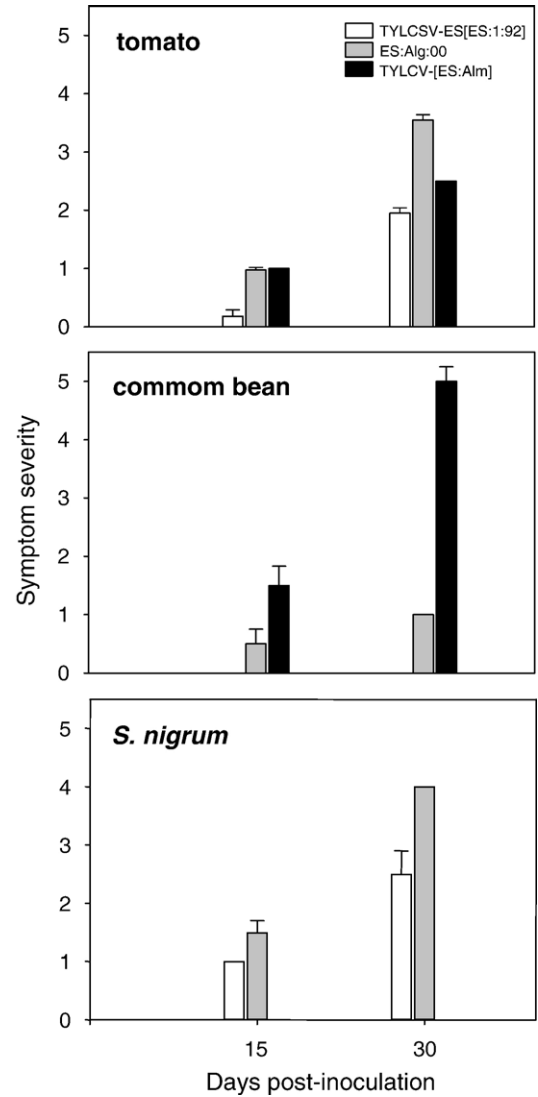

Fig. 4. Disease severity induced by the recombinant begomovirus ES:Alg:00 and representative isolates of the putative parental viruses in tomato, common bean, and *S. nigrum* plants. Severity is given in a 0 (no symptoms) to 5 (maximum disease severity) grade scale at 15 and 30 days post inoculation. Tomato, common bean, and *Solanum nigrum* plants were inoculated via *Agrobacterium tumefaciens*, with infectious clones of *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES[ES:1:92]), *Tomato yellow leaf curl virus* (TYLCV-[ES:Alm]), or ES:Alg:00. The mean ± SEM values of ten plants inoculated per treatment combination are shown.
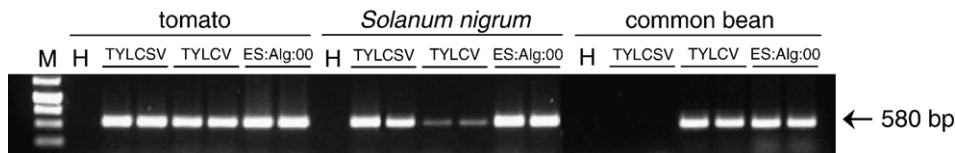

Fig. 3. Agarose gel electrophoresis of amplification products from polymerase chain reactions performed using primers TY1(+) and TY2(−) for the coat protein region, with nucleic acids extracted from young non-inoculated tissues of tomato, *Solanum nigrum*, and common bean plants (representative results from 2 plants shown in each case), 30 days after inoculation with infectious clones of *Tomato yellow leaf curl Sardinia virus*-ES[ES:1:92] (TYLCSV), *Tomato yellow leaf curl virus*-[ES:Alm] (TYLCV), or ES:Alg:00, via *Agrobacterium tumefaciens*. M, 1 kb ladder molecular size marker; H, healthy non-infected plant used as negative control. The position of the 580-bp fragment amplified is indicated.

infections were observed. Therefore, the previous results demonstrated that ES:Alg:00 has a wider host range than either putative parental virus and that this virus exhibits novel pathogenic characteristics.

Presence of ES:Alg:00-like isolates in commercial tomato crops

Presence of ES:Alg:00-like isolates could be demonstrated from plants of commercial tomato crops using PCR with primer combinations specific to ES:Alg:00, and sequence analysis of amplified products. Primers for PCR reactions were based on recombinant regions of ES:Alg:00, the IR and the 3′-end Rep ORF. We analyzed a total of 73 tomato samples collected during 2000, from commercial crops grown in the area in which the recombinant was found. Positive amplifications were obtained from 7 of them. Moreover, direct sequence analysis of the PCR DNA products obtained from one of these samples confirmed a 100% nucleotide sequence identity with the equivalent regions of ES:Alg:00. In contrast, analyses of tomato samples collected during extensive random samplings performed in the Málaga region during previous years (about 750 samples collected between 1997 and 1999) (Monci et al., 2002; Sánchez-Campos et al., 2002), did not provide evidence of the presence of similar infections. Therefore, our data suggested that ES:Alg:00-like isolates were already spreading into cultivated tomatoes, and that spread seems to have occurred only recently.

Discussion

Evidence is provided here of the relevance of the wild species *S. nigrum* as reservoir of begomoviruses that cause epidemics of TYLCD and of recombination as a force driving their evolution. Presence of isolates of all the begomovirus species and strains reported so far in southern Spain associated with TYLCD is demonstrated in the *S. nigrum* population analyzed. Moreover, co-infections were shown to be common, and the presence of a previously unreported begomovirus variant was demonstrated. Interestingly, this variant exhibited a recombinant nature and novel pathogenic properties that can result in enhanced long-term ecological fitness.

The colonization of begomoviruses in Spain is an interesting example of invasion success following multiple introductions, similar to those reported for animals or plants (Kolbe et al., 2004; Novak and Mack, 2001). Initial introduction of TYLCSV in the early 1990's was followed by subsequent introductions of TYLCV (Morilla et al., 2003; Navas-Castillo et al., 1999; Sánchez-Campos et al., 2002). This provided novel sources of variation and conditions for genetic exchange, that might help the invader begomoviruses to locally adapt (Monci et al., 2002). The results shown here are relevant because demonstrated that native hosts such as *S. nigrum* could be excellent reservoirs of genetic diversity for TYLCD-associated viruses. This might be important to understand epidemics and evolution of these viruses. Multiple and successive virus introductions probably resulted into the radially divergent population of begomoviruses shown here associated with *S. nigrum* (Fig. 1). Similarly,

presence of multiple infections of genetically divergent begomoviruses has been observed, for example, in the *Eupatorium yellow vein virus*-wild host species pathosystem (Ooi et al., 1997), reinforcing the role of wild species as reservoirs of begomovirus diversity. Our data suggested that in the case of TYLCV, *S. nigrum* seems to be a dead end host in the epidemics. We demonstrated that although this virus could infect *S. nigrum* plants, it accumulated at very low levels and could not be transmitted by whiteflies. Interestingly, we showed that mixed begomovirus infections are common in *S. nigrum* plants. This is important because *S. nigrum* is a field host that can survive for long periods (even more than two years) in the mild climatic conditions characteristic of southern Spain. The abundance of mixed infections should be cause for alarm, because this is the prerequisite for recombination to take place, a diversification mechanism largely exploited by begomoviruses (Morilla et al., 2004; Padidam et al., 1999; Preiss and Jeske, 2003). Recombination is a potent mechanism to create more fit genotypes (Bürger, 1999; Rhodes et al., 2003), that can help invaders to rapidly evolve and adapt to the novel environmental conditions (Dybdahl and Storfer, 2003; Lively and Dybdahl, 2000; Stavrinides and Guttman, 2004; Zhou et al., 1997). Therefore, as suggested for native hosts in other begomovirus complexes (e.g., Jovel et al., 2004; Sanz et al., 2000), *S. nigrum* plants can be an optimal niche for the development of better-adapted recombinant begomoviruses. In fact, we demonstrated the presence of a new previously undescribed begomovirus of recombinant nature infecting *S. nigrum* plants. Analysis of an isolate (ES:Alg:00) of this recombinant virus demonstrated that it resulted from a genetic exchange between isolates of extant TYLCD-associated viruses, the ES strain of TYLCSV and the type strain of TYLCV, found co-infecting *S. nigrum* plants. The recombination sites were located in a region of the IR close to the nick site for initiation of rolling circle replication (Gutiérrez, 1999; Hanley-Bowdoin et al., 2000), which is a hot spot for recombination in geminiviruses (Sanz et al., 1999; Stanley, 1995; Stenger et al., 1991), and in a region of the 3′-end of the Rep ORF. It should be highlighted that the type strain of TYLCV was only recently reported in Spain (Morilla et al., 2003). Therefore, recombination most probably occurred recently, which is supported by the close genetic relatedness of the recombinant to the putative parental viruses.

Recombination can impact evolution and epidemiology of plant viruses, as it can affect fitness and pathogenic characteristics (Fernández-Cuartero et al., 1994; Gibbs and Weiller, 1999; Monci et al., 2002; Pita et al., 2001; Stenger et al., 1994; Zhou et al., 1997). Survival and establishment of a recombinant in nature largely depends on it having selective advantages (Bonnet et al., 2005). Here, we demonstrated that the recombinant begomovirus characterized exhibited novel pathogenic properties that suggest enhanced ecological adaptation to the invaded area. Thus, in addition to be readily transmissible by *B. tabaci*, it exhibited a host range wider than either putative parental virus, which is consistent with selection for a better natural fit. Also, increased virulence was observed resulting in abnormally severe infections in tomato and *S. nigrum* plants. The novel pathogenic properties

of this recombinant virus indicate that it can interact differentially with host plant species. Therefore, further studies would be interesting to elucidate the nature and determinants of the interaction process between host and viral factors that control pathogenesis in this system. Interestingly, we found isolates resembling ES:Alg:00 infecting commercial tomatoes. Moreover, the detection of ES:Alg:00-like isolates in tomato samples collected during 2000, but not in those collected in previous years (1997 to 1999), may indicate that it has only recently spread into cultivated tomatoes.

Collectively, our data provide new knowledge to gain an insight into the bases of begomovirus epidemics and evolution. We demonstrated the outbreak of a new begomovirus with enhanced pathogenicity within the TYLCD complex, which is likely to spread throughout southern Spain. Since the appearance of more fit virus variants can have dramatic consequences in virus populations (Moonan et al., 2000; Pita et al., 2001; Revers et al., 1996; Zhou et al., 1997), studies are under way to elucidate the possible effect of this outbreak on the population of TYLCD-associated viruses. We also provide evidence for the role of native weeds like *S. nigrum* as reservoirs of genetic diversity for begomovirus epidemics. The common multiple infections present in this host might ensure that further begomovirus types might emerge after several rounds of recombination, in a symbiogenesis-like evolution process (Roossinck, 2005). Based on the singular genetic and biological properties of the recombinant begomovirus characterized here, and following new species demarcation criteria for begomoviruses proposed by the ICTV (Fauquet et al., 2003), we concluded that ES:Alg:00 represents an isolate of a new species of the genus *Begomovirus*. After consultation with the *Geminiviridae* Study Group of the ICTV, we proposed the name *Tomato yellow leaf curl Axarquia virus* (TYLCAxV) for this new virus species (Fauquet and Stanley, 2005), taking into account its relatedness with TYLCD-associated viruses, and the region of Málaga (Axarquía, from the arabic, الشرقية, ash-sharqiyya, the Eastern) from which the source sample was collected.

Materials and methods

General methods

Standard manipulations of nucleic acids and bacteria were performed according to protocols in Sambrook and Russell (2001).

Field surveys and virus sources

A survey was conducted during 4 years (between 2000 and 2003), on a native population of the wild host species *S. nigrum* present in the Málaga province (southern Mediterranean coast of Spain). In this survey, about 15 samples were collected randomly per year from *S. nigrum* plants exhibiting symptoms (yellowing, leaf distortion, dwarfing) typical of TYLCD-associated virus infection (Monci et al., 2002; Sánchez-Campos et al., 2000). Tomato samples were also

randomly collected in this area during 2000, from plants exhibiting symptoms of TYLCD in commercial plastic house crops. Samplings were performed during autumn and samples consisted of young leaf tissues which were stored at 4 °C until analyzed.

The infectious clone of an isolate of the type strain of TYLCV from southern Spain, TYLCV-[ES:Alm], was kindly supplied by E. R. Bejarano, Málaga University, Spain. The infectious clones of an isolate of the Mld strain of TYLCV (TYLCV-Mld[ES:72:97]), and of an isolate of the ES strain of TYLCSV (TYLCSV-ES[ES:1:92]), have been described (Navas-Castillo et al., 1999; Noris et al., 1994). The Mld strain differs genetically and biologically from the TYLCV type strain (Antignus and Cohen, 1994; Navas-Castillo et al., 2000).

Sample analyses

Tissue print hybridization to detect the viruses associated with TYLCD in the Mediterranean region was performed according to Accotto et al. (2000). Total nucleic acids were extracted as previously described (Monci et al., 2005). DNA fragments covering the IR were amplified from total nucleic acids extracts by polymerase chain reaction (PCR). The IR is informative about genetic diversity because it is the most variable part of begomovirus genome (Padidam et al., 1995; Sánchez-Campos et al., 2002). The PCR products were directly sequenced using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster city, CA) automatic sequencer. Thus, we obtained the sequence of a region of approximately 300 nucleotides covering the IR. Primers for amplification and sequencing were based on sequences of the begomoviruses reported in Spain associated with TYLCD: TYLCSV-ES, primers MA115 and MA116; TYLCV-Mld, primers MA117 and MA118 (Monci et al., 2002); and the type strain of TYLCV, primers MA250 (5′ GGTGTCCCTCAAAGCTCTATGG-CAATCG 3′, identical to nt 2629 to 2656 of TYLCV-[ES:Alm], GenBank accession number AJ489258) and MA118. PCR reactions with the different primer pair combinations were conducted with each analyzed sample. Recombination points at the 3′-end of the Rep ORF, similar to those described here for the isolate ES:Alg:00 (see below), were analyzed using the primer pair MA255 (5′ GAGGTATTCTTTAAATGAT-GATTGTG 3′, identical to nt 1668 to 1693 of TYLCSV-ES [ES:1:92], GenBank Z25751), and MA256 (5′ GATTTT-TAGTCCTCCTTTAGAAG 3′, complementary to nt 2109 to 2087 of TYLCV-[ES:Alm]). The primer pair Ty1(+)/Ty2(−), capable of amplifying a DNA fragment of about 580 bp in the CP ORF, was used for non-specific amplifications (Accotto et al., 2000). PCR reaction conditions were those described by Monci et al. (2002).

Construction, complete genome sequence and host range of an infectious clone of isolate ES:Alg:00

The isolation of the complete genome of a begomovirus isolate (referred to as ES:Alg:00 isolate) resembling ES:Sn8-

3:00 present in sample Sn8:00 (see Fig. 1), determination of its sequence, production of an infectious construct, host range studies, and *B. tabaci* transmission, were essentially done as described previously (Monci et al., 2002), except that: (i) the putative full-length genome copy was cloned into the *Bam*HI site of pBBR1MCS-5 (Kovach et al., 1995) to obtain the recombinant clone pES:Alg:00, and (ii) to construct the 1.4-mer tandemly repeated copy of ES:Alg:00 genomic DNA, the full-length insert excised with *Bam*HI from pES:Alg:00 was subcloned into the *Bam*HI site of a 0.4-mer pBin19 (Bevan, 1984) clone containing the *Eco*RI–*Bam*HI fragment excised from pES:Alg:00, to obtain p1.4ES:Alg:00. For host range studies, common bean (*Phaseolus vulgaris* L) cv. Donna (Nunhems Zaden, Haelen, The Netherlands), tomato (*Lycopersicon esculentum* Mill.) cv. Moneymaker (La Mayora germplasm bank), and *S. nigrum* plants were inoculated using infectious cultures of *Agrobacterium tumefaciens* (Monci et al., 2002). Symptom severity was monitored at 15 and 30 days post inoculation. For *B. tabaci* transmission experiments, virus was acquired from *S. nigrum* plants agroinoculated 3 weeks before, and transmission was conducted to healthy *S. nigrum* or tomato plants (three leaf growth stage) using 60 whiteflies per plant. Infection status of plants was assessed by hybridization and PCR. The identity of the virus present in systemically infected tissues with that inoculated was confirmed by PCR analysis using primers based on the IR and the Rep ORF (see above).

Sequence and phylogenetic analyses

Multiple sequence alignments were obtained with ClustalX software package (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/), with default parameters. Phylogenetic analyses were done by the neighbor-joining method of this software package and displayed using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). One thousand bootstrap replicates were used to assess the robustness of the final tree topology. Nucleotide sequence comparisons were also carried out using the Plotsimilarity program of the Genetic Computer Group Wisconsin package (http://www.accelrys.com/products/gcg_wisconsin_package). Nucleotide sequences of isolates TYLCSV-[IT:Sar], TYLCSV-Sic[IT:Sic], TYLCSV-ES[ES:1:92], TYLCV-Mld[ES:72:97], TYLCV-[ES:Alm], and TYLCMalV-[ES:421:99] (GenBank accession numbers X61153, Z28390,Z25751, AF071228, AJ489258, and AF271234, respectively) were used for comparisons, including the sequence of *Ageratum yellow vein virus* (GenBank X74516) as outgroup.

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