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Frequent occurrence of recombinants in mixed infections of tomato yellow leaf curl disease-associated begomoviruses

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Abstract

Begomoviruses are plant DNA viruses for which recombination plays a key role in driving evolution. However, little is known about how frequently begomovirus recombinants arise in mixed infected plants. To tackle this issue, co-infections of tomato with monopartite begomoviruses associated with the tomato yellow leaf curl disease, *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus*, have been studied as a model system. The frequency of recombinant genotypes in the progeny populations was evaluated at several times post inoculation. Recombinants constituted a significant proportion of the viral population. Interestingly, not all regions of the genome contributed equally to genetic exchange. In addition to the intergenic region, a known hot spot for recombination, a second hot spot region was found. Implication of secondary structure sequence features in cross-over sites is suggested, which might favor discontinuous DNA replication with the replication complex switching between homologous regions of DNA templates.

Keywords: *Begomovirus*; *Geminivirus*; Recombination; Tomato; *Tomato yellow leaf curl virus*; *Tomato yellow leaf curl Sardinia virus*

Introduction

During the infection cycle, plant viruses must adapt to heterogeneous environments. Even within a single plant, viruses need to invade different tissues and organs to establish a successful infections (French and Stenger, 2003; Li and Roossinck, 2004; Sacristán et al., 2003). Also, environmental changes can occur over time. Thus, insect vector populations can be altered after the introduction of new insecticides, or host plant population can be modified because of the deployment of resistant cultivars (McDonald and Linde, 2002; Van den Bosch et al., 2006). Therefore viruses must have the capacity to adapt in order to survive. Mutation and genetic exchange are the primary sources of genetic variation in plant virus populations to provide adaptive capacity (Fernández-Cuartero et al., 1994; García-Arenal et al., 2001; Roossinck, 1997). Genetic exchange via recombination or reassortment (for viruses with a segmented genome) provides a tool to combine sequences from different origins which might help viruses to quickly evolve (Posada et al., 2002). In many DNA and RNA viruses, genetic exchange is achieved through recombination (de Wispelaere et al., 2005; Froissart et al., 2004; Martin et al., 2005a). Homologous recombination can occur between donor and acceptor templates with significant sequence relatedness, while nonhomologous recombination involves dissimilar regions (Lai, 1992; Nagy and Simon, 1997). Important phenotypic effects could result from recombination events promoting viral adaptation and emergence associated with changes in virulence and host ranges (García-Arenal and McDonald, 2003; Monci et al., 2002; Zhou et al., 1997). As an increasing number of viral sequences becomes available for plant RNA and DNA viruses, clear evidence has been obtained for a key role of recombination in virus evolution (Chenault and Melcher, 1994; Fargette et al., 2006; García-

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Arenal et al., 2001; Padidam et al., 1999; Worobey and Holmes. 1999). Therefore, understanding the role and processes associated with recombination is essential to understanding the emergence of new viruses and to improve the effectiveness and durability of control practices (García-Arenal and McDonald, 2003; Posada et al., 2002). However, in contrast to RNA viruses (Aaziz and Tepfer, 1999; Bonnet et al., 2005; Bruyere et al., 2000; Fraile et al., 1997; García-Arenal et al., 2001; Lai, 1992), there is little information on the occurrence and frequency of recombinants in populations of plant DNA viruses (Froissart et al., 2005; Padidam et al., 1999; Schippenkoetter et al., 2001). We have addressed the analysis of this aspect in plant DNA virus populations using begomoviruses as an experimental system.

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) are plant single-stranded DNA viruses that are emerging worldwide, causing severe damage to economically important crops (Fargette et al., 2006; Rybicki and Pietersen, 1999; Seal et al., 2006). They have small twinned (geminate) icosahedral virions and are transmitted in nature by the whitefly *Bemisia tabaci* Gen. (Stanley et al., 2005). Most begomoviruses have bipartite genomes comprising two circular DNA components of about 2800 nucleotides (DNAs A and B). DNA A encodes a replication-associated protein (Rep), the coat protein (CP), and proteins such as the replication enhancer protein (REn), and the transcription activator protein (TrAP), that participate in the control of replication and gene expression. DNA B encodes proteins involved in virus movement. Open reading frames

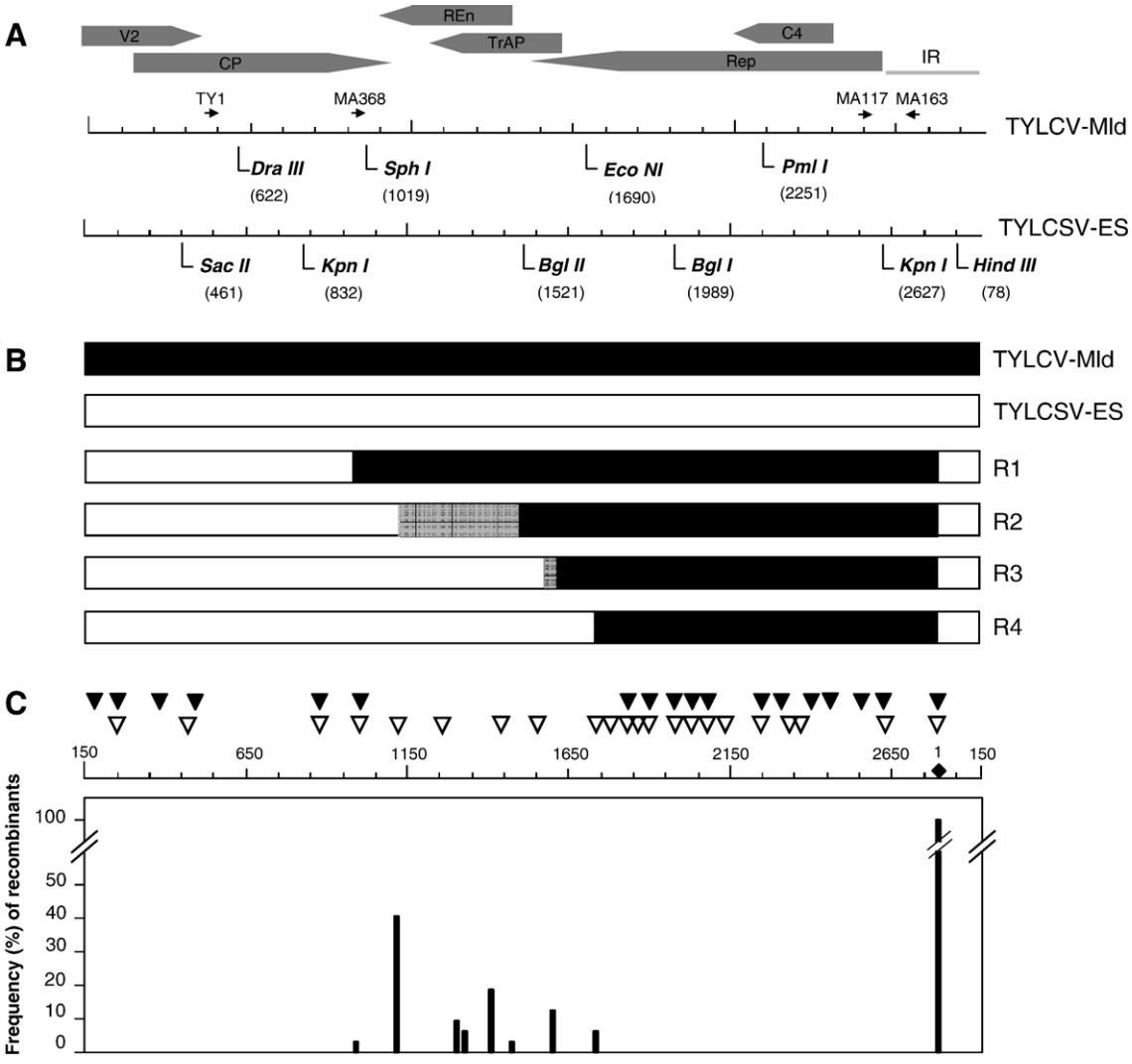

Fig. 1. (A) Schematic representation of the genomes of the [ES:Mur1:92] isolate of *Tomato yellow leaf curl Sardinia virus*-ES (TYLCSV-ES) (GenBank accession number Z25751) and of the [ES:72:97] isolate of *Tomato yellow leaf curl virus*-Mld (TYLCV-Mld) (GenBank AF071228), indicating the location of the V2, CP, Rep, C4, TrAP, and REn open reading frames and non coding region IR, and the nucleotide position of the target sequences of restriction endonuclease enzymes used in the restriction fragment length polymorphism (RFLP) analysis performed in this work. Position of primers TY1, MA368, MA117, and MA163 used for sequencing was also shown. (B) Summary of the RFLP analyses. Open boxes corresponding to TYLCSV sequences, black boxes to TYLCV sequences and grey boxes to the region in which the crossover sites of the different recombinant viruses comprised in each restriction pattern occur. (C) The frequency of recombinant sequences in the progeny virus population of plants at 400 dpi when co-inoculated with TYLCV and TYLCSV, having the crossover site at the genome position shown. The numbers refer to nucleotide position in the genome of TYLCV and the black diamond indicates the position of the conserved geminivirus stem–loop. The position of hairpin-like secondary structures predicted in the genome of TYLCSV and TYLCV are indicated with open and black triangles, respectively.

(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 by Hanley-Bowdoin et al., 2000). Monopartite begomoviruses have a single genomic component that resembles DNA A. Aspects of begomovirus replication have been reviewed thoroughly (Gutiérrez, 1999; Hanley-Bowdoin et al., 2000). Interestingly, multitasking in replication has been demonstrated, involving rolling circle replication (Saunders et al., 1991; Stenger et al., 1991) as well as recombination-dependent replication that recovers damaged and incomplete DNA for productive infection by homologous recombination (Preiss and Jeske, 2003).

Begomoviruses exploit gene flow, provided by recombination, as a mechanism to increase their evolutionary potential and local adaptation (Berrie et al., 2001; Monci et al., 2002; Pita et al., 2001; Preiss and Jeske, 2003; Rojas et al., 2005; Sanz et al., 2000; Zhou et al., 1997). Genomic sequence analyses have revealed that recombination has played an important role in begomovirus genetic diversity and evolution (Padidam et al., 1999). This conclusion derived from the analysis of begomoviruses from different regions, years and hosts. However, it remains unclear how frequently recombinants arise. In this study, the contribution of recombination to the population structure in a begomovirus system has been investigated. For this, two monopartite begomoviruses (*Tomato yellow leaf curl Sardinia virus*, TYLCSV, and *Tomato yellow leaf curl virus*, TYLCV) that cause severe *Tomato yellow leaf curl disease* (TYLCD) epidemics in tomato (*Solanum lycopersicum* L.) in warm and temperate regions worldwide (Moriones and Navas-Castillo, 2000) were used. These viruses differ in about 20% of their nucleotide sequence and are able to co-infect and replicate in single plants (Monci et al., 2002; Sánchez-Campos et al., 1999) and in single nuclei in infected cells (Morilla et al., 2004) which is a prerequisite for recombination. In fact, TYLCV and TYLCSV are known to produce viable recombinants in nature that are infectious and accumulate to similar level as the parental viruses in single infection (García-Andrés et al., 2006; Monci et al., 2002). Mixed infections of TYLCSV and TYLCV were obtained here by co-inoculating tomato plants, and the frequency and type of recombinants in the progeny virus population was investigated in time course analyses. Information was obtained about the impact of recombination in the virus population structure. Also, *B. tabaci* transmission of the newly emerged recombinants has been studied. To our knowledge, this is the first attempt to estimate recombinant frequencies in a begomovirus population during the course of a mixed infection of single plants in an experimental setting.

Results

A complex begomovirus population structure exists in mixed infected tomato plants

The virus population structure present in tomato plants co-infected with TYLCSV and TYLCV was studied by the isolation and characterization of full-length cloned viral DNA components from systemically infected tissues at several days post-inoculation (dpi). Two tomato lines were tested to evaluate the possible effect of the genetic background. RFLP analysis evidenced the presence of recombinant viruses (restriction patterns R1 to R4, Fig. 1B) in the progeny viral population, and further nucleotide sequence analysis of junction regions allowed to distinguish different recombinant virus types depending on the location of crossover sites ($P<0.05$, RDP analysis; Martin et al., 2005b) (Table 1). Recombinant type III corresponding to the restriction pattern R1, types IV to IX corresponding to R2, types X and XI corresponding to R3, and type XII corresponding to R4 were detected (Table 1). Types I and II in Table 1 correspond to TYLCSV and TYLCV parental viruses, respectively. In plant MmA, only the parental viruses were detected at early dates (20 and 60 dpi), with TYLCSV as the predominant species in the population. However, at 130 and 400 dpi, almost 50% (12 out of 25 clones) of the population comprised recombinant viruses. Both parental viruses were observed at 130 dpi, although TYLCV was not detected at 400 dpi. Similar results were obtained at 400 dpi in plant MmB and in plants of the tomato line Ty-1S. Thus, recombinant viruses occurred frequently in the population (28% and 52%, in MmB and Ty-1S, respectively; Table 1), and TYLCSV again largely predominated.

A control experiment was performed in which full-length genomic DNAs were isolated after TempliPhi-amplification on a mixture of TYLCV and TYLCSV viral DNA extracts. Analysis of clones showed that none of them displayed a recombinant profile (9 TYLCV clones and 16 TYLCSV clones were obtained), suggesting that *in vitro* amplification did not generate artifacts resembling recombinants produced *in vivo*

Table 1
Distribution of parental and recombinant clones in the progeny virus population resulting from co-inoculation of tomato cv. Moneymaker (Mm) and line Ty-1S plants with TYLCV and TYLCSV [a]

Restriction pattern	Virus type	Progeny begomovirus population					
		Mm A				Mm B	Ty-1S [b]
		20 dpi	60 dpi	130 dpi	400 dpi	400 dpi	400 dpi
TYLCSV	I	19	16	7	13	15	12
TYLCV	II	6	9	6	–	3	–
R1	III	–	–	–	–	–	1
R2	IV	–	–	9	10	3	–
	V	–	–	–	–	–	3
	VI	–	–	–	–	–	2
	VII	–	–	–	–	–	5
	VIII	–	–	–	–	–	1
	IX	–	–	–	–	–	1
R3	X	–	–	2	–	–	–
	XI	–	–	–	–	4	–
R4	XII	–	–	1	2	–	–
Detectable recombinant frequency		0/25 (0%)	0/25 (0%)	12/25 (48%)	12/25 (48%)	7/25 (28%)	13/25 (52%)

[a] Two Moneymaker (MmA and MmB) and three Ty-1S tomato plants were co-inoculated with TYLCV and TYLCSV and progeny virus in young tissues was analyzed at the indicated days post-inoculation (dpi) by restriction fragment length polymorphism analysis (restriction pattern) and nucleotide sequence analysis of the junction regions of recombinant molecules R1 to R4 (virus type).
[b] Results correspond to the analysis of progeny virus present in total DNA extracts obtained from a mixture of equal amounts of young leaf tissues from three co-inoculated plants.

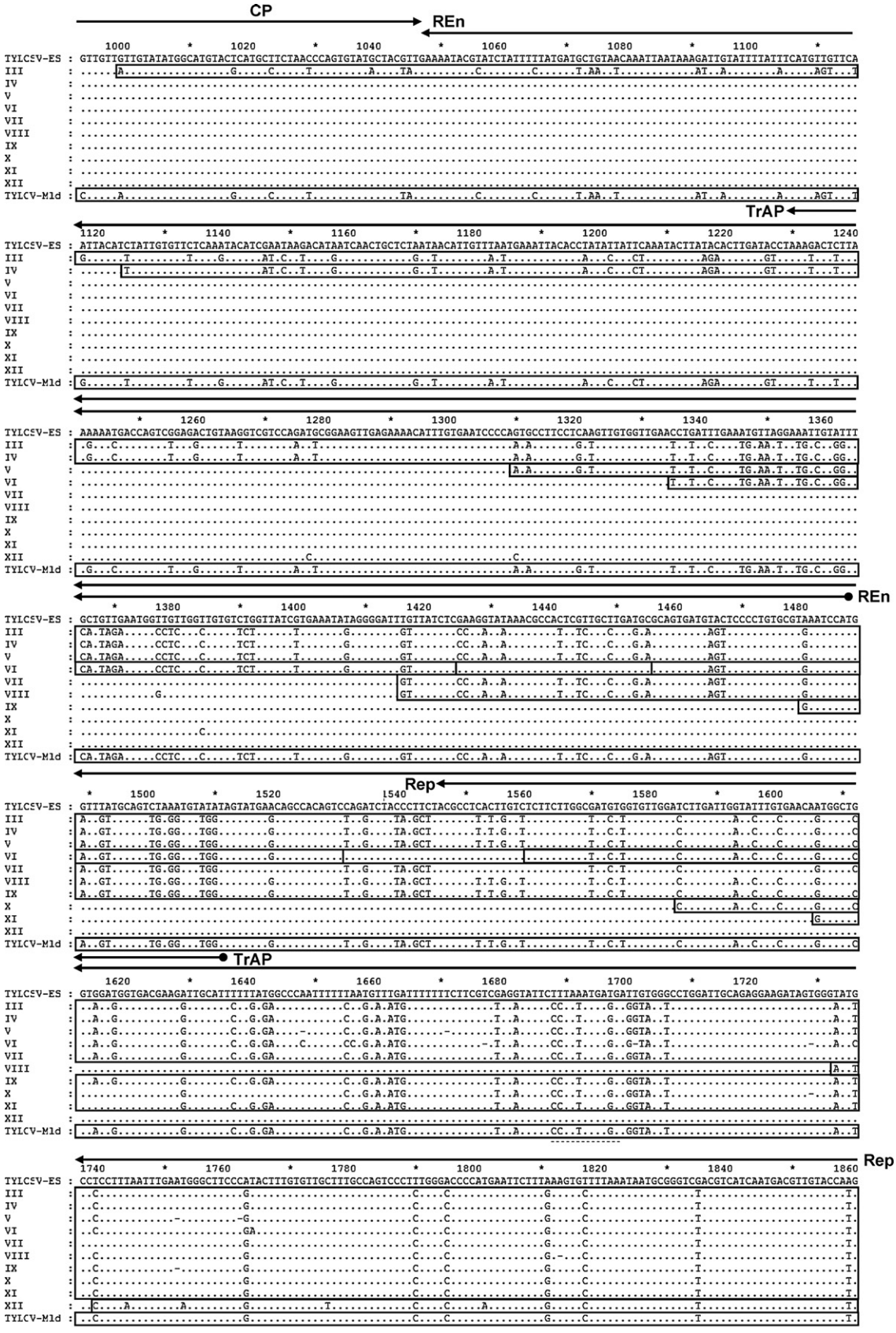

Fig. 2. Alignment of the nucleotide sequences around the cross-over sites located outside the IR of recombinants detected in tomato plants co-infected with the TYLCSV and TYLCV. The sequences of recombinant variants III–XII are compared with the parental viruses TYLCSV (top) and TYLCV (bottom). Dots indicate nucleotides identical to those of TYLCSV and boxed sequences are similar to those of TYLCV. The numbers indicate nucleotide position in the genome of TYLCSV of the following asterisk.

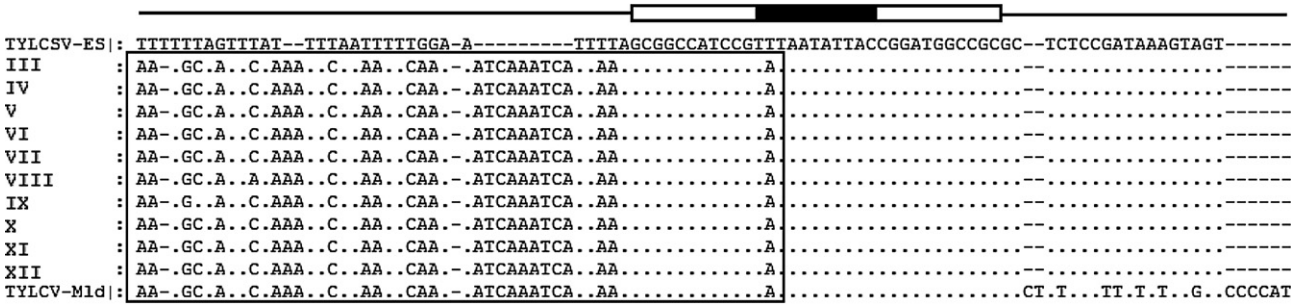

Fig. 3. Alignment of the nucleotide sequences around the cross-over sites located within the IR of recombinants detected in tomato plants co-infected with the TYLCSV and TYLCV. The sequences of recombinant variants III–XII are compared with the parental viruses TYLCSV (top) and TYLCV (bottom). Dots indicate nucleotides identical to those of TYLCSV and boxed sequences are similar to those of TYLCV. The IR region is schematically represented on the top of the figure, showing sequences involved in the stem (open boxes) and loop (black box) of the hairpin conserved in geminiviruses.

during co-infection of TYLCSV and TYLCV. Hence, the results show that recombination occurs *in vivo* and that recombinant frequency can be high in plants maintained for several months after infection.

All the recombinant viruses detected in plant MmA have one cross-over site in the IR and the second site was primarily within the REn ORF (Fig. 1, restriction pattern R2). Sequence analysis at the junction region demonstrated that all of these recombinants corresponded to the same virus type (Table 1, type IV). In addition, a few recombinant viruses had the second cross-over site within the 3′-terminus of the Rep ORF (Fig. 1, restriction patterns R3 and R4), and sequence analysis indicated that they corresponded to virus types X and XII, respectively (Table 1). Similar results were obtained at 400 dpi in plant MmB and in plants of Ty-1S, namely that all the recombinant viruses detected have one cross-over site in the IR and all but one of the second cross-over sites occurred in a short stretch of sequence encompassing the REn ORF and the 3′-terminus of the Rep ORF (Table 1, restriction patterns R2 and R3). A single recombinant virus was found in which the second cross-over site occurred outside of this region (Table 1 and Fig. 1, restriction pattern R1, virus type III). The specific distribution of recombinant virus types differed between MmA, MmB and Ty-1S plants at 400 dpi (Table 1). Interestingly, a closer analysis of nucleotide sequences provided evidence ($P<0.05$, RDP analysis; Martin et al., 2005b) of complex recombination histories for recombinant virus types VI and VIII, which exhibited short TYLCSV-like sequences embedded in TYLCV sequence, between nucleotides 1420–1460 and 1520–1550 in type VI, and nucleotides 1603–1722 in type VIII (Fig. 2). Also, it is worth noting that nucleotide sequence analysis at the IR junction region of recombinant viruses indicated that the cross-over site always occurred in the same position, within the stem–loop conserved in geminiviruses which contains the breaking and joining site for rolling-circle replication (Hanley-Bowdoin et al., 2000) (Fig. 3).

Table 2
Distribution of parental and recombinant clones in the progeny virus population present in Ty-1S tomato plants following *Bemisia tabaci* acquisition and transmission from Ty-1S tomato plants co-infected with TYLCV and TYLCSV

Restriction pattern	Virus type	Number of clones[a]
TYLCSV	I	1
TYLCV	II	–
R1	III	–
R2	IV	–
	V	4
	VI	–
	VII	17
	VIII	3
	IX	–
R3	X	–
	XI	–
R4	XII	–
Detectable recombinant frequency		24/25 (96%)

[a] The progeny begomovirus population was analyzed in a mixture of equal amounts of young tissues of six Ty-1S tomato test plants inoculated using viruliferous *Bemisia tabaci*. Source plants for virus acquisition were Ty-1S plants co-inoculated with TYLCV and TYLCSV, at 400 dpi (Table 1). Plant tissues were collected from test plants at 15 dpi.

Insect transmission of recombinant virus

Maintenance in nature of recombinant variants that emerge from begomovirus mixed infections will strongly depend on their capacity to be transmitted by *B. tabaci*, in addition to being functionally active. To test their ability to be transmitted, virus was acquired from co-infected tomato plants TY-1S at 400 dpi. Analysis of the progeny virus indicated that three of the recombinant types (V, VII and VIII) present in the source plant (Table 1) were transmitted to tomato plants along with the parental TYLCSV (Table 2). Interestingly, a change in the population structure present in the source plant was observed after transmission.

Are reciprocal recombinants viable?

The data of Fig. 1 indicate that none of the recombinants isolated here contained TYLCV sequences in the 3′ half of the IR, ORF V2 and much of the CP ORF. Therefore, we examined whether such recombinants represented non-functional or less aggressive hybrids that could be subject to negative selection in the population. For this, an infectious clone of a chimeric virus (Q7, Fig. 4) was constructed that simulated the result of a recombination event reciprocal to that observed in the recombinants corresponding to the restriction pattern R2. All

ten tomato plants agroinoculated with this chimeric virus became infected, accumulated viral DNA to levels similar to those observed for parental TYLCSV and TYLCV, and exhibited typical yellow leaf curl disease symptoms (data not shown). Moreover, progeny virus was readily transmissible by *B. tabaci* (all 10 tomato plants tested became infected) and retained the chimeric genome structure, as demonstrated by sequence analysis of the cross-over sites. Therefore, the results indicate that this reciprocal recombinant is biologically functional. Thus, reciprocal recombinants might be not found in the population either because they are less competitive or because the cross-over is less probable.

Nucleotide sequences and secondary structures at cross-over regions

Analyses of nucleotide sequences around the cross-over sites of the recombinant viruses detected in this study revealed only wild-type sequences originating from either TYLCV or TYLCSV. Therefore, recombination did not appear to result into additional sequence modifications. Also, in all the recombinants, cross-over sites occurred in regions with significant homology between templates, suggesting homologous recombination. Sequence inspection did not reveal obvious common primary sequence features in cross-over sites. However, prediction of potential secondary structures in the parental TYLCSV and TYLCV DNAs revealed significant structural conservation. As shown in Fig. 1C, potentially stable hairpin structures are predicted to occur throughout the sequences of both viruses. In the IR, the cross-over site is located within a region encompassing the stem–loop structure, conserved among geminiviruses, that is a known hot spot for recombination (Stanley, 1995; Stenger et al., 1991). Also, for the vast majority (93%) of recombinant viruses found in our study, the second cross-over site occurs within a region of sequence where stable hairpin secondary structures are predicted for TYLCSV, whereas a more relaxed sequence structure is predicted for TYLCV (Fig. 1C). Taken together, these data might suggest that, in addition to sequence homology, secondary structure features might favour the occurrence of recombination.

Discussion

Although the true frequency of recombination between TYLCSV and TYLCV could not be deduced from our data, the present work provides clear evidence for the frequent emergence of recombinants during mixed begomovirus infection of single plants. Insight into sequence features associated with the recombinants detected is also provided. Our data inform about the frequency of viable and competitive recombinants, showing that recombination could be a dominant force in shaping the population structure of these viruses. The frequent occurrence of recombinants has also been suggested for other plant DNA viruses like *Maize streak virus* (genus *Mastrevirus*, family *Geminiviridae*) (Schnippenkoetter et al., 2001) and *Cauliflower mosaic virus* (genus *Caulimovirus*, family *Caulimoviridae*) (Froissart et al., 2005). We have demonstrated that diverse types of recombinants can emerge, which reinforces the significance of recombination as a source of genetic diversity in begomoviruses. The observed potential of these viruses to generate new viral genetic variants through recombination could explain in part their adaptive capacity and emergence in nature (Fargette et al., 2006; Rojas et al, 2005; Rybicki and Pietersen, 1999).

Little is known about viral mixed infections in natural plant communities (Cooper and Jones, 2006), although previous reports suggest that they can be frequent for the begomoviruses studied here (Sánchez-Campos et al., 1999; García-Andrés et al., 2006). Hence, based on our results, the accumulation of recombinant variants within individual mixed infected plants would be anticipated, especially in long-lived plants that have greater opportunity to support mixed infections for long periods (Cooper and Jones, 2006). Once produced, the persistence of a new recombinant in nature will depend on its fitness. Here, we have shown that new recombinants can be maintained in single plants for long periods, suggesting that they are able to compete successfully within the population. Moreover, we also demonstrated their competence for transmission by the insect vector *B. tabaci*. This suggests that if such recombinants fit well in nature, could be maintained in the population and may eventually spread and contribute to epidemics. Interestingly, as shown for other viruses (Albiach-Martí et al., 2000; Ali et al., 2006), we observed a change in the population structure after insect transmission. Thus, either selection or random genetic drift due to population bottlenecks associated with insect transmission may operate during this process. This merits further study to better understand the possible impact of recombination on begomovirus populations. It is interesting to note that some correlation was found between recombinants obtained here experimentally and those detected in nature. Thus, the type IV recombinant variant is very similar to *Tomato yellow leaf curl Málaga virus* (Monci et al., 2002) and type XII strongly resembles *Tomato yellow leaf curl Axarquía virus* (García-Andrés et al., 2006), both natural recombinant viruses resulting from genetic exchange between TYLCSV and TYLCV and associated with epidemics in Spain (García-Andrés et al., 2007). Therefore, this reinforces the contention that the experimental methodology used here is robust and might provide a valuable tool to predict evolution in begomovirus populations.

The precise mechanisms controlling recombination in begomoviruses are unknown (Padidam et al., 1999; Seal et al., 2006) and the search for specific sequence features around recombination sites has been unsuccessful (Sanz et al., 2000). Here, we have shown here that recombination might be favored by nucleotide sequence identity around the cross-over sites and that precise homologous recombination occurs as observed for some RNA viruses (de Wispelaere et al., 2005; Jarvis and Kirkegaard, 1992). Also, we found that cross-over sites were not evenly distributed throughout the genome, as suggested by Fauquet et al. (2005). Inspection of the recombinant viruses failed to provide evidence for specific primary sequence features adjacent to the cross-over sites although, interestingly, potential hairpin-like secondary structures were associated with them. For all of the recombinant viruses detected here, one of the cross-over sites always occur in the IR, within the conserved

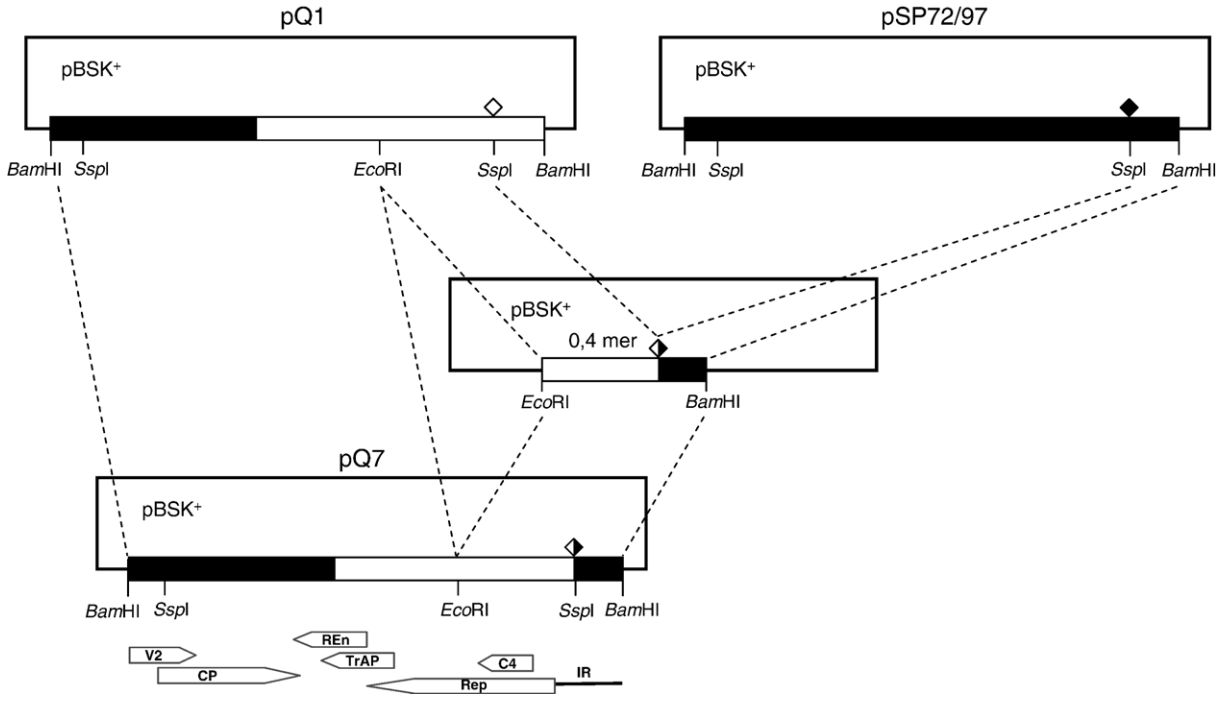

Fig. 4. Schematic representation of the construction of the chimeric Q7 genome. Clone pSP7/297 contains a complete copy of the TYLCV genome (black box) and pQ1 contains a complete copy of the genome of an artificially constructed chimeric virus with sequences from TYLCV (black box) and TYLCSV (open box). Black and open diamonds indicate TYLCV and TYLCSV sequences, respectively, within the conserved stem–loop in the intergenic region (IR). The positions of restriction endonuclease sites used to construct the recombinant genome are indicated. The location of viral open reading frames and the IR are shown at the bottom.

stem–loop structure where replication initiates in geminiviruses (Gutiérrez, 1999; Hanley-Bowdoin et al., 2000), which is a known hot spot for recombination (Stanley, 1995; Stenger et al., 1991). Noticeably, in all but one case, the second cross-over site occurred in a region where potential secondary structure features were predicted for TYLCSV while more relaxed structures were predicted for TYLCV. Therefore, both sequence homology and structure features might stimulate recombination similarly to RNA viruses (Nagy and Simon, 1997; Olsthoorn et al., 2002; White and Morris, 1995). Secondary structure elements might pause the replication complex and incomplete single-stranded DNA molecules derived could be recovered for productive infection by homologous recombination at more relaxed homologous regions in a recombination-dependent replication manner (Jeske et al., 2001; Preiss and Jeske, 2003). Further research is being conducted to substantiate this hypothesis because the existence of non functional or less competitive hybrids might also lead to modification of the apparent distribution of recombination sites throughout the viral genome (de Wispelaere et al., 2005; Martin et al., 2005a; Nagy and Bujarski, 1992).

All of the recombinant viruses contained TYLCV sequences encompassing most of the Rep ORF and the 5′ half of the IR embedded in the TYLCSV genome, consistent with the proposed modular organisation of begomovirus genomes with respect to their replication (Gutiérrez, 1999; Hanley-Bowdoin et al., 2000; Martin et al., 2005a). Although reciprocal recombinants were not observed, an artificially constructed recombinant was competent for infection, disease production and insect transmission. This suggests that the reciprocal recombinants might not be produced due to the absence of suitable sequence features to promote recombination, as discussed above. It is noteworthy that in most of the recombinants detected, the second cross-over site occurred within the REn ORF, suggesting that this region is particularly permissive for genetic exchange. The fact that REn proteins from diverse geminiviruses, including those from TYLCV and TYLCSV, have been demonstrated to be functionally interchangeable (Hormuzdi and Bisaro, 1995; Settlage et al., 2005; Sunter et al., 1994) might also support the propensity for recombination within this region.

The main purpose of this work is to provide baseline information for evaluating the potential impact of recombination in begomovirus populations in order to understand their evolution and thus design more durable control strategies. McDonald and co-workers have evidenced the relevance of gene flow provided by recombination in the evolutionary potential of viruses (García-Arenal and McDonald, 2003). Our data support the contention that the virus population in plants mixed infected with TYLCD-associated begomoviruses comprises a swarm of parental and *de novo* recombinants. The detailed appraisal of the diversity and adaptive capacity of these viruses will help to predict the emergence of new disease epidemics.

Materials and methods

Virus sources

Infectious clones of TYLCV-Mld[ES:72:97] (accession number AF071228) and TYLCSV-ES[ES:Mur1:92] (accession number Z25751) have been described elsewhere (Noris et al., 1994; Navas-Castillo et al., 1999). An infectious clone of a

chimeric virus (pQ7, Fig. 4), in which the 3′ half of the IR and ORFs V2 and CP of TYLCSV-ES have been replaced with the corresponding part of TYLCV-Mld, was constructed in pBluescript SK$^+$ (pBSK$^+$; Stratagene, La Jolla, CA). This was achieved by exchanging the *Ssp*I–*Bam*HI fragment of pQ1 (Sánchez-Campos, 2000), that comprised the 3′ half of the IR of TYLCSV-ES, with the corresponding fragment of TYLCV-Mld obtained from pSP7297 (Navas-Castillo et al., 1999). A partial repeat (1.4 copies) of the pQ7 insert was obtained by deletion of an *Eco*RI–*Bam*HI fragment from pQ7 and insertion the full-length insert into the *Bam*HI site. The resulting insert was excised with *Hin*dIII and *Sac*I and sub-cloned into the binary vector pBin19 (Bevan, 1984) to obtain p1.4Q7. *Agrobacterium tumefaciens* strain LBA 4404 (Höfgen and Willmitzer, 1988) was transformed with p1.4Q7, and infectivity was tested by agroinoculation of tomato (cv. Moneymaker) plants (see below). Maintenance of cross-over sites in the progeny virus was confirmed by PCR and direct sequencing of the amplification products, using primer pairs MA115 and M118 (Monci et al., 2002) for the IR, and TY1(+) (Accotto et al., 2000) and MA242 (García-Andrés et al., 2007) for REn and CP ORFs.

Plant inoculation and in vivo recombination assays

Liquid cultures of *A. tumefaciens*, adjusted to an OD of 1.0 at 600 nm, were used to agroinoculate test plants by stem inoculation (Monci et al., 2002). For recombination assays, equal amounts of *A. tumefaciens* cultures containing TYLCSV-ES and TYLCV-Mld clones were mixed, and 25 μl aliquots were used per plant to inoculate tomato plants at the three leaf growth stage. Two tomato lines were tested in independent experiments, cv. Moneymaker (La Mayora germplasm bank) and Ty-1S (kindly supplied by M. J. Díez, Universidad Politécnica de Valencia, Spain), both of which are susceptible to TYLCSV and TYLCV. Two tomato cv. Moneymaker plants (named MmA and MmB) and three tomato Ty-1S plants were inoculated. Plants were maintained in a growth chamber (26 °C during the day and 18 °C at night, 70% relative humidity, with a 16-h photoperiod at 250 μmol s^{-1} m^{-2} photosynthetically active radiation) and pruned when necessary. Samples were collected from test plants over a period of 400 days after inoculation. Cultivated tomatoes normally last for 4 to 5 months; however, long-duration crops that can last even for 1 year are also frequent under greenhouse conditions, e.g., in South-eastern Spain.

B. tabaci transmission

B. tabaci transmission experiments were performed with the Q biotype as described (Monci et al., 2002), using 50 viruliferous whiteflies per test plant. Inoculated plants were maintained in a growth chamber (conditions described above) until analyzed.

Cloning and analysis of viral genomes

Total DNA preparations were obtained from young tissues of test plants (Monci et al., 2005) and used as templates to amplify and clone the complete circular DNA genome of progeny begomoviruses. For amplification, the bacteriophage [phi]29 DNA polymerase was used according to Inoue-Nagata et al. (2004) with the commercial kit TempliPhi (Amersham Biosciences, England). The amplified DNA was digested with *Bam*HI that has a single site at equivalent positions in TYLCSV-ES and TYLCV-Mld DNA. Linearized genome-size DNA fragments were excised from agarose gels, purified using Ultrafree-DA kit (Millipore Corporation, Bedford, MA), and cloned into the *Bam*HI cloning site of pBSK$^+$. Twenty-five clones with genome-size inserts were randomly selected from each DNA extraction for restriction fragment length polymorphism (RFLP) analysis. *Bgl*I, *Bgl*II, *Dra*III, *Eco*NI, *Hin*dIII, *Kpn*I, *Pml*I, *Sac*II and *Sph*I, diagnostic for TYLCSV-ES and TYLCV-Mld sequences throughout the genome (Fig. 1A), were selected for restriction mapping. Clones containing recombinant begomovirus genomes based on RFLP analysis were further studied to determine the location of recombination sites as precisely as possible by sequence analyses of junction regions using primers MA117 (Monci et al., 2002), MA368 (5′ GCGTTGTTGTTGTATATGG 3′, identical to TYLCSV-ES nts 980–998), TY1(+) (Accotto et al., 2000) or MA163 (5′ TTTGAATTTTGAATTTTGAATTGC 3′, complementary to TYLCV-Mld nts 2733–2755) (Fig. 1A), and an automatic sequencer (ABI Prism 3700 DNA Analyzer, Applied Biosystems, Foster City, CA).

Control bacteriophage [phi]29 DNA polymerase amplification

A control experiment was performed to rule out the possibility of recombinant artifacts being generated during amplification using bacteriophage [phi]29 DNA polymerase as suggested for *Taq* polymerase during PCR amplifications (Bradley and Hillis, 1997). Equal amounts of viral DNA extracted from plants individually infected with either TYLCSV-ES or TYLCV-Mld were mixed and used as template in the TempliPhi amplification reaction. Following the procedure described above, the amplification product was used to produce 25 clones with genome-size inserts, which were analyzed by restriction mapping.

Sequence and secondary structure analyses

The ClustalX program (Thompson et al., 1997) was used for DNA multiple sequence alignment and the Genedoc program (Nicholas et al., 1997) for visualization of alignments. The DNA Mfold program (Zuker, 2003) was used to predict potential secondary structures in the DNA, using default parameters and temperature of 70 °C. Nucleotide sequences were checked for incongruent relationships, which might have resulted from recombination, using the Recombination Detection Program (RDP) version 2.0 (Martin et al., 2005b). This program estimates breakpoint positions and calculates probability scores using six different automated methods; a multiple comparison corrected *P*-value cutoff of 0.05 and default settings were used throughout and only events detectable with two or more different methods were retained.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this work were deposited in the GenBank/EMBL/DDBJ/databases under accession numbers EF423644 to EF423723 and EF428441 to EF428443.

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References

Aaziz, R., Tepfer, M., 1999. Recombination in RNA viruses and in virus-resistant transgenic plants. J. Gen. Virol. 80, 1339–1346.

Accotto, G.P., Navas-Castillo, J., Noris, E., Moriones, E., Louro, D., 2000. Typing of tomato yellow leaf curl viruses in Europe. Eur. J. Plant Pathol. 106, 179–186.

Albiach-Martí, M.R., Guerri, J., de Mendoza, A.H., Laigret, F., Ballester-Olmos, J.F., Moreno, P., 2000. Aphid transmission alters the genomic and defective RNA populations of *Citrus tristeza virus* isolates. Phytopathology 90, 134–138.

Ali, A., Li, H., Schneider, W.L., Sherman, D.J., Gray, S., Smith, D., Roossinck, M.J., 2006. Analysis of genetic bottlenecks during horizontal transmission of *Cucumber mosaic virus*. J. Virol. 80, 8345–8350.

Berrie, L.C., Rybicki, E.P., Rey, M.E.C., 2001. Complete nucleotide sequence and host range of *South African cassava mosaic virus*: further evidence for recombinations amongst begomoviruses. J. Gen. Virol. 82, 53–58.

Bevan, M., 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12, 8711–8721.

Bonnet, J., Fraile, A., Sacristán, S., Malpica, J.M., García-Arenal, F., 2005. Role of recombination in the evolution of natural populations of *Cucumber mosaic virus*, a tripartite RNA plant virus. Virology 332, 359–368.

Bradley, R.D., Hillis, D.M., 1997. Recombinant DNA sequences generated by PCR amplification. Mol. Biol. Evol. 14, 592–593.

Bruyere, A., Wantroba, M., Flasinski, S., Dzianott, A., Bujarski, J.J., 2000. Frequent homologous recombination events between molecules of one RNA component in a multipartite RNA virus. J. Virol. 74, 4214–4219.

Chenault, K.D., Melcher, U., 1994. Phylogenetic relationships reveal recombination among isolates of *Cauliflower mosaic virus*. J. Mol. Evol. 39, 496–505.

Cooper, I., Jones, R.A.C., 2006. Wild plants and viruses: under-investigated ecosystems. In: Maramorosch, K., Aaron, J.S. (Eds.), Advances in Virus Research Plant Virus Epidemiology. Academic Press, New York, pp. 1–47.

de Wispelaere, M., Gaubert, S., Trouilloud, S., Belin, C., Tepfer, M., 2005. A map of the diversity of RNA3 recombinants appearing in plants infected with *Cucumber mosaic virus* and *Tomato aspermy virus*. Virology 331, 117–127.

Fargette, D., Konate, G., Fauquet, C., Muller, E., Peterschmitt, M., Thresh, J.M., 2006. Molecular ecology and emergence of tropical plant viruses. Annu. Rev. Phytopathol. 44, 235–260.

Fauquet, C.M., Sawyer, S., Idris, A.M., Brown, J.K., 2005. Sequence analysis and classification of apparent recombinant begomoviruses infecting tomato in the Nile and Mediterranean basins. Phytopathology 95, 549–555.

Fernández-Cuartero, B., Bürgyan, J., Aranda, M.A., Salanki, K., Moriones, E., García-Arenal, F., 1994. Increase in the relative fitness of a plant–virus RNA associated with its recombinant nature. Virology 203, 373–377.

Fraile, A., Alonso-Prados, J.L., Aranda, M.A., Bernal, J.J., Malpica, J.M., García-Arenal, F., 1997. Genetic exchange by recombination or reassortment is infrequent in natural populations of a tripartite RNA plant virus. J. Virol. 71, 934–940.

French, R., Stenger, D.C., 2003. Evolution of *wheat streak mosaic virus*: dynamics of population growth within plants may explain limited genetic variation. Annu. Rev. Phytopathol. 41, 199–214.

Froissart, R., Wilke, C.O., Montville, R., Remold, S.K., Chao, L., Turner, P.E., 2004. Co-infection weakens selection against epistatic mutations in RNA viruses. Genetics 168, 9–19.

Froissart, R., Roze, D., Uzest, M., Galibert, L., Blanc, S., Michalakis, Y., 2005. Recombination every day: abundant recombination in a virus during a single multi-cellular host infection. PLoS Biol. 3, 389–395.

García-Andrés, S., Monci, F., Navas-Castillo, J., Moriones, E., 2006. Begomovirus genetic diversity in the native plant reservoir *Solanum nigrum*: evidence for the presence of a new virus species of recombinant nature. Virology 350, 433–442.

García-Andrés, S., Accotto, G.P., Navas-Castillo, J., Moriones, E., 2007. Founder effect, plant host, and recombination shape the emergent population of begomoviruses that cause the tomato yellow leaf curl disease in the Mediterranean basin. Virology 359, 302–312.

García-Arenal, F., McDonald, B.A., 2003. An analysis of the durability of resistance to plant viruses. Phytopathology 93, 941–952.

García-Arenal, F., Fraile, A., Malpica, J.M., 2001. Variability and genetic structure of plant virus populations. Annu. Rev. Phytopathol. 39, 157–186.

Gutiérrez, C., 1999. Geminivirus DNA replication. Cell. Mol. Life Sci. 56, 313–329.

Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S., Robertson, D., 2000. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit. Rev. Biochem. Mol. Biol. 35, 105–140.

Höfgen, R., Willmitzer, L., 1988. Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res. 16, 9877.

Hormuzdi, S.G., Bisaro, D.M., 1995. Genetic analysis of *Beet curly top virus*. Examination of the roles of *L2* and *L3* genes in viral pathogenesis. Virology 206, 1044–1054.

Inoue-Nagata, A.K., Albuquerque, L.C., Rocha, W.B., Nagata, T., 2004. A simple method for cloning the complete begomovirus genome using the bacteriophage [phi]29 DNA polymerase. J. Virol. Methods 116, 209–211.

Jarvis, T.C., Kirkegaard, K., 1992. Poliovirus RNA recombination: mechanistic studies in the absence of selection. EMBO J. 11, 3135–3145.

Jeske, H., Lutgemeier, M., Preiss, W., 2001. DNA forms indicate rolling circle and recombination-dependent replication of *Abutilon mosaic virus*. EMBO J. 20, 6158–6167.

Lai, M.M., 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. 56, 61–79.

Li, H., Roossinck, M.J., 2004. Genetic bottlenecks reduce population variation in an experimental RNA virus population. J. Virol. 78, 10582–10587.

Martin, D.P., van der Walt, E., Posada, D., Rybicki, E.P., 2005a. The evolutionary value of recombination is constrained by genome modularity. PLoS Genet. 1, 475–479.

Martin, D.P., Williamson, C., Posada, D., 2005b. RDP2: recombination detection and analysis from sequence alignments. Bioinformatics 21, 260–262.

McDonald, B.A., Linde, C., 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annu. Rev. Phytopathol. 40, 349–379.

Monci, F., Sánchez-Campos, S., Navas-Castillo, J., Moriones, E., 2002. 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. Virology 303, 317–326.

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

Morilla, G., Krenz, B., Jeske, H., Bejarano, E., Wege, C., 2004. Tête à Tête of *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus* in single nuclei. J. Virol. 78, 10715–10723.

Moriones, E., Navas-Castillo, J., 2000. *Tomato yellow leaf curl virus*, an emerging virus complex causing epidemics worldwide. Virus Res. 71, 123–134.

Nagy, P.D., Bujarski, J.J., 1992. Genetic recombination in *Brome mosaic virus*: effect of sequence and replication of RNA on accumulation of recombinants. J. Virol. 66, 6824–6828.

Nagy, P.D., Simon, A.E., 1997. New insights into the mechanisms of RNA recombination. Virology 235, 1–9.

Navas-Castillo, J., Sánchez-Campos, S., Díaz, J.A., Sáez-Alonso, E., Moriones, E., 1999. *Tomato yellow leaf curl virus*-Is causes a novel disease of common bean and severe epidemics in tomato in Spain. Plant Dis. 83, 29–32.

Nicholas, K.B., Nicholas Jr., H.B., Deerfield II, D.W., 1997. GeneDoc: analysis and visualization of genetic variation. Embnew News 4, 14.

Noris, E., Hidalgo, E., Accotto, G.P., Moriones, E., 1994. High similarity among the *Tomato yellow leaf curl virus* isolates from the west Mediterranean basin. The nucleotide sequence of an infectious clone from Spain. Arch. Virol. 135, 165–170.

Olsthoorn, R.C.L., Bruyere, A., Dzianott, A., Bujarski, J.J., 2002. RNA recombination in *Brome mosaic virus*: effects of strand-specific stem–loop inserts. J. Virol. 76, 12654–12662.

Padidam, M., Sawyer, S., Fauquet, C.M., 1999. Possible emergence of new geminiviruses by frequent recombination. Virology 265, 218–225.

Pita, J.S., Fondong, V., N. Sangare, A., Otim-Nape, G.W., Ogwal, S., Fauquet, C.M., 2001. Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. J. Gen. Virol. 82, 655–665.

Posada, D., Crandall, K.A., Holmes, E.C., 2002. Recombination in evolutionary genomics. Annu. Rev. Genet. 36, 75–97.

Preiss, W., Jeske, H., 2003. Multitasking in replication is common among geminiviruses. J. Virol. 77, 2972–2980.

Rojas, M.R., Hagen, C., Lucas, W.J., Gilbertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annu. Rev. Phytopathol. 43, 361–394.

Roossinck, M.J., 1997. Mechanisms of plant virus evolution. Annu. Rev. Phytopathol. 35, 191–209.

Rybicki, E.P., Pietersen, G., 1999. Plant virus problems in the developing world. Adv. Virus Res. 53, 127–175.

Sacristán, S., Malpica, J.M., Fraile, A., García-Arenal, F., 2003. Estimation of population bottlenecks during systemic movement of *Tobacco mosaic virus* in tobacco plants. J. Virol. 77, 9906–9911.

Sánchez-Campos, S. 2000. El rizado amarillo del tomate en España: caracterización de los geminivirus implicados y estudio de las epidemias. PhD thesis, University of Málaga, Spain.

Sánchez-Campos, S., Navas-Castillo, J., Camero, R., Soria, C., Díaz, J.A., Moriones, E., 1999. Displacement of *Tomato yellow leaf curl virus* (TYLCV)-Sr by TYLCV-Is in tomato epidemics in Spain. Phytopathology 89, 1038–1043.

Sanz, A.I., Fraile, A., García-Arenal, F., Zhou, X.P., Robinson, D.J., Khalid, S., Butt, T., Harrison, B.D., 2000. Multiple infection, recombination and genome relationships among begomovirus isolates found in cotton and other plants in Pakistan. J. Gen. Virol. 81, 1839–1849.

Saunders, K., Lucy, A., Stanley, J., 1991. DNA forms of the geminivirus *African cassava mosaic virus* consistent with a rolling circle mechanism of replication. Nucleic Acids Res. 19, 2325–2330.

Schnippenkoetter, W.H., Martin, D.P., Willment, J.A., Rybicki, E.P., 2001. Forced recombination between distinct strains of *Maize streak virus*. J. Gen. Virol. 82, 3081–3090.

Seal, S.E., vandenBosch, F., Jeger, M.J., 2006. Factors influencing begomovirus evolution and their increasing global significance: implications for sustainable control. Crit. Rev. Plant Sci. 25, 23–46.

Settlage, S.B., See, R.G., Hanley-Bowdoin, L., 2005. Geminivirus C3 protein: replication enhancement and protein interactions. J. Virol. 79, 9885–9895.

Stanley, J., 1995. Analysis of *African cassava mosaic virus* recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. Virology 206, 707–712.

Stanley, J., Bisaro, D.M., Briddon, R.W., Brown, J.K., Fauquet, C.M., Harrison, B.D., Rybicki, E.P., Stenger, D.C., 2005. *Geminiviridae*. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy, VIIIth Report of the ICTV. Elsevier/Academic Press, London, pp. 301–326.

Stenger, D.C., Revington, G.N., Stevenson, M.C., Bisaro, D.M., 1991. Replicational release of geminivirus genomes from tandemly repeated copies. Evidence for rolling circle replication of a plant viral DNA. Proc. Natl. Acad. Sci. U.S.A. 88, 8029–8033.

Sunter, G., Stenger, D.C., Bisaro, D.M., 1994. Heterologous complementation by geminivirus *AL2* and *AL3* genes. Virology 203, 203–210.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Van den Bosch, F., Akudibilah, G., Seal, S., Jeger, M., 2006. Host resistance and the evolutionary response of plant viruses. J. Appl. Ecol. 43, 503–516.

White, K.A., Morris, T.J., 1995. RNA determinants of junction site selection in RNA virus recombinants and defective interfering RNAs. RNA 1, 1029–1040.

Worobey, M., Holmes, E.C., 1999. Evolutionary aspects of recombination in RNA viruses. J. Gen. Virol. 80, 2535–2543.

Zhou, X.P., Liu, Y., L. Calvert, L., Munoz, C., Otim-Nape, G.W., Robinson, D.J., Harrison, B.D., 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J. Gen. Virol. 78, 2101–2111.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.