CHAPTER 3

RECOMBINATION IN THE TYLCV COMPLEX: A MECHANISM TO INCREASE GENETIC DIVERSITY. IMPLICATIONS FOR PLANT RESISTANCE DEVELOPMENT

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1. RECOMBINATION INCREASES GENETIC DIVERSITY AND DRIVES EVOLUTION OF PLANT VIRUSES

Mutation, reassortment, and recombination are the major sources of genetic variation of plant viruses (García-Arenal et al., 2001; Worobey & Holmes, 1999). During mixed infections, viruses can exchange genetic material through recombination or reassortment of segments (when the parental genomes are fragmented) if present in the same cell context of the host plant. Hybrid progeny viruses might then arise, some of them with novel pathogenic characteristics and well adapted in the population that can cause new emerging diseases. Genetic exchange provides organisms with a tool to combine sequences from different origins which might help them to quickly evolve (Cramer et al., 1998). In many DNA and RNA viruses, genetic exchange is achieved through recombination (Froissart et al., 2005; Martin et al., 2005). As increasing numbers of viral sequences become available, recombinant viruses are recognized to be frequent in nature and clear evidence is found for recombination to play a key role in virus evolution (Awadalla, 2003; Chenault & Melcher, 1994; Moonan et al., 2000; Padidam et al., 1999; Revers et al., 1996; García-Arenal et al., 2001; Moreno et al., 2004). Understanding the role of recombination in generating and eliminating variation in viral sequences is thus essential to understand virus evolution and adaptation to changing environments (de Wispelaere et al., 2005; Vignuzzi et al., 2006; Domingo, 2000; Eigen, 1993).

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Knowledge about the existence and frequency of recombination in a virus population might help understanding the extent at which genes are exchanged and new virus variants arise. This information is essential, for example, to predict durability of genetic resistance because new recombinant variants might be formed with increased fitness in host-resistant genotypes. Determination of the extent and rate at which genetic rearrangement through recombination does occur in natural populations is also crucial if we use genome and genetic-mapping information to locate genes responsible of important phenotypes such as genes associated with virulence, transmission, or breakdown of resistance. Therefore, better estimates of the rate of recombination will facilitate the development of more robust strategies for virus control (Awadalla, 2003).

Recombination appears to be common among members of the family Geminiviridae, which have single-stranded DNA genomes (Padidam et al., 1999). In this group of viruses, more notably among members of the genus Begomovirus, recombination seems to contribute greatly to the genetic diversification of viral populations (Zhou et al., 1997; Berrie et al., 2001; Pita et al., 2001; Monci et al., 2002; Chatchawankanphanich & Maxwell, 2002; Umaharan et al., 1998; Moffat, 1999; Harrison & Robinson, 1999; Sanz et al., 1999, 2000). Replication of these viruses, in addition to a rolling circle replication (RCR) mechanism (Saunders et al., 1991; Stenger et al., 1991), also involves a recombination-dependent replication (RDR) mechanism (Jeske et al., 2001). RDR provides geminiviruses with a tool by which damaged or incomplete DNA could be recovered for productive infection by homologous recombination and converted into full-size genomic DNA. The existence of this replication mechanism might explain in part the extent at which recombination occurs in geminivirus populations (Jeske et al., 2001; Preiss & Jeske, 2003). Recombination in begomoviruses is found at the strain (Hou & 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; Monci et al., 2002; García-Andrés et al., 2006), genus (Briddon et al., 1996; Klute et al., 1996), and family (Saunders & Stanley, 1999) levels. The potential of begomoviruses to generate genetic diversity through recombination can be relevant for their ecological fitness, because greater sequence heterogeneity provides a reservoir of virus variants in the population that enables rapid adaptation to changing environmental conditions. Thus, begomoviruses like those in the Tomato yellow leaf curl virus (TYLCV) complex exploit gene flow provided by recombination as a mechanism to increase their evolutionary potential and local adaptation.

2. RECOMBINATION HAS PLAYED AN IMPORTANT ROLE IN THE ORIGIN OF VIRUSES OF THE TYLCV COMPLEX

The tomato yellow leaf curl disease (TYLCD) causes severe damage to tomato production in many warm and temperate regions worldwide (Cohen & Antignus, 1994; Moriones & Navas-Castillo, 2000; Varma & Malathi, 2003).
Different virus species and strains of the same virus species have been associated with TYLCD, among them, TYLCV (Moriones and Navas-Castillo et al., 2000; Fauquet et al., 2003; Stanley et al., 2005). In this chapter, TYLCD-associated viruses are referred to as “TYLCV complex”. Recombination seems to have played an important role in the origin of viruses of the TYLCV complex. Two case studies are examined here in detail.

2.1. Case study I: The type strain of the species TYLCV is a recombinant virus which shares an ancestral parent with extant Asian begomoviruses

The earliest evidence of naturally occurring recombination within the genus *Begomovirus* was found when the genome of the Mld strain of the monopartite virus *Tomato yellow leaf curl virus* (TYLCV-Mld) (Antignus & Cohen, 1994) was compared with the genome of the type strain of TYLCV (Navot et al., 1991). The nucleotide sequences of the Rep gene and the intergenic region (IR) of the type and Mld strains of TYLCV were only 87% and 78% identical, respectively, whereas the rest of the genome shared 98% nucleotide identity. Harrison & Robinson (1999) suggested that the Rep–IR regions of both genomes were acquired from different parental viruses that could not be identified at that moment. However, when increasing number of begomovirus DNA-A sequences became available, Navas-Castillo et al. (2000) were able to identify the existent viruses more related to such parents. These authors compared the sequences of nine isolates of the TYLCV complex, three of the type strain of TYLCV (the original isolate from Israel, and isolates from the Dominican Republic and Cuba), five of the TYLCV-Mld strain (the original Mld isolate from Israel, and isolates from Spain, Portugal, and Japan), and one TYLCV isolate from Iran (now recognized as the IR strain of TYLCV). When phylogenetic relationships between nucleotide sequences of these isolates were analyzed, changes in the topological position of certain isolates occurred depending on the part of the genome compared (IR and open reading frames – ORFs – V1, V2 and C1–C4). Detailed comparisons throughout the genome using PLOTSIM-ILARITY diagrams (Wisconsin GCG software package) (Devereux et al., 1984) clearly indicated that four regions (named I–IV in Figure 1) were recognized for which differential distribution of nucleotide identity was observed. Region III comprised about 5′ half of the Rep gene (ORF C1), including the ORF C4 and part of the IR; region I comprised most of the rest of the genome, and regions II and IV were small regions separating region I from region III (Figure 1). In these comparisons, the nucleotide sequences of the TYLCV-Mld isolates from Spain, Portugal, and Japan on the one hand and those of the TYLCV type strain isolates from Israel, Cuba, and Dominican Republic on the other, proved to be almost identical between them throughout the genome. Also, when region I was analyzed, phylogenetic analyses revealed that all TYLCV isolates grouped in a single clade related to *Tomato yellow leaf curl Sardinia virus* (TYLCSV), another species of the TYLCV complex causing the TYLCD.
Figure 1. PLOTSIMILARITY diagrams (scanning window = 50) comparing the nucleotide sequences of TYLCV isolates (A) of the Mld (isolate ES:72:97) and type (isolate IL) strains, (B) of the type (isolate IL) and Iran (isolate IR) strains, (C) of the Mld (isolate ES:72:97) and Iran (isolate IR) strains, and (D) two isolates of the Mld strain (isolate ES:72:97 and IL). Separation between regions I–IV for which differential distribution of nucleotide identity is observed are indicated by vertical dotted lines. In brackets is indicated the first nucleotide of the region (numbers refer to nucleotide positions in the sequence of TYLCV-Mld[ES:72:97]). Positions of the open reading frames (ORFs) and of the intergenic region (IR) are indicated at the top of the figure. Horizontal broken lines are the mean similarity between the sequences compared. GenBank accession number of sequences used for comparison are AF071228 (TYLCV-Mld[ES:72:97]), AJ132711 (TYLCV-IR[IR]), X15656 (TYLCV-[IL:Reo:86]), and X76319 (TYLCV-Mld[IL]). (Adapted from Navas-Castillo et al., 2000.)
However, when comparing sequences in region III, significant changes occurred in the phylogenetic relationships of certain TYLCV isolates. Thus, based on sequences of this region most TYLCV isolates grouped in a single clade related to TYLCSV, but TYLCV (type strain) and TYLCV-IR isolates grouped separately, together with begomovirus isolates Bangalore-2 and Bangalore-4. These two latter viruses were considered at that moment to belong to the begomovirus species, *Tomato leaf curl virus* (ToLCV) (Moriones & Navas-Castillo, 2000), however now they are known to belong to the Asian begomovirus species *Tomato leaf curl Karnataka virus* (ToLCKV) and *Tomato leaf curl Bangalore virus* (ToLCBV), respectively (Stanley et al., 2005). Therefore, the genomes of TYLCV, TYLCV-Mld, and TYLCV-IR begomovirus isolates reflect a modular composition, with genome fragments having diverse phylogenetic origins that were probably put together after successive recombination events.

2.2. Case study II: The type strain of TYLCSV is a recombinant virus which shares an ancestral parent with extant African begomoviruses

Tomato yellow leaf curl Sardinia virus (TYLCSV) is another monopartite begomovirus species of the TYLCV complex that comprises isolates infecting tomato in the Mediterranean Basin, both in southern Europe and northern Africa (Noris et al., 1994; Moriones & Navas-Castillo, 2000). In the TYLCSV clade, at least three different types of sequences can be distinguished, represented by the type strain, originally described from Sardinia (TYLCSV), the Sicily strain (TYLCV-Sic), and the Spain strain (TYLCV-ES). Through comparison of the genome of isolates of the type and Sic strains from Italy following a similar procedure to that described above for TYLCV (i.e., search for topological changes in the phylogenetic trees, and analysis with PLOTSIMILARITY diagrams), evidence was obtained for differences in the phylogenetic origin of the different genomic regions of these isolates probably as a result of recombination events. Two regions could be distinguished when the genomes of these isolates were compared (Figure 2A): region I, in which the percentage of nucleotide identity between TYLCSV and TYLCSV-Sic is 96%, and region II, which includes a shorter fragment that comprises part of the IR, and the 5’ end of ORF C1, in which the percentage of nucleotide identity is significantly lower, about 64%. When nucleotide sequences in region I were phylogenetically analyzed, TYLCSV type strain clustered in the TYLCSV-clade, closely related to TYLCSV-Sic, TYLCSV-ES, and the rest of viruses of the TYLCV complex (Figure 2B). However, after comparison of nucleotide sequences in region II, a significant topological change occurred in the position of the TYLCSV type strain isolate (Figure 2C). Thus in this case, TYLCSV type strain isolate did not group with isolates of the TYLCV complex, but in a clade that comprised all the cassava-infecting begomoviruses from Africa, being the closest related sequence that of an isolate of *South African cassava mosaic virus* (SACMV) (Figure 2C). Therefore, these results strongly suggested that the TYLCSV-type strain resulted...
Figure 2. PLOTSIMILARITY diagram comparing the nucleotide sequences of isolates of the type (isolate Sar) and Sic (isolate Sic) strains of Tomato yellow leaf curl Sardinia virus (TYLCSV). Regions I and II for which differential distribution of nucleotide identity is observed are indicated. Positions of the open reading frames (ORFs) and of the intergenic region (IR) are indicated at the top of the figure (A). Phylogenetic relationships for viruses in the TYLCV-complex and the DNA A
from a recombination exchange of genetic material occurred between TYLCSV and African begomovirus ancestors.

2.3. Begomoviruses of the TYLCV complex are evolving through genetic exchange in their travel across Asia and Africa

The above examples of putative recombinations involving begomoviruses of the TYLCV complex from the Middle East and the Mediterranean Basin seem to reflect exchange of genomic fragments with begomoviruses present in Asia (e.g., India) and Africa. Therefore, it could be hypothesized that an ancestral “TYLCV” evolved and generated new variants (species or strains) by exchanging genetic material through recombination with other begomoviruses in its travel across different areas of the Old World. Because begomoviruses in the TYLCV complex belong to a clade of begomoviruses of the Old World that affect tomato, and this crop was introduced in this region from America only recently, several scenarios can be suggested. One possibility is that a number of TYLCV-like viruses could have already existed in the wild or cultivated hosts of the Old World before the introduction of tomato. When tomato was grown in different areas, it could have become infected with these preexisting viruses. Alternatively, an ancestor of the begomoviruses of the TYLCV complex infected tomato and, in its travel through the regions of Asia, Africa, and Europe (regions in which tomato has become a major crop), different virus lineages evolved by acquisition of genomic fragments from other begomoviruses by genetic exchange through recombination. Thus, emergence of new begomoviruses could have occurred which shared tomato as common host. As tomato has become a major crop, it could act as a bridge for begomoviruses between other local crops or wild reservoirs, favoring contact between viruses otherwise separated. Spread of *Bemisia tabaci* biotypes highly polyphagous like the biotype B could also have favored exchange of viruses between tomato and other cultivated or wild hosts and thus facilitating recombination to occur. Evidently, it is reasonable that an intermediate situation between the two scenarios proposed is what occurred and probably is occurring. New information about viruses infecting wild and cultivated hosts

Figure 2. (continued) component of representative isolates of viruses infecting cassava from Africa. Relationships were inferred by using the neighbor-joining method on the sequences of the region I (B) and II (C) deduced from the PLOTSIMILARITY analysis. Support for nodes in a bootstrap analysis with 1,000 replications is shown for figures over 500. Horizontal branch lengths are drawn to scale with the bar indicating 0.1 nucleotide replacements per site; vertical distances are arbitrary. Abbreviations and GenBank accession numbers are as follow: ACMV, *African cassava mosaic virus*, J02057; SACMV, *South African cassava mosaic virus*, AF155806; EACMV, *East African cassava mosaic virus*, Z83257; EACMMV, *East Africa cassava mosaic Malawi virus*, AJ006460; TYLCSV-ES, *Tomato yellow leaf curl Sardinia virus*-Spain, Z25751; TYLCV, *Tomato yellow leaf curl Sardinia virus*, X15655; TYLCSV-Sic, *Tomato yellow leaf curl Sardinia virus*-Sicily, Z28390; TYLCMLV, *Tomato yellow leaf curl Mali virus*, AJ006460; TYLC-Mld, *Tomato yellow leaf curl virus*-Mild, X76319. As outgroup, an isolate of *Tomato mottle virus* (ToMoV) was used (GenBank L14460).
in regions of the Old World could provide some clues about the origin and evolution of this complex of viruses.

In addition to the well-documented examples of recombination shown above, other examples that involved begomoviruses of the TYLCV complex have also been reported in the literature. Thus, an interspecific recombination has been described for begomoviruses infecting tomato in central Sudan (Idris & Brown, 2005). In this case, two recombinant fragments were identified in the genome of the isolate SD:Gez:96 of *Tomato leaf curl Sudan virus* from Gezira (ToLCSDV-[SD:Gez:96]) when compared with the isolate SD:96 from Sudan of the Gezira strain of TYLCV (TYLCV-Gez[SD:96]). Also, Padidam et al. (1999) detected several other putative recombination events also involving viruses of the TYLCV complex by employing a statistical technique for detecting gene conversion based on the program GENECONV. Their analyses, using 64 geminivirus DNA A sequences, identified 420 statistically significant recombinant events, 36 of them being listed and identified. Six of the listed recombination events involved TYLCV or TYLCSV, some of them between strains of the same species (e.g., TYLCSV/TYLCSV-Sic) whereas others had as a partner a non-TYLCV virus from Africa or Asia (e.g., TYLCV/Chayote yellow mosaic virus, TYLCSV/Indian cassava mosaic virus). Surprisingly, GENECONV identified as recombinant a fragment shared between a Spanish isolate of TYLCSV and *Bean dwarf mosaic virus*, a begomovirus species from the New World.

Rybicki (1994) already pointed out that recombination is probably a powerful tool in the evolution of begomoviruses, not only in the long term but also in the short to medium term. In this sense, Padidam et al. (1999) evaluated the hypothesis that recombination among begomoviruses is contributing to the increasing emergence of new species and suggested that such studies could facilitate understanding of how viruses could evolve in response to changes in the ecosystem. In the next sections of this chapter, we will present data that evidenced the extent at which recombination is contributing to the diversification and adaptation of begomoviruses of the TYLCV complex during their colonization of southern Spain (Western Mediterranean Basin). Emergence and spread of new recombinant viral species belonging to the TYLCV complex is shown from field data. Also evidences from laboratory experiments are provided that support frequent emergence of new recombinant virus variants during single host plant infection cycles, in mixed infections between TYLCV and TYLCSV.

### 3. RECOMBINATION IS DRIVING POPULATION EVOLUTION OF VIRUSES OF THE TYLCV COMPLEX INVADING NEW GEOGRAPHICAL AREAS: THE CASE OF THE WESTERN MEDITERRANEAN BASIN

The two case studies described in the previous section are examples of ancient, and probably multiple, recombination events that contributed to the emergence of begomoviruses of the TYLCV complex. But if located at the right place and the right time, it could be possible to be witness to the occurrence of such a
recombination event and emergence of the new virus variant originated. This was the case during studies of the epidemics of begomoviruses of the TYLCV complex that recently colonized southern Spain. Following are data that evidence the relevance of recombination in the rapid evolution of such a population for its adaptation to a novel environment.

3.1. Mixed infections: A prerequisite for recombination to occur in a begomovirus population

Mixed infections can be frequent in nature associated with begomovirus epidemics. Thus for example, in a recent survey for viruses associated to TYLCD in epidemics in tomatoes of the Western Mediterranean Basin (Italy and Spain), it was shown the presence of isolates of eight different virus variants of the TYLCV complex occurring simultaneously in the epidemics (Figure 3) (García-Andrés et al., 2007a). Coexistence of isolates corresponding to different virus variants in single fields and even mixed infections in single plants are suggested, which is a prerequisite for recombination to occur. In fact, analysis of begomovirus-related sequences present in single samples demonstrated that several virus strains could coexist (e.g., in sample T570/02:F4:Sic; Figure 4). Therefore, opportunities for genetic exchange are evident, and appearance of novel variants as a result of recombination events can be predicted. As mentioned before, begomovirus replication involves two mechanisms, a RCR (Saunders et al., 1991; Stenger et al., 1991) and a RDR (Jeske et al., 2001; Preiss & Jeske, 2003). Recombinant variants can be produced through the latter mechanism if viruses coexist in the same cell. If viable and competitive, these de novo created recombinant viruses can emerge and perpetuate in the population during epidemics.

Recent introduction of begomoviruses into new areas provided an ideal model to analyze aspects of genetic adaptation and evolution of an invading virus population. This was the case of the colonization of southern Spain by begomoviruses of the TYLCV complex associated with TYLCD, which is well documented (Sánchez-Campos et al., 1999, 2002). This case is an interesting example of invasion success following multiple introductions, similar to those reported for animal or plants (Novak & Mack, 2001; Kolbe et al., 2004), in which recombination is providing tools for biological adaptation. Initial colonization with isolates of the ES strain of TYLCSV during the early 1990s, resulted in a relatively stable population in which reduced genetic diversity was observed, a typical result of a population bottleneck upon invasion of a new area (Sánchez-Campos et al., 2002). This could have been detrimental for the success of the invader begomovirus population. However, subsequent introductions of isolates of the type and Mld strains of TYLCV (Navas-Castillo et al., 1999; Morilla et al., 2003) resulted in novel sources of variation, and conditions for recombination to occur, thus providing to the begomovirus population tools to gain potential for local adaptation. This was the case of the novel recombinant variant named Tomato yellow leaf curl Málaga virus (TYLCMalV) that
Figure 3. Phylogenetic relationships for *Tomato yellow leaf curl disease* (TYLCD)-associated begomovirus isolates present in tomato samples randomly collected in Italy (italics/bold letters) and Spain (normal letters) between 1999 and 2003. Relationships were inferred based on a sequence of about 300 nucleotides encompassing the intergenic region (IR) by neighbor-joining analysis. Support for nodes in a bootstrap analysis with 1,000 replications is shown for figures over 500. Horizontal branch lengths are drawn to scale with the bar indicating 0.1 nucleotide replacements per site. Vertical distances are arbitrary. Names of isolates refer to host species origin (T, tomato), sample number/year, field (Fi means field i), and sampling region (Sicily, Sic, and Sardinia, Sar, in Italy; Málaga, Mlg, Almería, Alm, and Murcia, Mur, in Spain). Representative isolates are included of begomovirus species associated with TYLCD in the Mediterranean area, the type, Sic and ES strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV), type (isolates from Israel, Spain, and Italy) and Mld strains of *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl Málaga virus* (TYLCMaLV), and *Tomato yellow leaf curl Axarquía virus* (TYLCAxV) (GenBank accession numbers X61153, Z28390, Z25751, X15656, AJ489258, DQ144621, AF071228, AF271234, and AY227892, respectively) (boxed and bold letters). As outgroup, an isolate of *Tomato leaf curl virus* (ToLCV) was used (GenBank S53251).
Recombination in the TYLCV Complex

129

emerged as a result of a genetic exchange between isolates of the ES strain of TYLCSV and of the Mld strain of TYLCV. This natural recombinant variant showed to be better adapted ecologically than either parental virus and spread rapidly in the population (Monci et al., 2002). Recently, a novel recombinant between TYLCSV-ES and the type strain of TYLCV was detected in the population, which also seemed to be well adapted ecologically (García-Andrés et al., 2006). Therefore, recombination showed to be an important force driving the evolution of the population of these viruses for adaptation to the novel ecological conditions present in the invaded area.

3.2. Wild hosts: reservoirs of mixed infections for begomovirus epidemics

Native species, acting as reservoirs, can play an important role in the emergence of plant virus epidemics (Hull, 2002). For begomoviruses, studies are available that indicate presence in wild reservoirs (Funayama-Noguchi, 2001; Jovel et al., 2004; Ooi et al., 1997; Roye et al., 1997). To evaluate the possible importance of wild reservoirs as sources of begomovirus genetic diversity for epidemics, the begomovirus population present in the Solanum nigrum L. plant community found in southern Spain was examined. S. nigrum is a wild host widely distributed in the Mediterranean area, which can survive for long periods (even for more than 2 years) thanks to the mild climatic conditions present. Infections with

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Figure 4. Restriction fragment length polymorphism analysis with the restriction enzyme Bgl II on 10 almost full-length genome clones of Tomato yellow leaf curl disease-associated (TYLCD) begomoviruses present in sample T570/02:F4:Sic. The almost full-length genome fragments were PCR-amplified from total nucleic acids obtained from the sample by means of a primer pair designed on nucleotide sequences conserved among all the TYLCD-associated viruses reported from the Western Mediterranean Basin: MA241 (5′-GAATGGGCTTCCCATACTTTGTGTTGC-3′), corresponding to 1739 to 1765 nt of TYLCSV-ES[ES:1:92] (GenBank Z25751), and MA242 (5′-CAC-TATCTTCTCTGCAATCCAGG-3′), complementary to 1,719 to 1,696 nt of this same virus. PCR fragments thus obtained were cloned into pGEM-T (Promega Corporation, Madison, USA) to derive the clones analyzed here (lanes 1–10). Equivalent clones obtained from samples infected with known isolates of the type and Sic strains of Tomato yellow leaf curl Sardinia virus (TYLCSV) (lanes 11 and 12, respectively) and of the type strain of Tomato yellow leaf curl virus (TYLCV) (lane 13) were used as controls. A 1Kb molecular marker (lane M) was included.
TYLCD-associated begomoviruses are known in this plant species (Bedford et al., 1998; Salati et al., 2002; Sánchez-Campos et al., 2000). Our studies indicated that this wild host is an excellent reservoir of variants of viruses of the TYLCV complex for TYLCD epidemics. In fact, phylogenetic reconstruction of sequences of begomoviruses obtained from *S. nigrum* plants sampled in the Málaga region (southern Spain) between 2000 and 2003 demonstrated the presence of isolates of all the TYLCD-associated begomoviruses species and strains reported in Spain (Figure 5). Moreover, mixed infections in single *S. nigrum*
plants were evident, as observed for example in sample Sn8:00, in which TYLCSV-like and TYLCV-like sequences were detected (e.g., isolates ES:Sn8-1:00, ES:Sn8-2:00, respectively, Figure 5). Therefore, *S. nigrum* plants can be an optimal niche for genetic exchanges to give rise to better-adapted recombinant begomoviruses. In fact, we demonstrated the presence in this host species of isolates of a new previously undescribed begomovirus of recombinant nature, named *Tomato yellow leaf curl Axarquía virus*, TYLCAxV (e.g., isolate ES:Sn1:03, Figure 5). This virus variant was demonstrated to be the result from a genetic exchange between isolates of the ES strain of TYLCSV and of the type strain of TYLCV (found coinfecting *S. nigrum* plants, Figure 5). Novel pathogenic properties are demonstrated for TYLCAxV that suggested enhanced ecological adaptation (García-Andrés et al., 2006). We concluded therefore that presence of mixed begomovirus infections in wild reservoirs can be a cause for alarm, because novel recombinants might arise with unpredictable consequences for epidemics of viruses of the TYLCV complex.

4. RECOMBINANTS OCCUR FREQUENTLY IN MIXED INFECTIONS OF VIRUSES OF THE TYLCV COMPLEX

Sequence analyses of field isolates have revealed substantial evidence for widespread occurrence of recombination amongst begomoviruses (Padidam et al., 1999; Sanz et al., 2000; Berrie et al., 2001; Pita et al., 2001; Monci et al., 2002; Chatchawankanphanich & Maxwell, 2002). However, it remains unclear whether recombination represents a frequent phenomenon shaping begomovirus populations during a single host plant infection life cycle. We investigated this aspect for infections with TYLCSV and TYLCV as model system (García-Andrés et al., 2007b). These two viruses coinfect single plants in nature (Sánchez-Campos et al., 1999; Monci et al. 2002; García-Andrés et al., 2006), and even could share single nuclei of an infected plant (Morilla et al., 2004), a prerequisite for recombination to occur. Natural mixed infections were simulated in tomato and the frequency of recombinant genomes was evaluated at several times post coinfection. We found that recombinant-like molecules accumulated in the virus progeny of mixed-infected plants and rapidly constituted a significant proportion of the population (in most cases about 50% of the genomes analyzed, Figure 6A). We also found that parent TYLCSV and recombinant variants generated *de novo* coexisted, suggesting that the latter fit well in the population and were not outcompeted. However, at least in the experimental conditions analyzed, TYLCV was outcompeted, suggesting that it is less adapted to compete *in planta* with either TYLCSV or the recombinants arisen. This was surprising because TYLCV seems to be well adapted to compete during natural epidemics (Sánchez-Campos et al., 1999). Therefore, other factors in addition to competitiveness *in planta* are associated with the success of a begomovirus variant in nature (transmission, host range, etc.). Interestingly, only
Figure 6. Appearance and frequency of recombinant genomes generated de novo after co-inoculation of the [ES:1:92] isolate of *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES) (Navas-Castillo et al. 1999) and the [ES:72:97] isolate of *Tomato yellow leaf curl virus* (TYLCV-Mld) (Noris et al. 1994) in a single tomato cv. Moneymaker plant. Studies were based on restriction fragment length polymorphism analysis on clones containing full-length genome fragments amplified from virus population present in total nucleic acids extracted from the mixed-infected plant at several times post co-inoculation using the commercial kit TempliPhi (Amersham Biosciences, England). The amplified DNA was digested with the restriction enzyme BamHI that singly cut either TYLCSV-ES[ES:1:92] or TYLCV-Mld[ES:72:97] double-stranded DNA forms in equivalent genome positions, and the linearized genome size DNA fragments were cloned into the BamHI-cloning site of pBluescript SK+ (pBSK+, Stratagene, La Jolla, CA). Twenty-five clones per time post-inoculation studied were analyzed. The evolution of the relative proportion of parental (TYLCV and TYLCSV) and recombinant-like variants (Rec) (A) and the frequency of the different recombinant variants found (variants X, IV, and XII) (B) at different times post-inoculation analyzed is shown. For restriction mapping, enzymes were selected (BglII, BglII, DraIII, EcoNI, HindIII, KpnI, PmlI, SacII, and SphI) that used in single combinations provide information about TYLCSV or TYLCV sequence identity at different positions along the cloned genome.
three types of recombinant variants could be recovered in the plant coinfected with TYLCV and TYLCSV during the 400-day infection cycle analyzed. Therefore, constraints for recombination seemed to exist in these viral genomes. Moreover, frequency of the different recombinant variants found in the population could vary with time but, at least in the experimental conditions used, one type predominated through the entire experiment (type IV, Figure 6B). Although additional studies are needed to better understand the significance of recombination in single host infection cycles in this group of viruses, these data suggested that recombination seems to be a frequent phenomenon and could contribute significantly in generating genetic diversity and novel virus variants for local adaptation.

5. IMPLICATIONS OF RECOMBINATION FOR VIRUS CONTROL THROUGH PLANT RESISTANCE

Given the importance of recombination in the molecular evolution of viruses promoting biological adaptation, understanding the frequency at which it occurs, mechanisms involved, and ecological features that control the rate of recombination, might help to predict the emergence of new viruses. This knowledge can be used to improve effectiveness and durability of current control procedures (Bonnet et al., 2005; Lewis-Rogers et al., 2004). Efficient control of plant virus diseases is difficult, however the use of virus-resistant cultivars can provide an effective mean to limit the economic damage caused. Although the use of resistance is the most desirable plant virus control strategy, it often fails because resistance-breaking virus genotypes appear and increase their frequency in the virus population (Lecoq et al., 2004; García-Arenal et al., 2001). The durability of resistance is determined by the evolutionary potential of plant viruses (García-Arenal & McDonald, 2003) and recombination is one of the major forces driving virus evolution. In this regard, recombination events have been demonstrated to be associated with major changes in fitness and pathogenic characteristics of plant viruses, including expansion of their host range and increase in their virulence (Fernández-Cuartero et al., 1994; Stenger et al., 1994; Pita et al., 2001; Zhou et al., 1997; Gibbs et al., 2001; Monci et al., 2002; García-Arenal & McDonald, 2003; Rest & Mindell, 2003; García-Andrés et al., 2006). Thus, recombination can accompany or even be at the origin of major changes during virus adaptation. In fact, recombination is known to be a potent mechanism to create more fit genotypes (Bürger, 1999; Hu et al., 2003), that can help viruses to adapt to novel environmental conditions (Dybdahl & Storfer, 2003; Lively & Dybdahl, 2000; Stavrinides & Guttman, 2004; Zhou et al., 1997). Therefore, the risk of break of a begomovirus resistance owe to the appearance and spread of better-adapted recombinant variants exists and should be considered to predict the durability of a resistance.

The abundance of recombinant variants in a virus population should also be kept in mind for the evaluation of the potential impact of recombination in the
use of transgenic plants expressing viral sequences (Harrison, 2002; Aaziz & Tepfer, 1999b; Tepfer, 2002; de Wispelaere et al., 2005). The virus-resistant transgenic plants (VRTPs) hold the promise of enormous benefit for agriculture, however, questions concerning the potential ecological impact have been raised (Tepfer, 2002). Numerous transgenic crops resistant to a wide range of viruses have been developed (Beachy, 1997), many of them based on the application of the concept of pathogen-derived resistance (Sanford & Johnston, 1985). Different virus sequences have been used for the development of virus-derived transgenic resistance, including genes encoding coat proteins, replicases, movement proteins, proteases, or helper components (Lomonossoff, 1995). However, it is important to examine VRTP carefully and take into account the risk of the deployment from the point of view of biosafety. Interactions are possible in transgenic plants between products of the viral transgene (whether DNA, RNA, or protein) and an incoming virus, which can result in potential ecological risks like synergism, heteroencapsidation, or recombination (Tepfer, 1993, 2002; Robinson, 1996; Aaziz & Tepfer, 1999a). It has been demonstrated that recombination of a challenging virus with a transgene could have important biological consequences such as changes in virulence or host range (Kiraly et al., 1998; Frischmuth & Stanley, 1998). Therefore, if as mentioned above RDR occurs during geminivirus multiplication within plants, transgenic constructs that provide information for symptom expression, host range, tissue and vector specificities should be avoided (Jeske et al., 2001). In this sense, it is a fortunate coincidence that the resistance strategy that uses defective interfering DNAs as control elements was successful for geminiviruses (Frischmuth & Stanley, 1993; Jeske et al., 2001).

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