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The non-canonical tomato yellow leaf curl virus recombinant that displaced its parental viruses in Southern Morocco exhibits a high selective advantage in experimental conditions

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The non-canonical tomato yellow leaf curl virus recombinant that displaced its parental viruses in Southern Morocco exhibits a high selective advantage in experimental conditions

Short title: Selective advantage of an emerging TYLCV recombinant

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Abstract

Recombination events are frequently inferred from the increasing number of sequenced viral genomes, but their impact on natural viral populations has rarely been evidenced. TYLCV-IS76 is a recombinant (*Begomovirus*, *Geminiviridae*) between the Israel strain of Tomato yellow leaf curl virus (TYLCV-IL) and the Spanish strain of Tomato yellow leaf curl Sardinia virus (TYLCSV-ES) that was generated most probably in the late 1990s in Southern Morocco (Souss). Its emergence in the 2000s coincided with the increasing use of resistant tomato cultivars bearing the *Ty-1* gene, and led eventually to the entire displacement of both parental viruses in the Souss. Here, we provide compelling evidence that this viral population shift was associated with selection of TYLCV-IS76 viruses in tomato plants and particularly in *Ty-1*-bearing cultivars. Real-time qPCR monitoring revealed that TYLCV-IS76 DNA accumulation in *Ty-1*-bearing plants was significantly higher than that of representatives of the parental virus species in single infection or competition assays. This advantage of the recombinant in *Ty-1*-bearing plants was not associated with a fitness cost in a susceptible, nearly isogenic, cultivar. In competition assays in the resistant cultivar, the DNA accumulation of the TYLCV-IL clone—the parent less affected by the *Ty-1* gene in single infection—dropped below the qPCR detection level at 120 days post-infection (dpi) and below the whitefly vector (*Bemisia tabaci*) transmissibility level at 60 dpi. The molecular basis of the selective advantage of TYLCV-IS76 is discussed in relation to its non-canonical recombination pattern, and the RNA-dependent RNA polymerase encoded by the *Ty-1* gene.

Introduction

Genetic exchange via recombination has been reported frequently for both DNA and RNA viruses (Lefeuvre & Moriones, 2015; Wain-Hobson *et al.*, 2003; Worobey & Holmes, 1999). The variation generated by mutation and recombination constitutes the raw material on which natural selection and genetic drift act to shape populations, and is essential for virus adaptation in changing environments. Most population shifts have been reported to involve variants that differ from the preceding populations by mutations that did not necessarily involve recombination events. This is, for example, the case in population shifts detected at the host level in patients infected with human immunodeficiency virus type 1 (HIV-1) and treated with reverse transcriptase or protease inhibitors (Condra *et al.*, 1995; Larder, 1994; Richman *et al.*, 1994); parallel mutations detected in viral variants isolated from such patients

are consistent with positive selection under drug pressure. At the regional level, population shifts may be caused either by accidently imported variants, for example the Asian 1 lineage of dengue virus (DENV) in Viet Nam (Hang *et al.*, 2010), or by variants arising locally through evolutionary processes involving positive selection or genetic drift, for example the West Nile virus WN02 genotype that displaced the NY99 genotype in the United States between 2001 and 2004 (Moudy *et al.*, 2007). Viruses of the emerging WN02 genotype were thought to have derived from the NY99 genotype by mutations. Both in Dengue and West Nile viruses, the selective hypothesis proposed to explain the population shift was supported by experimental results that revealed differential virus accumulation between lineages for DENV and differential efficiency of mosquito transmission between genotypes for WNV.

In some rare cases, population shifts have been reported to involve recombinant viruses (Belabess *et al.*, 2015; Monci *et al.*, 2002; Shi *et al.*, 2013), and two such cases are derived from viruses of the genus *Begomovirus* (*Geminiviridae*), i.e. TYLCV and TYLCSV of the species *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus*, respectively. Following introduction of the Mild strain of TYLCV (TYLCV-Mld) into Spain, two tomato-infecting begomoviruses co-circulated in this country: TYLCV-Mld and the resident Spanish strain of TYLCSV (TYLCSV-ES). One year after the first detection of the invading TYLCV-Mld in 1998, a TYLCV-Mld/TYLCSV-ES recombinant was detected: TYLCMalV (species *Tomato yellow leaf curl Malaga virus*) (Monci *et al.*, 2002). This variant displaced TYLCV-Mld viruses in bean in the Almeria province, with 76% of monitored plants (64/84) being infected only with TYLCMalV viruses. It was proposed that its extended host range, which included bean—a species that could not be infected with TYLCSV—and two non-cultivated Solanaceous species that could not be infected with TYLCV, might have provided a selective advantage to the recombinant TYLCMalV (García-Andrés *et al.*, 2007a). The second population shift associated with a recombinant was reported from Morocco with the recombinant TYLCV-IS76 (Belabess *et al.*, 2015). Here again, TYLCSV was involved as the parental virus but recombination occurred with the Israel strain of TYLCV (TYLCV-IL). TYLCV-IS76 exhibited a non-canonical recombination pattern with a 76-nt TYLCSV-derived fragment that was much shorter than that of TYLCV/TYLCSV recombinants reported previously (Davino *et al.*, 2009, 2012; García-Andrés *et al.*, 2007b; Monci *et al.*, 2002). TYLCV-IS76 was not detected between 1999 and 2003 when TYLCV-IL and TYLCSV were co-circulating in Morocco, but was found to have completely displaced its parental viruses in Southern Morocco (Souss) by 2012. Interestingly, the period during which TYLCV-IS76 passed from low frequency (below detection level) to the replacement of its parental viruses

in the Souss (2004–2012), coincides with the period during which susceptible tomato cultivars were replaced to a large extent with cultivars bearing the *Ty-1* resistance gene. Likewise, the partial displacement of TYLCSV by the invading TYLCV in Spain has coincided with the general deployment of Ty-1 resistant cultivars (Garcia-Andrés *et al.*, 2009). In both cases, it is thought that the population shift may have been driven by the selection exerted by tomato cultivars bearing the *Ty-1* gene associated with hindrance of intra-plant viral accumulation (Michelson *et al.*, 1994). In the case of the displacement observed in Spain, the selective hypothesis has been supported by intra-plant viral DNA accumulation data showing that the *Ty-1* gene was more effective with TYLCSV than with TYLCV (Garcia-Andrés *et al.*, 2009). In the case of the displacement observed in Morocco, selection has been favored over drift as the main evolutionary mechanism, since tomatoes are grown year-round in the Souss, maintaining high viral populations and preventing narrow bottlenecks.

Hence, the objective of this study was to confirm the selective hypothesis by testing, under experimental conditions, whether the fitness of TYLCV-IS76 is higher than that of representatives of its parental virus species, TYLCV-IL and TYLCSV-ES. Taking viral DNA accumulation as a proxy for fitness, we show here with real time PCR that intra-tomato plant accumulation of TYLCV-IS76 recombinants was significantly higher than that of the parental viruses in a *Ty-1*-bearing cultivar, irrespective of the infection status (single- or multiple infection) or the number of days post-infection (dpi) at which plant samples were collected (10–120 dpi). Moreover, the fitness advantage in the resistant cultivar was not associated with a fitness cost in a susceptible nearly isogenic cultivar. Taken together, the results support the hypothesis that the viral population shift observed in the Souss has been driven by positive selection in the resistant tomato cultivars. The most striking feature of the TYLCV-IS76 phenotype is the drastic and deleterious effect on the accumulation of TYLCV-IL viruses, which dropped below both the qPCR detection level and the transmissibility level, as assessed with the whitefly vector *Bemisia tabaci* (Gennadius, 1889). Using a genetically engineered virus recombinant, we show that the non-canonical recombination pattern of TYLCV-IS76 is the major determinant of its selective advantage.

Results

Fitness advantage of TYLCV-IS76 in grafted and non-grafted Ty-1-resistant tomato plants

The population shift that resulted in the complete dominance of the recombinant TYLCV-IS76 in the Souss coincided with the increased use of Ty-1-resistant plants, which is thought

to have positively selected for this recombinant viral strain (Belabess *et al.*, 2015). The "selection" hypothesis was tested here by assessing the viral DNA accumulation of a TYLCV-IS76 clone in a Ty-1-resistant plant and comparing it to that of representatives of the parental species, i.e. TYLCV-IL and TYLCSV-ES clones. The Ty-1-resistant tomato cultivar Pristyla was used because it is grown in more than half of the Souss area cultivated with Ty-1-resistant cultivars of round-shaped tomatoes. As all the tomato plants grown by farmers in the Souss are grafted, Pristyla was tested not only as seedlings but also as grafted plants. Maxifort was used as the rootstock because it is the most commonly used rootstock in the Souss. The plants were agroinoculated with TYLCV-IS76, TYLCV-IL and TYLCSV-ES clones in various combinations.

The inoculation of 20 grafted Pristyla plants per viral clone was 100% successful for the three agroinfectious clones (Table 1; experiment 1). None of the infected plants exhibited the Tomato yellow leaf curl (Tylc) symptoms induced by these viral clones in susceptible tomato plants. For convenience, viral DNA accumulation of a viral clone, for example the TYLCV-IL clone, is hereafter referred to as DNA accumulation of TYLCV-IL. In competition assays, DNA accumulation of TYLCV-IS76 was significantly higher than that of both parental viruses at 30 dpi (Fig. 1). The most striking differences were detected between TYLCV-IS76 and TYLCV-IL, with a mean accumulation ratio [TYLCV-IS76/TYLCV-IL] of 700 in the triple infection treatment, and 500 in double infection. The mean accumulation ratio [TYLCV-IS76/TYLCSV-ES] was about 10 in both triple- and double infection treatments. In single infection treatments, the DNA accumulation of TYLCV-IS76 was also higher than those of TYLCV-IL and TYLCSV-ES, which indicated that the fitness advantage of the TYLCV-IS76 clone revealed in competition assays is not associated with an accumulation cost in single infection. By comparing the viral DNA accumulation of each virus between single- and competition infections, contrasting interactions were detected. Whereas the mean DNA accumulations of TYLCV-IS76 and TYLCSV-ES were similar or higher in competition assays than in single infection, the mean DNA accumulation of TYLCV-IL in double- or triple infection with TYLCV-IS76 was more than 100 times lower than that in single infection. The deleterious effect on the TYLCV-IL clone was detected already at 10 dpi, with mean DNA accumulation ratios [single/competition infections] of 4 and 8 in double- and triple infection, respectively (results not shown). Interestingly, co-infection was always profitable to the DNA accumulation of TYLCSV-ES and, unlike the TYLCV-IS76 clone, TYLCSV-ES clone did not exhibit any deleterious effect on the TYLCV-IL clone.

Like for the grafted Pristyla plants, agroinoculation of non-grafted Pristyla plants was 100% successful and did not induce any Tylc symptoms, irrespective of the agroinfectious clone (Table 1; experiment 2). The DNA accumulation patterns determined with non-grafted Pristyla plants were similar to those determined with grafted plants (compare Figs. 1 and 2a). Thus, the DNA accumulation of TYLCV-IS76 was significantly higher than those of TYLCV-IL and TYLCSV-ES, irrespective of infection status (single- or competition infections) or collection time of the samples (10, 20, 30, 60 or 90 dpi), which confirms the fitness advantage of TYLCV-IS76. Interestingly, whereas the differential of DNA accumulation between the three viruses tended to increase over time in the competition infection, with, for example, an increase in the mean accumulation ratio [TYLCV-IS76/TYLCV-IL] from 15 at 10 dpi to 3600 at 60 dpi, the differential tended to decrease in single infections with, for example, a decrease in the mean accumulation ratio [TYLCV-IS76/TYLCSV-ES] from 1000 at 10 dpi to 10 at 90 dpi. These differing accumulation dynamics between the single- and competition infections were due mainly to the DNA accumulation of TYLCV-IL in triple-infected plants, which was drastically lower than that in single-infected plants, irrespective of sampling time. The deleterious effect was strong enough to be detectable at an early stage of infection, with a TYLCV-IL DNA accumulation ratio [single-/competition infection] of 10 at 10 dpi; the deleterious effect apparently did not decrease over time according to the 60 dpi ratio of mean accumulations, which was 1500. Unlike DNA accumulation of TYLCV-IL, DNA accumulations of TYLCV-IS76 and TYLCSV-ES were not much affected by co-infection, and their accumulations were not significantly different between single- and triple-infected plants at the latest common collection date, i.e., 60 dpi.

Taken together, the DNA accumulation patterns of TYLCV-IS76 and parental viruses in Pristyla plants (Figs. 1 and 2a) were consistent with the hypothesis of a critical role played by Ty-1 resistant plants in the emergence, and eventually the complete dominance, of TYLCV-IS76 viruses in the Souss.

The fitness advantage of TYLCV-IS76 in Ty-1-resistant plants is not associated with a fitness cost in susceptible plants

To further confirm the selective hypothesis of displacement of the parental viruses by TYLCV-IS76 recombinant viruses in the Souss agroecosystem, we tested if the fitness advantage of TYLCV-IS76 in Ty-1-resistant plants could be associated with any cost with

respect to its DNA accumulation in susceptible non-*Ty-1*-bearing plants. Hence, the DNA accumulations of the three viruses were tested in a susceptible cultivar that is nearly isogenic with Pristyla. For this and the following tests, non-grafted tomato plants were used as a more straightforward model that did not require the assistance of a commercial nursery. This simplification was possible because, as reported above, the fitness advantage of TYLCV-IS76 seen in grafted Pristyla plants was also found in non-grafted Pristyla plants (compare Figs. 1 and 2a). Thus, the resistant and susceptible non-grafted tomato plants were agro-inoculated in the same experiment with the TYLCV-IS76, TYLCV-IL and TYLCSV-ES clones, and leaf samples were collected between 10 and 90 dpi (Table 1; experiment 2).

The Tylc symptoms induced by TYLCV-IS76 in plants of the susceptible cultivar were similar to those induced by TYLCV-IL and TYLCSV-ES. The viral DNA contents of the susceptible plants were significantly higher than those of the resistant plants for the three viruses (compare Fig. 2a and Fig. 2b, p-value< 2.2e-16, for single- and triple-infected plants). The DNA accumulation of TYLCV-IS76 in the susceptible cultivar was either higher than those of both parental viruses or similar to that of the parental virus exhibiting the highest DNA accumulation (Fig. 2b). Hence, the fitness advantage of TYLCV-IS76 in Pristyla was not associated with an apparent cost with respect to its accumulation in the susceptible cultivar. In spite of its high accumulation in the susceptible plants, TYLCV-IS76 DNA accumulation was relatively less affected than those of parental viruses in the Ty-1-resistant cultivar. Indeed, the mean DNA accumulation ratios between susceptible plants and resistant plants were always lower for TYLCV-IS76 than for parental viruses, irrespective of infection status, and date of sampling, except in the single infection treatment at 30 dpi where the ratios were similar between TYLCV-IL and TYLCV-IS76 clones, around 29. Interestingly, the mean DNA accumulation ratios [TYLCV-IS76/parental virus (TYLCV-IL or TYLCSV-ES)] were highest in samples of the earliest collection dates (10 dpi or 20 dpi) in both the competition- and single infection assays in the resistant cultivar, which suggests that TYLCV-IS76 has an advantage at the onset of infection. It is noteworthy that competition is deleterious to the accumulation of TYLCV-IL DNA in susceptible plants although not as drastically as in resistant plants. Indeed, the ratio of TYLCV-IL DNA mean accumulations between single- and triple-infected plants at 10, 30 and 60 dpi was 10, 10 and 80 in susceptible plants, and 11, 42 and 1500 in resistant plants, respectively.

The fitness advantage of TYLCV-IS76 is related to the Ty-1 resistance gene

To test if the fitness advantage of TYLCV-IS76 viruses in Pristyla was due mainly to the *Ty-1* gene rather than to the genetic background of the cultivar Pristyla or its specific interaction with the *Ty-1* gene, we used the cultivar "F", which has the same *Ty-1* allele as Pristyla but in a different genetic background. As the major feature of the fitness advantage of TYLCV-IS76 was its deleterious effect on DNA accumulation of TYLCV-IL, the test was limited to three treatments: single infection with the TYLCV-IL clone, single infection with the TYLCV-IS76 clone, and a competition test with both these clones; five F plants were infected for each treatment and sampled at 30 dpi (Table 1; experiment 3). DNA accumulation of TYLCV-IS76 was significantly higher than that of TYLCV-IL in the single- and competition infections (Wilcoxon test, *P*-value = 0.007) (Fig. 3), and the mean accumulation ratios [TYLCV-IS76/TYLCV-IL] were of the same order of magnitude as those detected with Pristyla, i.e., 200 in co-infected plants and 6 in single-infected plants. These results support the view that the selective advantage of TYLCV-IS76 over TYLCV-IL was due primarily to the *Ty-1* gene that both cultivars have in common, and only marginally dependent (if at all) on their different genetic background.

From 60 dpi, Pristyla tomato plants tend to be dead ends for TYLCV-IL when co-infected with TYLCV-IS76

The deleterious effect of TYLCV-IS76 viruses on TYLCV-IL viruses in Pristyla plants was observed until 60 dpi (Fig. 2a). To test if it is maintained beyond this date, and might result in the elimination of TYLCV-IL viruses, viral DNA accumulations were further monitored up to 120 dpi (Table 1; experiment 4). Fifteen Pristyla plants double-infected with the TYLCV-IL and TYLCV-IS76 clones, and 20 plants triple-infected with the TYLCV-IL, TYLCV-IS76 and TYLCSV-ES clones were sampled at 10, 20, 30 dpi, and 9 of them were sampled randomly at 120 dpi. Fifteen plants infected with the TYLCV-IL clone alone and 15 plants co-infected with the TYLCV-IL and TYLCSV-ES clones were tested in parallel as negative controls in which TYLCV-IL viruses were not subjected to the deleterious effect of TYLCV-IS76 viruses. As in the previous experiments (Figs. 1–3), DNA accumulation of TYLCV-IL was drastically affected by the presence of TYLCV-IS76 in both the double- and triple-infected plants (Fig. 4). Thus, at 30 dpi, DNA accumulation of TYLCV-IL in these plants was more than 200 times lower than that of TYLCV-IL in plants without TYLCV-IS76. Interestingly, the amount of TYLCV-IL DNA dropped below the detection level at 120 dpi in plants co-infected with TYLCV-IS76 in both competition treatments; it remained detectable in only one of the nine triple-infected plants tested. In the plants infected only with TYLCV-

IL viruses, the mean DNA accumulation at 120 dpi was similar to that at 30 dpi. According to the 120 dpi samplings, it seems that TYLCSV-ES has also a negative impact on the DNA accumulation of TYLCV-IL, as the mean DNA accumulation of TYLCV-IL in competition with TYLCSV-ES was significantly lower than that of TYLCV-IL in single infection.

It was expected that the negative impact of TYLCV-IS76 viruses on TYLCV-IL viruses may compromise the transmission of TYLCV-IL from coinfected plants by the whitefly vector *B. tabaci*. A preliminary transmission test was conducted with two Pristyla plants in which the DNA accumulation of TYLCV-IS76 were 2500 and 4000 times higher than that of TYLCV-IL, respectively at 56 dpi. TYLCV-IS76 was the only virus detected among infected test plants (6/15 plants of the susceptible cultivar and 3/15 plants of the resistant cultivar).

Recombination is the determinant of the antagonistic impact of TYLCV-IS76 on TYLCV-IL

It was inferred from Bayesian analysis that the recombination event leading to this TYLCV-IS76 viruses occurred most probably in the late 1990s (Belabess *et al.*, 2015). Therefore, as the genuine parents of TYLCV-IS76 viruses were not available, the clones used as representatives of the parental species in the experimental studies were selected among those that exhibited the highest nucleotide identity with them. Thus, the TYLCV-IL-derived fragment genome of the TYLCV-IS76 clone differed from that of the selected TYLCV-IL clone by 27 mutations. Conversely, the TYLCSV-ES-derived fragment genome of the TYLCV-IS76 clone differed from that of the selected TYLCSV-ES clone by two mutations. Hence, to determine if recombination alone may have provided a fitness advantage to the TYLCV-IS76 recombinant over its genuine and unknown parental TYLCV-IL, we used the agroinfectious clone TYLCV-IL to engineer TYLCV-IS76'—a recombinant with the same recombination profile as TYLCV-IS76 but which is 100% identical to the genome of its parental TYLCV-IL in its TYLCV-IL-derived region and 100% identical to the genome of the clone TYLCV-IS76 in its TYLCSV-ES derived region.

The infectivity of the engineered TYLCV-IS76' was similar to that of its parental clone TYLCV-IL in both cultivars: TYLCV-IS76' and TYLCV-IL infected 100% of inoculated Pristyla plants (20 and 15 inoculated plants respectively), and 100% of nearly isogenic susceptible plants (5 for each clone) (Table 1; experiments 5 and 6). TYLCV-IS76' produced the typical Tylc symptoms of TYLCV-IL in the susceptible cultivar. Moreover, in single-infected resistant plants, the DNA accumulation of TYLCV-IS76' was similar to that of TYLCV-IL at 30 dpi (Fig. 5), which, altogether indicates that recombination at position 76

had no negative impact on infectivity or viral DNA accumulation of the engineered recombinant in comparison to its TYLCV-IL progenitor. Consistently, the sequence of four full-length TYLCV-IS76' genomes cloned from a 30 dpi plant co-infected with the TYLCV-IL clone were 100% identical to that of the agroinoculated clone. In competition tests, the DNA accumulation of TYLCV-IS76' was significantly higher than that of its progenitor TYLCV-IL in both cultivars at 30 dpi. Taken together, and, similarly to the wild type TYLCV-IS76 (Figs 1-3), the fitness advantage of the TYLCV-IS76' revealed in competition assays with TYLCV-IL is not associated with a cost with respect to its accumulation in single infection. As a control, Pristyla plants were co-infected in parallel with the wild type TYLCV-IS76 and TYLCV-IL (Fig. 5). Interestingly, the DNA accumulation of TYLCV-IL was similarly low in both competition tests, indicating that the 27 discriminating mutations between the engineered and the wild type recombinants had no significant impact on the deleterious effect induced by recombination. However, the slight but significant difference in virus accumulation between the natural Moroccan recombinant and the recombinant engineered with a TYLCV-IL clone from Réunion is determined obviously by mutations (Fig.5).

Discussion

The TYLCV-IS76 recombinants that have emerged in the Souss region of Southern Morocco have virtually replaced their parental viruses since the deployment of Ty-1-cultivars in the 2000s. The experimental fitness comparisons of the recombinant and parental type viruses presented here provide compelling results to support the selective advantage of TYLCV-IS76 over parental type viruses and, most importantly, its selection by Ty-1-cultivars without any detectable fitness cost in non-*Ty-1*-bearing cultivars.

Measuring nucleic acid accumulation has already been used to show the fitness advantage of a cucumber mosaic virus recombinant produced under greenhouse conditions (Fernandez-Cuartero *et al.*, 1994). However, although natural emerging recombinants have been previously reported (Davino *et al.*, 2009; García-Andrés *et al.*, 2006; Monci *et al.*, 2002; Shi *et al.*, 2013; Zhou *et al.*, 1997), a mechanistic basis for such population shifts has only been proposed with TYLCMalV, the recombinant which partially displaced its parental viruses in bean. It was reported to have a slightly higher infectivity in Ty-1 resistant tomato plants, and an extended host range in comparison with those of representatives of its parental species (Monci *et al.*, 2002). However, its transmission efficiency from single-infected plants was not

higher than that of its parents, and its viral DNA accumulation assessed from dot blot hybridizations was only 14% of TYLCV—the parent exhibiting the highest accumulation. Similar results were obtained with two TYLCV-IL/TYLCSV-Sar recombinant viruses from Italy exhibiting the same recombination profiles as TYLCAxV—the other TYLCV/TYLCSV recombinant from Spain (García-Andrés *et al.*, 2006)—and TYLCMalV; their transmission efficiency was not higher than that of the parental viruses, and their replication in agroinfiltrated *Nicotiana benthamiana* leaves was estimated to be about 10 times lower than that of a representative clone of the parental TYLCSV species (Davino *et al.*, 2009). The low accumulation of these canonical recombinants may explain why plants infected only by such recombinants were not detected in Italy, and why the replacement of parental viruses by recombinants has not been reported from Spain.

According to a host range study on tomato, common bean (cv. Contender) and *Solanum nigrum*, the TYLCV-IS76 clone did not exhibit any extended host range compared to that of the parental clones (data not shown).

Contrasted efficiency of the Ty-1 gene to control TYLCV-IS76 and its parental viruses

The viral DNA contents of the susceptible plants were significantly higher than those of the resistant plants for the three viruses (compare Figs. 2a and 2b). This result is consistent with the reported negative impact of the *Ty-1*-resistance gene on viral DNA accumulation previously monitored by southern- or dot-blot hybridization (Barbieri *et al.*, 2010; Garcia-Andrés *et al.*, 2009; Michelson *et al.*, 1994) and monitored here with qPCR for the first time. More specifically, the *Ty-1*-gene was more effective to restrain the TYLCSV-ES clone than the TYLCV-IL clone which is consistent with squash-blot results obtained previously with representatives of TYLCSV-ES and TYLCV-Mld (Garcia-Andrés *et al.*, 2009). TYLCV-IS76 was the less affected virus in the resistant cultivar, particularly in competition test. Moreover, it is noteworthy that the deleterious effect of TYLCV-IS76 on TYLCV-IL DNA accumulation observed is much higher in Pristyla- than in susceptible plants (Fig. 2), which is consistent with the supposed triggering effect of Ty-1-resistant plants on the emergence of TYLCV-IS76 recombinants in Morocco.

Recombinant and parental viruses exhibit contrasting viral DNA accumulation and competitiveness

Comparison of virus accumulation between single- and multiple-infected plants revealed that the three viral clones were affected differently by competition. Competition was always deleterious for DNA accumulation of TYLCV-IL (Figs. 1 and 2) except in the double infection with TYLCSV-ES at 30 dpi (Fig. 1). On the contrary, competition was always beneficial or neutral to DNA accumulation of TYLCSV-ES, except in triple-infected susceptible plants at 10 dpi (Fig. 2). These results are consistent with the relatively higher frequency of TYLCSV than TYLCV-Mld amplicons generated and cloned from 400 dpi samples of tomato plants of susceptible (García-Andrés *et al.*, 2007b) and Ty-1 resistant (Garcia-Andrés *et al.*, 2009) cultivars co-infected with these viruses.

Competition is mostly beneficial or neutral to DNA accumulation of TYLCV-IS76 except at the earliest sampling times, i.e. 10 and 20 dpi in the susceptible cultivar and 10 dpi in the resistant cultivar (Fig. 2). Interestingly, a positive cooperative interaction was detected with the TYLCSV-ES clone at 30 dpi in the Ty-1 cultivar (Figs. 1 and 2a). Similar synergies in DNA accumulations have been detected with two begomoviruses infecting tomato in India (Chakraborty *et al.*, 2008), as well as two begomoviruses of cassava (Fondong *et al.*, 2000).

The negative impact of the TYLCSV-ES clone on the DNA accumulation of the TYLCV-IL clone and its positive cooperative interaction with the TYLCV-IS76 clone, are consistent with the higher number of TYLCSV (6) than TYLCV-IL infected plants (1) within the 301 Tylc virus-positive tomato plants sampled in the Souss after the displacement of parental viruses by the recombinant virus (Belabess *et al.*, 2015).

Methylation may explain the low fitness of TYLCV-IL in competition tests

The 76-nt TYLCSV-ES-derived region of TYLCV-IS76 was identified as the molecular determinant of the dramatic deleterious effect on the DNA accumulation of TYLCV-IL clone in the Ty-1 resistant cultivar. Although no particular function has been associated to this region in geminiviruses, it has been shown to be one of the favored regions for siRNA targeting and methylation in the case of the A component of three bipartite begomoviruses, mungbean yellow mosaic India virus (MYMIV) (Yadav & Chattopadhyay, 2011), tomato leaf curl New Delhi virus (ToLCNDV) (Sahu *et al.*, 2014), and pepper golden mosaic virus (PepGMV) (Rodríguez-Negrete *et al.*, 2009). Intriguingly, this was not the case of the IR of the monopartite begomoviruses, tomato yellow leaf curl China virus (Yang *et al.*, 2011) and TYLCV (Butterbach *et al.*, 2014)]. However, as the DNA accumulation of TYLCV-IL was affected in co-infection experiments, with both TYLCV-ES and TYLCV-IS76, it is possible

that co-infection mimics the infection with a bipartite begomovirus. Thus, the 76-nt discriminating nucleotides of TYLCV-IL genome may be targeted by silencing mechanisms triggered by the co-infecting viruses. As the level of silencing may depend on the concentration of the co-infecting virus, it is supposed to be higher with TYLCV-IS76 whose DNA accumulation is 10 times higher than that of TYLCSV-ES. The silencing hypothesis is consistent with the results of Butterbach *et al.*, (2014) showing that the Ty-1 resistance against TYLCV is associated with increasing cytosine methylation of the viral genome suggestive of enhanced transcriptional gene silencing (TGS). The involvement of TGS in the deleterious effect of TYLCV-IS76 on TYLCV-IL DNA accumulation should be tested experimentally.

Selection-driven displacement scenario of Tylc-associated viruses leading to the dominance of TYLCV-IS76 viruses in the Souss

According to previous reports and the results presented here on the fitness advantage of TYLCV-IS76, the following scenario is proposed to account for the replacement of parental viruses by the recombinant TYLCV-IS76 viruses in Southern Morocco (Souss). At the beginning of the 2000s, when the susceptible cultivars started to be replaced with Ty-1-cultivars in Morocco, the population of viruses associated with Tylc symptoms was composed mainly of TYLCV-IL, TYLCV-Mld and TYLCSV viruses, and some canonical TYLCV-IL/TYLCSV recombinants, but TYLCV-IS76 viruses had not been detected (Belabess *et al.*, 2015). TYLCSV viruses are thought to have been displaced by TYLCV-Mld viruses, because according to field surveys conducted in Spain and to experimental studies, TYLCV-Mld has a better ecological performance than TYLCSV (Sánchez-Campos *et al.*, 1999), and was shown to accumulate to a greater extent than TYLCSV in Ty-1-cultivars (Garcia-Andrés *et al.*, 2009). TYLCV-Mld viruses are thought to have been displaced by TYLCV-IL viruses because the non-recombinant viruses detected in the surveys conducted in Northern Morocco between 2008 and 2014, were mostly TYLCV-IL viruses (Belabess *et al.*, 2015), which is consistent with surveys conducted in Réunion, where the introduction of TYLCV-IL viruses has displaced the resident TYLCV-Mld viruses (Péréfarres *et al.*, 2014). According to Bayesian inferences, the recombination event that led to TYLCV-IS76 viruses most probably occurred at the end of the 1990s, which suggests that they were already present at the beginning of 2000s but probably at low prevalence, at least in tomato (Belabess *et al.*, 2015). Then, in accordance with the results of the present study, where a TYLCV-IS76 clone was shown to have a selective advantage over parental clones and particularly a TYLCV-IL clone,

TYLCV-IL viruses have been displaced by TYLCV-IS76 viruses. The complete displacement of TYLCV-IL suggests that the selective advantage of TYLCV-IS76 was highly efficient, which is fully consistent with the drastic deleterious effect of both TYLCV-IS76-type recombinants (IS76 and IS76') on the DNA accumulation of TYLCV-IL in co-infected Ty-1- plants (Figs. 4 and 5). Moreover, as suggested by a preliminary transmission test, the transmission of TYLCV-IL from co-infected Ty-1 plants seems to be compromised, which is fully consistent with the previously reported correlation between viral DNA accumulation and transmission efficiency of Tylc-associated viruses (Lapidot *et al.*, 2001).

Hence, the co-occurrence of the replacement of susceptible cultivars by resistant ones and the complete displacement of Tylc-associated viruses by TYLCV-IS76 viruses may be more than a coincidence. Indeed, as the selective advantage of TYLCV-IS76 was far higher in Ty-1- than in susceptible cultivars in both single- and competition tests, our results strongly support the hypothesis that the deployment of Ty-1- cultivars has played a role in the observed viral population shift.

Although the results presented here provide compelling support for the selective hypothesis to explain the entire displacement of the parental viruses by the TYLCV-IS76 recombinants, no new light is shed on the origin of the non-canonical TYLCV-IS76 recombinant. The reasons why it was detected in Morocco but not in other countries where TYLCV/TYLCSV recombinants are frequently reported (Spain and Italy) are presently not clear. As a first step, it will be useful to test if TYLCV-IS76 viruses can be generated in tomato plants co-infected with representatives of its two parental species, and, if that is the case, would the resistant plants bearing the *Ty-1* gene be more conducive to an increase of their intra-plant frequency in comparison to susceptible plants.

Materials and Methods

Plant material

Nearly isogenic susceptible and Ty-1 resistant cultivars of tomato were used to test the effect of the *Ty-1* gene on virus accumulation. The resistance conferred by this gene was recently showed to act through TGS (Butterbach *et al.*, 2014). The resistant cultivar "Pristyla" carrying the Ty-1 resistance allele in a heterozygote state (Ty-1/ty-1) is a hybrid cultivar obtained by crossing a Ty-1 bearing line Ar (Ty-1/Ty-1) with a susceptible line B; the line Ar was derived from the susceptible line A following Ty-1 introgression. The nearly isogenic susceptible

cultivar of Pristyla is the hybrid A x B. The effect of the *Ty-1* gene was tested also with the resistant cultivar "F", which has the same *Ty-1* gene with Pristyla but introgressed in a different genetic background; the *Ty-1* allele of F was also in a heterozygote state. The term "resistant" was chosen to designate *Ty-1*- bearing cultivars because the *Ty-1* gene induces resistance to TYLCV DNA accumulation (De Castro *et al.*, 2005; Michelson *et al.*, 1994). Seven-day-old seedlings were transplanted into individual pots for the tests. Grafted and non-grafted Pristyla plants were tested. Grafting was performed by Nimaplants nursery (Nîmes, France) 20 and 22 days after sowing Pristyla and the rootstock "Maxifort" respectively.

All plants were grown in containment growth chambers under 14h light at 26±2°C, and 10h dark at 24±2°C, and were watered with 15:10:30 NPK fertilizer + oligoelements.

Agroinfectious clones

Three agro-infectious clones were used: a clone of the recombinant TYLCV-IS76, and a clone of each of the two parental viruses, TYLCV-IL and TYLCSV-ES. The agroinfectious clone TYLCV-IS76[MA:SouG8:10] (GenBank accession number LN812978) has been described previously (Belabess *et al.*, 2015). Representatives of the parental species were selected among those which exhibited the highest nucleotide identity with the TYLCV- and TYLCSV-derived sequences of the recombinant TYLCV-IS76. The clone TYLCV-IL[RE:STG4:04] (GenBank accession number AM409201) (Belabess *et al.*, 2015) exhibits 99% nucleotide identity with the TYLCV-derived fragment of the TYLCV-IS76 clone. An agroinfectious clone was constructed previously as follows. A 0.9-mer genome obtained by digestion with *Nco*I and *Eco*RI was ligated into the corresponding restriction sites of the vector pCAMBIA0380. The full-length genome was excised from the plasmid pGEMT with *Nco*I and ligated into the *Nco*I restriction site of the recombined pCAMBIA0380. The construction was introduced into bacteria of the C58 MP90 strain of *Agrobacterium tumefaciens* via electroporation. The clone TYLCSV-ES[MA:Aga5a:12] (GenBank accession number LN846598) (Belabess *et al.*, 2015) differs at only two nucleotide positions (32 and 44) from the TYLCSV-derived region of the TYLCV-IS76 clone; an agro-infectious clone has been constructed previously (Belabess *et al.*, 2015).

Construction of recombinant TYLCV-IS76' by site-directed mutagenesis

The recombinant TYLCV-IS76' was engineered starting from TYLCV-IL[RE:STG4:04] with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, USA) and

primers (Table S1 in Supplementary Material). An agroinfectious clone of TYLCV-IS76' was prepared according to the partial tandem method described above.

Agro-inoculation, and randomized experimental design

Tomato plants were agroinoculated 14 days after sowing or grafting with various combinations of agroinfectious clones as described in Table 1. The transformed *A. tumefaciens* clones were grown at 28°C in liquid LB medium containing kanamycin (50 mg/mL) and gentamycin (20 mg/mL). After about 26 h, when suspensions reached optical densities (OD) of about 3–5, the agrobacteria cultures containing the infectious TYLCV-IL, TYLCSV-ES and TYLCV-IS76 genomes were adjusted to identical ODs with LB medium. The equally concentrated cultures were centrifuged for 20 min at 1,000 *g*, and each pellet was resuspended in water (the same volume as the centrifuged volume) containing 150 µM acetosyringone and 10 mM $MgCl_2$. For mixed virus infections the same procedure was used except that the same volumes of equally concentrated agrobacteria cultures containing the infectious viral genomes were mixed before centrifugation. The resuspension volume of the mixed inocula was such that each virus was inoculated at the same agrobacterial concentration in single and mixed infections. Non-grafted plants (Table 1; experiments 2–6) were agroinfiltrated via transepidermal delivery of the agrobacterial suspension using a needle-less syringe applied to the underside of cotyledons. The young leaves of grafted plants (Table 1; experiment 1) were scratched at the injection spot before agro-infiltration. Some plants used as negative controls were agroinfiltrated with bacteria of the C58 MP90 strain of *A. tumefaciens* containing an empty pCAMBIA2300 plasmid. Plants were arranged in a complete randomized block design.

Total DNA extraction

Leaf samples collected from each plant at each collection date were taken from the youngest leaf for which five leaflets were visible, and consisted of five 4-mm diameter leaf disks, one per leaflet. Total DNA from each sample was extracted according to the protocol of Dellaporta et *al*. (1983) with previously reported modifications (Urbino *et al.*, 2013), and stored at –20°C until use.

Real-time PCR quantification of each virus

The content of intra-plant viral DNA was determined with real-time PCR (qPCR) and was used as a proxy for fitness, as previously applied to TYLCV (Péréfarres *et al.*, 2014; Urbino *et*

al., 2013; Vuillaume *et al.*, 2011) and other viruses (Carrasco *et al.*, 2007; Gómez *et al.*, 2009; Hillung *et al.*, 2015; Tromas *et al.*, 2014). Primer pairs were designed on both sides of the origin of replication (OR) for the specific detection of each of the parental clones (TYLCV-IL and TYLCSV-ES), and on both sides of locus 76 for detection of the TYLCV-IS76 and TYLCV-IS76' clones (Table S1 in Supplementary Material). The specificity of each primer pair was tested with viral DNA of non-targeted viral clones. Moreover, the primer pair targeting recombinants did not produce any positive detection with DNA extracts from plants double-infected with TYLCV-IL and TYLCSV clones, or with a mix of plasmid DNA extracts of the parental viral clones, indicating that no recombinants were generated *in vitro*.

The viral DNA content of each agroinfected plant was quantified in duplicate using the LightCycler 480 SYBR Green I qPCR mix (Master, Roche, Germany) as described in Supplementary material. All PCR fluorescence data were analyzed as described in Supplementary material.

Transmission tests

The transmission tests were carried out with Q1 type *B. tabaci* whiteflies of the putative species Mediterranean (Med) (Angers, France), which is the most common species in Morocco (Tahiri *et al.*, 2006, 2013). Approximately 300 adults newly emerged were given a 2-day acquisition access period (AAP) on two Ty-1-resistant tomato plants (cv. Pristyla), 56 days after their co-agroinfection with the TYLCV-IL and TYLCV-IS76 clones (Table 1; experiment 6). At the end of the AAP, 120 whiteflies were shifted to 16-day-old Pristyla plants (15 plants) for a 5-day inoculation access period (IAP), and similarly 120 whiteflies were shifted to 15 plants of the susceptible cultivar. The transmission success of each virus was assessed with Multiplex PCR tests (Belabess *et al.*, 2015), which can distinguish between TYLCV-IL and TYLCV-IS76 viruses.

Statistical analysis

All statistical analyses were performed using R Studio software, version 3.0.3. (R_Development_Core_Team, 2010). Viral DNA accumulations were compared between or within plants using the log transformation of CVr data. As there was no statistical difference between the results of experiments 5 and 6 (Table 1), the data obtained for the samples collected at 10 and 30 dpi of these two experiments were pooled for statistical analysis. ANOVA tests were used to analyze virus accumulation data for statistical significance. A

non-parametric test, Wilcoxon test, was performed with data from experiment 3 because of the low numbers of plants.

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673

674 **Table 1**: Origin of leaf samples from which the viral DNA content was quantified by real
675 time PCR. Samples were collected from tomato plants of the Ty-1-resistant cv. Pristyla, from
676 a susceptible nearly isogenic cultivar, and from the Ty-1-resistant cultivar F. Resistant
677 cultivars were heterozygous for the *Ty-1*-resistant allele (Ty-1/ty-1). Plants were infected in
678 single or mixed infection with TYLCV-IL (IL), TYLCSV-ES (ES), TYLCV-IS76 (IS76) or
679 TYLCV-IS76' (IS76') agroinfectious clones.

Experiment	Tomato cultivar (*Ty1* gene alleles)	Viral infection status	Number of infected plants/ Number of inoculated plants	Number of plants tested at each sampling time (dpi)*					
				10	**20**	**30**	**60**	**90**	**120**
1	Pristyla (*Ty1/ty1*) grafted on cultivar Maxifort	IL	20/20	20		20			
		ES	20/20	20		20			
		IS76	20/20	20		20			
		IL+ES	20/20	20		20			
		IL+IS76	19/20	19		19			
		IS76+ES	20/20	20		20			
		IL+ES+IS76	34/40	34		34			
2	Pristyla (*Ty1/ty1*)	IL	33/35	30	30	30	9	9	
		ES	28/35	28	28	28	9	9	
		IS76	60/60	30	30	30	10	10	
		IL+ES+IS76	39/60	26	26	26	10		
	Susceptible nearly isogenic cultivar (*ty1/ty1*)	IL	35/35	30	30	30	9	9	
		ES	33/35	30	30	30	10	9	
		IS76	60/60	30	30	30	8	8	
		IL+ES+IS76	52/60	30	30	30	9		
3	F (*Ty1/ty1*)	IL	5/5			5			
		IS76	5/5			5			
		IL+IS76	5/5			5			
4	Pristyla (*Ty1/ty1*)	IL	15/15	15	15	15			8
		IL+ES	15/15	15	15	15			8
		IL+IS76	15/15	15	15	15			9
		IL+ES+IS76	20/20	20	20	20			9
5	Pristyla (*Ty1/ty1*)	IL	15/15	13		15			
		IS76'	20/20	15		20			
		IL+IS76	17/18	16		17			
6	Pristyla (*Ty1/ty1*)	IL	4/5	3		4			
		IS76'	5/5	5		5			
		IL+IS76	5/5	5		5			
		IL+IS76'	49/51	15		15			
	Susceptible nearly isogenic cultivar (*ty1/ty1*)	IL	5/5	5		4			
		IS76'	5/5	5		4			
		IL+IS76'	20/20	20		15			

680 *: days post inoculation

Figure legends

Figure 1: DNA accumulations of TYLCV-IL (IL), TYLCSV-ES (ES) and TYLCV-IS76 (IS76) clones in agro-infected tomato plants of the Ty-1-resistant cv. Pristyla grafted on a tomato rootstock of the cv. Maxifort. The infection status of plants is indicated at the top of the figure. Viral DNA was quantified with real-time PCR from leaf samples collected at 30 dpi from 20 plants per treatment, except in the triple infection and the TYLCV-IL/TYLCV-IS76 double infection treatments, in which 34 and 19 plants were tested, respectively (Table 1; experiment 1). The logarithm of the Calibrated Value (logCVr) reflects viral DNA accumulation. Within the boxes, the horizontal line indicates the median value (50% quantile), the box itself delimits the 25% and 75% quantiles, and lines represent the normal range of the values; the points above and/or below correspond to outlying values. The red, blue and purple dotted lines represent the mean of logCVr values obtained with mock-inoculated plants tested with TYLCV-IL, TYLCSV-ES and TYLCV-IS76 specific primers, respectively. The red and purple dotted lines cannot be distinguished because the corresponding logCVr values are virtually the same: –6.99 and –6.97, respectively. Boxplots with different letters indicate significant differences of viral DNA accumulations (Tukey's test, $P = 0.05$): small letters correspond to comparisons between single-infected viruses or between different viruses in co-infected plants, whereas capital letters correspond to the comparisons of the same virus between treatments.

Figure 2: DNA accumulations of TYLCV-IL, TYLCSV-ES and TYLCV-IS76 in agro-infected non-grafted tomato plants of (a) the Ty-1-resistant cv. Pristyla (Ty-1/ty-1) and (b) a susceptible nearly isogenic cultivar (ty-1/ty-1). The infection status of plants, single- or triple infection, is indicated at the top of the figure. Viral DNA was quantified with real-time PCR from leaf samples collected between 10 and 90 dpi from at least 26 plants for the 10, 20 and 30 dpi samplings, and from at least 8 plants for the 60 and 90 dpi samplings (Table 1; experiment 2). Representation of viral DNA accumulations, box-plots, positive thresholds and statistics as in Fig. 1. Small letters correspond to the differences between the three viruses among samples collected at the same time from plants of the same cultivar, and with the same infection status, whereas capital letters correspond to the comparisons of the same virus between single- and triple-infected plants of the same cultivar and sampled at the same time.

Figure 3: DNA accumulations of TYLCV-IL (IL) and TYLCV-IS76 (IS76) in agro-infected non-grafted tomato plants of the Ty-1-resistant cv. F (Ty-1/ty-1). The infection status of plants, single- or double infection, is indicated at the top of the figure. Viral DNA was quantified with real-time PCR from leaf samples collected at 30 dpi from five plants per treatment (Table 1; experiment 3). Representation of DNA accumulations, box-plots and positive thresholds as in Fig. 1.

Figure 4: DNA accumulations of TYLCV-IL in non-grafted tomato plants of the *Ty-1*-resistant cv. Pristyla, either single-infected or co-infected with TYLCV-IS76 (IS76), TYLCSV-ES (ES) or both. The infection status of plants is indicated at the top of the figure. Viral DNA was quantified with real-time PCR from leaf samples collected between 10 and 120 dpi from at least 15 plants for the 10, 20 and 30 dpi samplings, and from at least 8 plants for the 120 dpi samplings (Table 1; experiment 4). Representation of viral DNA accumulations, box-plots, positive thresholds and statistics as in Fig. 1. Different letters indicate significant differences of TYLCV-IL DNA accumulations between plant samples of the four treatments collected at the same time.

Figure 5: DNA accumulations of TYLCV-IL (IL), TYLCV-IS76 (IS76) and TYLCV-IS76' (IS76') in agro-infected non-grafted tomato plants of the Ty-1-resistant cv. Pristyla (*Ty-1*/*ty-1*) and a susceptible nearly isogenic cultivar (*ty-1*/*ty-1*). The infection status of the plants is indicated at the top of the figure. Viral DNA was quantified with real-time PCR from leaf samples collected at 10 and 30 dpi from 15–20 plants per treatment (Table 1; experiments 5 and 6). As there was no statistical difference between the results of experiments 5 and 6 (Table 1), the data obtained for the samples collected at 10 and 30 dpi of these two experiments were pooled for statistical analysis. Representation of viral DNA accumulations, box-plots, positive thresholds and statistics as in Fig. 1. The positive threshold for TYLCV-IS76' and TYLCV-IS76 is the same because the same primer pair was used for both viruses. Small letters correspond to the differences between different viruses among samples collected at the same time from plants of the same cultivar and with the same infection status, capital letters correspond to the comparisons of the same virus between single- and double-infected plants of the same cultivar and sampled at the same time, and bold capital letters correspond to the comparisons of IS76 and IS76' virus in the resistant cultivar and sampled at the same time.

Figure 1

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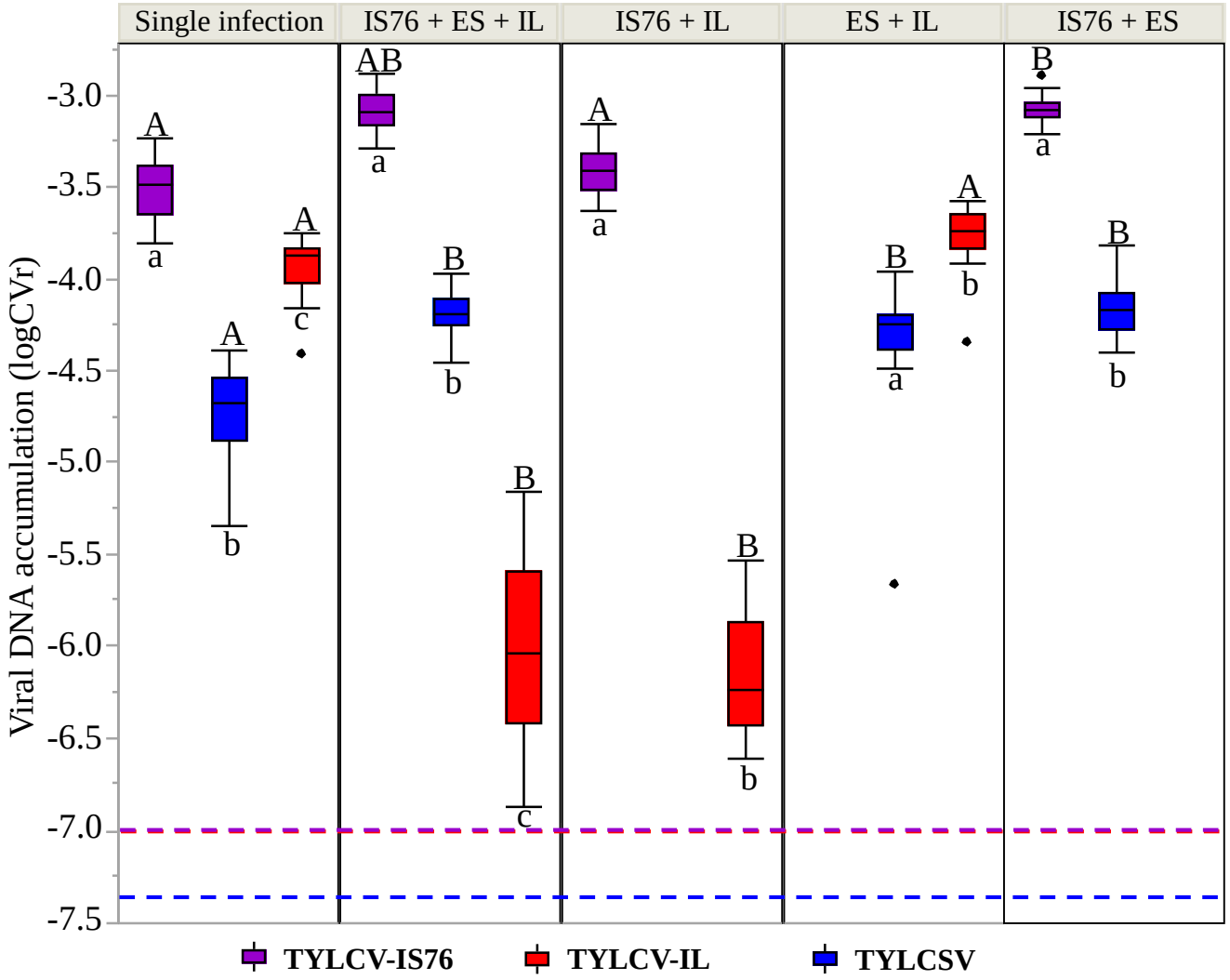

Figure 2

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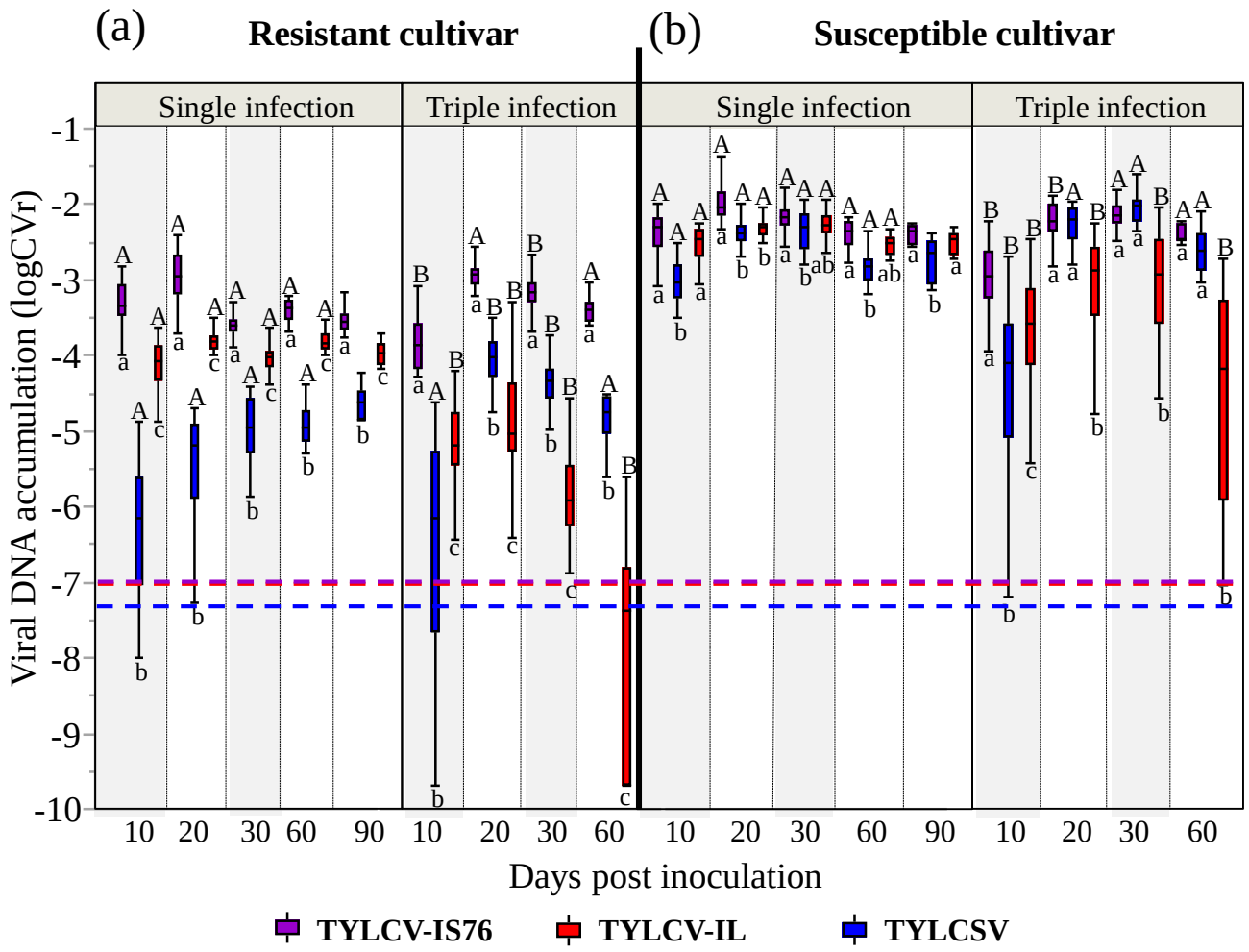

Figure 3

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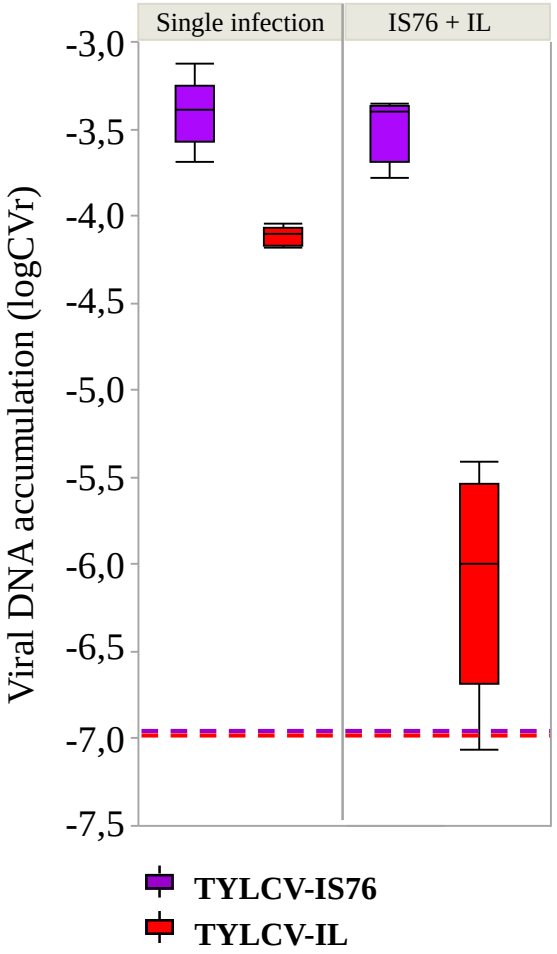

Figure 4

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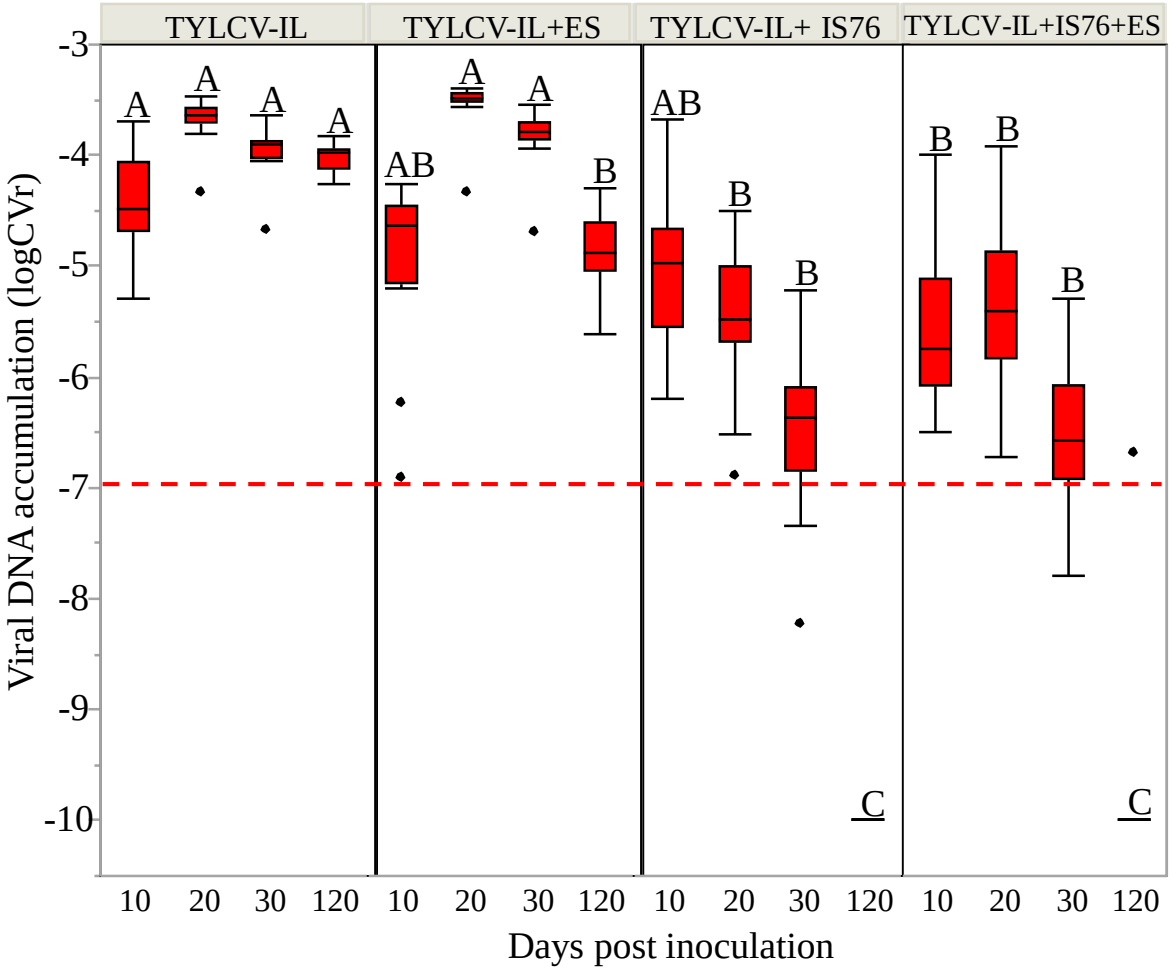

Figure 5

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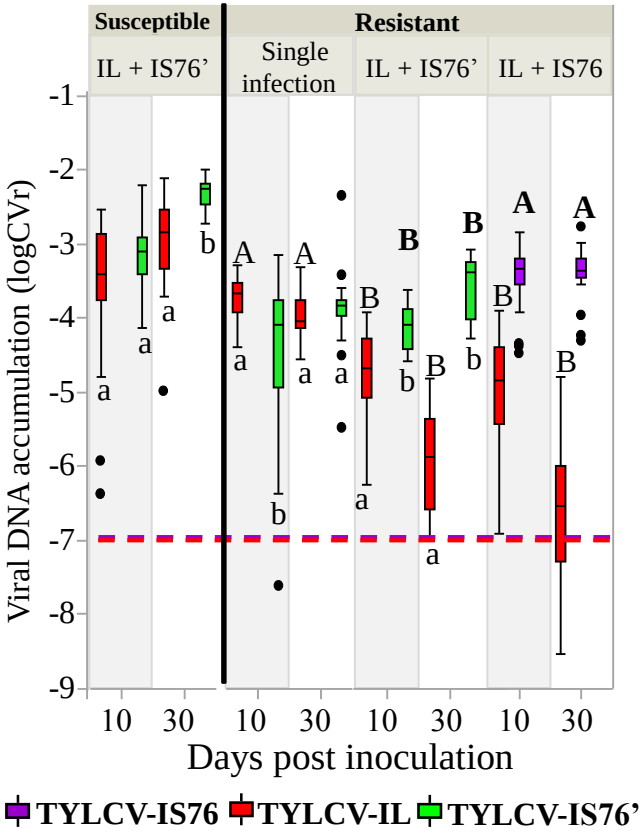

Supplementary material

1) Construction of recombinant TYLCV-IS76' by site-directed mutagenesis

The 66 nts of TYLCV-IL[RE:STG4:04] located between positions 15 and 82 (see Figure below) were deleted and subsequently substituted with the homologous TYLCSV fragment from TYLCV-IS76[MA:SouG8:10]. Primers for deletion and insertion were designed using the web-based QuikChange Primer Design Program available online at www.agilent.com/genomics/qcpd (Table S1). Mutagenesis reactions were carried out according to the manufacturer's instructions with the following changes: the reaction was performed in a final volume of 50 µl containing 2 ng of plasmid DNA, 0.4 µM of each primer, and 2 µL of the dNTP mix supplied with the kit. The amplification consisted of a denaturation step at 95°C for 2 min followed by 20 cycles of 30 s at 95°C, 30 s at 48°C, 11 min 33 s at 68°C, and a final extension step of 10 min at 68°C. The amplification product was digested with 2 µL of *Dpn*I at 37°C for 5 h, and introduced into XL10-Gold competent cells. Plasmid DNA was purified with the Wizard sv plus minipreps DNA purification system (Promega), and sequenced with primers TYLC-384R (Belabess et al. 2015), T7P, SP6 and with other insert-specific primers (Beckman Coulter Genomics).

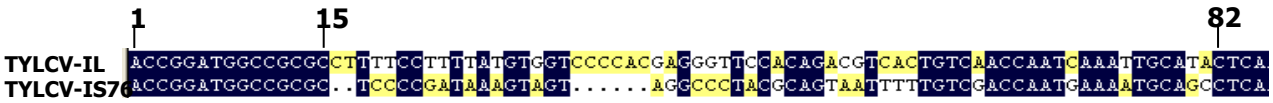

Figure: Comparison of the genomic sequences of the TYLCV-IL[RE:STG4:04] (GenBank accession no. AM409201) and the recombinant TYLCV-IS76[MA:SouG8:10] (LN812978) within the region in which the genomes of the TYLCV-IS76 viruses are derived from representatives of TYLCSV-ES. Nucleotide coordinates shown at the top of the alignment refer to the genome of the TYLCV-IL clone. Nucleotides discriminating both genomes are labeled in yellow.

2) qPCR and fluorescence data analysis

Two microliters of a 1/100 dilution of total DNA extract of each plant sample was added to 5 µL qPCR mix (LightCycler 480 SYBR Green I Master, Roche, Germany) in a final volume of 10 µL. The amplification reactions were run in 384-well optical plates in a LightCycler 480 (Roche, Germany). Cycling parameters were 95°C for 10 min followed by 40 cycles of 10 s at 95°C, 20 or 40 s at 60 or 63°C (see Table S1), and 15 s at 72°C. The amount of plant DNA of each extract was estimated by qPCR quantification of the nuclear-encoded large subunit ribosomal RNA gene (*Solanum lycopersicum* L. 25S ribosomal RNA gene). The qPCR conditions were as reported above and in Table S1. All PCR fluorescence data were analyzed by the LinReg computer program (Ruijter et al. 2009) according to the 2nd derivative max function provided with the LightCycler480 Software. The starting concentration of target, N_0, is expressed in fluorescence units per sample. N_0 is calculated as follows from (i) the value of the fluorescence threshold (F_t) for each plate computed by LightCycler480 Software, (ii) the mean PCR efficiency calculated for each plate (E_{mean}), and (iii) the fractional number of cycles needed to reach the fluorescence threshold (C_t):

$$\mathbf{N_0 = F_t / E_{mean}^{Ct}}$$

As the number of DNA samples to be compared did not always fit into a single qPCR plate, an inter-plate calibrator was tested on all plates. This was prepared from a mix of total DNA extracts from each of ten plants triple-infected with TYLCV-IL, TYLCSV-ES and TYLCV-IS76 viruses. These control samples were kept at –20°C as single-use aliquots.

As the DNA extraction efficiency may not be the same between extractions, the viral DNA content of each sample was standardized relative to its plant DNA content. The estimation of viral DNA content was also standardized to amplicon size , because size is expected to influence the amount of bound SYBR™ Green I per amplicon molecule (Zipper et al. 2004; Rutledge & Côté 2003). Given the above, a relative calibrated value (CVr) allowing comparison between different viral accumulations was calculated:

$$CVr = \frac{N0\ virus}{N0\ 25S\ RNA} \Big/ virus\ amplicon\ size$$

A plant was considered infected with TYLCV-IL, TYLCV-IS76 or TYLCSV-ES viruses when the CVr value obtained with the specific qPCR test was above the 95%-quantile of the distribution of the CVr values of, respectively, 66, 89 and 94 samples from mock-inoculated plants

References

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Ruijter, J.M. et al., 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research*, 37, p.e45.

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Table S1: Description of primers, primer products and amplification conditions for site-directed mutagenesis and qPCR

Tests	Targeted viral clone (GenBank acc.)	Primer	Sequence (genome region and coordinates)*	Amplicon size (bp)	Hybridization T°/time	Primer concentration	Efficiency
Site-directed mutagenesis*	**TYLCV-IL** [RE:STG4:04] (AM409201)	Del F	5'-TACCGGATGGCCGCGCCTCAAACGTTAGATAAG-3' (IR 2781-98)	-	48°C/30s	400nM	-
		Del R	5'-CTTATCTAACGTTTGAGGCGCGGCCATCCGGTA-3' (IR 98-2781)	-			
		Ins F	5'-TACCGGATGGCCGCGCtccccgataaagtagtaggccctacgcag taatttttgtcgaccaatgaaaatgcagcCTCAAACGTTAGATAAG-3' (IR 2781-98)	-	48°C/30s	400nM	-
		Ins R	5'-CTTATCTAACGTTTGAGgctgcattttcattggtcgacaaaaa ttactgcgtagggcctactactttatcggggaGCGCGGCCATCCGGTA-3' (IR 98-2781)	-			
qPCR	**TYLCV-IL** [RE:STG4:04] (AM409201	IL-2690F	5'-AATGGCTATTTGGTAATTTCG-3' (IR 2690-2710)	146	63°C/40s	800nM	83%
		IL-55R	5'-CGTCTGTGGAACCCTCG -3' (IR 55-39)				
	TYLCSV-ES [MA:Aga5a:12] (LN846598)	ES-2666F	5'-AGATTGGTAGCTCTTATATACTTG-3' (IR 2666-2689)	230	60°C/40s	800nM	99%
		ES-118R	5'-GAAGCCAAGTTTATAACAAAGT -3' (IR 118-97)				
	TYLCV-IS76 [MA:SouG8:10] (LN812978) **TYLCV-IS76'**	ES-19F	5'-CCGATAAAGTAGTAGGCCCTACGCA-3' (IR 19-43)	135	63°C/20s	300nM	95%
		IL-153R	5'-AGTGGGTCCCACATATTGCAAGAC-3' (IR/V2 153-130)				
	25S RNA tomato gene	25S RNA1137F	5'-AGAACTGGCGATGCGGGATG-3' (1137-1156)	161	60°C/20s	300nM	90%
		25S RNA1297R	5'-GTTGATTCGGCAGGTGAGTTGT-3' (1297-1276)				

*Sequence of DNA primers designed for the deletion (Del) and the insertion (Ins) steps of the site-directed mutagenesis used to construct the recombinant TYLCV-IS76′ clone. Capital letters correspond to the unchanged genomic region whereas lower case letters correspond to the modified region (nucleotides 16–82 in the TYLCV-IL[RE:STG4:04] clone (GenBank accession no. AM409201)).