Population shifts in begomoviruses associated with tomato yellow leaf curl disease in western Mediterranean countries

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Abstract

Tomato yellow leaf curl disease (TYLCD) was reported in western Mediterranean basin since the late 1980s. Based on intensive plant samplings performed in Spain, Italy and Morocco at different periods between the 1990s and 2014, several begomoviruses (family Geminiviridae) were identified as the cause of TYLCD. They comprise the native Tomato yellow leaf curl Sardinia virus (TYLCSV), two strains of Tomato yellow leaf curl virus (TYLCV) introduced from the Middle East, and several types of TYLCV/TYLCSV recombinants including the invasive recombinant TYLCV-IS76 in which the proportion of genome inherited from TYLCSV was unusually short. Although parental and recombinant TYLCD-associated begomoviruses were present in each country, country specificities were detected with respect to their relative prevalence, the infection profiles of individual tomato plants, and the recombination patterns of TYLCV/TYLCSV recombinants. Considering geographic proximities and trade activities between these countries, and the efficient transmission of begomoviruses which are persistently transmitted by the polyphagous and tiny whitefly vector *Bemisia tabaci*, it was not known if these specificities would be maintained over time. To address this question, tomato samples collected in the three countries between 2015 and 2019 were analysed with PCR tests previously designed to distinguish species, strains and recombinants of TYLCD associated begomoviruses, and with an original PCR test distinguishing potentially invasive recombinants with short TYLCSV-inherited fragments (Srec) from those with longer TYLCSV fragments (Lrec). The results show that country specificities are still present, the most striking being the contrasted geographic distribution of Srec recombinants, with TYLCV-IS76 detected in Morocco but not in Italy and TYLCV-IS141 and a new Srec recombinant (TYLCV-IS60) both detected in Italy and not in Morocco. Nevertheless, besides country specificities, a general population shift was revealed with TYLCV/TYLCSV recombinants. Indeed, all the recombinant positive samples, irrespective of their geographic origin, were Srec-positive but Lrec-negative, which suggest that the emergence of Srec recombinants occurred at the expenses of the Lrec recombinants. These results are discussed in relation to the positive selection of Srec recombinants by Ty-1 resistant tomato plants.

1. Introduction

Tomato (*Solanum lycopersicum*) is one of the New World (NW) originated vegetables that is presently grown worldwide including Mediterranean countries. During its global spread, it encountered Old World (OW) viruses and particularly viruses belonging to the genus *Begomovirus* of the family *Geminiviridae*. Indeed, according to the International Committee on Taxonomy of Viruses (https://ictv.global/taxonomy), 112 Begomoviruses were isolated from tomato worldwide and most of them (69) from the OW. Noteworthy, a typical feature of OW begomoviruses is their ability to induce leaf curl, a feature which is highlighted in all the 69 names, as for example Tomato yellow **leaf curl** virus (TYLCV) or Tomato yellow **leaf curl** Sardinia virus (TYLCSV). The symptoms induced by the 43 NW

begomoviruses are much more diverse and only 8 of them were characterized by the terms “leaf curl” in their names.

Following the introduction of tomato in the western Mediterranean region, it encountered at least two OW begomoviruses, TYLCSV and TYLCV. They both cause tomato yellow leaf curl disease (TYLCD), an economically important disease of tomato. TYLCSV was reported first (Kheyr-Pour et al., 1991; Luisoni et al., 1989; Noris et al., 1994) and is generally considered to be indigenous (Lefeuvre et al., 2010). TYLCV is a foreign begomovirus most probably from eastern Mediterranean countries (Lefeuvre et al., 2010). Two TYLCV strains were reported from western Mediterranean countries, TYLCV-Israel (TYLCV-IL) and the so-called mild strain, TYLCV-Mld. In Spain, the earliest plant samples in which TYLCV-Mld was detected were collected in 1997 (NavasCastillo et al., 1997) and TYLCV-IL, in 2002 (Morilla et al., 2003). In Italy, the earliest collected samples in which TYLCV was detected are from 2002 for the IL strain (Accotto et al., 2003) and 2012 for the Mld strain (Belabess et al., 2015). In Morocco, the earliest plant samples in which TYLCV-IL and TYLCV-Mld were detected, were collected in 1998 (Belabess et al., 2015; Peterschmitt et al., 1999). Although all three countries host TYLCD-associated viruses of the same species, country specificities were detected. For example, the displacement of TYLCSV induced by the arrival of TYLCV was different between Spain and Italy. Indeed, whereas in Spain the displacement was virtually total, in Italy, virus populations reached an equilibrium in which the two virus species coexist (Davino et al., 2006; Davino et al., 2009; Garcia-Andres et al., 2007; SanchezCampos et al., 1999).

Since the spread of TYLCV into western Mediterranean countries, the diversity of begomoviruses has increased considerably, and not only because of representatives of a new virus species, i.e. *Tomato leaf curl New Delhi virus* (ToLCNDV) (Juárez et al., 2013), but also due to recombinants between TYLCV and TYLCSV as reported from Spain (Garcia-Andres et al., 2006; Monci et al., 2002), Italy (Belabess et al., 2015; Davino et al., 2008; Granier et al., 2019; Panno et al., 2018) and Morocco (Belabess et al., 2015).

The earliest collection of plant samples detected positive for TYLCV/TYLCSV recombinants is in 1998 in Spain and Morocco (Belabess et al., 2015; Monci et al., 2002) and 2002 in Italy (Davino et al., 2008). Although the collection dates of these recombinant positive samples were fairly similar, the earliest dates at which plants were detected with only recombinants, i.e. testing negative for parental viruses, are very different. Indeed, whereas in Spain such plants were detected 1 year after the first detection of recombinants (Monci et al., 2002), in Morocco and Italy the delay was at least 10 years (Belabess et al., 2015). In natural conditions, when a recombinant is detected in a plant without parental viruses, it generally means that it is fit enough to spread autonomously from plant to plant and is potentially emerging. It seems therefore that the agroecological environment of Spain was more conducive to the emergence and spread of TYLCV/TYLCSV recombinants than the Italian and Moroccan environment. The contrasted practice of bean growing between Southern Spain and Sicily was suggested to be critical (Davino et al., 2009); in Southern Spain, beans (Phaseolus vulgaris) are regularly grown between tomato crops, but not in Sicily. Thus, this specific growing practice of beans may have favoured the emergence of the bean adapted recombinant Tomato yellow leaf curl Malaga virus (TYLCMalV) in Spain (Monci et al., 2002).

Another country specificity is related to the recombination profiles of the autonomously spreading TYLCV/TYLCSV recombinants. The two spreading recombinants reported from Spain, i.e., TYLCMalV (Monci et al., 2002) and Tomato yellow leaf curl Axarquia virus (TYLCAxV) (Garcia-Andres et al., 2006) have both inherited a long TYLCSV fragment of at least 1000 nts, including at least the two viral strand-encoded genes V1 and V2 and their promoter region up to the origin of replication. Although this type of recombinants were also reported from Italy (Davino et al., 2012) and are suspected from Morocco (Belabess et al., 2015), they were not found to spread autonomously. Unlike in Spain, the spreading recombinants that emerged in Italy and Morocco inherited a much shorter TYLCSV fragment, located between the origin of replication and the 5’ end of the V2 gene. Intriguingly, while the emerging

recombinants reported from Italy have a TYLCSV-inherited fragment of 141 nts (Belabess et al., 2015; Granier et al., 2019; Panno et al., 2018), the emerging recombinant reported from Morocco has only a 76 nts fragment inherited from TYLCSV (Belabess et al., 2015). These recombinants were named TYLCV-IS76 and TYLCV-IS141 according to the position of their typical recombination breakpoints (Belabess et al., 2015; Belabess et al., 2018). Noteworthy, TYLCV-IS76 recombinants were recently detected in Spain (Fortes et al., 2023; Torre et al., 2019) and due to their high nucleotide identity with Moroccan representatives, they presumably originate from Morocco. Noteworthy, TYLCV-IS76 and TYLCV-IS141 exhibit resistance breaking features. Indeed, they were both detected on symptomatic tomato plants bearing the Ty-1 resistance gene (Belabess et al., 2015; Panno et al., 2018).

Considering geographic proximity and trade activities between Morocco, Italy and Spain, and the efficient and persistent transmission of these viruses with the polyphagous and tiny whitefly vector *Bemisia tabaci*, it was not known if these specificities would be maintained or rather fade over time due to potential gene flow induced by natural or anthropogenic virus spread. To address this question, we analysed the infection profiles of tomato samples collected from the three countries between 2015 and 2019. The infection profile of each plant was determined with the multiplex PCR tests described by Belabess et al (Belabess et al., 2015). Additionally, to monitor more specifically the prevalence of recombinants with a resistance breaking potential like TYLCV-IS76 and TYLCV-IS141, a new test was designed to distinguish such recombinants characterized by their short TYLCSV inherited fragment (Srec), from those that inherited longer TYLCSV fragments like TYLCMalV and TYLCAxV (Lrec). Although the results show that country specificities are maintained with contrasted prevalence of recombinant and parental type viruses between countries, they also show a general population shift with the emergence of Srec recombinants at the expenses of the Lrec recombinants which were not detected in any of the samples from the three countries.

2. Material and Methods

2.1. Plant material

Leaf samples were collected from protected tomato crops between 2015 and 2019 in Morocco, Spain and Italy (Table 1).

In Morocco, 3 samples were collected in December 2015 in Agadir, and 48 samples were collected in 2017 from 6 locations along the Atlantic coast between Casablanca and Agadir. These 48 samples were from 12 farms, with 4 samples per farm collected on tomato plants exhibiting TYLCD symptoms. Collection sites from North to South were Azmour with 1 farm sampled the 26th of April 2017, Chtouka ouled laissaoui with one farm sampled the 16th of March 2017, Ouled Frej with 3 farms sampled the 27th of February 2017, Oualidia with 4 farms sampled the 1st of March 2017, Safi doukkala had lhrara with one farm sampled the 1st of March 2017, and Agadir with 2 farms sampled the 1st of January 2017.

In Italy, 37 samples were collected between 2015 and 2019. Four samples are from Sardinia and 33 samples from Sicily. Samples from Sardinia were previously reported (Granier et al. 2019) but were further analysed here with a PCR test distinguishing Lrec and Srec type recombinants.

In Spain, a total of 17 samples were collected between 2015 and 2018 in the South-East of the country within the tomato-growing regions of Murcia and Almeria

2.2. DNA extraction

The 48 leaf samples collected in 2017 in Morocco were lyophilized and total DNA was extracted with a reported protocol (Dellaporta et al., 1983) modified as follows. The amount of lyophilized plant material used per extraction ranged between 1.25 and 20 mg. Plant material was ground in 400 µL extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 1% SDS, 36 mM Na_2SO_3, and 0.1 mg/ml RNase), incubated at 65°C for 10 min and centrifuged (16,000 g, 10 min). One volume of

isopropanol was added to 300 µL of the supernatant and nucleic acids were precipitated by centrifugation (16,000 g, 20 min); the pellet was washed with ethanol 70% and then resuspended in 50 µL water. As no PCR product was detected with 32 samples, a second DNA extraction was done with a silica-based DNA extraction kit (DNeasy Plant Mini Kit, QIAGEN S.A., Courtaboeuf, France) with 10 mg of lyophilized plant material per extraction.

Total DNA from all the other samples from Morocco, and those from Italy and Spain was extracted with the silica-based DNA extraction kit "NucleoSpin Plant II, Mini kit for DNA from plants" (Macherey Nagel SAS, Hoerdt, France), except for the samples from Sardinia for which the Qiagen-DNeasy Plant Mini kit was used.

2.3. PCR tests and primer design

Plant samples were first analysed with the two multiplex PCR tests MxPCR1 and MxPCR2, with which the presence of TYLCV-IL, TYLCV-Mld, TYLCSV, and recombinants derived from them (Belabess et al., 2015) can be specifically detected. Due to the fact that all reported TYLCV/TYLCSV recombinants bear a recombination breakpoint at the origin of replication (OR), the test is expected to detect all recombinants between TYLCV-IL and TYLCSV and between TYLCV-Mld and TYLCSV. Another typical feature of these reported recombinants is that the sequence contiguous to OR and located at the complementary sense gene side was always inherited from TYLCV, whereas the sequence located at the other side is always inherited from TYLCSV; reciprocal recombinants have not been reported so far.

Samples detected positive for the presence of TYLCV/TYLCSV recombinants, were analysed further with PCR tests designed to distinguish between recombinants that inherited a short TYLCSV fragment (Srec) like TYLCV-IS76 or TYLCV-IS141, from those that inherited long TYLCSV fragments (Lrec) (Fig. 1). Noteworthy, two reverse primers were used to distinguish the Lrec recombinants derived from TYLCV-IL (Lrec-IL) like TYLCAxV (Garcia-Andres et al., 2006), from those derived from TYLCV-Mld (Lrec-Mld) like TYLCMalV (Monci et al., 2002). Specificity of PCR primers were determined in-silico with a range of TYLCV-IL, TYLCV-Mld and TYLCSV genomes available in GenBank. Some of the primers were degenerated according to the intra-species nucleotide diversity of reported genomes. PCR primers used to distinguish Srec and Lrec recombinants are presented in Fig 1 and Table 2.

The cycling protocol for the amplification of Lrec recombinants was as follow: 5 min denaturation at 95 °C, followed by 30 cycles, each consisting of 45 s at 95 °C, 45 s at 50 °C, and 90 s at 72 °C. The PCR program was ended by a 10 min incubation at 72 °C. The same protocol was used for Lrec-IL and Lrec-Mld. The PCR products were resolved by electrophoresis in a 1% agarose gel and visualized with ethidium bromide staining.

The cycling protocol for the amplification of Srec recombinants was as follows: 5 min denaturation at 95 °C, followed by 30 cycles, each consisting of 45 s at 95 °C, 45 s at - 52 °C with reverse primers IM650R and TY282Rd, 54°C with primer TY282Rnd and 55°C with primer TY153Rnd - and 45 s elongation at 72 °C. The PCR program was ended by a 5- or 10-min incubation at 72 °C. The PCR products were resolved by electrophoresis in a 1% to 2% agarose gel according to the different length of the fragment, and visualized with ethidium bromide staining.

The Srec/Lrec discriminating PCR test was validated with total DNA extracted from tomato plants agroinoculated with infectious viral clones. Thus, the Srec PCR test was validated from total DNA of a tomato plant of the cultivar Pristyla (Gautier Semences, France) agroinfected with TYLCV-IS76 (Belabess et al., 2015), and total DNA from a tomato plant of the cultivar Calvi (Gautier Semences) agroinfected with TYLCV-IS141 (TYLCV-IS141[IT:Sic1; 13]) (Urbino et al., 2020). The Lrec-IL PCR test was validated with a total DNA from a tomato plant of the cultivar Pristyla co-agroinoculated with TYLCV and TYLCSV (Belabess et al., 2018); the DNA extract was from plant R11 in which Lrec recombinants were detected by Sanger sequencing in a sample collected 180 days post inoculation [see Fig. 8 of

(Belabess et al., 2018)]. The Lrec-Mld PCR test was validated with a recombinant plasmid DNA containing the full-length genome of a TYLCVMalV clone (Genbank accession number LN846611) (Belabess et al., 2015); the viral clone was obtained from a bean plant (H5) sampled in March 2012 in the Souss region of Morocco.

2.4. Sequencing of PCR products and sequence analysis

Some of the samples in which Srec type recombinants were detected by PCR, were further analysed by sequencing to determine non-OR recombination breakpoints. To do this, the PCR products generated with Srec-specific PCR primers were sequenced by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany) using the PCR primer IM650R, complementary to the viral sense strand of the viral genome (Fig. 1).

The samples from Spain in which TYLCV was detected were further analysed by sequencing to test if the detected TYLCV isolates may belong to any of the two groups that were previously identified by sequence comparisons of viruses isolated from tomato plants sampled in Southeast Spain in 2015 and 2016 (Torre et al., 2018). These authors distinguished two TYLCV groups, group 1 and group 2. Group 1 viruses are related to the two early TYLCV-IL isolates of Southeast Spain, both sampled on pepper (*Capsicum annuum*), one in 1999 in Almeria (TYLCV-IL[ES:Alm:Pep:99], GenBank accession number AJ489258) (Morilla et al., 2005) and one in 2003 in Malaga (TYLCV-IL [ES:Mlg:TY11:Pep2003], GenBank accession number KC953602). Group 2 viruses are closely related to two TYLCV-IL isolates sampled on tomato in Morocco, TYLCV-IL[MA:El Jadida:Tom:2013] (GenBank accession number LN846613 and LN846613) (Belabess et al., 2015). To test the relatedness of TYLCV isolates with viruses of group 1 or 2, we PCR-amplified and sequenced a DNA fragment of the TYLCV genome. The fragment was amplified with the forward primer IL-2629F (5'-GGTGTCCCTCAAAGCTCTAWG-3') of the multiplex PCR test MxPCR2 (Belabess et al., 2015) in association with the reverse primer IM650R also used for Sanger-sequencing (Table 2). The region amplified by PCR was selected because of its relatively high density of group1/group2 discriminating nucleotides, with 11 positions within a 592-nucleotide fragment of the PCR product. Sequences were aligned with homologous genomic sequences from TYLCV isolated in Spain and for which complete viral genomes are available in Genbank. The alignment was performed with the Optimal Alignment method of DNAMAN (version 5.0; Lynnon BioSoft, Quebec, Canada). A phylogenetic tree was set up with a Jukes and Cantor distance matrix using the neighbour-joining method (Saitou and Nei, 1987) as implemented in the DNAMAN software.

2.5. Cloning and sequencing of complete viral genomes

A new Srec type recombinant bearing a recombination breakpoint at position 60 was detected by sequencing PCR products. To characterize the full recombination profile of this new type of Srec recombinants, their complete genome was cloned from total DNA extracted from three plant samples with the DNeasy Plant Minikit. Circular DNA molecules were amplified using a TempliPhi Amplification kit (GE Healthcare, UK) according to the manufacturer's instructions, and digested with restriction enzymes that are expected to be potentially unique according to reported sequences of parental type genomes of TYLCV and TYLCSV. Digestion of RCA products with SacI generated linear fragment of about 2.8kb, a size expected with full-length begomovirus genomes. The restricted fragments were ligated into the plasmid pBC and ligation products were introduced into the bacterial strain *Escherichia coli* DH5alpha. The cloned fragments were sequenced with M13 primers and virus-specific primers. Sequences were analysed with Chromas (Technelysium Pty Ltd, Australia) and DNAMAN.

3. Results

3.1. Srec and Lrec recombinants can be distinguished with a discriminating PCR test

Amplification products generated with Srec/Lrec discriminating PCR primers are presented in Fig. 1. Srec primers do not generate any amplification product with Lrec infected plants and vice versa. No amplification product was detected with a mock-inoculated plant infiltrated with an agrobacterium clone carrying an empty PC2300 binary vector.

3.2. Infection profile of samples collected in Morocco

Among the 51 samples analysed, 46 were detected positive for at least one PCR-targeted virus and three infection profiles were distinguished (Table 1): TYLCSV (1 sample), IL/Sar (43 samples), and coinfection with IL/Sar and Mld/Sar (2 samples). Moroccan samples can be characterized as follow. (i) The parental virus types TYLCV-IL and TYLCV-Mld were not detected and the TYLCSV type was detected in only one sample. (ii) All the 46 PCR-positive samples, were positive for IL/Sar except the sample detected positive for TYLCSV only. (iii) The recombinant-positive samples were negative for recombinant-types that inherited long TYLCSV fragments (Lrec-IL), but positive for Srec recombinants. (iv) The recombination breakpoints within the sequence of the Srec PCR products were analysed with 5 samples of different regions and collection years, and were shown to be all at position 76 like that of the typical recombination breakpoint of TYLCV-IS76.

The 4 samples collected in Ouled-Frej from symptomatic Ty-1 resistant tomato plants of the cultivar Pristyla tested negative for all targeted begomoviruses. It is possible that the PCR tests were not sensitive enough to detect the low virus amounts of Ty1 resistant Pristyla plants which were reported to be about 10 times lower than the amounts in nearly isogenic susceptible tomato plants (Belabess et al., 2016). This hypothesis is consistent with the observation of a relatively faint band observed in lane IS76 of the agarose gel shown in Fig. 1A, corresponding to an amplification product obtained with the Srec specific primers, from a Pristyla tomato plant agroinoculated with a clone of TYLCV-IS76. It is also possible that the milder TYLCD symptoms exhibited by Ty-1 resistant plants may have been confused with other types of symptoms.

3.3. Infection profiles of samples collected in Italy

Among the 37 samples analysed, 31 were detected positive for at least one PCR-targeted virus. The infection profiles are relatively heterogeneous. Indeed, unlike in Morocco where only one parental-type virus was detected (TYLCSV) and only one type of TYLCV/TYLCSV recombinant (TYLCV-IS76), in Italy, all three parental type viruses were detected as well as two types of recombinants, TYLCV-IS141 and a new one described below, TYLCV-IS60 (IS60). Moreover, whereas only three infection profiles were detected in Moroccan samples, up to 9 infection profiles were detected in Italian samples. In spite of these differences between infection profiles of Italian and Moroccan samples, some common features can be noted. Thus, like in Morocco, only one sample was infected with a parental virus alone (TYLCSV), whereas all the other samples detected PCR-positive for at least one of the targeted begomovirus-types, were positive for the presence of IL/Sar or Mld/Sar recombinants. Moreover, like in Morocco, all the recombinant-positive samples are negative for Lrec recombinants, but positive for Srec recombinants.

To further characterize the Srec recombinants, 20 of the 31 Srec-positive samples were further analysed by sequencing; samples were selected from each collection year and from different sampling locations. The sequencing results revealed that 14 samples contained Srec recombinants of the IS141-type with a recombination breakpoint around position 141, whereas 5 samples contained a new type of Srec recombinants with a recombination breakpoint located within the 57 to 62 position-range of nucleotides that are identical between TYLCV and TYLCSV genomes (Fig. 2). Hence it was named TYLCV-IS60 according to the coding used to name previous Srec-type recombinants (Belabess et al., 2015), 60 being one of the two median positions of the 6 non-discriminating nucleotides range in which

recombination events occurred. IS60 recombinant genomes were cloned from three samples collected in October 2018 and were fully sequenced (GenBank accession numbers OR473055, OR473056, OR473057). Besides the recombination breakpoints at the origin of replication and around position 60, no other recombination breakpoint was detected. The IS60 genomes are 2783 or 2784 nts in length and differ from each other at 1, 8 or 9 positions. Whereas IS141-type recombinants were detected every year since 2015, IS60-type recombinants were detected only in 2016, 2018 and 2019.

3.4. Infection profiles of samples collected in Spain

Among the 17 samples analysed, 15 were detected virus- positive and all of these 15 plants exhibited a mono-infection profile. Thus, thirteen samples were positive only for the presence of TYLCV-IL, and two only for TYLCV-IS76-type recombinants. Whereas TYLCV-IL was detected every year of sampling between 2015 and 2018, TYLCV-IS76-type recombinants were detected in 2018 only.

The TYLCV-IL-positive samples were analysed further by sequencing a PCR product containing nucleotide positions that can be used to discriminate two TYLCV strains previously reported from Southeast Spain as group 1 and 2 (Torre et al., 2018). Viruses infecting the 5 TYLCV-IL positive plants collected in 2015 and 2016 clustered with group 1 viruses (Fig. 3); 2 TYLCV-IL-positive samples collected later (2018) were found to be infected with group 1 viruses too. Viruses infecting all the other TYLCV-IL-positive samples, i.e., 2 collected in 2017 and 3 collected in 2018, clustered with group 2 viruses. Interestingly, one of the three samples collected in 2017 was found to be co-infected with group 1 and group 2 viruses. Indeed, the Sanger-sequencing electropherogram obtained with this sample exhibited double peaks at group1/group2 discriminating positions.

Like the 4 samples collected in Ouled-Frej (Morocco) that tested negative in spite of their collection on symptomatic plants, 2 samples collected in Murcia in Oct 2018 revealed the same inconsistency (Table 1). As suggested for the Ouled-Frej samples, the PCR tests were not sensitive enough to detect the potentially lower virus amount that is expected in resistant plants. It is also possible that the milder TYLCD symptoms exhibited in Ty-1 resistant plants may have been confused with other types of symptoms.

4. Discussion

Begomovirus populations associated with TYLCD in western Mediterranean countries are composed of viruses belonging to the alien species *Tomato yellow leaf curl virus*, the native species *Tomato yellow leaf curl Sardinia virus*, and various interspecies recombinants. Although representatives of each of these three TYLCD-associated virus types were reported from Spain, Italy and Morocco, country specificities were previously detected with plant samples collected over time but mostly before 2015; sampling efforts extended up to 2003 in Spain (Garcia-Andres et al., 2007), up to 2008 in Italy (Davino et al., 2009) and up to 2014 in Morocco (Belabess et al., 2015). Considering geographic proximity and trade activities between these countries and the efficient whitefly transmission of these viruses, it was not known if these specificities would be maintained. This question was addressed here by analysing the infection profile of samples collected between 2015 and 2019 in the three countries.

Due to their resistance breaking feature in Ty1 resistant plants (Belabess et al., 2015; Fortes et al., 2023; Panno et al., 2018), Srec recombinants were given special attention by distinguishing them from the Lrec recombinants, commonly reported from Spain and Italy (Davino et al., 2012; Garcia-Andres et al., 2006; Monci et al., 2002). Thus, using an original Srec/Lrec discriminating PCR test together with the previously reported multiplex PCR tests (Belabess et al., 2015), we were able to specifically

determine the proportion of samples containing Srec recombinants among the samples detected positive for TYLCD-associated begomoviruses.

4.1. TYLCV-IS76 is virtually the only TYLCD-associated begomovirus on the Atlantic coast of Morocco

The dominance of Srec recombinants in the samples collected from Morocco and their identification as TYLCV-IS76 is fully consistent with previous results (Belabess et al., 2015). Indeed, these authors showed that TYLCV-IS76 has replaced its parental viruses in the region of Agadir (Souss) and that it started to spread northwards. The northward spread is confirmed here with Srec recombinants being the only begomovirus type detected in the 10 farms located in the north of Agadir along the Atlantic coast up to Casablanca. The detection of TYLCSV in 2015 in Agadir is consistent with previous results showing that TYLCSV was more frequently detected than TYLCV among the rare tomato samples in which parental type viruses were detected after the invasion of TYLCV-IS76 (Belabess et al., 2015). Further studies are needed with plant samples from northern and eastern Morocco to complete the picture of tomato begomoviruses in Morocco.

4.2. Srec recombinants are present in virtually all begomovirus-positive samples in Italy

Most of the samples collected from Italy are from Sicily. One of the major results with Sicilian samples is that all the plants samples were positive for TYLCV/TYLCSV recombinants, comprising 11 samples in which the TYLCV parent was of the IL strain, 1 sample in which it was of the Mld strain, and 15 samples in which both type of recombinants were detected, i.e., IL/Sar and Mld/Sar. These infection profiles can be compared with those determined from tomato samples collected in Sicily before 2015 (Belabess et al., 2015; Davino et al., 2008; Davino et al., 2009) because similar PCR approaches were used (Fig. 4). However, as the PCR techniques used by Davino et al were not designed to distinguish IL and Mld strains (Davino et al., 2008; Davino et al., 2009), the comparison was done after grouping IL and Mld strain-positive samples - distinguished in our study and in that of Belabess et al. - in a unique TYLCV group, and similarly, after grouping IL/Sar and Mld/Sar recombinants in a unique TYLCV/TYLCSV group. In 2002, shortly after the invasion of TYLCV, only three types of infection profiles were detected. Subsequently, the number of infection profiles increased with a maximum of 6 infection profiles in 2004 and 2008. Such highly diverse infection profiles were confirmed from samples collected in Sicily between 2006 and 2009, at the rate of 100 samples per year (Davino et al., 2012). In spite of high sampling efforts, samples that were positive only for TYLCV/TYLCSV recombinants ("TYLCV/TYLCSV") were not detected before 2009. An important change has apparently occurred between 2009 and 2012, because among the 9 samples collected in the 2012-2013 period, 2 samples exhibited the "TYLCV/TYLCSV" infection profile (Belabess et al., 2015). In samples collected later, between 2015 and 2019, that were analysed in this study, "TYLCV/TYLCSV" was the dominant infection profile, suggesting that recombinants with a relatively higher fitness have emerged. Noteworthy, while the recombinants reported from Sicily before 2012 were all of the Lrec type (Davino et al., 2012; Davino et al., 2009), the recombinants detected in the samples collected during 2015-2019, were all of the Srec type (Table 1) and 5 out of the 9 samples collected in 2012-2013, were positive for Srec too (Belabess et al., 2015). All together these results suggest that the relatively higher fitness of the recombinants detected since 2012 is determined by their typical recombination profile. This conclusion is supported by the identification of TYLCV-IS141 in most of the samples that were analysed further by sequencing [Table 1 and (Belabess et al., 2015)], a recombinant whose fitness was reported to be similar to that of TYLCV-IS76, the invasive recombinant that emerged in Morocco (Urbino et al., 2020).

Unlike Sicilian samples which were all negative for TYLCSV, the four Sardinian samples were all positive for TYLCSV. However, like in Sicily, all the samples detected positive of TYLCV/TYLCSV recombinants were positive for Srec but negative for Lrec. Although further conclusions cannot be drawn from such

a limited number of samples, these results show at least that the prevalence of TYLCD associated begomoviruses in Sardinia cannot be extrapolated from the results obtained from Sicily where TYLCSV was not detected in any of the 33 samples analysed.

4.3. TYLCV-IS60, a new Srec recombinant spreading in Sicily

Interestingly, besides TYLCV-IS141, a second Srec recombinant was detected in Sicily during the period 2015-2019, i.e., TYLCV-IS60. Although TYLCV-IS60 was detected less frequently than TYLCV-IS141 (Table 1), it exhibits features of an emerging virus. Indeed, the 4 samples that were positive for TYLCV-IS60 were not positive for any of the targeted begomoviruses, indicating that TYLCV-IS60 is already circulating autonomously. Moreover TYLCV-IS60 was detected from samples collected in three of the five years of sampling, and it was detected in at least two distinct collection sites, namely Marina di Ragusa and Vittoria.

4.4. Emergence of TYLCV-IS76 and its presumed back recombinant in Southeast Spain

The dominant infection profile of the samples collected in the Murcia and Almeria provinces is "TYLCV" with 13 samples being positive for TYLCV only, among the 15 PCR-positive samples (Table 1). This result is consistent with previous reports with samples collected earlier from the same Spanish region. Thus, the five tomato samples collected in 2013 in Almeria province and analysed with the same PCR approach were PCR positive only for TYLCV (Belabess et al., 2015). Likewise, among 370 tomato samples collected in the two Spanish provinces in 2003 and detected positive for tomato begomoviruses by tissue blot hybridization, 368 were positive only with the DNA probe specific to TYLCV; 2 were positive with both the TYLCV and the TYLCSV probes and no sample was detected positive for TYLCSV only (Garcia-Andres et al., 2007). In this later study in which tomato samples were also analysed from the Malaga province, 45 tomato samples from the three provinces, detected positive by tissue-blot hybridization, were selected to be analysed further by sequencing a PCR product comprising the non-coding intergenic region and the 5'-proximal parts of Rep and V2 open reading frames. Among them, only three samples bore TYLCV/TYLCSV recombinant genomes, two which inherited the TYLCV fragment from a Mld strain parent and one from an IL strain parent. This low proportion of recombinants stands in contrast with the 2 samples detected positive for IL/Sar recombinants among the 15 PCR positive samples analysed here. The much higher prevalence of TYLCV/TYLCSV recombinants in tomato is apparently related with the length of the TYLCSV fragment inherited by the recombinants. Whereas the 2 recombinants detected in 2018 have inherited a short 76 nts fragment of TYLCSV, like that of the previously reported TYLCV-IS76 viruses, the three recombinants detected in 2003 inherited a much longer TYLCSV fragment that extended beyond the initiation codon of the V2 gene. While the TYLCV-IS76-positive sample form El Ejido confirms the emergence of TYLCV-IS76 in the Almeria province (Fortes et al., 2023; Torre et al., 2019), the detection of TYLCV-IS76 in a sample from Murcia shows that its distribution is not limited to the Almeria province (Table 1).

The high nucleotide identity between TYLCV-IS76 recombinants from Spain and Morocco (Fortes et al., 2023; Torre et al., 2019) suggest that their presence in Spain is due to an introduction from Morocco where it was initially detected in tomato plants sampled in 2010. Such a scenario may also explain the presence of a new type of TYLCV-IL viruses in Southeast Spain because of their close relation to TYLCV-IL viruses detected in Morocco from tomato plants sampled in 2013 in El Jadida (Fig. 3). Based on a high sequence identity with the TYLCV-inherited region of TYLCV-IS76, El Jadida-TYLCV-IL-like viruses were presumed to derive from a TYLCV-IS76 recombinant through a back recombination event (Belabess et al., 2015; Torre et al., 2018). Their detection in more than 50% of the TYLCV-IL-positive samples collected in 2017 and 2018, confirm their emergence in Southeast Spain since their first

detection from tomato samples collected in 2015 and 2016 (Torre et al., 2018). As these authors have analysed only samples from the province of Murcia, it was not known if the El Jadida-TYLCV-IL-like viruses have emerged elsewhere. Here, with samples collected in 2017 in the province of Almeria (Table 1), we show that their presence is not limited to the province of Murcia. The successful emergence of viruses of the El Jadida-TYLCV-IL-type is consistent with their fitness advantage shown with a Spanish clone inoculated in Ty-1 resistant tomato plants (Torre et al., 2018). Indeed, the El-Jadida type clone was significantly more accumulated than TYLCV-IL and TYLCV-IS76 in mono-infected plants. Considering the emergence of the El Jadida-TYLCV-IL-like viruses in Spain, it is puzzling that they were not detected in any of the plants that were sampled in 2015 and 2017 in the region of El Jadida; indeed, all the Moroccan samples tested negative for TYLCV-IL (Table 1). It is possible that their emergence was impeded in Morocco due to the dominance of TYLCV-IS76 which was shown to be more competitive than the El Jadida-TYLCV-IL when representatives of both types were co-inoculated in tomato plants, irrespective of the cultivar, with or without the Ty-1-resistance gene (Torre et al., 2018).

4.5 Concluding remarks

The populations of TYLCD-associated begomoviruses that are described in this study reveal that country specificities were still present between 2015 and 2019. The specificities concerning parental type viruses (TYLCV, TYLCSV) is their virtual absence in the sampled region of Morocco along the Atlantic coast, the relatively frequent detection of both parents in Italy but with potential regional specificities (Sicily/Sardinia), and frequent detection of TYLCV in Spain. The specificities concerning recombinants is revealed by the dominance of TYLCV-IS76 in Morocco but with no detection of TYLCV-IS141, the dominance of TYLCV-IS141 in Italy but with no detection of TYLCV-IS76, and confirmation of the emergence of TYLCV-IS76 and the El Jadida-TYLCV-IL-type in Southeast Spain.

However, besides these country specificities, a general trend was revealed with the original PCR test developed here to distinguish Srec and Lrec recombinants. Indeed, while TYLCV/TYLCSV recombinants detected in tomato samples collected before 2010 were all of the Lrec type (Belabess et al., 2015; Davino et al., 2008; Davino et al., 2012; Davino et al., 2009; Garcia-Andres et al., 2007; Garcia-Andres et al., 2006; Monci et al., 2002), all the tomato samples detected positive for TYLCV/TYLCSV recombinants in this study are positive for Srec type recombinants and negative for Lrec. Thus, since the first detection of Srec type from Morocco in 2010 and from Sicily in 2012 (Belabess et al., 2015), the prevalence of Srec recombinants has increased at the expenses of Lrec recombinants.

The higher competitiveness of TYLCV-IS76 as compared to Lrec recombinants observed in controlled conditions using vector transmission (Jammes et al., 2023) is consistent with these inverse dynamics of prevalence of Srec versus Lrec recombinants. The large regional scale at which this trend is detected may be explained by the cultivar switch that occurred in the three countries from susceptible to Ty-1 resistant cultivars, as reported from Morocco (Belabess et al., 2015; Belabess et al., 2016). More recent samplings are needed to monitor further the evolution of TYLCD-associated begomoviruses in these countries and particularly the dynamics of TYLCV-IS76 and El Jadida-TYLCV-IL-type in Spain, the fate of the new Srec recombinant circulating in Sicily (TYLCV-IS60), and the potential persistence of parental type viruses in the less intensive tomato production areas such as the North East of Morocco.

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Table 1: Begomovirus infection profiles of tomato plants sampled in Morocco, Italy and Spain. Total DNA of plant samples were analysed by PCR and DNA sequencing. The presence of Tomato yellow leaf curl virus (TYLCV), Tomato yellow leaf curl Sardinia virus (TYLCSV) and the interspecies recombinants derived from them were tested with the multiplex PCR MxPCR1 and MxPCR2 (Belabess et al., 2015); note that the test distinguishes the Israel and Mild strain of TYLCV and the recombinants derived from them, TYLCV-IL/TYLCSV (IL/Sar) and TYLCV-Mld/TYLCSV (Mld/Sar). "Sar/Ty" corresponds to potentially reciprocal recombinants TYLCSV/TYLCV. Samples from Spain detected positive for TYLCV-IL were analysed further by sequencing to specify to which formerly identified group they may belong to (Torre et al., 2018), i.e., group 1 (g1) or group 2 (g2) as shown in Fig.3. Samples detected positive for recombinants were analysed further by PCR tests to distinguish them according to the size of the fragment inherited from TYLCSV, either long (Lrec-IL or Lrec-Mld) or short (Srec) (Fig. 1, Table 2). Positions of recombination breakpoints were identified by sequencing PCR products; the number of "+" indicates the number of samples that were analysed by sequencing. Recombination breakpoints (RB) were detected at position 60, 76, or 141. [1]Samples for which recombinant genomes were totally sequenced following cloning; the three sequences of the Sardinian TYLCV-IS141 were reported previously (Granier et al., 2019). In the columns corresponding to PCR tests, + and - indicate that a PCR product of the expected size was detected or not detected, respectively; an empty box means that the PCR test was not done. The boxes containing a "+" were coloured in yellow to facilitate the reading of the table. In the column describing the samples, NA means not available.

Table 2: **Description of PCR primers and primer products.**

Figure 1: **Presentation of PCR primers designed to distinguish Srec and Lrec recombinants**. (A) The pair of PCR primers targeting Srec recombinants that inherited short TYLCSV derived fragments of less than 623 nts (Srec), comprise the forward primer Sar19F, complementary to TYLCSV genome sequences, and the reverse primer IM650R, complementary to TYLCV genome sequences. The reverse primers Ty153R and Ty282R were used to further analyse Srec-positive samples for the presence of IS76- or IS141-type recombinants. PCR products were resolved on a 1.5% agarose and stained with ethidium bromide (M, Benchtop ladder, Promega, France). (B) The forward PCR primer targeting Lrec recombinants that inherited TYLCSV-derived fragments of more than 623nts, is Sar599F, a primer complementary to TYLCSV genome sequences. To specifically detect Lrec derived from TYLCV-IL and those derived from TYLCV-Mld, two reverse primers were used, IL-2258R and Mld-2310R, respectively. PCR products were resolved on a 1% agarose gel (M, Gene ruler 1kb, Fisher Scientific SAS, France). All PCR primers are described in Table 2. Noteworthy, as the discriminating region between TYLCV-IL and TYLCV-Mld is located only between position 2000 and the end of the genome, it was not possible to infer from the Srec PCR product whether the TYLCV parent of the Srec was of the IL or the Mld strain. PCR specificity was validated with total DNA of agroinfected tomato plants containing representatives of the targeted recombinant types, except for the Lrec-Mld type for which a recombinant plasmid DNA was used as described in section 2.3 of the Material and Methods. IS76, TYLCV-IS76; IS141, TYLCV-IS141; X, non-used wells; PC, total DNA from a tomato plant of the cultivar Pristyla infiltrated with an agrobacterium clone carrying an empty PC2300 binary vector.

Figure 2: **DNA alignments showing the typical recombination breakpoint of the TYLCV-IS60 recombinant.** Partial DNA sequences of TYLCSV-Sic (GenBank accession number GU951759), TYLCV-IL from Italy (GenBank accession number DQ144621) and one representative of the TYLCV-IS60 (GenBank accession number OR473055) were aligned. Number 1 is the nucleotide position of the origin of replication, and positions 57 to 62 is the range of non-discriminating nucleotides bearing the recombination breakpoint RB60 typical of TYLCV-IS60. The range of non-discriminating positions bearing RB76 is shown for information. The numbering is according to the TYLCSV sequence.

Figure 3: **Neighbour-joining tree showing the genetic distance among isolates of Tomato yellow leaf curl virus (TYLCV) originating from Southeast Spain**. The tree was constructed from the alignment of a 592-nt-genomic fragment between the origin of replication and the first third of the capsid protein

gene. The sequences used for the comparison were from viruses isolated in this study (highlighted in yellow) as well as from other isolates for which sequences were downloaded from Genbank. All the isolates were from tomato plants (*Solanum lycopersicum*) except the isolates collected in 1999 and 2003 which were collected from pepper (*Capsicum annuum*) (written in blue). Sequence data from this study were obtained by sequencing a PCR product generated with the primer pair IL-2629F/ IM650R, and the sequences of the 592-nt fragment are available in Supplementary material 1. Virus isolates from this study are identified with a code number followed by the location and date of their collection. Isolates written in black without yellow highlights were collected in the province of Murcia and are identified with their GenBank accession number and the code numbers used previously, in which 15 and 16 correspond to the year of collection (2015, 2016) and AG and MZ to the sampling locations, i.e., Aguilas and Mazaron, respectively (Torre et al., 2018). Isolates written in blue correspond to the earliest collected TYLCV in Southeast Spain for which total genome sequences were reported. Isolates written in red correspond to the two TYLCV isolates from El-Jadida (Morocco) that were reported to be the most closely related to isolates of a new type detected in Southeast Spain (Torre et al., 2018). The scale measures the Jukes and Cantor distance between sequences. Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting the nodes; only percentages ≥ 90% are represented. The two highly supported groups correspond to the previously identified group 1 and group 2 (Torre et al., 2018).

Figure 4: **Dynamics of the relative frequency of plants exhibiting particular profiles of begomovirus infections in Sicily.** The infection profiles of the samples collected between 2015 and 2019 were extracted from Table 1, whereas those of the samples collected earlier were reported previously (Belabess et al., 2015; Davino et al., 2008; Davino et al., 2009). As similar PCR approaches were used in previous and present studies and as all the begomovirus types targeted in the previous studies were also targeted here, the results are presented on the same graph. The figure presents the percentage of plants exhibiting each infection profile over time. The number of plants analysed in each collection period are indicated in brackets under the graph. The PCR tool used by Davino et al. was not specific enough to distinguish the two strains of TYLCV (IL and Mld) and their recombinants with TYLCSV. Hence, for the sake of homogeneity and clarity, the figure does not distinguish the IL and Mld strains for the samples analysed in this study (Table 1) and by (Belabess et al., 2015); IL and Mld strains were combined in a unique TYLCV group and similarly the recombinants IL/Sar and Mld/Sar were combined in a unique TYLCV/TYLCSV recombinant group. Noteworthy, it is only from the sampling of 2012-2013 that some samples were detected positive only for TYLCV/TYLCSV recombinants.

Supplementary material 1: DNA sequences of the 592-nt-fragment used to compare the 13 TYLCV isolates detected in tomato samples collected in Southeast Spain (Table 1) with samples collected in other studies and particularly in (Torre et al., 2018). Only 12 of the 13 sequences generated from TYLCV isolates from Spain are presented, because one of the three samples collected in the province of Almeria in 2017 (Table 1) was co-infected with group 1 and group 2 viruses; indeed, the electropherogram revealed double peaks at nucleotide positions at which the two types of viruses can be distinguished.

References

Accotto, G.P., Bragaloni, M., Luison, D., Davino, S., Davino, M., 2003. First report of Tomato yellow leaf curl virus (TYLCV) in Italy. Plant Pathology 52, 799.

Belabess, Z., Dallot, S., El-Montaser, S., Granier, M., Majde, M., Tahiri, A., Blenzar, A., Urbino, C., Peterschmitt, M., 2015. Monitoring the dynamics of emergence of a non-canonical recombinant of Tomato yellow leaf curl virus and displacement of its parental viruses in tomato. Virology 486, 291-306.

Belabess, Z., Peterschmitt, M., Granier, M., Tahiri, A., Blenzar, A., Urbino, C., 2016. 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. Journal of General Virology 97, 3433-3445.
Belabess, Z., Urbino, C., Granier, M., Tahiri, A., Blenzar, A., Peterschmitt, M., 2018. The typical RB76 recombination breakpoint of the invasive recombinant tomato yellow leaf curl virus of Morocco can be generated experimentally but is not positively selected in tomato. Virus Res. 243, 44-51.
Davino, S., Davino, M., Accotto, G.P., 2008. A single-tube PCR assay for detecting viruses and their recombinants that cause tomato yellow leaf curl disease in the Mediterranean basin. Journal of Virological Methods 147, 93-98.
Davino, S., Miozzi, L., Panno, S., Rubio, L., Davino, M., Accotto, G.P., 2012. Recombination profiles between Tomato yellow leaf curl virus and Tomato yellow leaf curl Sardinia virus in laboratory and field conditions: evolutionary and taxonomic implications. Journal of General Virology 93, 2712-2717.
Davino, S., Napoli, C., Davino, M., Accotto, G.P., 2006. Spread of Tomato yellow leaf curl virus in Sicily: partial displacement of another geminivirus originally present. Eur J Plant Pathol 114, 293-299.
Davino, S., Napoli, C., Dellacroce, C., Miozzi, L., Noris, E., Davino, M., Accotto, G.P., 2009. Two new natural begomovirus recombinants associated with the tomato yellow leaf curl disease co-exist with parental viruses in tomato epidemics in Italy. Virus Res. 143, 15-23.
Dellaporta, S.L., Wood, J., Hicks, J.B., 1983. A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1, 19-21.
Fortes, I.M., Fernández-Muñoz, R., Moriones, E., 2023. Crinivirus Tomato Chlorosis Virus Compromises the Control of Tomato Yellow Leaf Curl Virus in Tomato Plants by the Ty-1 Gene. Phytopathology 113, 1347-1359.
Garcia-Andres, S., Accotto, G.P., Navas-Castillo, J., Moriones, E., 2007. Founder effect, plant host, and recombination shape the emergent population of begomoviruses that cause the tomato yellow leaf curl disease in the Mediterranean basin. Virology 359, 302-312.
Garcia-Andres, S., Monci, F., Navas-Castillo, J., Moriones, E., 2006. Begomovirus genetic diversity in the native plant reservoir Solanum nigrum: Evidence for the presence of a new virus species of recombinant nature. Virology 350, 433-442.
Granier, M., Tomassoli, L., Manglli, A., Nannini, M., Peterschmitt, M., Urbino, C., 2019. First Report of TYLCV-IS141, a Tomato Yellow Leaf Curl Virus Recombinant Infecting Tomato Plants Carrying the Ty-I Resistance Gene in Sardinia (Italy). Plant Dis 103, 1437-1437.
Jammes, M., Urbino, C., Diouf, M.B., Peterschmitt, M., 2023. Refining the emergence scenario of the invasive recombinant Tomato yellow leaf curl virus -IS76. Virology 578, 71-80.
Juárez, M., Tovar, R., Fiallo-Olivé, E., Aranda, M.A., Gosálvez, B., Castillo, P., Moriones, E., Navas-Castillo, J., 2013. First Detection of Tomato leaf curl New Delhi virus Infecting Zucchini in Spain. Plant Dis 98, 857-857.
Kheyr-Pour, A., Bendahmane, M., Matzeit, V., Accotto, G.P., Crespi, S., Gronenborn, B., 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Research 19, 6763-6769.
Lefeuvre, P., Martin, D.P., Harkins, G., Lemey, P., Gray, A.J.A., Meredith, S., Lakay, F., Monjane, A., Lett, J.M., Varsani, A., Heydarnejad, J., 2010. The Spread of Tomato Yellow Leaf Curl Virus from the Middle East to the World. Plos Pathogens 6.
Luisoni, E., Milne, R.G., Caciagli, P., Accotto, G.P., Conti, M., Gallitelli, D., Martelli, G.P., Antignus, Y., Cohen, S., Marras, F., Idini, G., Loche, P., Piras, S., Leoni, S., 1989. A geminivirus associated with a severe yellow leaf curl disease of tomato in Sardinia, in: Group, I.S.o.H.S.V.V.W. (Ed.), Recent Advances in Vegetable Virus Research (6th Conference), Asilomar, California, pp. 13-14.
Monci, F., Sanchez-Campos, S., Navas-Castillo, J., Moriones, E., 2002. A natural recombinant between the geminiviruses Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. Virology 303, 317-326.
Morilla, G., Antunez, C., Bejarano, E.R., Janssen, D., Cuadrado, I.M., 2003. A new tomato yellow leaf curl virus strain in southern Spain. Plant Dis 87, 1004-1004.

Morilla, G., Janssen, D., Garcia-Andres, S., Moriones, E., Cuadrado, I.M., Bejarano, E.R., 2005. Pepper (Capsicum annuum) is a dead-end host for Tomato yellow leaf curl virus. Phytopathology 95, 1089-1097.
NavasCastillo, J., SanchezCampos, S., Diaz, J.A., SaezAlonso, E., Moriones, E., 1997. First report of tomato yellow leaf curl virus is in Spain: Coexistence of two different geminiviruses in the same epidemic outbreak. Plant Dis 81, 1461-1461.
Noris, E., Hidalgo, E., Accotto, G.P., Moriones, E., 1994. High Similarity among the Tomato Yellow Leaf Curl Virus Isolates from the West Mediterranean Basin - the Nucleotide-Sequence of an Infectious Clone from Spain. Archives of Virology 135, 165-170.
Panno, S., Caruso, A.G., Davino, S., 2018. The nucleotide sequence of a recombinant tomato yellow leaf curl virus strain frequently detected in Sicily isolated from tomato plants carrying the Ty-1 resistance gene. Archives of Virology 163, 795-797.
Peterschmitt, M., Granier, M., Aboulama, S., 1999. First report of Tomato yellow leaf curl geminivirus in Morocco. Plant Dis 83, 1074.
Saitou, N., Nei, M., 1987. The neighbor joining method: A new method for reconstructing phylogenetic trees. Molecular and Biological Evolution 4, 406-425.
SanchezCampos, S., NavasCastillo, J., Camero, R., Soria, C., Diaz, J.A., Moriones, E., 1999. Displacement of tomato yellow leaf curl virus (TYLCV)-Sr by TYLCV-Is in tomato epidemics in spain. Phytopathology 89, 1038-1043.
Torre, C., Aguero, J., Aranda, M.A., 2019. First Evidence of Tomato Yellow Leaf Curl Virus-Israel IS76 Recombinant Isolates Associated with Severe Yellow Leaf Curl Epidemics in Resistant Tomatoes in Spain. Plant Dis 103, 780-781.
Torre, C., Donaire, L., Gomez-Aix, C., Juarez, M., Peterschmitt, M., Urbino, C., Hernando, Y., Aguero, J., Aranda, M.A., 2018. Characterization of Begomoviruses Sampled during Severe Epidemics in Tomato Cultivars Carrying the Ty-1 Gene. International Journal of Molecular Sciences 19.
Urbino, C., Regragui, Z.F., Granier, M., Peterschmitt, M., 2020. Fitness advantage of inter-species TYLCV recombinants induced by beneficial intra-genomic interactions rather than by specific mutations. Virology 542, 20-27.

Table 1

Origin	Sampling date	Region	Tomato genotype or cultivar (TYLCD resistance status)	TYLCD symptoms	Number of samples/ infection profile	MxPCR2 (Belabess et al. 2015)			MxPCR1 (Belabess et al. 2015)			PCR tests (Fig.1)			Partial genome sequence		
						TYLCV -IL	TYLCV - Mld	Sar/Ty	TYLCSV	IL/Sar	Mld/Sar	Lrec - IL	Lrec - Mld	Srec	RB60	RB76	RB141
Morocco (Atlantic coastal region)	Dec 2015	Agadir	Breeding material (NA)	NA	1	-	-	-	+	-	-						
					1	-	-	-	-	+	-	-		+		+	
					1	-	-	-	-	-	-						
	Jan-Apr 2017	Oualidia	Antalya (S)	+	6	-	-	-	-	+	-	-		+			
					2	-	-	-	-	+	+	-	-	+			
			Calvi (S)		8	-	-	-	-	+	-	-		+		+	
		Safi doukkala had lhrara	Calvi (S)		4	-	-	-	-	+	-	-		+		+	
		Chtouka ouled laissaoui	Calvi (S)		4	-	-	-	-	+	-	-		+			
		Ouled Frej	Calvi (S)		8	-	-	-	-	+	-	-		+			
			Pristyla (Ty-1)		4	-	-	-	-	-	-						
		Azmour	Dynamite (S)		4	-	-	-	-	+	-	-		+		+	
		Agadir	Calvi (S)		4	-	-	-	-	+	-	-		+		+	
		Agadir	Wafira (S)		4	-	-	-	-	+	-	-		+			
Italy (Sardinia)	Sept 2016	NA	Parioli (Ty-1)	-	1	-	-	-	+	-	-						
				+	2	-	-	-	+	+	-	-		++[1]			++[1]
					1	+	-	-	+	+	-	-		+[1]			+[1]
Italy (Sicily)	Nov 2015	NA	Breeding material (NA)	NA	2	-	-	-	-	+	+	-	-	+			++
	Dec 2016	Gela	Pakita (NA)	yellowing	1	+	-	-	-	+	+	-	-	+			
		Marina di Ragusa	Ikram (S)	+	1	-	-	-	-	+	-	-		+	+		
		Gela	Creativo (Ty-1)	yellowing	1	+	-	-	-	+	-	-		+			+
	Dec 2017	Pachino	Breeding material (NA)	NA	6	-	-	-	-	-	-						
					1	+	+	-	-	+	+	-	-	+			+
					1	-	-	-	-	-	+		-	+			+
			Creativo, C113 (Ty-1)		2	-	-	-	-	+	-	-	-	+			++

	Oct 2018	NA	NA (NA)	NA	3	-	-	-	-	+	-	-	-	+	+++[1]		
					2	-	-	-	-	+	-	-	-	+			++
	Oct 2019	Vittoria	Breeding material (NA)	NA	9	+	-	-	-	+	+	-	-	+			++
					2	-	-	-	-	+	-	-	-	+	+		
					2	-	-	-	-	+	+	-	-	+			
Spain (Southeast)	Dec 2015	Almeria	6304 La Canada (NA)	NA	1	+ (g1)	-	-	-	-	-						
			Breeding material (NA)		1	+ (g1)	-	-	-	-	-						
	Feb 2016	Almeria (El Ejido)	Ateneo (Ty-1)	+	2	+ (g1)	-	-	-	-	-						
			Caniles (Ty-1)	+	1	+ (g1)	-	-	-	-	-						
	Sep 2017	Almeria	Breeding material (NA)	+	1	+ (g1,g2)	-	-	-	-	-						
					2	+ (g2)											
	Oct 2018	Almeria (El Ejido)	7966 (NA)	?	1	-	-	-	-	+	-	-		+		+	
		Murcia	L4238.0 (Ty-2?), C4239.0 (Ty-1)	+	2	-	-	-	-	-	-						
		Murcia	Calvi (S), Pristyla (Ty-1)	+	2	+ (g1)											
		Murcia	Myriade (S); Faustyno, C113 (Ty-1)	+	3	+ (g2)	-	-	-	-	-	-					
		Murcia	Vinety (Ty-1)	+	1	-	-	-	-	+	-	-		+		+	

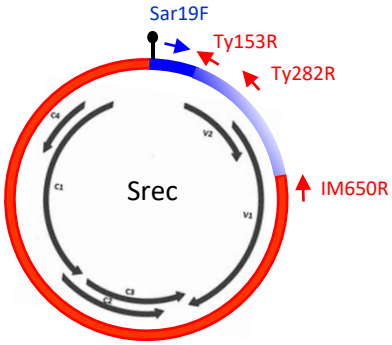
Table 2: Description of PCR primers and primer products

Name of PCR test Primer Name	Sequence	Targeted region (Genomic coordinates)[2]	Recombinant type detected	Size of expected product
Srec specific PCR				
Sar19Fd[1]	5'-CCGATAAAGWAGTRGGHCCTACGCA-3'	IR (19-43)	Forward primer to detect SRec	-
Sar19Fnd[1]	5'-CCGATAAAGTAGTGGGCCCTACGCA-3'	IR (19-43)		
Ty282Rd[1]	5'-CACGKGCYCTTACAACRGATATA-3'	V2 (296-274)	Reverse primer to detect SRec with TYLCSV-derived fragments of less than 274 nucleotides like IS76 and IS141	278bp
Ty282Rnd[1]	5'- CACGGGCCCTTACAACAGATATA -3'	V2 (296-274)		
TY-153Rnd[1]	5'-AGTGGGTCCCACATATTGCAAGAC-3'	IR/V2 (153-130)	Reverse primer to detect SRec with TYLCSV-derived fragments of less than 130 nucleotides like IS76	135bp
IM-650R	5'-TCGATTTAACACAGAACCTC -3'	V1 (642-623)	Reverse primer to detect SRec with TYLCSV-derived fragments of less than 530 nucleotides	624bp
Lrec specific PCR				
Sar599F	5'- GGTATTACWCATAGAGTWGGTAAAC-3'	V1 (599-623)	Forward primer to detect LRec-IL and LRec-Mld	-
IL-2258R	5'-TCARCTAGAGGAGGTCARCAATCT-3'	C1 (2250-2227)	Reverse primer to detect LRec-IL	1652bp
Mld-2310R	5'-CGATAAGGACGGARACACCG-3'	C1 (2310-2291)	Reverse primer to detect LRec-Mld	1712bp

[1] Primers reported in Belabess et al. (2018). [2] Nucleotide coordinates of the primers are referred to the genome of TYLCV-IS76 [MA:SouG8:10] (GenBank accession number LN812978), except for primer Sar599F for which they refer to the genome of TYLCSV-ES [MA:Aga5a:12] (GenBank accession number LN846598) and primer Mld-2310R to the genome of TYLCV-Mld[RE:02] (GenBank accession number AJ865337). Position 1 is always the position of the first nucleotide to be replicated at the origin of replication.

Figure 1

A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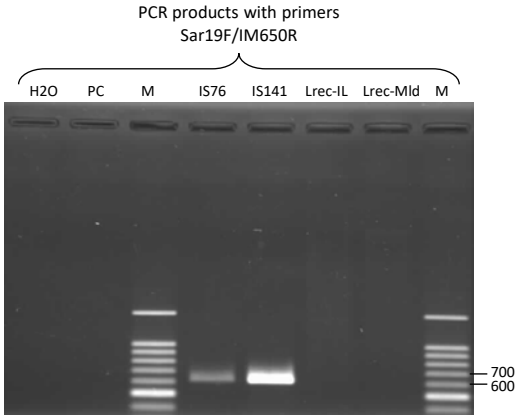
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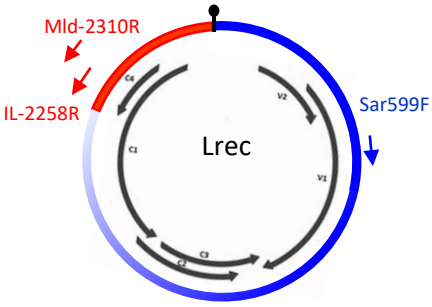
B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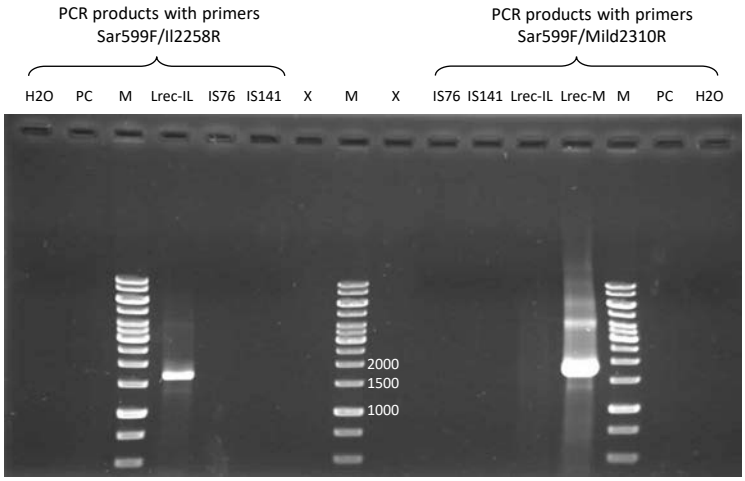
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PCR products with primers
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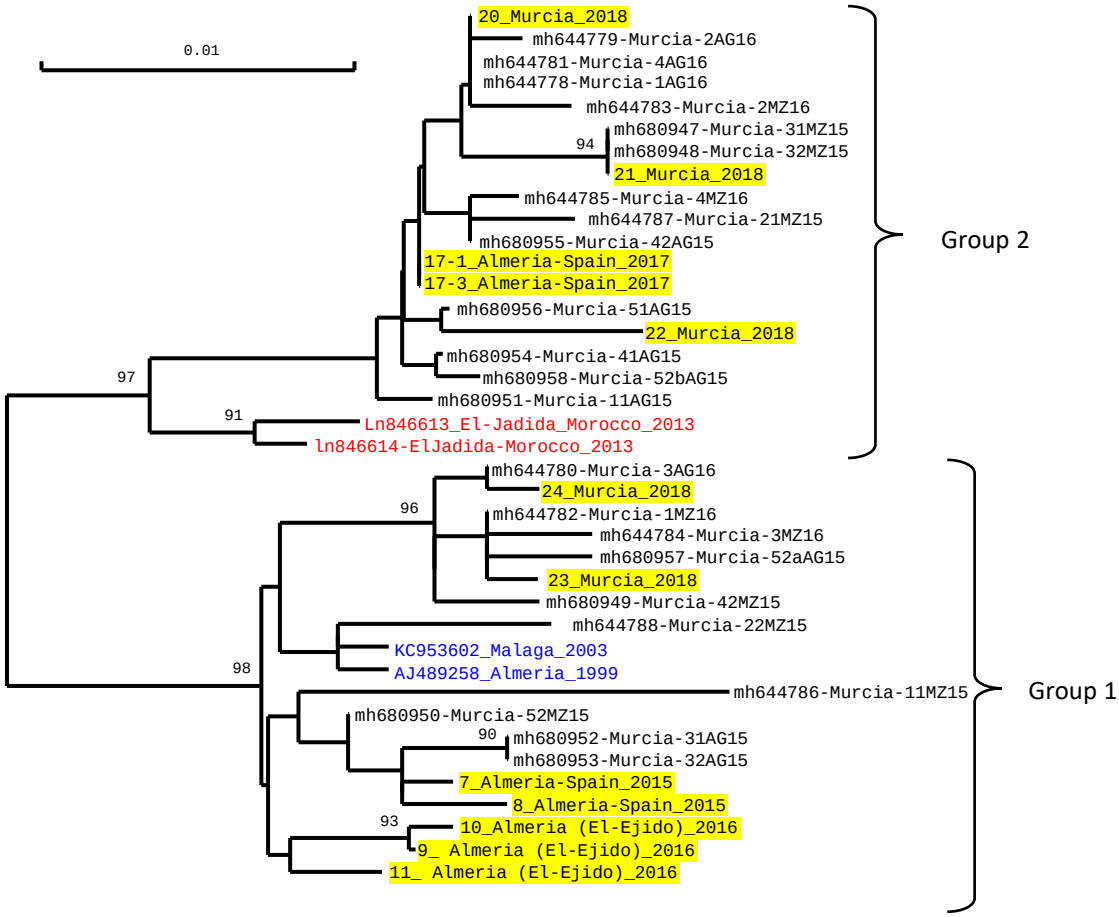
Figure 2

Figure 3



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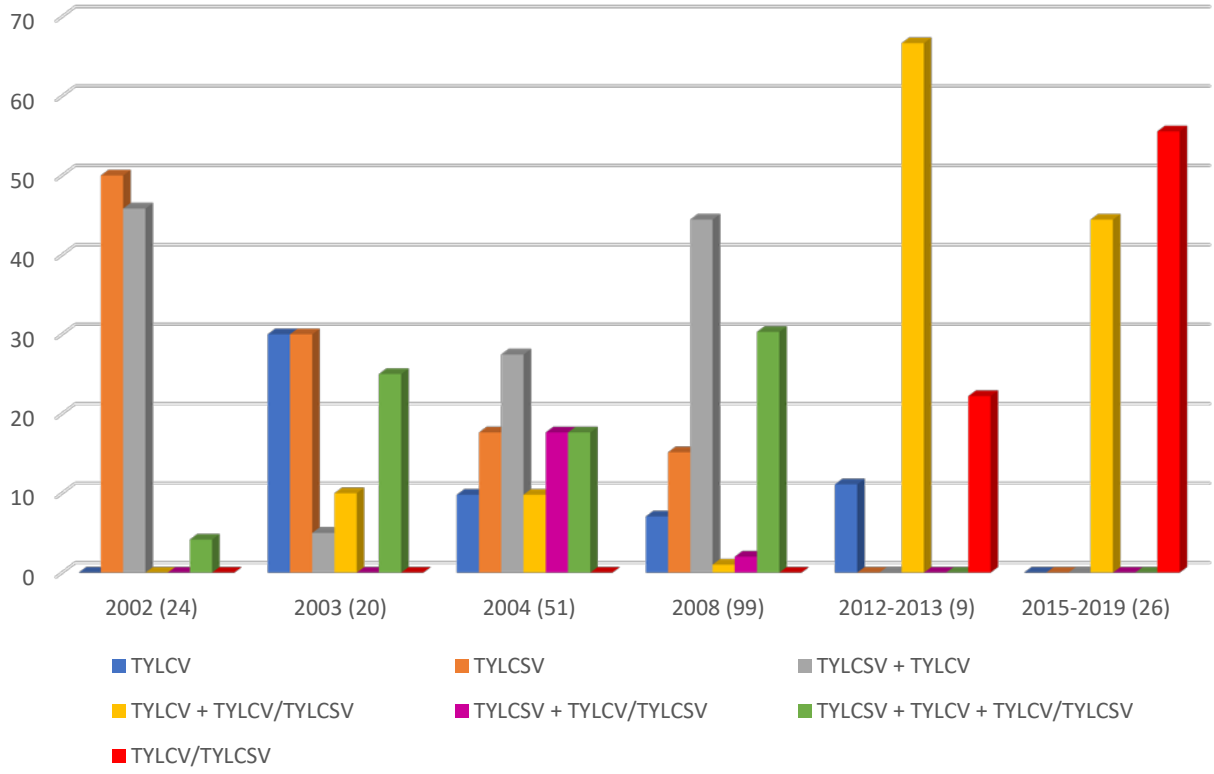
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Supplementary material 1

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