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 *Begomovirus solanumflavusardiniaense* (Tomato yellow leaf curl Sardinia virus, TYLCSAV), two strains of *Begomovirus coheni* (Tomato yellow leaf curl virus, TYLCV) introduced from the Middle East, and several types of TYLCV/TYLCSaV recombinants including the invasive recombinant TYLCV-IS76 in which the genome fragment inherited from TYLCSaV 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/TYLCSaV 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, 105 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 emerging resistance-breaking recombinants bearing short TYLCSaV-inherited fragments like TYLCV-IS76 (Srec) from formerly reported recombinants with longer TYLCSaV 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-IMS60-2400) both detected in Italy and not in Morocco. Nevertheless, besides country specificities, a general population shift was revealed regarding TYLCV/TYLCSaV 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* L.) 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, ICTV_Master_Species_List_2023_MSL39), 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 (TYLCSaV). The names of the 43 NW begomoviruses indicate much more diverse symptoms like mosaic, mottle, distortion, rugose, streak… 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, TYLCSaV and TYLCV. They both cause tomato yellow leaf curl disease (TYLCD), an economically important disease of tomato. TYLCSaV was reported first (Kheyr-Pour et al., 1991; Luisoni et al., 1989; Noris et al., 1994) and is generally considered to be indigenous to the Western Mediterranean region (Lefeuvre et al., 2010). TYLCV is a foreign begomovirus that probably arose somewhere in the Middle East (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 and TYLCV-IL were detected, were collected in 1997 (NavasCastillo et al., 1997) and 2002 (Morilla et al., 2003), respectively. 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 TYLCSaV 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 the subsequent introduction of a non-native virus species from Asia, i.e. *Tomato leaf curl New Delhi virus* (ToLCNDV) (Juárez et al., 2013), but also due to recombinants between TYLCV and TYLCSaV 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/TYLCSaV recombinants was 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/TYLCSaV recombinants than the Italian and Moroccan environment. The contrasted practice of bean (*Phaseolus vulgaris* L.) cultivation between Southern Spain and Sicily was suggested to be critical (Davino et al., 2009); in Southern Spain, this host of TYLCD-associated viruses is regularly cultivated between tomato crops, but not in Sicily. Thus, this specific cultivation 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 TYLCV/TYLCSaV recombinants detected in plants tested negative for parental-type viruses reflecting their autonomous spread. The two autonomously 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 TYLCSaV 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. After 2000, TYLCD-resistant-tomato cultivars bearing the *Ty-1* resistance gene have progressively replaced susceptible cultivars. This replacement coincided with the emergence of the autonomously spreading recombinant TYLCV-IS76 in Morocco that displaced its parental viruses (Belabess et al., 2015). This coincidence together with a relatively higher experimental competitiveness of TYLCV-IS76 in *Ty-1* resistant plants (Belabess et al., 2016) is consistent with a *Ty-1*-resistance driven emergence of TYLCV-IS76 by its positive selection. Intriguingly, although *Ty-1* cultivars were similarly deployed in Spain and Italy, TYLCV-IS76 did not emerge in these countries; TYLCV-IS76 recombinants were reported in Spain (Fortes et al., 2023; Torre et al., 2019), but due to their high nucleotide identity with Moroccan representatives, they presumably originate from Morocco (Torre et al., 2018). However, in Italy, a similar recombinant emerged, TYLCV-IS141. Its detection from *Ty-1*-resistant plants (Granier et al., 2019; Panno et al., 2018) and its relatively higher experimental competitiveness in *Ty-1* plants (Urbino et al., 2020) suggest that its emergence was triggered by the deployment of *Ty-1* resistant cultivars. In comparison to the autonomously spreading recombinants that emerged in Spain, those that emerged in Italy and Morocco inherited a much shorter TYLCSaV 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 TYLCSaV-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 TYLCSaV (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)..

Considering geographic proximity and trade activities between Morocco, Italy and Spain, and the efficient and persistent transmission of these viruses with the tiny polyphagous winged whitefly vector *Bemisia tabaci*, it was not known if these specificities would be maintained or rather fade over time due to natural or anthropogenic-induced 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 first determined with the multiplex PCR tests described by Belabess et al (Belabess et al., 2015). Then, 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 TYLCSaV inherited fragment (Srec), from those that inherited longer TYLCSaV 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 not detected in any of the samples from the three countries. These results are discussed in relation to the deployment of Ty-1-resistant cultivars and their positive selection of Srec recombinants.

2. Material and Methods

2.1. Plant material

Leaf samples were collected from net-house protected tomato crops between 2015 and 2019 in Morocco, Spain and Italy (Table 2, Supplementary material 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 in April, Chtouka ouled laissaoui with one farm sampled in March, Ouled Frej with 3 farms sampled in February, Oualidia with 4 farms

142 sampled in March, Safi doukkala had lhrara with one farm sampled in March, and Agadir with 2 farms
143 sampled in January.

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

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

149 2.2. DNA extraction

150 The 48 leaf samples collected in 2017 in Morocco were lyophilized and total DNA was extracted with
151 a reported protocol (Dellaporta et al., 1983) modified as follows. The amount of lyophilized plant
152 material used per extraction ranged between 1.25 and 20 mg. Plant material was ground in 400 µL
153 extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 1% SDS, 36 mM Na_2SO_3, and
154 0.1 mg/ml RNase), incubated at 65°C for 10 min and centrifuged (16,000 g, 10 min). One volume of
155 isopropanol was added to 300 µL of the supernatant and nucleic acids were precipitated by
156 centrifugation (16,000 g, 20 min); the pellet was washed with ethanol 70% and then resuspended in
157 50 µL water. As PCR amplifications failed for 32 of the samples, total DNA was extracted again for these
158 32 samples using a silica-based DNA extraction kit (DNeasy Plant Mini Kit, QIAGEN S.A., Courtaboeuf,
159 France) with 10 mg of lyophilized plant material per extraction.

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

164 2.3. PCR tests and primer design

165 Plant samples were first analysed with the two multiplex PCR tests MxPCR1 and MxPCR2, with which
166 the presence of TYLCV-IL, TYLCV-Mld, TYLCSaV, and recombinants derived from them (Belabess et al.,
167 2015) can be specifically detected. Due to the fact that all reported TYLCV/TYLCSaV recombinants bear
168 a recombination breakpoint in the stem loop bearing the origin of replication (OR), the test is expected
169 to detect all recombinants between TYLCV-IL and TYLCSaV and between TYLCV-Mld and TYLCSaV.
170 Another typical feature of these reported recombinants is that the sequence contiguous to OR and
171 located at the complementary sense gene side IS always inherited from TYLCV, whereas the sequence
172 located at the other side is always inherited from TYLCSaV; reciprocal recombinants have not been
173 reported so far.

174 Samples detected positive for the presence of TYLCV/TYLCSaV recombinants with MxPCR1, were
175 analysed further with original PCR tests designed in this study to distinguish between recombinants
176 that inherited a short TYLCSaV fragment (Srec) like TYLCV-IS76 or TYLCV-IS141, from those that
177 inherited long TYLCSaV fragments (Lrec) like TYLCAxV (Garcia-Andres et al., 2006) or TYLCMalV (Monci
178 et al., 2002).

179 The pair of PCR primers targeting Srec recombinants, comprise the previously reported forward primer
180 Sar19F (Belabess et al., 2018), complementary to TYLCSaV genome sequences, and an original reverse
181 primer IM650R, complementary to TYLCV genome sequences (Fig 1A and Table 1). The previously
182 designed reverse primers Ty153R and Ty282R (Belabess et al., 2018) were used to further analyse Srec-
183 positive samples for the presence of IS76- or IS141-type recombinants. The original forward PCR primer
184 targeting Lrec recombinants is Sar599F, a primer complementary to TYLCSaV genome sequences (Fig
185 1C and Table 1). To distinguish Lrec having a TYLCV-IL parental virus like TYLCAxV (Garcia-Andres et al.,
186 2006) from Lrec having a TYLCV-Mld parental virus like TYLCMalV (Monci et al., 2002), two original
187 reverse primers were used, IL-2258R and Mld-2310R, respectively (Table 1). Noteworthy, as the TYLCV-

188 IL and TYLCV-Mld genomes can be distinguished only between position 2000 and the end of the
189 genome, it was not possible to infer from the Srec PCR products, located between position 1 and 650,
190 whether the TYLCV parent of the Srec was of the IL or the Mld strain. The specificity of the five original
191 primers of the Srec/Lrec PCR tests (Sar599F, IM650R, IL2258R and Mld2310R), was determined in silico
192 with full genomic sequences available in GenBank, 121 from TYLCV-IL clones, 18 from TYLCV-Mld
193 clones and 21 from TYLCSaV clones. The nucleotides determining the specificity of the primers were
194 written with bold letters in Table 1. Noteworthy, some of the primers were degenerated to take into
195 account the intra-species nucleotide diversity.

196 Considering the position of the primers described above, the Srec recombinants are TYLCV/TYLCSaV
197 recombinants that inherited a TYLCSaV fragment starting from the OR and extending at the maximum
198 up to position 622, just before the 3' terminal nucleotide of the TYLCV specific primer IM650R (Table
199 1). Lrec recombinants are TYLCV/TYLCSaV recombinants that inherited a TYLCSaV fragment that
200 extended at least up to position 623, corresponding to the 3' terminal nucleotide of primer Sar599F.
201 Lrec-IL are Lrec recombinants for which the TYLCV parent is from the IL strain whereas Lrec-Mld are
202 Lrec recombinants with a TYLCV-Mld parent.

203 The cycling protocol for the amplification of Srec recombinants was as follows: 5 min denaturation at
204 95 °C, followed by 30 cycles, each consisting of 45 s at 95 °C, 45 s at - 52 °C with reverse primers IM650R
205 and TY282Rd, 54°C with primer TY282Rnd and 55°C with primer TY153Rnd - and 45 s elongation at 72
206 °C. The PCR program was ended by a 5- or 10-min incubation at 72 °C. The PCR products were resolved
207 by electrophoresis in a 1% to 2% agarose gel according to the different lengths of the expected
208 fragments, and visualized with ethidium bromide staining.

209 The cycling protocol for the amplification of Lrec recombinants was as follow: 5 min denaturation at
210 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
211 program was ended by a 10 min incubation at 72 °C. The same protocol was used for Lrec-IL and Lrec-
212 Mld. The PCR products were resolved by electrophoresis in a 1% agarose gel and visualized with
213 ethidium bromide staining.

214 The Srec/Lrec discriminating PCR tests were validated with total DNA extracted from tomato plants
215 agroinoculated with infectious viral clones. Two DNA extracts were used as positive controls for Srec,
216 one from a tomato plant of the cultivar Pristyla (Gautier Semences, France) agroinfected with TYLCV-
217 IS76 (Belabess et al., 2015), and one from the cultivar Calvi (Gautier Semences) agroinfected with
218 TYLCV-IS141 (TYLCV-IS141[IT:Sic1; 13]) (Urbino et al., 2020). The positive control for Lrec-IL was an
219 extract of total DNA from a tomato plant of the cultivar Pristyla co-agroinoculated with TYLCV-IL and
220 TYLCSaV (Belabess et al., 2018); this DNA was previously extracted from a leaf sample collected from
221 plant R11, 180 days post inoculation, and the presence of Lrec recombinants in this sample was
222 determined by Sanger sequencing of a PCR product generated with primers designed to detect
223 TYLCV/TYLCSaV recombinants [see Fig. 8 and Material and Methods of (Belabess et al., 2018)]. The
224 positive control for Lrec-Mld was a recombinant plasmid DNA containing the full-length genome of a
225 TYLCVMalV clone (Genbank accession number LN846611) (Belabess et al., 2015); the viral clone was
226 obtained from a bean plant (H5) sampled in March 2012 in the Souss region of Morocco. As negative
227 plant control, we used total DNA extracted from a tomato plant of the cultivar Pristyla infiltrated with
228 an agrobacterium clone carrying an empty PC2300 binary vector. The specificity of each primer pair
229 was tested with DNA extracts of target and non-target recombinants, and with the negative plant
230 control.

231 2.4. Sequencing of PCR products and sequence analysis

232 Some of the samples in which Srec type recombinants were detected by PCR, were further analysed
233 by sequencing to determine non-OR recombination breakpoints. To do this, the PCR products
234 generated with the Srec-specific PCR primer pair Sar19/IM-650R or the products generated with the
235 primer pair Ty-2225F/Ty-400R were sequenced by Sanger sequencing (Eurofins Genomics, Ebersberg,

236 Germany) using the PCR primer IM650R, and both PCR primers in the case of the second PCR product
237 (Table 1).

238 Additional sequencing of PCR products was done for plant samples detected positive for TYLCV but
239 only for those collected in Spain. Two TYLCV genotypes, group 1 and group 2, were previously
240 identified from tomato plants sampled in Southeast Spain in 2015 and 2016 (Torre et al., 2018). Group
241 1 viruses are related to the two early TYLCV-IL isolates of Southeast Spain, both sampled on pepper
242 (*Capsicum annuum*), one in 1999 in Almeria (TYLCV-IL[ES:Alm:Pep:99], GenBank accession number
243 AJ489258) (Morilla et al., 2005) and one in 2003 in Malaga (TYLCV-IL [ES:Mlg:TY11:Pep2003], GenBank
244 accession number KC953602). Group 2 viruses are closely related to two TYLCV-IL isolates sampled on
245 tomato in Morocco, TYLCV-IL[MA:El Jadida:Tom:2013] (GenBank accession number LN846613 and
246 LN846613) (Belabess et al., 2015). The most parsimonious evolutionary scenario suggested that the
247 TYLCV isolates of Group 2 are back recombinant isolates derived from TYLCV-IS76 (Torre et al., 2018).
248 The relatedness to group 1 or 2 of the TYLCV isolates from Spain, was based on a 803-nucleotide
249 fragment amplified with the forward primer IL-2629F (5'-GGTGTCCCTCAAAGCTCTAWG-3') of the
250 multiplex PCR test MxPCR2 (Belabess et al., 2015) and the original reverse primer IM650R (Table 1).
251 Using IM-650R as sequencing primer, 11 group 1/group2 discriminating nucleotide positions could be
252 determined. Sequences were aligned with 27 homologous genomic sequences from TYLCVs for which
253 complete viral genomes are available in Genbank, 25 from Spain and the 2 from Morocco
254 representative of group 2. The alignment was performed with the Optimal Alignment method of
255 DNAMAN (version 5.0; Lynnon BioSoft, Quebec, Canada). A phylogenetic tree was set up with a Jukes
256 and Cantor distance matrix using the neighbour-joining method (Saitou and Nei, 1987) as implemented
257 in the DNAMAN software. The sequences used for the alignment are described on the phylogenetic
258 tree presented in the section Results.

259 2.5. Cloning and sequencing of complete viral genomes

260 A new Srec type recombinant bearing a recombination breakpoint at position 60 was detected in Sicily
261 by sequencing PCR products. To characterize the full-length genome of this new type of Srec
262 recombinant, it was cloned from total DNA extracted from three plant samples with the DNeasy Plant
263 Minikit. Circular DNA molecules were amplified using a TempliPhi Amplification kit (GE Healthcare, UK)
264 according to the manufacturer's instructions, and digested with restriction enzymes that are expected
265 to be potentially unique according to reported sequences of parental type genomes of TYLCV and
266 TYLCSaV. Digestion of RCA products with *Sac*I generated linear fragment of about 2.8kb, a size
267 expected with full-length begomovirus genomes. The restricted fragments were ligated into the
268 plasmid pBC and ligation products were introduced into the bacterial strain *Escherichia coli* DH5alpha.
269 The cloned fragments were sequenced with M13 primers and virus-specific primers (not
270 communicated). Sequences were analysed with Chromas (Technelysium Pty Ltd, Australia) and
271 DNAMAN.

272 2.6. Recombination analysis

273 A recombination analysis was performed on full-length genomes of the new Sicilian Srec recombinant
274 bearing a recombination breakpoint at position 60. Recombination analysis was performed with the
275 Recombination Detection Program RDP4.101 (Martin et al., 2015) with default settings. The genomes
276 used for the input alignment are from clones of potential parental viruses present in Sicily, i.e., TYLCV-
277 IL, TYLCV-Mld, TYLCSaV and the recombinant TYLCV-IS141. The genomes of these viruses were from
278 clones isolated either from Italy or nearby countries in the Western Mediterranean region. The
279 genomes used for the alignment are from 17 TYLCV-IL clones (GenBank accession numbers AJ489258,
280 DQ144621, EF060196, KC953602, LN846613, LN846615, LN846616, LN846617, MH644779,
281 MH644782, MH644786, MH644787, MH644788, MH680947, MH680949, MH680953, MH680958), 3
282 TYLCV-Mld clones (AF071228, AF105975, AJ519441), 16 TYLCSaV clones (AJ519675, AY702650,
283 AY736854, GU951759, JN859134, JN859136, KC 953603, KC953604, LN846595, LN846596, LN846597,
284 LN846598, L27708, X61153, Z28390, Z25751) and 3 TYLCV-IS141 clones (MH817479, MG489968,

285 MF405078); The genome of a TYLCV-Mld clone (GenBank accession number KJ913683) isolated in
286 Dominican Republic was added to the alignment because it showed the highest genetic relatedness
287 with the RB60-bearing recombinant according to preliminary analysis. A similarity plot was produced
288 with the application SimPlot++ (Samson et al., 2022) to show the complete recombination profile of
289 the RB60 bearing recombinant.

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291 **3. Results**

292 3.1. Srec and Lrec recombinants can be distinguished with a discriminating PCR test

293 An original PCR test was designed to distinguish TYLCV/TYLCSaV recombinants that inherited short
294 TYLCSaV derived fragments of less than 623 nts (Srec), from those that inherited TYLCSaV-derived
295 fragments of more than 623nts (Lrec).

296 Amplification products generated with Srec/Lrec discriminating PCR primers are presented in Fig. 1B
297 and Fig. 1D. Srec primers do not generate any amplification product with Lrec infected plants and vice
298 versa. No amplification product was detected with a mock-inoculated plant infiltrated with the
299 agrobacterium clone carrying an empty PC2300 binary vector. A PCR product of higher-than-expected
300 size was resolved for the positive control of Lrec-Mld (Fig. 1D). As the control DNA for Lrec-Mld is a
301 full-length viral genome cloned in an *Escherichia coli* plasmid, we suppose that this long product was
302 generated with elongations that went all around the recombinant plasmid.

303 The Srec/Lrec discriminating PCR test was subsequently used to analyse tomato samples detected
304 positive for the presence of TYLCV/TYLCSaV recombinants according to the results obtained with the
305 MxPCR1 test (Belabess et al., 2015) targeting recombination breakpoints at the origin of replication.

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322 **Figure 1**: **Presentation of PCR primers designed to distinguish Srec and Lrec recombinants**. (A) PCR
323 primers targeting recombinants that inherited short TYLCSaV derived fragments of less than 623
324 nts (Srec). Primer positions are schematically presented on a circular viral genome; black arrows
325 correspond to the commonly reported open reading frames and the short vertical line at the top of
326 the circle correspond to the stem loop bearing the origin of replication. (B) PCR products obtained
327 with Srec-targeting primers resolved on a 1.5% agarose and stained with ethidium bromide (M,
328 Benchtop ladder, Promega, France). (C) PCR primers targeting recombinants that inherited
329 TYLCSaV-derived fragments of more than 623nts (Lrec). The reverse primer IL-2258R, specifically
330 hybridize to Lrec having a TYLCV-IL parental virus (Lrec-IL), whereas Mld-2310R specifically hybridize
331 to Lrec having a TYLCV-Mld parent (Lrec-Mld). (D) PCR products obtained with Lrec-targeting
332 primers resolved on a 1% agarose gel (M, Gene ruler 1kb, Fisher Scientific SAS, France). All PCR
333 primers are described in Table 1. IS76, TYLCV-IS76; IS141, TYLCV-IS141; PC, total DNA from a mock
334 inoculated plant.

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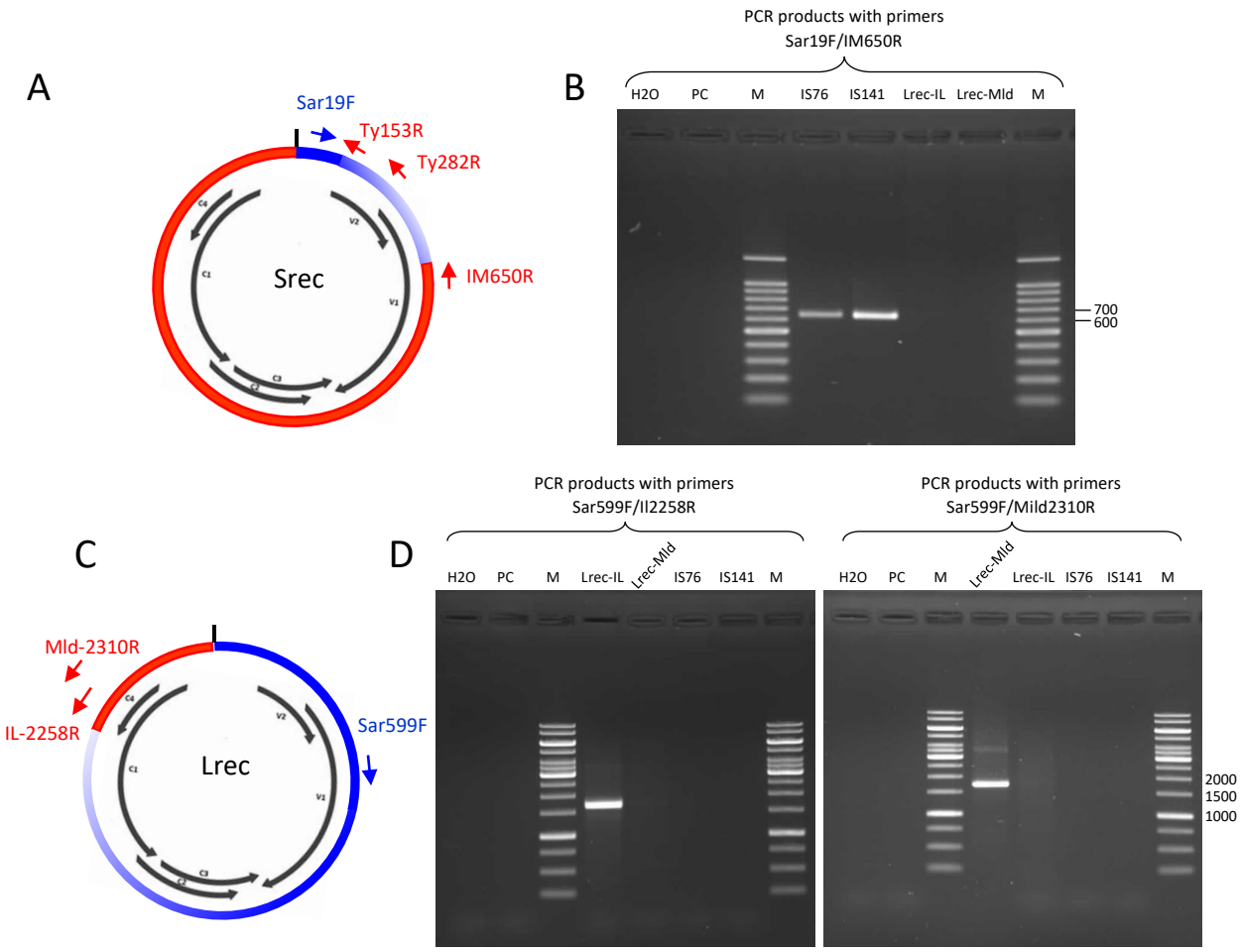

350 **Table 1**: **Description of PCR primers and primer products.**

Name of PCR test Primer Name	Sequence	Targeted region (Genomic coordinates)[3]	Recombinant type detected or analyzed further	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 TYLCSaV-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 TYLCSaV-derived fragments of less than 130 nucleotides like IS76	135bp
IM-650R[2]	5'-T**C**GA**T**TTAA**C**ACAGAACC**TC** -3'	V1 (642-623)	Reverse primer to detect Srec with TYLCSaV-derived fragments of less than 530 nucleotides	624bp
Lrec specific PCR				
Sar599F[2]	5'- GG**T**ATTACWCA**T**AGAGT**W**GGTAA**AC**-3'	V1 (599-623)	Forward primer to detect Lrec-IL and Lrec-Mld	-
IL-2258R[2]	5'-TC**A**RC**TA**G**A**GG**A**GG**T**CARCA**AT**C**T**-3'	C1 (2250-2227)	Reverse primer to detect Lrec-IL	1652bp
Mld-2310R[2]	5'-**C**GA**T**AA**G**GACGGARA**CACCG**-3'	C1 (2310-2291)	Reverse primer to detect Lrec-Mld	1712bp
PCR and Sanger sequencing primers to detect RB positions of IMS60-2400 recombinants				
Ty-2225F	GCAGATTGCTGACCTCCTCT	C1/C4 (2225-2244)	Production of a PCR fragment to be sequenced for RB detections	978bp
Ty-400R	GTTTGTGCCTTGGACAATGG	V1/V2 (419-400)		

351 [1] Primers reported in Belabess et al. (2018). [2] Primers designed in this study; the nucleotide positions determining the specificity to the targeted parental
352 genome (Fig. 1A, C) are in bold. [3] Nucleotide coordinates of the primers are referred to the genome of TYLCV-IS76 [MA:SouG8:10] (GenBank accession
353 number LN812978), except for primer Sar599F for which they refer to the genome of TYLCSaV-ES [MA:Aga5a:12] (GenBank accession number LN846598)
354 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
355 nucleotide to be replicated at the origin of replication. RB: recombination breakpoint.

356 3.2. Infection profiles of samples collected in Morocco

357 Among the 51 samples analysed, 46 were detected positive for at least one PCR-targeted virus and
358 three infection profiles were distinguished (Table 2): TYLCSaV (1 sample), IL/Sar (43 samples), and
359 coinfection with IL/Sar and Mld/Sar (2 samples). Moroccan samples can be characterized as follows.
360 (i) The parental virus types TYLCV-IL and TYLCV-Mld were not detected and the TYLCSaV type was
361 detected in only one sample. (ii) All the 46 PCR-positive samples, were positive for IL/Sar except the
362 sample detected positive for TYLCSaV only. (iii) The recombinant-positive samples were negative for
363 recombinant-types that inherited long TYLCSaV fragments (Lrec), but positive for Srec recombinants.
364 (iv) The recombination breakpoints within the sequence of the Srec PCR products were analysed for 5
365 plant samples by sequencing. For representativity reasons, the sequencing was performed on samples
366 of different regions and collection years. Recombination breakpoints were shown to be all at position
367 76, like that of the typical recombination breakpoint of TYLCV-IS76.

368

369

370 **Table 2: Begomovirus infection profiles of tomato plants sampled in Morocco, Italy and Spain.**
371 Total DNA of plant samples were analysed by PCR and DNA sequencing. The presence of Tomato
372 yellow leaf curl virus (TYLCV), Tomato yellow leaf curl Sardinia virus (TYLCSaV) and the interspecies
373 recombinants derived from them were tested with the multiplex PCR MxPCR1 and MxPCR2
374 (Belabess et al., 2015); note that the test distinguishes the Israel and Mild strain of TYLCV and the
375 recombinants derived from them, TYLCV-IL/TYLCSaV (IL/Sar) and TYLCV-Mld/TYLCSaV (Mld/Sar).
376 IL/Sar and Mld/Sar correspond to recombinants in which the sequence contiguous to the origin of
377 replication on the virus sense gene side is inherited from TYLCSaV. Inversely, “Sar/Ty” corresponds
378 to potential reciprocal recombinants TYLCSaV/TYLCV. Samples from Spain detected positive for
379 TYLCV-IL were analysed further by sequencing to specify to which formerly identified group they
380 may belong to (Torre et al., 2018), i.e., group 1 (g1) or group 2 (g2) as shown in Fig.4. Samples
381 detected positive for recombinants were analysed further by PCR tests to distinguish them
382 according to the size of the fragment inherited from TYLCSaV, either long (Lrec-IL or Lrec-Mld) or
383 short (Srec) (Fig. 1, Table 1). Positions of recombination breakpoints were identified by sequencing
384 PCR products (last 3 columns); the number of "+" indicates the number of samples that were
385 analysed by sequencing. Recombination breakpoints (RB) were detected at position 60, 76, or 141.
386 In the columns corresponding to PCR tests, + and - indicate that a PCR product of the expected size
387 was detected or not detected, respectively; an empty box means that the PCR test was not done.
388 The boxes containing a “+” were coloured in yellow to facilitate the reading of the table. In the
389 column describing the samples, NA means not available. The TYLCD resistance status of tomato
390 genotypes or cultivar is described with the presence of the resistance genes *Ty-1* and *Ty-2*, and with
391 S meaning susceptible. [1]Samples for which recombinant genomes were totally sequenced following
392 cloning; the three sequences of the Sardinian TYLCV-IS141 were reported previously (Granier et al.,
393 2019).

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	TYLCSaV	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 Ihrara	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]
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	+	-	-	-	+	+	-	-	+			++
					1	-	-	-	-	+	-	-	-	+			+
					1	-	-	-	-	+	-	-	-	+	+		
					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	-	-	-	-	+	-	-		+		+	

399 The 4 samples collected in Ouled-Frej from symptomatic *Ty-1* resistant tomato plants of the cultivar
400 Pristyla tested negative for all targeted begomoviruses whereas all the 8 samples from the susceptible
401 cultivar Calvi were detected positive. It is possible that the PCR tests were not sensitive enough to
402 detect the low virus amounts of Ty1 resistant Pristyla plants which were reported to be about 10 times
403 lower than the amounts in nearly isogenic susceptible tomato plants (Belabess et al., 2016). This
404 hypothesis is consistent with the observation of a relatively faint band observed in lane IS76 of the
405 agarose gel shown in Fig. 1A, corresponding to an amplification product obtained with the Srec specific
406 primers, from a Pristyla tomato plant agroinoculated with a clone of TYLCV-IS76. It is also possible that
407 the milder TYLCD symptoms exhibited by *Ty-1* resistant plants may have been confused with other
408 types of symptoms. Finally, it cannot be excluded that these samples are infected with TYLCD-
409 associated begomoviruses that are not detectable with the PCR approach used.

410 3.3. Infection profiles of samples collected in Italy

411 Among the 37 samples analysed, 31 were detected positive for at least one PCR-targeted virus. The
412 infection profiles are relatively heterogeneous. Indeed, unlike in Morocco where only one parental-
413 type virus (TYLCSaV) and only one type of TYLCV/TYLCSaV recombinant (TYLCV-IS76) were detected, in
414 Italy, all three parental type viruses were detected as well as two types of recombinants, TYLCV-IS141
415 and a new one described below, TYLCV-IMS60-2400 (IMS60-2400). Moreover, whereas only three
416 infection profiles were detected in Moroccan samples, up to 9 infection profiles were detected in
417 Italian samples. In spite of these differences between infection profiles of Italian and Moroccan
418 samples, some common features can be noted. Thus, like in Morocco, only one sample was infected
419 with a parental virus alone (TYLCSaV), whereas all the other virus-positive samples, were positive for
420 the presence of IL/Sar or Mld/Sar recombinants. Moreover, like in Morocco, all the recombinant-
421 positive samples are negative for Lrec recombinants, but positive for Srec recombinants.

422 To further characterize the Srec recombinants, the Srec PCR products from 20 of the 30 Srec-positive
423 samples were analysed by sequencing; for representativity reasons, samples were selected from each
424 collection year and from each reported sampling locations. The sequencing results revealed that 15
425 samples contained Srec recombinants of the IS141-type with a recombination breakpoint around
426 position 141 (RB141), whereas 5 samples contained a new type of Srec recombinants with a
427 recombination breakpoint located within the 57 to 62 position-range of identical nucleotides between
428 TYLCV and TYLCSaV parental genomes (Fig. 2A). The recombination breakpoint was named RB60, 60
429 being one of the two median positions of the 6 non-discriminating nucleotide range in which
430 recombination events occurred. Three RB60-bearing recombinant genomes were cloned from three
431 samples collected in October 2018 and were fully sequenced (GenBank accession numbers OR473055,
432 OR473056, OR473057). The genomes are 2783 or 2784 nts in length and differ from each other at 1, 8
433 or 9 positions. The potential presence of other recombination breakpoints was analysed with the
434 program RDP4. Two recombinant regions were detected (Table 3). One was detected in the intergenic
435 region of the viral sense gene side between RB60 and the stem loop bearing the origin of replication
436 known as a recombination hot spot in begomoviruses (Lefeuvre et al., 2007). A longer one was
437 detected between the stem loop and an RB at position 2400 (RB2400) including the 5' terminal regions
438 of the C1 and C4 genes and the intergenic region on the complementary sense gene side. Considering
439 the low probabilities of false positives obtained with respectively 5 and 7 methods of recombination
440 detection in RDP4, the recombinant origin of these two regions was strongly supported. The new
441 recombinant was named IMS60-2400 in which "I" stand for the IL strain of TYLCV, the major parent,
442 and "M" and "S" for the minor parents, i.e., the Mild strain of TYLCV and TYLCSaV; 60 and 2400 refer
443 to median nucleotide positions within the non-discriminating regions in which the two non-OR
444 recombination events occurred (Fig. 2A, 2B). The two samples detected positive for RB60-bearing

445 recombinants by partial sequencing but for which full-length genomes were not cloned and sequenced
446 (Table 2), were shown to contain both the RB60 and RB2400 by sequencing a PCR product generated
447 and sequenced with the primer pair Ty-2225F/Ty-400R (Table 1).

448 Six samples collected in Pachino on symptomatic plants from breeding material tested negative. As
449 suggested with symptomatic samples from *Ty-1* resistant plants collected in Ouled-Frej (Morocco), the
450 6 Pachino samples may have been collected from resistant plants in which the virus amount may be
451 too low to be detected. It is also possible that the supposed TYLCD symptoms were caused by some
452 other stresses such as other plant pathogens, or even pesticides. Finally, it cannot be excluded that
453 these samples are infected with TYLCD-associated begomoviruses that are not detectable with the PCR
454 approach used.

455

456 **Table 3: Detection of recombinant regions in the genome of TYLCV-IMS60-2400** (Genbank
457 accession number OR473055) with methods available in RDP4: RDP (R), GENECONV (G), BOOTSCAN
458 (B), MAXIMUM CHI SQUARE (M), CHIMAERA (C), SISTER SCAN (S) and 3SEQ (3S). The reported P-
459 value is for the method in bold type and is the best P-value of the detected region. Upper-case
460 letters were used for methods detecting the potential recombinant region with a false positive
461 probability of less than 10^{-9}, whereas lower-case letters were used when probability is between 10^{-}
462 ^{4} and 10^{-2}. The inferred recombinant regions are characterized by the positions of their terminal
463 nucleotides; positions are according to the genome of TYLCV-IMS60-2400, position 1 being the
464 origin of replication. Genes and intergenic regions contained in the recombinant regions are
465 indicated. IR, intergenic region.

Positions of the recombinant region	Genomic regions	Major parent	Minor parent	Detection methods	P value
2766 -59	IR	TYLCV-IL (DQ144621)	TYLCSaV (AY736854)	**R**,G, m,c,3s	$1,2 \times 10^{-19}$
2400-2723	C1, C4, IR	TYLCV-IL (DQ144621)	TYLCV-Mld (KJ913683)	R,G,B,M,C,S,**3S**	$1,7 \times 10^{-34}$

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467

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Figure 2: **DNA alignments showing the typical recombination breakpoints of the TYLCV-IMS60-2400 recombinant.** (A) Alignment showing the recombination breakpoint RB60. The partial DNA sequences are from the genome of three viral clones, TYLCV-IMS60-2400 (GenBank accession number OR473055) TYLCSaV-Sic from Tunisia (GenBank accession number AY736854), and TYLCV-IL from Italy (GenBank accession number DQ144621). 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. The range of non-discriminating positions bearing RB76 is shown for information. (B) Alignment showing the recombination breakpoint RB2400. The partial DNA sequences are from the genome of three viral clones, TYLCV-IMS60-2400, TYLCV-IL and TYLCV-Mld from Dominican Republic (GenBank accession number KJ913683). Positions 2399 to 2402 is the range of non-discriminating nucleotides bearing the recombination breakpoint RB2400. Nucleotide positions are numbered according to the genome of TYLCV-IMS60-2400.

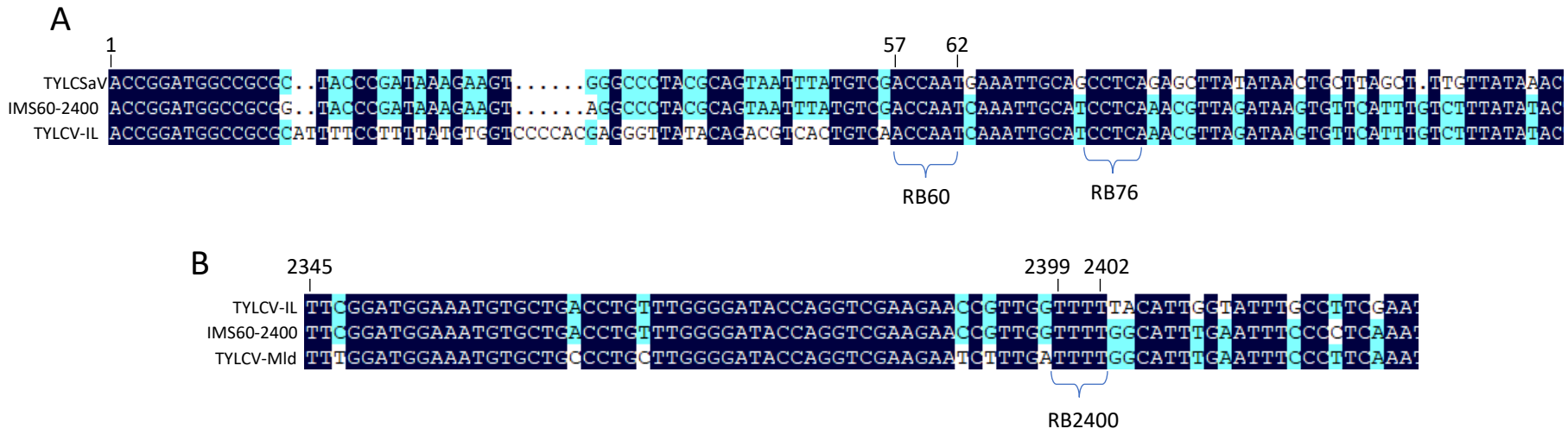
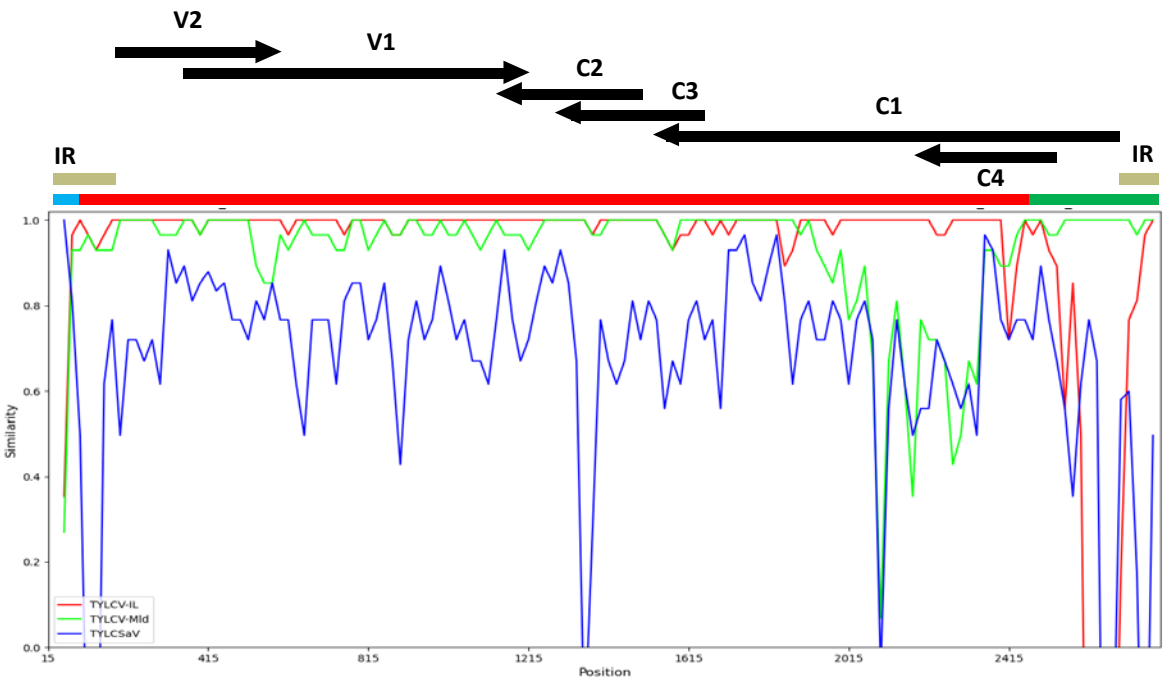


Figure 3: Similarity plot showing the recombination profile of TYLCV-IMS60-2400. The similarity plot was produced with the SimPlot++ application. The distance between sequences was determined with the Jukes Cantor model within a 30-nucleotide window slid by 20-nucleotide steps along the genome. The parental viruses were those inferred from the RDP analysis (Table 3). The recombination profile of the IMS60-2400 recombinant is presented in a linear form with the horizontal bar at the top of the similarity plot. The parental origin of the recombinant regions is presented with the colour code used in the similarity plot. The intergenic region and the six commonly reported ORFs of monopartite begomoviruses are shown at the top of the figure.



The infection profiles determined with the samples collected in Sicily were compared with those determined from tomato samples collected there before 2015 (Belabess et al., 2015; Davino et al., 2008; Davino et al., 2009) (Supplementary material 2). This comparison was possible because similar PCR approaches were used to determine the infection profiles. 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/TYLCSaV group. In 2002, shortly after the invasion of TYLCV, only three types of infection profiles were detected (Fig. 4). 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). However, in spite of high sampling efforts, samples that were positive only for TYLCV/TYLCSaV recombinants ("TYLCV/TYLCSaV") were not detected before 2009, whereas such samples were detected from 2012.

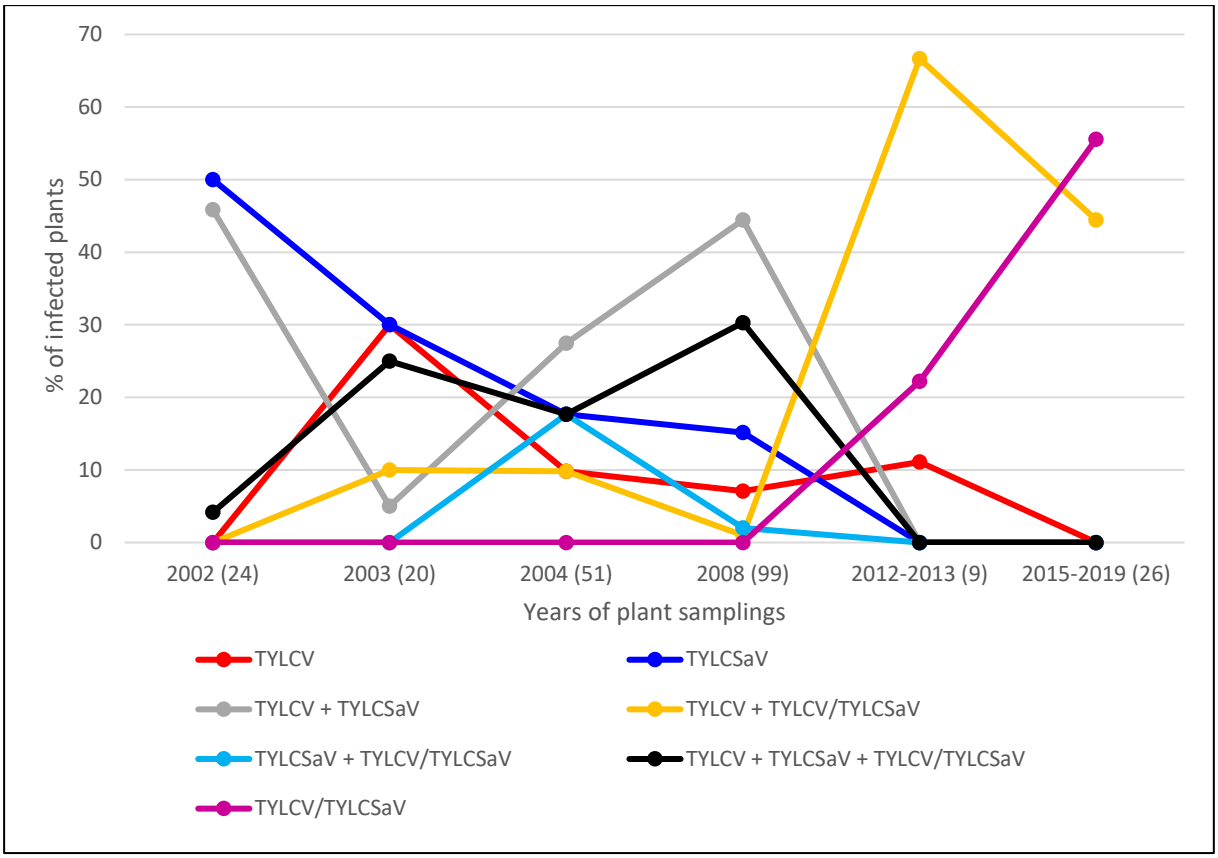


Figure 4: **Evolution of the relative frequency of plants exhibiting particular profiles of begomovirus infections in Sicily.** The figure presents for each sampling year the percentage of plants corresponding to each infection profile described in the legend. The number of plants analysed in each collection period are indicated in brackets under the graph. The infection profile data of the samples collected between 2015 and 2019 were extracted from Table 1, whereas those of samples collected earlier were extracted from previous reports (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, it was relevant to present all results on the same graph. The data used to generate Fig. 4 are available in Supplementary material 2. 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 TYLCSaV. 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/TYLCSaV recombinant group. Noteworthy, it is only from the sampling of 2012-2013 that some samples were detected positive only for TYLCV/TYLCSaV recombinants.

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 (13 samples), TYLCV-IS76-type recombinants were detected in 2018 only (2 samples).

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

556 Like the 4 samples collected in Ouled-Frej (Morocco) that tested negative in spite of their collection on
557 symptomatic plants, 2 samples collected in Murcia in Oct 2018 revealed the same inconsistency (Table
558 1). As suggested for the Ouled-Frej samples, the sensitivity of the PCR tests may have been insufficient
559 to detect the potentially lower virus amount that is expected in resistant plants. It is also possible that
560 the milder TYLCD symptoms exhibited in *Ty-1* resistant plants have been confused with symptoms
561 caused by other plant pathogens or pesticides. Finally, it cannot be excluded that these samples are
562 infected with TYLCD-associated begomoviruses that are not detectable with the PCR approach used.

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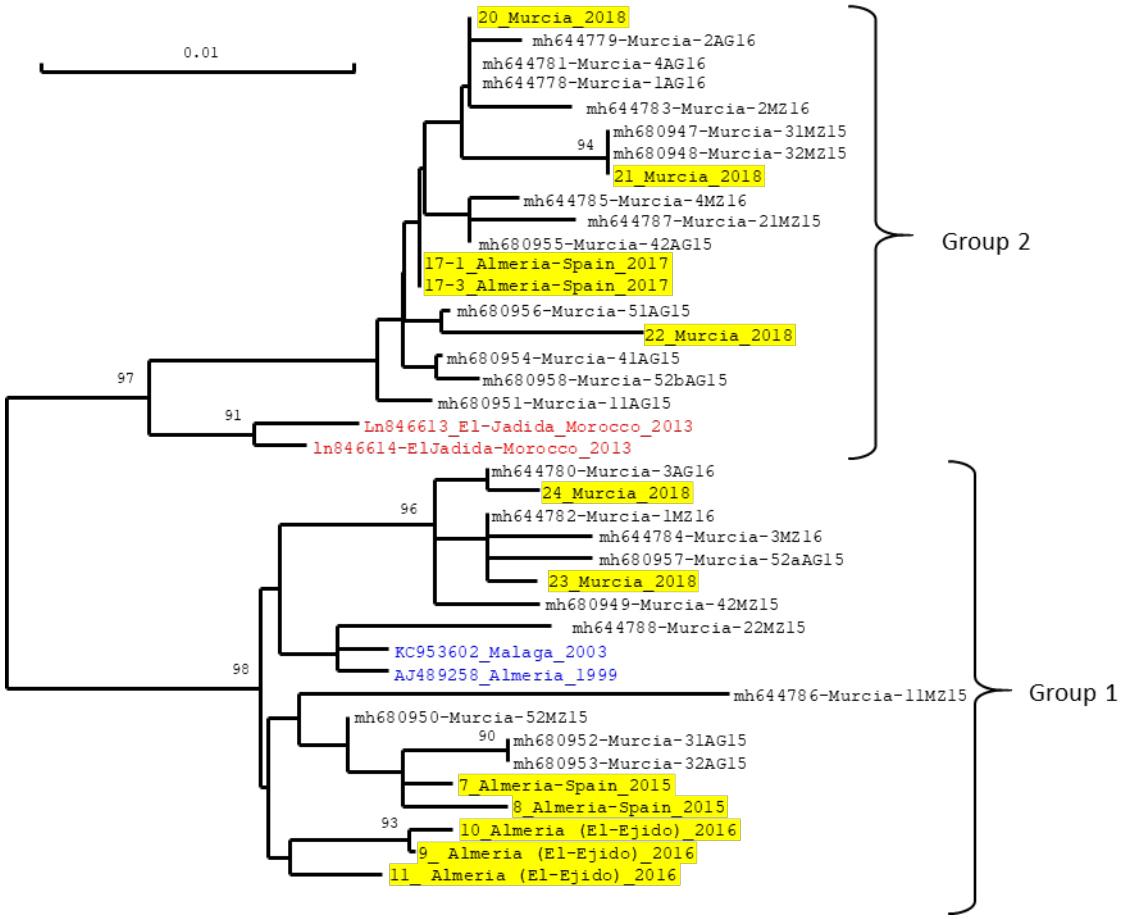

580

581 **Figure 5**: **Neighbour-joining tree showing the genetic distance among isolates of Tomato yellow**
582 **leaf curl virus (TYLCV) originating from Southeast Spain**. The tree was constructed from the
583 alignment of a 592-nt-genomic fragment between the origin of replication and the first third of the

584 capsid protein gene. The sequences used for the comparison were from the TYLCV-IL viruses
585 isolated in this study (highlighted in yellow) as well as from other TYLCV-IL isolates for which
586 sequences were downloaded from Genbank. All the isolates were from tomato plants (*Solanum*
587 *lycopersicum*) except the isolates collected in 1999 and 2003 which were collected from pepper
588 (*Capsicum annuum*) (written in blue). Sequence data from this study were obtained by sequencing
589 a PCR product generated with the primer pair IL-2629F/ IM650R, and the sequences of the 592-nt
590 fragment are available in Supplementary material 3. Virus isolates from this study are identified
591 with a code number followed by the location and year of collection. Isolates written in black without
592 yellow highlights were collected in the province of Murcia and are identified with their GenBank
593 accession number and the code numbers used previously, in which 15 and 16 correspond to the
594 year of collection (2015, 2016) and AG and MZ to the sampling locations, i.e., Aguilas and Mazaron,
595 respectively (Torre et al., 2018). Isolates written in blue correspond to the earliest collected TYLCV
596 in Southeast Spain for which total genome sequences were reported. Isolates written in red
597 correspond to the two TYLCV isolates from El-Jadida (Morocco) that were reported to be the most
598 closely related to group 2 isolates identified as a new type of TYLCV isolates in Southeast Spain
599 (Torre et al., 2018). The scale measures the Jukes and Cantor distance between sequences.
600 Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting
601 the nodes; only percentages ≥ 90% are represented. The two highly supported groups correspond
602 to the previously identified group 1 and group 2 (Torre et al., 2018).

603

604 **4. Discussion**

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

615 Due to their resistance breaking feature in Ty1 resistant plants (Belabess et al., 2015; Fortes et al.,
616 2023; Panno et al., 2018), Srec recombinants were given special attention by distinguishing them with
617 an original PCR test from Lrec recombinants commonly reported from Spain and Italy (Davino et al.,
618 2012; Garcia-Andres et al., 2006; Monci et al., 2002). Thus, together with the previously reported
619 multiplex PCR tests (Belabess et al., 2015), we were able to specifically determine the proportion of
620 samples containing Srec recombinants among the samples detected positive for TYLCD-associated
621 begomoviruses.

622 The symptomatic samples that tested negative with all the PCR tests used reveal the potential
623 limitation of the a priori PCR approach. Indeed, besides the possibility of insufficient sensitivity of the
624 PCR tests and confusing symptoms, it cannot be excluded that potential TYLCD-associated viruses were
625 not detectable with the primer sets used. Higher sequencing investments are needed to detect
626 potential TYLCD-associated viruses that may have been missed by the PCR approach used. Such missing
627 risk is even higher with recombinant genomes as shown with TYLCV-IMS60-2400. Indeed, from the
628 sequence of its complete genome, its amplification with MxPCR1 primers is a posteriori quite puzzling
629 because it has a TYLCV-IL-derived sequence at the nucleotide positions targeted by the TYLCV-Mld-

630 specific primer, and a TYLCV-Mld-derived sequence at the nucleotide positions targeted by the TYLCV-
631 IL specific primers.

632 4.1. TYLCV-IS76 is virtually the only TYLCD-associated begomovirus on the Atlantic coast of Morocco

633 The dominance of Srec recombinants in the samples collected from Morocco and their identification
634 as TYLCV-IS76 is fully consistent with previous results (Belabess et al., 2015). Indeed, these authors
635 showed that TYLCV-IS76 has replaced its parental viruses in the region of Agadir (Souss) and that it
636 started to spread northwards. The northward spread is confirmed here with Srec recombinants being
637 the only begomovirus type detected in the 10 farms located in the north of Agadir along the Atlantic
638 coast up to Casablanca. The detection of TYLCSaV in 2015 in Agadir is consistent with previous results
639 showing that TYLCSaV was more frequently detected than TYLCV among the rare tomato samples in
640 which parental type viruses were detected after the invasion of TYLCV-IS76 (Belabess et al., 2015).
641 Interestingly the "original" group 2-type virus from Morocco (Belabess et al., 2015) that was detected
642 from two tomato samples collected in 2013 in the area surveyed in this study (El Jadida on the Atlantic
643 Coast) was not detected again from the Moroccan samples, whereas such viruses were detected from
644 Spain (Table 2). This striking difference of group 2 virus prevalence between the two countries suggest
645 that the dominance of TYLCV-IS76 has blocked its spread in Morocco. Therefore, considering the
646 relative competitiveness determined experimentally between TYLCV-IS76 and group 2 viruses (Torre
647 et al., 2018), it seems that the relatively higher fitness of group 2 viruses in single infection of *Ty-1*-
648 resistant plants could not compensate the relatively higher fitness of TYLCV-IS76 in coinfection in
649 susceptible and *Ty-1* resistant plants. Finally, further studies are needed with plant samples from
650 northern and eastern Morocco to complete the picture of tomato begomoviruses in Morocco.

651 4.2. Major shift in Sicilian populations of TYLCD-associated begomoviruses between 2009 and 2012

652 The comparison of the infection profiles of tomato plants sampled over time in Italy (Fig. 4) reveals
653 two important changes that occurred between 2009 and 2012. Firstly, whereas TYLCV/TYLCSaV
654 recombinants were never found in any plant without parental viruses before 2010 in spite of important
655 sampling efforts with 100 plants per year from 2006 to 2009 (Davino et al., 2012), recombinants were
656 found alone in plants sampled after 2011. Indeed among the 9 samples collected in the 2012-2013
657 period, 2 samples were positive only for TYLCV/TYLCSaV recombinants (Belabess et al., 2015) and
658 among samples collected later (2015- 2019) and analysed in this study, "TYLCV/TYLCSaV" is the
659 dominant infection profile (15 samples out of 27, Supplementary material 2). This evolution of
660 infection profiles over time suggests that recombinants with a relatively higher fitness have emerged.
661 The second change that occurred between 2009 and 2012 is the emergence of Srec recombinants.
662 Indeed, while the recombinants reported from Sicily before 2012 were all of the Lrec type (Davino et
663 al., 2012; Davino et al., 2009), the recombinants detected in the samples collected during 2015-2019,
664 were all of the Srec type (Table 2) and 5 out of the 9 samples collected in 2012-2013, were positive
665 for Srec too (Belabess et al., 2015). The two changes observed since 2012, i.e. the occurrence of plant
666 detected positive only for TYLCV/TYLCSaV recombinants and the emergence of Srec recombinants, are
667 presumably linked. Indeed, the relatively higher fitness experimentally assessed for Srec recombinants
668 (Belabess et al., 2016; Jammes et al., 2023) and particularly TYLCV-IS141 (Urbino et al., 2020) - the Srec
669 recombinant frequently detected in Sicily (Belabess et al., 2015; Granier et al., 2019; Panno et al., 2018)
670 - is consistent with its independent spread. As a reminder, the higher fitness of TYLCV-IS141 was
671 deduced from its relatively higher accumulation in susceptible and *Ty-1* resistant plants as comparted
672 to TYLCV-IL, the most competitive parental virus in *Ty-1*-resistant plants (Urbino et al., 2020).

673

674

675 4.3. TYLCV-IMS60-2400, a new Srec recombinant spreading in Sicily

676 Interestingly, besides TYLCV-IS141, a second Srec recombinant was detected in Sicily during the period
677 2015-2019, i.e., TYLCV-IMS60-2400. Although TYLCV-IMS60-2400 was detected less frequently than
678 TYLCV-IS141 (Table 1), it exhibits features of an emerging virus. Indeed, the 5 samples that were
679 positive for recombinants bearing the RB60 recombination breakpoint were not positive for any of the
680 targeted begomoviruses, indicating that these recombinants are already circulating autonomously.
681 Moreover TYLCV-IS60-type recombinants were detected from samples collected in three of the five
682 years of sampling, and it was detected in at least two distinct collection sites, namely Marina di Ragusa
683 and Vittoria.

684 4.4. Emergence of TYLCV-IS76 and its presumed back recombinant in Southeast Spain

685 The dominant infection profile of the samples collected in the Murcia and Almeria provinces is "TYLCV-
686 IL" with 13 samples being positive for TYLCV-IL only, among the 15 PCR-positive samples (Table 2). This
687 result is consistent with previous reports with samples collected earlier from the same Spanish region.
688 Thus, the five tomato samples collected in 2013 in Almeria province and analysed with the same PCR
689 approach were PCR positive only for TYLCV-IL (Belabess et al., 2015). Likewise, among 370 tomato
690 samples collected in the two Spanish provinces in 2003 and detected positive for tomato
691 begomoviruses by tissue blot hybridization, 368 were positive only with the DNA probe specific to
692 TYLCV; 2 were positive with both the TYLCV and the TYLCSaV probes and no sample was detected
693 positive for TYLCSaV only (Garcia-Andres et al., 2007). In this later study in which tomato samples were
694 also analysed from the Malaga province, 45 tomato samples from the three provinces, detected
695 positive by tissue-blot hybridization, were selected to be analysed further by sequencing a PCR product
696 comprising the non-coding intergenic region and the 5'-proximal parts of Rep and V2 open reading
697 frames. Among them, only three samples bore TYLCV/TYLCSaV recombinant genomes, two which
698 inherited the TYLCV fragment from a Mld strain parent and one from an IL strain parent. This low
699 proportion of recombinants stands in contrast with the 2 samples detected positive for IL/Sar
700 recombinants among the 15 PCR positive samples analysed here. The much higher prevalence of
701 TYLCV/TYLCSaV recombinants in tomato is apparently related with the length of the TYLCSaV fragment
702 inherited by the recombinants. Whereas the 2 recombinants detected in 2018 have inherited a short
703 76 nts fragment of TYLCSaV, like that of the previously reported TYLCV-IS76 viruses, the three
704 recombinants detected in 2003 inherited a much longer TYLCSaV fragment that extended beyond the
705 initiation codon of the V2 gene. While the TYLCV-IS76-positive sample form El Ejido confirms the
706 emergence of TYLCV-IS76 in the Almeria province (Fortes et al., 2023; Torre et al., 2019), the detection
707 of TYLCV-IS76 in a sample from Murcia shows that its distribution is not limited to the Almeria province
708 (Table 2).

709 The high nucleotide identity between TYLCV-IS76 recombinants from Spain and Morocco (Fortes et al.,
710 2023; Torre et al., 2019) suggests that their presence in Spain is due to an introduction from Morocco
711 where it was initially detected in tomato plants sampled in 2010. Such a scenario may also explain the
712 presence of a new type of TYLCV-IL viruses in Southeast Spain because of their close nucleotide identity
713 to TYLCV-IL viruses detected in Morocco from tomato plants sampled in 2013 in El Jadida (Fig. 5). Based
714 on a high sequence identity with the TYLCV-inherited region of TYLCV-IS76, El Jadida-TYLCV-IL-like
715 viruses were presumed to derive from a TYLCV-IS76 recombinant through a back recombination event
716 (Belabess et al., 2015; Torre et al., 2018). Their detection in more than 50% of the TYLCV-IL-positive
717 samples collected in 2017 and 2018, confirm their emergence in Southeast Spain since their first
718 detection from tomato samples collected in 2015 and 2016 (Torre et al., 2018). As these authors have
719 analysed only samples from the province of Murcia, it was not known if the El Jadida-TYLCV-IL-like
720 viruses have emerged elsewhere. Here, with samples collected in 2017 in the province of Almeria

721 (Table 2), we show that their presence is not limited to the province of Murcia. The successful
722 emergence of viruses of the El Jadida-TYLCV-IL-type is consistent with their fitness advantage shown
723 with a Spanish clone inoculated in *Ty-1* resistant tomato plants (Torre et al., 2018). Although this fitness
724 advantage was only observed in single infection but not in co-infection where TYLCV-IS76 is the most
725 competitive in both susceptible and *Ty-1* resistant plants, the emergence of El Jadida-TYLCV-IL-type
726 viruses was possible in Spain presumably because of the relatively low prevalence of TYLCV-IS76. This
727 assumption is consistent with the failure of its emergence in the region of El-Jadida where it was
728 initially reported but where TYLCV-IS76 is the dominant TYLCD-associated begomovirus.

729 4.5 Concluding remarks

730 The populations of TYLCD-associated begomoviruses that are described in this study reveal that
731 geographic specificities were still present between 2015 and 2019. The specificities concerning
732 parental type viruses (TYLCV, TYLCSaV) is their virtual absence in the sampled region of Morocco along
733 the Atlantic coast, the contrasted situation in Italy where TYLCSaV was detected in Sardinian samples
734 but not in Sicilian samples, and frequent detection of TYLCV in Spain. The specificities concerning
735 recombinants is revealed by the dominance of TYLCV-IS76 in Morocco but no detection of TYLCV-IS141,
736 the dominance of TYLCV-IS141 in Italy but no detection of TYLCV-IS76, and by the confirmation of the
737 emergence of TYLCV-IS76 and the El Jadida-TYLCV-IL-type in Southeast Spain.

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

746 The higher competitiveness of TYLCV-IS76 as compared to Lrec recombinants observed in controlled
747 conditions using vector transmission (Jammes et al., 2023) is consistent with these inverse dynamics
748 of prevalence of Srec versus Lrec recombinants. The large regional scale at which this trend is detected
749 may be explained by the cultivar switch that occurred in the three countries from susceptible to *Ty-1*
750 resistant cultivars, as reported from Morocco (Belabess et al., 2015; Belabess et al., 2016). More
751 samplings and originating from larger areas of the surveyed countries, are needed to confirm and
752 refine the population shifts detected here. More generally, with such a new survey, the evolution of
753 TYLCD-associated begomoviruses may be monitored further in these countries and particularly the
754 dynamics of TYLCV-IS76 and El Jadida-TYLCV-IL-type viruses in Spain, the fate of the new Srec
755 recombinant circulating in Sicily (TYLCV-IMS60-2400), and the potential persistence of parental type
756 viruses in the less intensive tomato production areas such as the North East of Morocco. Finally, to
757 gain a more comprehensive picture of TYLCD-associated virus populations, metagenomic approaches
758 may be used to address the risk of missing viruses that may not be detectable with the PCR approaches
759 as suggested with some symptomatic plants sampled in this study and the relatively complex
760 recombination profile of TYLCV-IMS60-2400.

761

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Conflict of interest disclosure

The authors declare that they have no financial conflicts of interest in relation to the content of the article

Author contributions

Mohamed Faize (MF) organized the collection of Moroccan samples. Martine Granier (MG) and Sandie Passera extracted total DNA from plant samples. MG performed the laboratory analysis of the samples. Michel Peterschmitt (MP) designed the study, analysed the data and wrote the original draft. MF, Cica Urbino and MP reviewed and edited the manuscript.

Data

DNA sequences of the three complete genomes, generated in this study for the newly reported TYLCV-IMS60-2400 recombinant, are available in GenBank under accession numbers OR473055, OR473056, OR473057. The partial sequences of other viruses generated in this study and used to construct the distance tree shown in Fig. 5 are available in the Supplementary material 3. The data used to prepare Fig. 4 are available in Supplementary material 2.

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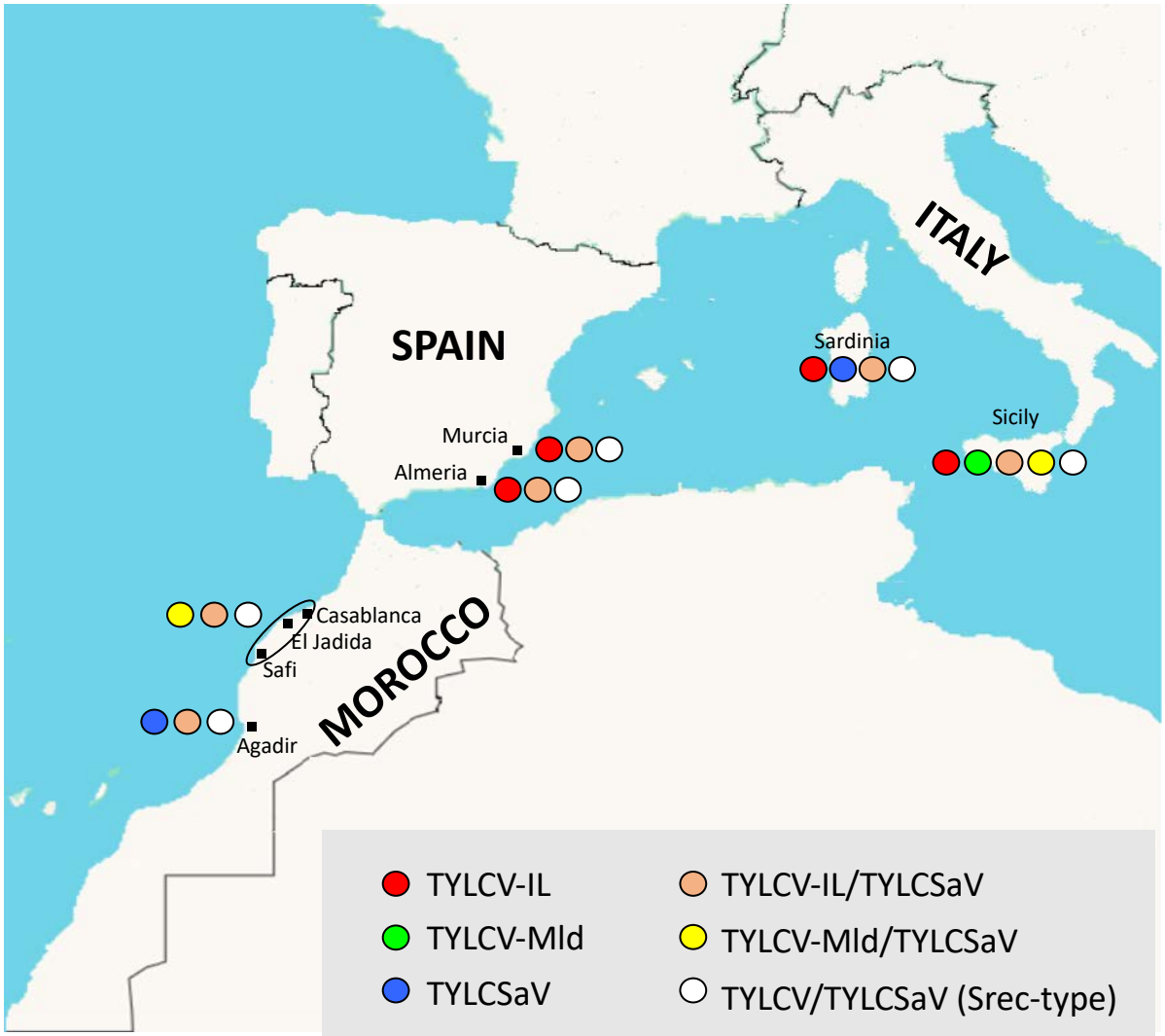
Supplementary material 1: Geographical distribution of begomoviruses associated with tomato yellow leaf curl disease, detected in samples collected from Spain Italy and Morocco.



Supplementary material 2: The data presented in this table were used to generate Fig. 4. They present the number of tomato plants of each infection profiles sampled in different years in Sicily. The data obtained with the plants sampled in the years 2002 and 2004 are from [(Davino et al., 2008), Table 2], those from years 2004 and 2008 are from[(Davino et al., 2009), Table 2], those from years 2012- 2013 are from [(Belabess et al., 2015), Supplementary Table S8], and those from years 2015- 2019 are from this study (Table 1). Table cells corresponding to plants detected positive for TYLCV/TYLCSAV recombinants are highlighted in orange. The penultimate table line shows the total number of plants detected positive regardless of their infection profile.

Infection profiles	Number of plants of each infection profile per sampling years (percentage)					
	2002	2003	2004	2008	2012-2013	2015-2019
TYLCV	0 (0)	6 (30)	5 (10)	7 (7)	1 (11)	0 (0)
TYLCSaV	12 (50)	6 (30)	9 (18)	15 (15)	0 (0)	0 (0)
TYLCSaV + TYLCV	11 (46)	1 (5)	14 (27)	44 (44)	0 (0)	0 (0)
TYLCV + TYLCV/TYLCSaV	0 (0)	2 (10)	5 (10)	1 (1)	6 (67)	12 (44)
TYLCSaV + TYLCV/TYLCSaV	0 (0)	0 (0)	9 (18)	2 (2)	0 (0)	0 (0)
TYLCSaV + TYLCV + TYLCV/TYLCSaV	1 (4)	5 (25)	9 (18)	30 (30)	0 (0)	0 (0)
TYLCV/TYLCSaV	0 (0)	0 (0)	0 (0)	0 (0)	2 (22)	15 (56)
Total	24 (100)	20 (100)	51 (100)	99 (100)	9 (100)	27 (100)
Total TYLCV/TYLCSaV	1 (4)	7 (35)	23 (45)	33 (33)	8 (89)	27 (100)

Supplementary material 3: DNA sequences of the 592-nt-fragment with which the 13 TYLCV-IL isolates detected in tomato samples collected in Southeast Spain (Table 2) were identified as belonging to the previously reported group 1 or group 2 (Torre et al., 2018) (Fig. 5). Only 12 of them are presented, because one of the three samples collected in the province of Almeria in 2017 (Table 2) 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.

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CCGAAGGCTGAACTTCGACAGCCCATACAGCACCCGTGCTGCTGTCCCCATTGTCCAAGG
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