Short Communication

Natural occurrence of begomovirus recombinants associated with tomato yellow leaf curl disease co-existing with parental viruses in tomato crops and weeds in Tunisia

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SUMMARY

Tomato yellow leaf curl virus disease (TYLCVD) is the main limiting factor for tomato production in the Mediterranean Basin, and particularly in Tunisia where climatic and crop production conditions, as well as the occurrence of many weeds, provide suitable conditions for the presence and spread of TYLCVD all year round.

In Tunisia, epidemics in tomato-growing regions have been associated with two begomoviruses: Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl Sar dinia virus (TYLCSV) but the presence of recombinants between them has never been investigated.

A large-scale survey was conducted on leaves sampled from late-season tomato crops exhibiting severe curling symptoms, from beans, and from nine nearby weed species in the South and Sahel regions of Tunisia to investigate: (i) the natural occurrence of the TYLCV and TYLCSV species; (ii) the presence of the two recombinant types, RecA and RecB, derived from these species. Identification of TYLCV and TYLCSV was based on a multiplex PCR with primers generating a typical pattern on gels (750 bp and 366 bp fragments, respectively). Recombinants were identified by simplex PCR, which amplified the intergenic region, the most common recombination site described for begomoviruses, and sequencing. The occurrence of TYLCSV and TYLCV was confirmed in both Tunisian regions. Their co-existence was shown on tomato and in new weed species. This is the first time that recombinants between TYLCV and TYLCSV species showing 93% to 95% nucleotide identity with tomato yellow leaf curl recombinant viruses, are reported in Tunisia in tomato and black nightshade.

Key words: Tomato, Begomovirus, TYLCV, TYLCSV, recombinant virus.

Tomato yellow leaf curl disease (TYLCVD) is one of the most devastating disorders of tomato crops worldwide (Czosnek and Latcrerot, 1997) which can also affect many other crops and weeds (Brown et al., 2011). It causes serious damage and severely constrains production of susceptible crops, particularly in some Mediterranean areas where suitable climatic conditions in addition to complementary agricultural structures allow year-round crop production. There, TYLCVD is more likely to entrench and spread.

In Tunisia, production of ornamentals and vegetables, particularly tomato (Solanum lycopersicum L.), is particularly constrained by TYLCD which causes significant yield losses in the main tomato-growing regions (Cherif and Russo, 1983). The tomato crop is of major economic importance with a total production of about 26,000 ha/year (i.e. 17% of the land used for cultivating vegetables) (Anonymous, 2010). From November to June, tomatoes are mainly produced in geothermally heated greenhouses in the south of the country and in unheated tunnels in the Sahel. During the rest of the year, tomatoes are mainly grown in open fields in Cap Bon and in the central region.

The causal agents of this whitefly-transmitted virus disease are members of a virus complex belonging to the genus Begomovirus, family Geminiviridae, which are also found on a wide range of host plant species worldwide. Until recently, TYLCVD epidemics in Mediterranean countries were mainly associated with two viruses: Tomato yellow leaf curl virus (TYLCV) first described in Israel, and Tomato yellow leaf curl Sardinia virus (TYLCSV) (Kheyr-Pour et al., 1991). Distinct strains of the same virus species and/or different virus species can be associated with TYLCVD either as single or recombinant forms (Brown et al., 2011). The begomoviruses associated with TYLCVD are characterized by small circular single-stranded (ss) DNA genomes which replicate in their host cell via double stranded intermediates by a rolling-circle mechanism analogous to that used by ssDNA phages (Saunders et al., 1991; Stanley, 1995) and by recombination-dependent replication (RDR) as confirmed.
by Jeske et al. (2001). Their genome is also characterized by the presence of an intergenic region (IR) of about 300 nucleotides including a stem-loop and a conserved nona-nucleotide sequence (TAATATTAC) placed in this loop, where the breaking and joining site for rolling-circle replication occurs (Hanley-Bowdoin et al., 2000). This IR is a well-known recombination site in geminiviruses (Stanley, 1995; Navas-Castillo et al., 2000; Monci et al., 2002; García-Andrés et al., 2006), particularly in the recombinant progeny of TYLCV and TYLCSV (Garcia-Andrés et al., 2007; Davino et al., 2009).

In Tunisia, TYLCVD was first reported in 1983 (Cherif and Russo, 1983). The associated virus was first observed by electron microscopy and the cytological changes observed were similar to those previously observed in diseased tomato plants suffering from tomato yellow leaf curl disease in Israel (Cherif and Russo, 1983). The presence of TYLCSV species was then identified through molecular characterisation from symptomatic tomato plants collected in 2001 and 2002 (Fekih-Hassen et al., 2003) in the Sahel and the southern areas of Tunisia. The Tomato yellow leaf curl virus-Israel species (TYLCV-[IL]) was molecularly identified in tomato, pepper (Capsicum annuum) and faba bean (Vicia faba) in the 2004 and 2006 growing seasons in different locations of Sahel (Gharsallah-Chouchane et al., 2007). Thus, in Tunisia, TYLCVD may be associated with distinct species.

Recently, the simultaneous presence of TYLCV and TYLCSV has been reported in mixed infections in the same host plant (Pellegrin et al., 2008), but the occurrence of recombinants between these two viruses had not been looked for. Therefore, in this study we investigated: (i) the occurrence of both viruses (i.e. TYLCSV and TYLCV), alone or together, in Tunisian tomato, faba bean and pepper crops and in nearby weed species; (ii) the presence of potential recombinants between these two parental TYLCV species. Our research has focused on the presence of two recombinant types (namely, RecA and RecB) of TYLCV and TYLCSV, commonly found in nearby Mediterranean countries, particularly in Spain (Garcia-Andrés et al., 2007) and Italy (Davino et al., 2009).

To this aim, a large survey was conducted sampling leaves from late field tomato crops exhibiting severe begomovirus-like curling and yellowing symptoms, but also from faba bean and pepper crops and nine weed species showing different degrees of leaf yellowing and curling in the Sahel and southern Tunisian areas in 2009 (Table 1). Leaves from 124 plants sampled were stored separately in a freezer at −20°C until molecular characterisation.

### Table 1. Results of the survey conducted in the late tomato crop season in the Sahel and southern regions of Tunisia, in 2009, with TYLCV species and detection of recombinants.

<table>
<thead>
<tr>
<th>Region</th>
<th>Plant species</th>
<th>Single infection by TYLCV (366 bp)</th>
<th>TYLCV (750 bp)</th>
<th>Mixed infection TYLCV and TYLCSV (366 bp and 750 bp)</th>
<th>Recombinant TYLCV and TYLCSV (570bp)</th>
<th>GenBank accession Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sahel</td>
<td>Vegetables</td>
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<td></td>
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<tr>
<td>Tomato (Solanum lycopersicum)</td>
<td>1/63</td>
<td>8/63</td>
<td>46/63</td>
<td>8/63 recA</td>
<td>GU322870-873</td>
<td></td>
</tr>
<tr>
<td>Faba bean (Vicia faba)</td>
<td>0/4</td>
<td>0/4</td>
<td>3/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pepper (Capsicum annuum)</td>
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<td>0/5</td>
<td>5/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeds</td>
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<td>0/19</td>
<td>6/19</td>
<td>12/19</td>
<td>0/19</td>
<td></td>
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<tr>
<td>Lantana camara</td>
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<tr>
<td>Malva parviflora</td>
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<td>0/3</td>
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<tr>
<td>Sisymbrium thio</td>
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<td>0/2</td>
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<td>Melilotus officinalis</td>
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<td>Sonchus arvensis</td>
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<td>Amaranthus lividus</td>
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<td></td>
</tr>
<tr>
<td>Tomato (Solanum lycopersicum)</td>
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<td>0/8</td>
<td>4/8</td>
<td>0/8</td>
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</tr>
<tr>
<td>Faba bean (Vicia faba)</td>
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<td>0/1</td>
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<tr>
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<td>0/4</td>
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<td>1 recA</td>
<td>GU322874</td>
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<tr>
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<td>0/1</td>
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</tr>
</tbody>
</table>

Total number of positive samples observed/tested: 10/124, 14/124, 86/124, 9 recA

* rec = recombinant
at 5 min intervals. An equal volume of phenol-chloroform isoamyl alcohol (25:24:1) was then added and centrifuged at 13,000 rpm for 5 min. From this stage on, the protocol used followed that described by Carling (2004). Extracted DNA was quantified using 1% agarose gel and diluted when necessary before amplification.

Detection of TYLCSV and TYLCV in our plant sampling was based on multiplex PCR using primers TY209, TY575, TY613, TY1363 (Pellegrin et al., 2008) which generate a typical amplified fragment size pattern (750 bp for TYLCV, 366 bp for TYLCSV) on gels. Detection of potential recombinants between TYLCV and TYLCSV in samples previously shown to be infected by one or both parental viral species (Pellegrin et al., 2008) was based on separate PCRs using primers designed to target the IR (Davino et al., 2008). The following viral DNAs were amplified (primer pairs in brackets): recombinant type A, namely RecA (TY2463+/TY247-) of ca. 570 bp with the sequence from TYLCV to the left and TYLCSV to the right of the recombination site in the IR; recombinant type B, namely RecB (TY2222+/TY2255-) of ca. 800 bp with the sequence from TYLCSV to the left and TYLCV to the right of the recombination site, as detailed in Davino et al. (2008). DNA amplicons from samples infected by parental viral forms were compared with the amplicon reference that corresponded to a 570 bp recombinant isolate of TYLCAxV-Sic1-[IT: Sic2/2:04] (GenBank accession No. NC_011024) provided by Dr. G.P. Accotto (Fig. 1). Some amplified products (570 bp) from our samples were either directly sequenced using the primer set (TY2463+/TY247-) or cloned before sequencing. These amplicons were purified (Wizard PCR DNA purification system, Promega, USA), and ligated into the pGEM-T Easy vector (Promega, USA) following the manufacturer’s recommendations. Briefly, Escherichia coli competent cells (Promega strain JM 109) were transformed with the resultant recombinant plasmids and cultured onto solid Luria-Bertani medium for blue/white colony selection. Recombinant plasmids were tested by EcoRI digestion, which gives the linear plasmid form and an approximately 600 bp insert fragment, then purified with Qiaprep spin miniprep kit (Qiagen, USA) before sequencing. For each insert, two independent clones were sequenced on both directions using T7 and Sp6 primers.

All sequencing was done with an automated ABI3730 DNA sequencer (Applied Biosystems, USA) and the resulting consensus sequences were aligned before being deposited in GenBank and analysed. They were aligned with Clustal W (Thompson et al., 1994) and compared among themselves and with sequences from GenBank database using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Multiplex PCR reactions tested on the 124 leaves collected revealed that 110 samples (including all host plants collected except for Sonchus arvensis) were positive, that is, were infected with TYLCV and/or TYLCSV (Table 1). Plants infected by a single virus species (14 by only TYLCV, 10 by only TYLCSV) were more rarely observed than plants infected by both species (86/110 infected). In south Tunisia and Sahel 50 and 73% of symptomatic tomato plants, respectively, were found to be infected by both TYLCV and TYLCSV. Such mixed infections were also observed in the other vegetable crops (faba bean, pepper) but above all, and with a very high occurrence, in all weed species (i.e. except one) collected (Table 1) regardless of the severity of the symptoms shown.

Out of the 110 samples infected with TYLCV and/or TYLCSV and tested for a potential recombinant type virus, eight tomato samples and one S. nigrum sample yielded a 570 bp amplicon identical in size to the 570 bp IR amplified fragment of the reference recombinant A (Fig. 1). The amplified product derived from S. nigrum was directly sequenced (accession No. GU322874). Four out of the eight amplified fragments derived from tomato samples were cloned and sequenced (GU322870-GU322873). When aligned and compared to TYLCV and TYLCSV nucleotide sequences retrieved from GenBank (EF101929 and DQ317784, respectively), the five sequences obtained representing recombinant virus sequences RecA respectively, were shown to match the TYLCV sequence to the left of the IR and the TYLCSV sequence to the right of this region characterised by the conserved TAATATTAC nona-nucleotide sequences and the nicking site for initiation of virus DNA replication indicated by an open box in Fig. 2.

Significant nucleotide identity (nID) between these five sequences (GU322870-GU322874) and more sequences from GenBank were also found (Table 2). They
displayed more than 93% nucleotide identity with different tomato yellow leaf curl recombinant viruses (Table 2). Thus, among the sequences derived from tomato samples, GU322870 and GU322872 shared 93% nID with the Tomato yellow leaf curl Axarquia virus (TYLCxV; DQ317696.1), GU322873 shared 94% nID with the Tomato yellow leaf curl Malaga virus (TYLCMaV; DQ317720.1) and GU322871 95% nID with TYLCV/TYLCSV recombinant Ragusa (EU719096.1). The sequence from the infected weed S. nigrum (GU322874) shared 94% nID with the TYLCV/TYLCSV recombinant Ragusa (EU719096.1). Our results confirm that TYLCV and TYLCSV species are widely distributed and established in Tunisia, either in single or mixed infections. They naturally and
predominantly co-occur in the same plant, as previously reported by Pellegrin et al. (2008), in the two major Tunisian agricultural areas studied. Mixed infections are widely distributed in tomato, faba bean, pepper and many weed species. The following weed species: Solanum nigrum, Chenopodium album, Lantana camara, Amaranthus lividus, Emex spinosa, Melilotus officinalis and Sisymbrium trifolium, which are significant components of natural and agricultural ecosystems, are here described for the first time as being infected by both TYLCV and TYLCSV species in Tunisia.

Although the strains were sometimes different from those in Tunisia, such mixed infections in crops and weeds have been already widely reported in Mediterranean countries, particularly in Spain (García-Andrés et al., 2007) and Italy (Davino et al., 2009). Co-occurrence of different strains and species is a prerequisite for the emergence of new recombinant viruses associated with TYLCV and TYLCSV. Thus, in the present study and for the first time in Tunisia, natural recombinants between TYLCV and TYLCSV on tomato and S. nigrum, two solanaceous plant species, were observed in the major tomato growing areas in both heated and unheated tunnels. No recombinants have yet been detected in the other weed species analysed, although mixed infections have been widely reported. But it is likely that some identical or distinct TYLCV recombinants will also be isolated and characterised in some of these plants in the future. García-Andres et al. (2007) provided clear evidence for frequent emergence of recombinant species during mixed begomovirus infection in the same plant.

These recombination events might be a major driving force for the evolution of begomoviruses through the generation of new variants, and their ability to emerge in nature and break resistance in crop plants (Monci et al., 2002; García-Arenal and McDonald, 2003).

Based on our results, further field surveys will be undertaken to investigate: (i) whether the recombinant species may be more damaging to tomato crops than the parental species; (ii) the importance of weed species, as these might play a critical role in the ecology and epidemiology of viruses as potential reservoir plants. Furthermore, they offer suitable conditions for the presence and spread of TYLCV all year round, in periods when no tomato crops are available. Further targeted surveys must therefore be undertaken to advance our knowledge of the role of weeds in TYLCV epidemics and emergence of new tomato yellow leaf curl recombinant viruses.

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