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Spread of *Tomato yellow leaf curl virus* in Sicily: partial displacement of another geminivirus originally present

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Abstract

The geminivirus *Tomato yellow leaf curl virus* (TYLCV) was reported for the first time in Italy in 2002. We have followed its spread in Sicily, where *Tomato yellow leaf curl Sardinia virus* (TYLCSV), another tomato-infecting geminivirus, is endemic and has been causing severe crop losses since 1989. The presence of the two viruses was monitored in the main tomato growing area, the Ragusa province, analyzing samples with yellow leaf curling symptoms. At first (spring–summer 2002) both viruses were always found in mixed infections, but in 2003 and 2004 18–35% of plants were found infected by TYLCV alone and 8–28% by TYLCSV alone, with 41–69% carrying both viruses. TYLCV has spread quickly in the area, demonstrating, as in other parts of the world, its high virulence and invasiveness; however it has not, so far, completely displaced TYLCSV. An infectious clone of TYLCV from Sicily (TYLCV-IT) was sequenced. The nucleotide sequence was 97% identical to other TYLCV strains of the 'severe' type, found in many countries worldwide.

Introduction

Tomato yellow leaf curl disease (TYLCD) is caused by a complex of virus species that are responsible for severe losses in tomato crops worldwide (Czosnek and Laterrot, 1997). The list of countries suffering severe TYLCD epidemics is continuously increasing (Moriones and Navas-Castillo, 2000). TYLCD symptoms on tomato plants include curling and size reduction of leaves, yellowing, stunting of the plants and flower abortion. The causal agents are species of the genus *Begomovirus*, family Geminiviridae, transmitted by the whitefly *Bemisia tabaci*. Naming of species in this genus has undergone several revisions, and the recent nomenclature (Fauquet et al., 2003) is used in this work. In the Mediterranean basin the species *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) cause important losses in tomato crops (Moriones and Navas-Castillo, 2000). Their virions have a circular, single-stranded DNA monopartite genome about 2.8 kb in length, containing six partially overlapping open reading frames (ORFs), two on the virion sense strand (CP and V2) and four on the virus antisense strand (C1, C2, C3 and C4), separated by an intergenic region (IR) of about 300 nt.

In Italy TYLCSV is endemic in Sicily and Sardinia, where it has been reported since 1989 (Credi et al., 1989; Crespi et al., 1995), and causes occasional outbreaks in other regions. TYLCV was first detected in tomato samples with yellow leaf curl symptoms, collected in Ragusa province, Sicily, in spring 2002 (Accotto et al., 2003). This species was found in mixed infections with TYLCSV. Partial

sequence of the capsid protein gene confirmed the identification of TYLCV, but could not attribute the new isolates to the 'severe' or 'mild' type.

In the present work we followed the population dynamics of the two species in the main tomato growing area of Sicily, and characterized one isolate of TYLCV by cloning and sequencing its genome, and constructing an infectious clone.

Materials and methods

Plastic tunnels were surveyed in Ragusa province between spring 2002 and summer 2004. Samples were collected from plants showing yellow leaf curl symptoms. These symptoms were visible on 20–80% of the plants in the crops. Freshly cut leaf petioles were spotted on positively charged nylon membranes, which were hybridized with digoxigenin-labelled probes specific for TYLCV or TYLCSV obtained by incorporating dig-dUTP into PCR reactions containing the degenerate primers TY1(+) and TY2(−), located on the CP gene (Accotto et al., 2000). Each membrane was first treated with the TYLCSV-specific probe, deprobed, and then rehybridized with the TYLCV-specific probe. Hybridization conditions, washings, and detection were as previously described (Accotto et al., 2000). When DNA extracts were needed for PCR or cloning purposes, samples (fresh or dried leaves) were extracted with a procedure previously described (Noris et al., 1994).

A TYLCV-infected sample, collected in 2004 and known from previous hybridization experiments not to contain TYLCSV, was selected as source for cloning the TYLCV genome. Viral DNA was amplified using the TempliPhi kit (Amersham), which exploits the ability of bacteriophage phi 29 DNA polymerase to amplify any circular single-stranded or double-stranded DNA in the presence of random primers. Reaction products were digested with *Sph*I. This restriction site was chosen since, according to TYLCV GenBank data, is present once in the sequence, and therefore produces a linearized dsDNA suitable for cloning the full-length genome of TYLCV isolates. The 2.8 kb linearized molecule was excised from agarose gels, purified (High Pure PCR Product Purification Kit, Roche), and cloned into *Sph*I–linearized pUC118 plasmid, following standard protocols (Sambrook et al., 1989). Out of 12 clones containing a 2.8 kb insert, one (p#8/4) was selected for sequencing and construction of an infectious clone for agroinoculation. Cycle sequencing, on both DNA strands, was performed with an ABI-Prism 3730XL machine, using two universal primers (M13/pUC forward and reverse) and the following TYLCV-specific ones (3 of viral-sense polarity, and 3 of antisense polarity): Ty328(+) (5′-GGTCGCTTCGACATAGTCAC-3′), Ty1831(+) (5′-TAAATAATGCGGGTCTACG-TC-3′), Ty 2463(+) (5′-TGGTTCCCCATTCTC-GTGG-3′), Ty2482(−) (5′-CCACGAGAATGG-GGAACCA-3′), Ty1794(−) (5′-TGGCAAAGC-AACACAAAGTA-3′) and Ty255(−) (5′-GCTC-GTAAGTTTCCTCAACGGAC-3′).

Sequence analysis and comparisons were performed using the programmes available at the web sites of the National Centre of Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the European Bioinformatic Institute (http://www.ebi.ac.uk/).

To test infectivity of the cloned DNA, a construct suitable for agroinoculation (Grimsley et al., 1987) was prepared. Starting from p#8/4, a series of subcloning steps were performed. First, a deletion of about 700 bp was produced by digesting with *Xba*I (one site in the insert and one in the vector) and religating, producing a plasmid where only one *Sph*I site was left. A full-length genomic DNA was then inserted in this site, obtaining a plasmid that contains a 1.8-mer insert (about 4.7 kb) of the viral genome. This insert was recovered using *Hind*III and *Kpn*I and then transferred into the pBin19 binary vector previously linearized with the same two enzymes, obtaining pBin-Ty8/4. Finally, this plasmid was transformed into the LBA4404 strain of *Agrobacterium tumefaciens*. Agroinoculation experiments were performed on seedlings of tomato, *Nicotiana benthamiana*, *Datura stramonium* and *Solanum nigrum* using standard procedures (Kheyr-Pour et al., 1991). After 3 weeks, plants were observed for symptoms and analysed by the tissue print hybridization described above, using the TYLCV-specific probe.

Results

Out of more than 2000 symptomatic plants individually tested during the surveys in tomato

protected crops, 1943 plants were infected by one or both viruses (Figure 1 and Table 1).

The use of CP-specific rather than full-length probes notably reduced the cross-hybridization cases previously observed (Accotto et al., 2000). However, in a few cases, samples producing a strong hybridization signal with one probe reacted very weakly with the other. When plant extracts were available, PCR assays run with specific primers indicated that the weak signal was generally due to probe cross-hybridization rather than to the presence of the second virus (not shown). Those cases were therefore considered as infections by a single virus.

In 2002 some farmers noticed plants with symptoms more severe than usual, and this prompted us to start new surveys in the area, with particular attention to detection of new virus types. TYLCV was first detected in spring 2002, in plants where TYLCSV was also present. All samples collected during spring and summer 2002 contained either TYLCSV (89.5%) or mixed infections of TYLCSV and TYLCV (10.5%); TYLCV was never detected in single infections (Table 1). A few months later, a second survey indicated that TYLCV, the incoming virus, had increased dramatically its incidence, and was found present for the first time in single infections in 17% of the plants. Incidence of mixed infections also increased, reaching almost 70% of cases. Only 13.3% of plants were singly infected by TYLCSV.

In 2003 and 2004, the incidence of plants infected by TYLCV alone varied between 17.9% and 34.6%. Interestingly, the proportion of mixed infections was always very high in all surveys done after spring/summer 2002, ranging between 40.7% and 69.7%. TYLCSV, at the last time point examined, was still found in single infection in 13.4% of the plants examined.

The complete nucleotide sequence of clone p#8/4 (deposited in the GenBank database under Accession No. DQ144621) comprised 2781 nucleotides and was organized in two ORFs in the virion-sense and four in the complementary-sense strand, separated by an IR of 313 nucleotides, which included the TAATATTAC motif common to all geminiviruses. Comparisons with data available in the GenBank showed that #8/4 DNA sequence is highly similar to other accessions of TYLCV (Table 2). It showed highest similarity to TYLCV

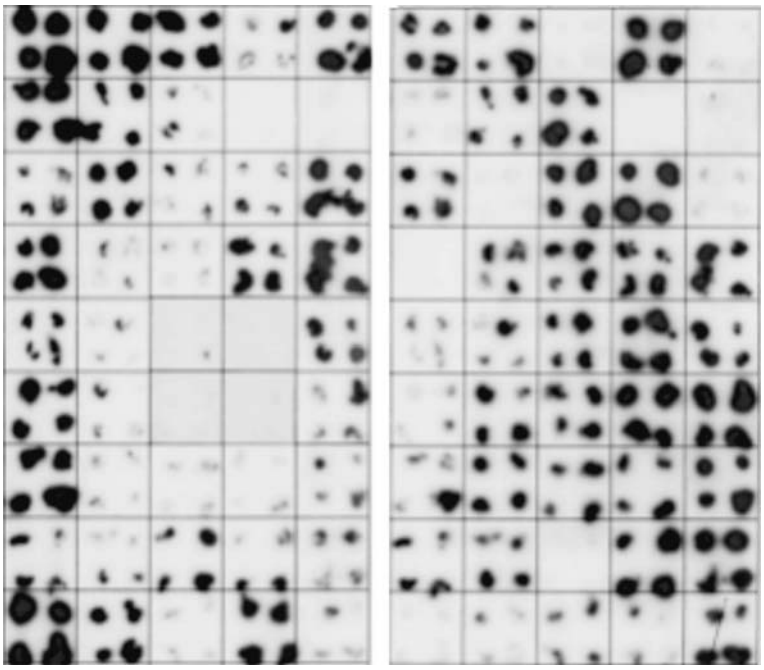

Figure 1. Tissue print of a selection of tomato field samples hybridized with probes specific to TYLCSV (left) or TYLCV (right). Each square represents a single plant with four stem prints. Although most plants are infected by both viruses, some cases of single infections are visible.

Table 1. Number of tomato plants infected by TYLCSV and/or TYLCV (percentages in parentheses)

	TYLCSV	TYLCV	Both	Total
2002 spring/summer	119 (89.5)	0 (0.0)	14 (10.5)	133 (100)
2002 autumn	22 (13.3)	28 (17.0)	115 (69.7)	165 (100)
2003 summer	14 (7.8)	62 (34.6)	103 (57.5)	179 (100)
2003/04 winter	348 (28.0)	389 (31.3)	505 (40.7)	1242 (100)
2004 summer	30 (13.4)	40 (17.9)	154 (68.8)	224 (100)
TOTAL	533	519	891	1943

Plants were sampled between 2002 and 2004 in the Ragusa province (Sicily) and virus presence determined by hybridization with specific probes (see text).

isolates of the 'severe' type, rather than to those of the 'mild' type. Therefore this new isolate from Sicily, hereafter named TYLCV-[IT], can be classified as a TYLCV of the 'severe' type, close to those from Israel, Egypt, Spain, Cuba, Dominican Republic, U.S.A., Puerto Rico and Japan (Figure 2).

For infectivity assays, the pBin-8/4 plasmid, carrying 1.8 copies of the #8/4 viral sequence, was constructed and then used in agroinoculation experiments. Typical TYLCV symptoms were observed two weeks after inoculation on tomato, *N. benthamiana* and *D. stramonium*. Infection was confirmed using tissue print hybridization (not shown). No infection was detected on *S. nigrum*.

Discussion

The first outbreaks of TYLCD in Sicily date back to 1989 (Credi et al., 1989) in the most important area of greenhouse tomato cultivation on the south coast in the province of Ragusa. This coastal strip produces about 60% of the fresh market tomatoes in Italy. The causal agent was TYLCSV (Crespi et al., 1995), not TYLCV, the other well known tomato *Begomovirus* present in the Mediterranean basin (Accotto et al., 2000).

Although the symptoms on tomato plants caused by TYLCSV and TYLCV are very similar, the use of probes and PCR showed that, until 2001, only TYLCSV was present in the area, but that in 2002 TYLCV additionally became present (Accotto et al., 2003). The population dynamics of these two viruses was studied here thereafter. At first TYLCV only appeared in mixed infections and in a limited number of plants, probably introduced with the new spring crops. TYLCV then spread very quickly. In the survey conducted in autumn 2002, mixed infections increased, TYLCSV alone decreased dramatically, and TYLCV appeared for the first time in single infections. Tomato crops surveyed in autumn were planted in August, therefore they differed from (although overlapped with) those sampled in spring/summer.

With such a scenario, the complete displacement of TYLCSV by TYLCV could have been expected in 2003. Actually, a further increase of TYLCV incidence in single infections was observed in summer 2003, while single infections of TYLCSV dropped to 7.8%. However, in the last two surveys (see Table 1) although a high incidence of TYLCV was observed, there was no indication of complete displacement of TYLCSV. It appeared that the viral populations reached an equilibrium, in which

Table 2. Percentage of DNA (for total genome and IR) or protein similarity between TYLCV-[IT] and one isolate of the 'severe' type (TYLCV, X15656), one of the 'mild' type (TYLCV-Mld, X76319), or TYLCSV (X61153)

	Nucleotides		Amino acids					
	Total	IR	V1	CP	C1	C2	C3	C4
TYLCV	97.8	97	100	98.1	98	98.5	97.8	98
TYLCV-Mld	91.7	92	100	100	93.0	98.5	97.8	58.0
TYLCSV	76.8	61.9	95.3	89.7	88.6	80.7	79.9	55.6

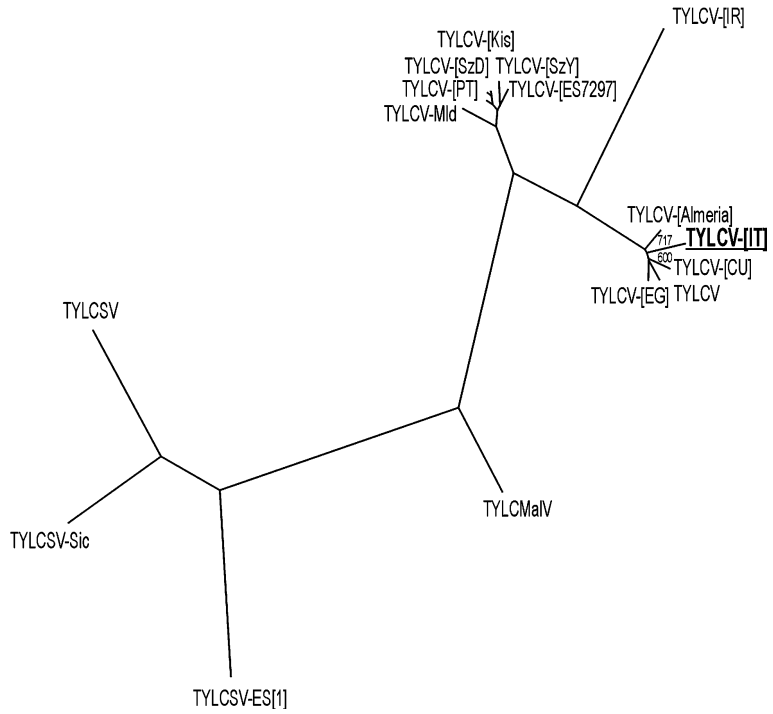

Figure 2. Unrooted phylogenetic tree based on full nucleotide sequences of selected isolates of TYLCV and TYLCSV. Multiple alignments were done using CLUSTAL X, and analysed by the neighbour-joining method, with 1000 bootstrap replications. All bootstrap values exceeded 90%, except those indicated. The genomic sequences of the following begomoviruses were used: TYLCV (X15656) and TYLCV-Mld (X76319) from Israel, TYLCV-[PT] (AF105975) from Portugal, TYLCV-[SzD] (AB116635), TYLCV-[Kis] (AB116634) and TYLCV-[SzY] (AB116632) from Japan, TYLCV-[Almeria] (AJ489258), TYLCSV-ES[1] (Z25751), TYLCV-[ES7297] (AF071228) and TYLCMalV (NC_004569) from Spain, TYLCV-[IR] (AJ132711) from Iran, TYLCV-[EG] (AY594174) from Egypt, TYLCV-[CU] (AJ223505) from Cuba, TYLCV-[IT] (DQ144621, this work), TYLCSV (X61153) and TYLCSV-Sic (Z28390) from Italy. Branch lengths are proportional to sequence distances.

the two virus species coexist, mostly in double infections, but also as single infections in a limited percentage of plants.

Historically, a similar event happened in southern Spain some years ago. TYLCSV had caused severe infections in tomato since 1992 (Noris et al., 1994), but in 1996 TYLCV was reported and quickly spread in the area (Navas-Castillo et al., 1997). A study conducted in the province of Malaga from 1996 to 1998 (Sanchez-Campos et al., 1999) showed that TYLCV was at first present in single infections in 10.3% of infected plants, but increased to 47% in 1997 and 75.2% in 1998. In the same three years TYLCSV single infections decreased dramatically (57.7%, 20.9% and 1.9%, respectively). Meanwhile, the number of plants infected by both viruses varied between 32% and 23%.

The population dynamic observed in Spain is quite different to that observed in Sicily, and it is intriguing to attempt to understand the reasons. The areas of Ragusa and Malaga share similar ecological and agricultural characteristics: intensive vegetable production year-round under plastic, use of similar tomato cultivars, difficulties in whitefly control and regular epidemics of TYLCD. However, one difference in agronomic practices might be important: in the Malaga province beans (*Phaseolus vulgaris*) are grown between tomato crops. TYLCV, but not TYLCSV, can infect beans, and cause epidemics of a disease named 'bean leaf crumple' (Navas-Castillo et al., 1999). Thus, as suggested by Sanchez-Campos et al. (1999), beans may be a reservoir for TYLCV, which can be maintained and multiplied, ready to be transmitted to young tomato plants in springtime. In Sicily, bean is not used as intercrop, therefore this may reduce the pressure against TYLCSV maintenance and both viruses can survive in the same territory.

Another factor that may influence virus population composition may be the vector (Sanchez-Campos et al., 1999), but whether this occurs in our case is not clear. In Sicily the B and Q biotypes of *B. tabaci* are present, with B widely distributed but Q on the increase (Simon et al., 2003). These two biotypes are also present in southern Spain (Guirao et al., 1997). Laboratory experiments using whitefly populations and virus strains found in Spain showed that TYLCSV was less efficiently transmitted than TYLCV by both biotypes (Sanchez-Campos et al., 1999), and this may partially explain the rapid displacement of TYLCSV in that region.

Virus strains present in Sicily are different from those in Spain (TYLCSV-ES[1] and TYLCV-[ES7297]). Therefore laboratory experiments with local Sicilian viruses and whitefly populations need to be performed.

The most striking characteristic emerging from the survey conducted here is the high percentage of plants mixed infected with TYLCSV and TYLCV (68.8% in the last survey). In other regions and crops the presence of more than one geminivirus in single plants is also frequent (Sanz et al., 2000; Varma and Malathi, 2003), but the reason for such prevalence of mixed infections is unclear. Recently it was shown that TYLCV and TYLCSV can be found not only in the same tomato plant, but infecting the same cell (Morilla et al., 2004). Double infections create the conditions for appearance of new viral types through recombination, as previously observed in southern Spain (Monci et al., 2001). The situation in Sicily therefore merits attention in future.

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