Tomato Infectious Chlorosis Virus Causes Leaf Yellowing and Reddening of Tomato in Italy

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Since autumn 2000, severe and widespread chlorosis, sometimes associated with redness, has been observed in greenhouse tomatoes in different regions of Italy. A total of 104 samples were analyzed for tomato infectious chlorosis virus (TICV), by a one-step RT-PCR procedure. In some areas of central Italy and Sardinia, the symptom was consistently correlated with the presence of TICV. The RT-PCR procedure enabled rapid and reliable detection of TICV from field samples. Sequence analysis of the amplified 501-bp fragment, part of the HSP70 coding region, revealed an identity of 99% with the TICV sequence in the GenBank database. A digoxigenin-labeled DNA probe was also produced and successfully tested in dot blot assays. This is the first report of TICV causing epidemics in Europe.
KEY WORDS: Closteroviridae; crinivirus; tomato infectious chlorosis virus; TICV; tomato; whiteflies.

The crinivirus tomato infectious chlorosis virus (TICV) is transmitted by the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood), and in tomato causes yellowing and/or reddening leaf symptoms (10). Although TICV was found for the first time in northern Italy in 1991 as a sporadic case (3,4), and occasionally detected in the following years in cultivated tomato and artichoke crops in the same area (2), no significant spread of the yellowing syndromes occurred. Over the same period a serious disease of tomato caused by TICV occurred in Orange County, California (USA) (5). The disease induced interveinal yellowing and necrosis in the leaves and severe crop loss in the entire region and was associated with the occurrence of high populations of *T. vaporariorum*. A recent report on TICV distribution (11) records the virus in California, North Carolina (USA), Italy and Taiwan.

Since autumn 2000 a serious yellow leaf disorder of tomato, associated with high *T. vaporariorum* infestation, was observed in several greenhouses in Latina province (Lazio, central Italy) and Cagliari province (Sardinia) (see Table 1); the disease was accompanied by the continuous presence of *T. vaporariorum* throughout the winter, which was unusually mild. Intermediate and lower leaves of tomato plants showed brittleness, rolling and marked interveinal yellowing and/or reddening; plants appeared less vigorous, and in some cases fruits showed delayed ripening.

Samples with similar symptoms were also collected in Ragusa province (Sicily) and in several areas of Liguria (northern Italy), although in these two areas no relevant disease outbreaks were noted (see Table 1).

The situation suggested the presence of a crinivirus, but symptoms induced in tomato by TICV and by another tomato-infecting crinivirus, tomato chlorosis virus (ToCV), are very similar (12) and often misidentified as nutritional deficiency. ToCV, transmitted by *T. vaporariorum*, *Bemisia tabaci* (Gennadius) and *T. abutilonea* (Haldeman) (13), has recently been reported from Portugal (7), Spain (9) and Italy (1) some-

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times causing severe diseases. The problem is further complicated by the existence of a third crinivirus infecting tomato, not yet characterized, found in the Canary Islands (11). The three viruses are distinguishable using nucleic acid-based assays.

Since RT-PCR (reverse transcriptase polymerase chain reaction) is considered the most sensitive assay for TICV (6), a one-step RT-PCR was developed. For sample extraction, $\sim$ 0.1 g of leaf tissue was used to prepare total RNA using RNAWiz reagent (Ambion). Total RNA was diluted to $\sim$ 0.01–0.05 $\mu g\ \mu l^{-1}$ and 5 μl was used for RT-PCR.

Two TICV-specific oligonucleotide primers were designed on the available TICV sequence (GenBank acc. no. U67449), to amplify a 501-bp fragment corresponding to part of the coding sequence of the heat shock protein (HSP70) homologue, excluding the phosphate 1 and 2 motifs: TICV-32(+) (5'-TCAGTGCGTACGTTAATGGG-3') and TICV-532(-) (5'-CACAGTATACAGCAGCGGCA-3'). RT-PCR was performed using the One Step RT-PCR System (Invitrogen): total RNAs were first heat-denatured at 65°C for 5 min and then chilled rapidly on ice; the reaction mixture was then added to the PCR tubes. The first step of reverse transcription was done at 50°C for 30 min. After a brief denaturation step at 94°C, 35 cycles (15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C) were performed, ending with a terminal extension step of 5 min at 72°C, in a Perkin Elmer 2400 Thermal Cycler. The whole procedure, including RNA extraction, RT-PCR conditions and gel electrophoresis, was identical to that for ToCV (7), since it was expressly designed to allow detection of TICV and ToCV at the same time.

Reliability of the detection assay was tested using, as templates, total RNAs extracted from a *Nicotiana benthamiana* plant infected with the TICV isolate from Liguria, Italy (T260; described by 3) (positive control), from ToCV-infected tomato, from cucurbit yellow stunting disorder virus (CYSDV)-infected cucumber and from healthy controls. The specific 501-bp fragment was amplified in the positive control but not from ToCV- or CYSDV-infected samples or healthy material (Fig. 1, panel a). When total RNAs from field tomatoes were used (Fig. 1, panel b), the TICV-specific DNA fragment was promptly amplified from some samples, without an aspecific reaction in any sample.

In an attempt to develop a test more suitable than RT-PCR for mass screening, a digoxigenin-labeled probe was synthesized by PCR with the PCR DIG Probe Synthesis kit (Roche) using the TICV-specific primers and a RT-PCR-amplified fragment from a Sardinian isolate as template. In dot blot hybridization (Fig. 1, panel c), 1–5 μg of total RNA was spotted on a positively charged nylon membrane (Roche). Hybridization, high stringency washings and chemiluminescent detection were performed as described before (7). The probe hybridized specifically with TICV-T260, and no reaction was observed with healthy or ToCV-infected plant extracts. Most of the tomato samples that were TICV-infected in RT-PCR could be detected by dot blot, making this test suitable for mass screening. This discrepancy can be explained by the higher sensitivity of the RT-PCR with respect to hybridization techniques.

The data from surveys in the different areas are summarized in Table 1. In Sardinia TICV was found in 93% of the samples tested; 17 samples were also infected by ToCV (29% infection) with 15 of the 17 in mixed infection with TICV; the geminivirus tomato yellow leaf curl Sardinia virus, reported from this area since 1989 (8), was also detected in mixed infections (data not shown). In Lazio, TICV, but no ToCV, was detected on each of the two farms surveyed; all samples tested were TICV-infected. Visual incidence of the yellowing disease in the greenhouses of these two areas was very high (40% to 100%). Suspicious tomato leaf yellowing was also observed in other greenhouses in southern Italy (Campania): two samples were tested from that area and TICV was present in both.

In the eight farms surveyed in Sicily, by contrast, although yellowing symptoms were evident and the vectors were present, no TICV was found, and ToCV was found in only five cases out of 26. Work is in progress to determine if the symptoms observed are associated with some other not yet identified crinivirus infecting tomato or are the result of some nutritional deficiency.

TABLE 1. Occurrence of TICV in tomatoes showing leaf yellowing and/or reddening, collected in greenhouses in some tomato-growing areas in Italy

Region	Interveinal yellowing (visual incidence)	Cultivar	Number of:		
			Greenhouses inspected	Symptomatic samples collected[y]	Samples infected by TICV[x]
Sardinia	40–95%	Camone, Carson, Cencara, Cherry, Colibrì, Conchita, Cuore di Bue, Donador, Furino, Gabriela, Jabot, Kamonium, Kelly, Secolo, T54, Tradiro	33	59	55
Sicily	10–50%	Bonny, Calida, Chiqui, Ciliegino, Cuore di Bue, Inkram, Naomi	8	26	0
Liguria	sporadic	Cuore di Bue	3	5	5
Lazio	50–100%	nd[z]	2	12	12
Campania	sporadic	nd	1	2	2

[z] not determined.
[y] 1–15 samples were collected from each greenhouse inspected.
[x] As determined by RT-PCR.

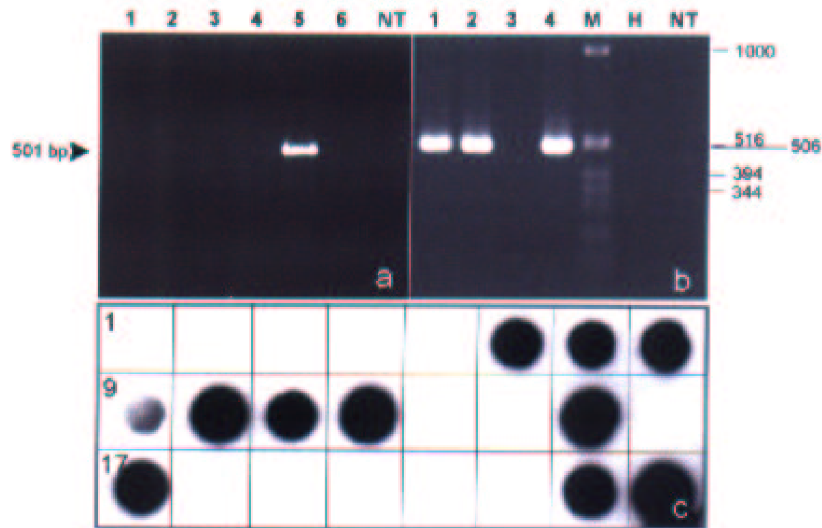

Fig. 1. TICV diagnostic procedures.
Panel a. RT-PCR reliability test using the primers TICV-32(+) and TICV-532(-) in a one-step procedure. Ethidium bromide stained 1.2% agarose gel in TBE. The arrow indicates the amplified fragment; 1 = CYSDV-infected cucumber; 2 = healthy cucumber; 3 = ToCV-infected tomato; 4 = healthy tomato; 5 = TICV-infected *Nicotiana benthamiana*; 6 = healthy *N. benthamiana*. NT = no template. Size markers are shown in panel b.
Panel b. Ethidium bromide stained 1.2% agarose gel for the detection of TICV by one-step RT-PCR in field tomato samples. 1, 2, 4 = field tomato samples under test (see Table 1); 3 = ToCV-infected field tomato sample; M = 1kb DNA ladder (Life Technologies/Invitrogen), sizes in bp at right; H = healthy tomato sample.
Panel c. Dot blot hybridization of tomato total RNA extracts with TICV-specific digoxigenin-DNA probe. Field samples from Sicily (1, 2, 4, 5); Sardinia (6, 7, 8, 9, 10, 11, 12, 17, 18, 22, 23, 24); healthy tomato (3, 13, 14, 19, 20, 21); and TICV (15) and ToCV (16) positive controls.

In Liguria, incidence of infection has not been high but both TICV and *T. vaporariorum* are present and the danger of serious infection is acute, especially as ornamentals and vegetables – many of them hosts of TICV (12) – are cultivated in proximity.

The TICV-specific 501 bp fragments amplified from three samples (TICV-L from Lazio, TICV-S from Sardinia and TICV-T260 from Liguria) were purified using a High Pure PCR kit (Roche) and sequenced in both directions using the same primers designed for amplification. The sequences obtained showed more than 99% identity to that of TICV strain 'California' (Genbank acc. no. U67449), confirming identification. When aligning TICV-T260 (GenBank acc. no. AY048855), TICV-L (AY048856) and TICV-S (AY048857) with the sequence U67449, three base changes were found. Whereas there was a silent base change at position 486, the changes at position 316 and 419 resulted in amino acid changes of threonine to alanine and alanine to valine, respectively. TICV-L contained a further silent base change at position 75. Numbers refer to positions in the U67449 sequence.

This report provides additional information about the increasing damage that whitefly-transmitted viruses are causing to protected crops worldwide. The cause of the sudden emergence of crinivirus diseases in Italy is not obvious and probably complex. Particular environmental conditions associated with the presence of virus reservoirs in particular crops, relaxation of defence measures against whiteflies, and difficulty in recognizing the symptoms, may all have played a part. Here we describe RT-PCR and hybridization diagnostic techniques for TICV which, together with those described for ToCV (7), provide useful tools for detection of these viruses.

The whitefly *T. vaporariorum*, distributed throughout the world (2), and TICV, represent a severe threat to tomato crops in Europe and worldwide. As a consequence, searching for sources of resistance or tolerance and breeding them into cultivated tomato will be the most effective way to cope with the problem in the future.

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