Multiplex Detection of Criniviruses Associated with Epidemics of a Yellowing Disease of Tomato in Greece

C. I. Dovas and N. I. Katis, Plant Pathology Laboratory, Faculty of Agriculture, Aristotle University of Thessaloniki, 54 006, Thessaloniki, Greece; and A. D. Avgelis, National Agricultural Research Foundation, Plant Protection Institute, Plant Virology Laboratory, 71 003 Heraklio, Crete, Greece

ABSTRACT

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Since 1997, a yellowing disease has been observed in greenhouse tomato (*Lycopersicon esculentum*). By 2001, the disease was widespread, including open field tomato crops, and in most cases its incidence was 80 to 90% or even 100%. Epidemics in glasshouses were mainly associated with high populations of the whitefly *Trialeurodes vaporariorum* and *Bemisia tabaci*, the major whitefly pests in vegetable crops in Greece. The main leaf symptoms were severe yellowing, rolling, and brittleness. Samples from symptomatic plants were analyzed by polymerase chain reaction (PCR) and shown to be infected with *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV) (family *Closteroviridae*, genus *Crinivirus*). TICV was found in 164 of 183 symptomatic samples, while ToCV was less representative (25/183). Sequence comparisons of the amplified 229-bp and 466-bp products revealed 99 and 100% identity with the reported sequences of TICV and ToCV, respectively. A reverse transcription (RT) multiplex PCR assay using a simple sample preparation procedure was developed to allow rapid, specific, and simultaneous detection of both ToCV and TICV sequences in two steps. The method involves a one-tube RT-PCR step in which the combination of primers amplifies part of the heat shock protein to homologue gene of both ToCV and TICV, followed by a multiplex nested PCR amplification. This is the first report of TICV and ToCV in Greece and, as far as we know, the first report of TICV in Europe.

Additional keywords: diagnosis, RT-PCR

In 1997, a low incidence of yellowing symptoms appeared in greenhouse tomato plants in Southern Greece and the island of Crete. By 2001, the distribution and incidence had increased to the majority of tomatoes grown in greenhouses in Greece. Similar symptoms also appeared, although in lower incidence, in open field-grown tomato crops. Yellowing symptoms were obvious in all cultivated tomato hybrids and were initially attributed to nutritional deficiencies. However, the fact that the first symptomatic plants were near greenhouse openings and were followed by rapid and random disease distribution, led us to investigate a viral etiology. Yellowing symptoms differed considerably from those caused by *Tomato yellow leaf curl virus* (TYLCV-Is) (family *Geminiviridae*, genus *Begomovirus*) (2), and from four major endemic viral diseases of tomato crops in

Greece: *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), and *Tomato spotted wilt virus* (TSWV). Symptoms of affected plants appeared to be more similar to those caused by tomato criniviruses (family *Closteroviridae*) such as *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV) (23). Both viruses were first reported during the last decade in the United States (6,19), and ToCV has been reported to occur in the Mediterranean countries, Portugal (13), Spain (16), and Italy (1).

The aim of the present research was to clarify the etiology and the incidence of the causal agent of the tomato yellows observed. In addition, polymerase chain reaction (PCR)-based detection methods were optimized and developed toward highly sensitive large-scale applications to test tomato leaves for the presence of TICV and ToCV, focusing on sample preparation and multiplex detection of both viruses.

MATERIALS AND METHODS

Field surveys and samples tested.

Fully developed young leaves showing severe yellowing were collected from tomato plants grown in greenhouses or in open fields from five major tomato-growing areas, i.e., Crete (Kundura, Platanos, Chania, Messara Valley, Ierapetra),

Peloponnesus (Arkadia, Messinia, Ilia, Achaia, Argolida, Lakonia, Poros), Epirous (Preveza), Macedonia (Pella), and the island of Rodos (Fig. 1). In each surveyed crop, the tomato hybrid was recorded, the incidence of yellowing disease was expressed as the percentage of symptomatic plants over 200 randomly selected plants, and samples from three plants showing typical symptoms were collected for mechanical transmission tests and PCR analysis. Samples were maintained at 4°C or lyophilized until they were tested or analyzed. The assayed 183 samples included the following tomato hybrids from 49 glasshouses (147 samples) and 12 fields (36 samples): Baya, Belladonna, Troya, Iron, 9/84, Noa, 717 Fitro, Preveza, Alma, Jumbo, Naysica, Galli, Carina, Electra, Cortina, Nefeli, Amati, and Vapsa.

Two Italian ToCV-infected tomato samples from Italy were a kind gift from A. M. Vaira (Istituto di Fitovirologica Applicata, Torino, Italy).

Mechanical inoculations. Tissue from affected plants extracted in phosphate buffer, pH 7.0, (1 g/10 ml) was used to sap inoculate a wide range of indicator plants including: *Gomphrena globosa*, *Chenopodium amaranticolor*, *C. quinoa*, *Zinnia elegans*, *Celosia cristata*, *Cucumis sativus*, *C. melo*, *Cucurbita pepo*, *Ocimum basilicum*, *Phaseolus aureus*, *P. vulgaris* cv. Bountiful, *Vicia faba*, *Vigna sinensis*, *Cap-sicum annuum*, *Lycopersicon esculentum* cv. Noa F1, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. megalosiphon*, *N. rustica*, *N. tabacum* cv. W.B., Xanthi, Samsun, *Petunia hybrida*, and *Solanum melongena*. The test plants were grown and observed in an insect-proof glasshouse (22 ± 2°C, 75% relative humidity).

Sample preparation for PCR. The extraction protocol was a modification of that reported previously (10,17) and consisted of the following steps: tomato petioles were macerated in extraction buffer (1.59 g of Na₂CO₃ per liter, 2.93 g of NaHCO₃ per liter, pH 9.6, 6% PVP-40, 0.5% bovine serum albumin, and 0.05% Tween 20) at a 1:20 dilution. A small aliquot of this crude extract was centrifuged in 1.5-ml tubes at 5,000 × g for 2 min, and 2 µl was transferred into 200 µl of GES buffer (0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM EDTA) containing 0.5% Triton X-100 and 1% β-mercaptoethanol. The mixture was then incubated at 94°C for 5 min,

Corresponding author: N. I. Katis
E-mail: katis@agro.auth.gr

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placed on ice, and 1 µl was added to 24 µl of reverse transcription (RT)-PCR reaction mixture.

Oligonucleotides. Primers used in this study are presented in Table 1. In the initial amplification, a pair of degenerate primers (HS-11 and HS-12) that can amplify both ToCV and TICV genomes were used. Both primers were designed based on the primer sequences reported previously (16), and they were derived from highly conserved regions of the heat shock protein (HSP) 70 homologue genes reported for these viruses (GenBank accession numbers: AF024630, AF215817, AF215818, AF233435, AF234029, U67449). Sequence alignment was performed using the program CLUSTAL V. Two pairs of specific primers, one for each virus (TIC-3/TIC-4 and ToC-5/ToC-6), were designed for use in nested PCR. The criteria used for the de-

sign of each primer pair were the relative position within the target sequence, specific detection of each virus, range of sizes of the amplicons produced for multiplex detection, similarity of reaction kinetics, and a higher annealing temperature than primers HS-11 and HS-12. Since only one sequence fragment of HSP70 homologue gene has been reported for TICV, more sequence information was needed for sequence comparison and selection of primer TIC-4. For this reason, total RNA was extracted from two TICV-infected tomato samples originating from Crete and Preveza, respectively (15). Two microliters of RNA extract was used in one-step RT-PCR using the HS-11/HS-12 primer pair, and 587-bp PCR products were sequenced using primer HS-11 according to the conditions described in the "PCR product sequencing" paragraph.

First round PCR amplification. The 25-µl reaction mixture contained: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.25 mM of each deoxyribonucleoside triphosphate (dNTP), 5.0 mM DTT, 0.5 µg purified BSA (New England Biolabs, Beverly, MA), 12 units RNASEOUT (Life Technologies, Inc., Gaithersburg, MD), 1.0 unit Avian Myeloblastosis Virus Reverse Transcriptase (Finnzymes Oy, Espoo, Finland), and 0.6 units Dynazyme II DNA Polymerase (Finnzymes), and a final concentration of 1 µM for each primer (HS-11 and HS-12). The cycling profile was as follows: first step at 42°C for 60 min, second step at 50°C for 2 min, third step at 94°C for 5 min, 35 cycles segmented in step 1: 30 s at 95°C, step 2: 30 s at 43°C, step 3: 15 s at 72°C, followed by a final extension step at 72°C for 2 min. Amplifications were carried out in a GeneAmp 2400 thermal Cycler (Perkin Elmer, Norwalk, CT) utilizing thin-walled reaction tubes.

Nested PCR amplification. Part of the collected samples, consisting of 90 symptomatic tomato samples from 11 different locations in Greece (Table 2) and two ToCV-infected samples from Italy, were used to test the performance (polyvalence and specificity) of the primers designed in this study. Twenty microliter nested PCR reactions were performed. The reaction mixture contained 20 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 0.5 unit *Taq* DNA Polymerase (Life Technologies). A final concentration of 0.2 µM for each primer pair was used for the detection of TICV (TIC-3/TIC-4) or ToCV (ToC-5/ToC-6). The cycling profile consisted of a first denaturing step at 95°C for 1 min, 40 cycles segmented in 20 s at 95°C, 15 s at 60°C, and 10 s at 72°C, followed by one final extension step at 72°C for 2 min. The reaction products were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer (1× 0.04 M Tris-acetate, 0.001 M EDTA), stained with ethidium bromide, and visualized under UV light.

Sequencing of PCR products. Specificity of both nested primer pairs was confirmed by direct sequencing the PCR products. Two isolates of each TICV and ToCV were amplified by RT-PCR with primer pairs HS-11/HS-12 and subsequently using the nested-PCR conditions described above for single-virus detection with primer pairs TIC-3/TIC-4 and ToC-5/ToC-6. Nested-PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA), and both strands were sequenced with an ABI Prism 3700 DNA Analyzer, using ABI PRISM BigDye Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Multiplex PCR. All collected samples were tested by multiplex PCR with a protocol identical to the single-virus assays described previously except for modifica-

Fig. 1. Map of Greece. Black circles show areas where tomato samples with yellowing symptoms were collected for the present study.

Table 1. Primer pairs used for the detection of *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV), and respective amplicons produced by reverse transcription-polymerase chain reaction (RT-PCR)

Name	Sequence	Virus detected	Assay	Amplicon size
HS-11	5'-GG(G/T)TT(A/G)GA(G/T)TT(C/T)GGTACTAC-3'	ToCV,	One step	587 bp
HS-12	5'-CC(G/T)CCACCAAA(A/G)TCGTA-3'	TICV	RT-PCR	
TIC-3	5'-GGGTTAGAGTTCGGTACTACTTTTCAGT-3'	TICV	Nested-PCR	223 bp
TIC-4	5'-CGTCGAAAGATTTCATCGACT-3'	TICV	PCR	
ToC-5	5'-GGTTTGGATTTCGGTACTACATTCAGT-3'	ToCV	Nested-PCR	463 bp
ToC-6	5'-AAACTGCCTGCATGAAAGTCTC-3'	ToCV	PCR	

tions in primer concentrations used in nested PCR. A titration of primer pairs was necessary for equal amplification of both amplicons in doubly infected samples. A final concentration of 0.2 μ M for each of TIC-3 and TIC-4 and 0.4 μ M for each of ToC-5 and ToC-6 was selected for routine use.

RESULTS AND DISCUSSION

Tomatoes are one of the most important vegetable crops in Greece, with the majority of tomatoes used for fresh consumption obtained from open fields (19,100 ha) and a small proportion of early tomatoes from the greenhouse (2,600 ha). About 24,100 ha are field cultivated for canned tomatoes. The last few years, a yellowing disease appeared to be endemic in the greenhouse tomatoes, usually in high incidence, and more rarely in the open field. In 2001, a large-scale survey found that many cultivated tomato hybrids had similar yellowing leaf symptoms. The main symptoms were irregular chlorotic areas on the leaves, which gradually progressed into interveinal chlorosis, rolling, and brittleness. Symptoms initially appeared in the lower, but also in the middle leaves, and eventually on the upper part of the plant. Affected plants produced smaller fruits, which ripened later, although without any symptoms. These symptoms were similar to those caused by criniviruses such as TICV and ToCV, both of which first appeared in the United States (21,22). Initially, symptomatic plants were scattered in the affected crops, and the initial foci were usually in the greenhouse openings or at the outer lines of the open field crops. Disease incidence increased gradually, and by the end of the growing season, almost all plants were affected both in the field and in the greenhouse.

We were unable to reproduce yellowing symptoms by mechanical inoculation onto indicator tomato plants (hybrid Noa) using

sap from affected tomato plants. However, in a few cases, isolates of CMV, PVY, and TSWV were mechanically isolated and detected by serological identification.

To investigate the etiology of the yellowing disease of tomato and the implication of the TICV and ToCV criniviruses, we designed specific primers for their reliable detection. Additionally, a multiplex nested PCR was developed for large-scale detection applications. A fraction (90 samples) of the symptomatic tomato samples collected from 11 different locations in Greece and two ToCV-infected samples from Italy were tested by uniplex nested PCR. Ten and 84 samples were found infected by ToCV and TICV, respectively (four samples were infected by both viruses). In all cases, nested PCR yielded the amplification products expected for each virus. Direct sequencing of four nested PCR products amplified from TICV- and ToCV-infected tomato samples revealed 99 and 100% identity to the corresponding HSP70 homologue region of ToCV (EMBL no. AF024630, AF233435, AF215817, AF215818, and AF234029) and TICV (EMBL no. U67449), respectively,

confirming their viral origin. Two sequences were used for the selection of primer TIC-4 and deposited in EMBL/GenBank database (Accession numbers: AJ344212 and AJ344213). Multiplex PCR successfully detected both viruses in all 90 samples infected either by a single virus or both viruses simultaneously, and all results were in agreement with uniplex PCR tests performed previously. From all of the 183 collected samples tested by multiplex PCR, 25 and 164 samples were infected by ToCV and TICV, respectively, and six samples were infected by both viruses. Figure 2 presents selected nested multiplex PCR results of TICV and ToCV detection from 13 samples from different geographic regions.

This large-scale survey clearly showed the close association of TICV and ToCV criniviruses with the yellowing disease of tomatoes in Greece. All tested samples from plants showing the yellowing disease were positive to one or both criniviruses. About 70% of the inspected crops showed high incidence of the disease with over 40% of symptomatic plants (Table 2). TICV, a *Trialeurodes vaporariorum*-borne

Fig. 2. Partial results from multiplex nested polymerase chain reaction (PCR) amplification of field grown and greenhouse tomato plants naturally infected with *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV) (lanes 1 to 11). Sample 1 is doubly infected by TICV and ToCV. H, healthy controls; M, 100-bp DNA ladder.

Table 2. Incidence of the yellowing disease in greenhouse and open field grown tomato crops surveyed during 2001 in Greece, and associated criniviruses: *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV)

Site	No. of fields inspected	No. of fields with incidence level (%) ^a					Fields infected by TICV/ToCV	Cultivar
		<20	20-40	40-60	60-80	>80		
Rhodos	3		1	1		1	0/3	Belladonna, Troya
Crete	12	...	1	2	3	6	9/4	Baya, Belladonna, Iron Noa, 9/84, 717 Fitro
Arkadia	13	...	4	1	1	7	13/0	Preveza, Alma, Jumbo Naysika, Galli, Arion
Messinia	4	...	2	2	...	...	4/0	Baya
Iliia	9	1	...	...	...	8	9/0	Belladonna, Iron, Noa, Cortina, Electra
Achaia	3	1	...	1	...	1	3/0	Belladonna, Nefeli Cortina
Argolida	1	...	...	1	...	...	1/0	Unknown
Laconia	1	...	...	1	...	...	1/0	Unknown
Poros	3	1	2	...	...	...	2/2	Baya
Preveza	11	2	4	3	1	1	11/0	Jambo, Galli, Baya, Amati, Vapsa, Noa
Pella	1			1			0/1	Unknown
Total	61	5	14	13	5	24	53/10	

^a Incidence of symptomatic plants exhibiting the yellowing disease of 200 plants evaluated; ... indicates that no field with this incidence level was observed.

virus, has been identified in the majority of the sampling areas (53 fields out of 61 tested, about 87%), whereas ToCV, a virus transmitted by *T. vaporariorum*, *Bemisia tabaci*, and *T. abutilonea*, was only present in 10 fields, in Rhodos, Crete, Poros, and Pella (Macedonia), and it was found in mixed infections with TICV in two fields in Crete and Poros (Attica). Interestingly, ToCV was not detected in any of the 12 open field tomato crops (data not shown) showing yellowing symptoms where TICV was always present. Although the impact of criniviruses, either alone or in mixed infection with other viruses (TYLCV-Is, TSWV, CMV), on tomato production has not been determined yet, it seems that at least under our conditions, favoring their incidence, they will be a limiting factor in tomato production. Crinivirus epidemics were always associated with crops heavily infested by both *T. vaporariorum* and *T. tabaci*. In Greece, although there are no systematic studies on their incidence, *B. tabaci* is considered to be more frequent in open field crops and in the greenhouses during late summer to early autumn. On the other hand, *T. vaporariorum* is more abundant in greenhouse crops during winter and spring. However, differences in their distribution may occur in southern Greece, including islands, and northern Greece due to different environmental conditions. The other vector species of TICV, *T. abutiloneus*, so far has not been recorded in Greece (14). On the contrary, in countries of the Mediterranean basin such as Spain (16), Portugal (13), and Italy (1), where *B. tabaci* was the predominant whitefly present, ToCV was the only crinivirus present, and it is considered the causal agent of the tomato yellowing disease.

TICV and ToCV were identified in more than 18 tomato hybrids cultivated in Greece, and no differences could be observed in symptomatology and disease incidence. A similar situation was observed among the cultivated tomato cultivars in southern Spain (16).

Whiteflies, mainly *B. tabaci* and *T. vaporariorum*, have become major crop pests all over the world, particularly in tropical and subtropical areas (3,5). Both species have become serious pests in Mediterranean countries; therefore, criniviruses together with TYLCV might now represent major threats to this important crop. During the dry and warm season, the tobacco whitefly, *B. tabaci*, seems to be the major pest in most of the Mediterranean countries, resulting in great yield losses both by direct damage and the transmission of TYLCV and ToCV in tomato crops (13,16).

The yellowing disease of tomato represents a new threat to tomato production all over Greece. Initially, yellowing symptoms were attributed to mineral deficiencies and possible virus implication was overlooked.

Soon thereafter, as the first "nest" of heavily affected greenhouses was noticed in the Koundura area (southwestern Crete) in late 2000, investigations were directed to isolate and identify the viruses implicated. During the 1990s, aphid-borne viruses such as AMV, CMV, and PVY and the thrips-borne TSWV appeared to be the most widespread tomato viruses in Greece (4,8,9,20). Although symptoms similar to those caused by TYLCV were first observed sporadically in Crete at the beginning of the 1990s, it was only in 2000 and 2001 that epidemics appeared in southern Crete. Soon thereafter, the disease also appeared in areas of continental Greece such as Attiki, Preveza, and the southern Peloponnese (2; A. D. Avgelis, unpublished data), and recently an epidemic has been observed on the island of Rodos (E. Papadopoulou, C. I. Dovas, N. I. Katis, unpublished data). This survey (extended in all the important tomato producing areas) confirmed the spread and occurrence of a new viral tomato disease. Two criniviruses were implicated with the disease: TICV and ToCV. This is the first report of these viruses in Greece.

During the last 10 years, yellowing symptoms were also widespread in cucumber and melon in glasshouses and open field crops all over Greece. A close relationship was found between the presence of criniviruses and yellowing symptoms. Initially, *Beet pseudo-yellowing virus* (BPYV) was identified as the only causal agent of the disease in the western Peloponnese (12) and on the island of Crete (18). However, in a more extended survey, BPYV was found to occur in all areas where yellowing symptoms were observed, but *Cucurbit yellowing stunting disorder virus* (CYSDV) was also isolated from affected cucumber and melon crops in the islands of Rhodos, Poros, and in the area of Leonidio in the mainland (eastern Peloponnese) (I. Bourbourakas and N. I. Katis, unpublished data). BPYV is transmitted by *T. vaporariorum* (6), whereas CYSDV is transmitted by *B. tabaci* (22). The data concerning the criniviruses observed in tomato and cucurbit crops in Greece indicate that *T. vaporariorum* is probably the most prevalent whitefly. However, large-scale surveys of whiteflies throughout the country will answer this question.

Until now, specific detection of tomato criniviruses such as TICV (11) and simultaneous detection of both TICV and ToCV (16) were based on RT-PCR involving double tube PCR formats and total RNA extracts as templates. Primers were designed for a comprehensive detection of ToCV and TICV by RT-PCR based methods. A previously developed simple sample preparation (17) alleviating the need for laborious preparation of purified RNA was coupled to a one-tube RT-PCR protocol using a pair of degenerate primers for the amplification of part of the HSP region of

both viruses (7). In this study, to increase detection sensitivity and simultaneous differentiation of ToCV and TICV, a multiplex nested PCR assay followed, utilizing primers designed to anneal into the amplification product from the first reaction. Differential detection of each virus was made possible by the size of its respective PCR product. Sample preparation for PCR testing has proven to be difficult, due to the effect of putative PCR-inhibitors of plant origin. Total nucleic acid extraction is costly and not practical for routine diagnosis when analysis of many samples is required. The simple sample preparation method combined with one-tube RT-PCR protocol allows simple, fast, and cost-effective analysis of a large number of samples. Application of nested PCR compensated for the potential decrease in sensitivity due to the high dilutions required during sample preparation. In addition, the use of two pairs of specific primers in nested PCR producing two different-sized amplicons allows specific detection and differentiation of both viruses. Multiplex PCR methods are less labor demanding and more economical than single PCR methods. Our experiments confirmed the specificity of primer pairs TIC-3, -4 and ToC-5, -6 for their respective target viruses in both single-virus and multiplex PCR assays. Based on this finding, the multiplex system was used for testing of all collected samples. The primers amplified successfully more than 164 isolates of TICV and 25 isolates of ToCV from different geographic regions in Greece along with two ToCV isolates from Italy. The nested PCR described here for multiplex detection of both viruses could be useful for study of the tomato yellowing etiology and their epidemiology since large numbers of samples can be tested. However, thorough examination of the primers polyvalence should be conducted by testing more virus isolates to evaluate the reported assay for worldwide monitoring distribution and host range of both viruses. To our knowledge, this is the first report of a multiplex nested PCR assay that can reliably detect and discriminate two criniviruses such as TICV and ToCV.

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In our article "Multiplex Detection of Criniviruses Associated with Epidemics of a Yellowing Disease of Tomato in Greece" by C. I. Dovas, N. I. Katis, and A. D. Avgelis, pages 1345-1349, we claimed that during that work there was the first report of *Tomato infectious chlorosis virus* (TICV) in Europe. However, TICV had already been found in Italy (Wisler et al., 1998). In addition, during 2002, two more papers reporting the presence of TICV in Italy (Vaira et al., 2002) and Spain (Font et al., 2002) appeared.

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