Variability Among Italian *Citrus tristeza virus* Isolates Revealed by SSCP Analysis, Cloning and Sequencing

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ABSTRACT. Since 2001, thousands of citrus plants of 15 varieties affected by *Citrus tristeza virus* (CTV) have been detected in Italy. CTV isolates differ by their biological characteristics, particularly in the intensity of symptoms on different cultivars, in transmissibility by aphids vectors and in their ability to cross-protect. A study was instigated to examine CTV isolates present in Italy to determine the degree of genetic variability in the population of CTV found in Italy. Genetic variability of CTV isolates was analyzed using single strand conformation polymorphism (SSCP) analysis, cloning and phylogenetic analysis of nucleotide sequences. The SSCP patterns revealed that many isolates found in the last four years in Italy could be related to mild isolates present in different countries of the Mediterranean Basin, while others are similar to Asian and American isolates. Our data suggest the presence of five independent introductions of CTV in Italy, likely by illegal import of CTV-infected budwood.

Index words. Italian-CTV-isolates, RT-PCR, SSCP, cloning, sequencing.

Citrus tristeza virus (CTV) is the causal agent of one of the most important diseases of citrus. More than 80 million trees around the world have been killed by this virus (18). The existence of CTV strains which induce variable symptoms on several citrus species or scion/rootstock combinations has been widely documented (4, 12, 21).

CTV is a *Closterovirus* with a single-stranded, positive sense genomic RNA (gRNA) of approximately 20 kilobases (kb). Virions are composed of RNA encapsidated by two capsid proteins of 25 and 27 kDa, comprising 95% and 5% of the virion, respectively (8, 14). CTV is a phloem-limited virus transmitted by aphids in a semipersistent manner.

The major gRNA of isolates T36 and T30 from Florida (1, 14, 20), VT from Israel (17), T385 from Spain (25) and SY568 from California (26) have been sequenced. All had similar genome structures of 12 open-reading-frames (ORFs) and untranslated regions (UTR) at their 5' and 3' termini. The two 5' proximal ORFs encode replication-related proteins and are translated directly from the gRNA. The remaining 10 ORF, encoding various structural and non-structural proteins, are expressed via subgenomic RNAs (sgRNA), which are 3' conterminal (13). Infected trees also may also contain defective RNAs (D-RNA), which have both gRNA termini but lack variable portions of the central regions (2, 16). CTV infected tissue also may contain a population of sequence variants resulting from the error-prone nature of RNA dependent RNA polymerases (3, 22).

The extent of the damage to citrus due to CTV infection depends on the isolate characteristics and on the cultivars. Some isolates are essentially symptomless, but most of them cause one or more of the following symptoms: i) decline and death of most citrus varieties grafted on sour orange rootstocks, ii) stem-pitting in many citrus varieties regardless of the rootstock and iii) seedling yellows, a chlorosis that develops in seedlings of sour orange, grapefruit or lemon (9). The molecular basis of these symptoms is presently unknown.

Biological characterization by symptoms produced in a standard panel of citrus indicator species (10) allows classification of CTV isolates into several biogroups (11), but this is a slow and expensive procedure that cannot be used for routine identification.

Single-strand conformation polymorphism (SSCP) analysis is a simple method that differentiates DNA fragments of the same size but of different nucleotide sequence. (19). In Italy CTV was probably introduced as early as 1980 and by 2001 CTV infection was epidemic (6), having caused the death of a great number of trees. The uncontrolled movement of citrus budwood and the presence of the vector *Aphis gossypii* has contributed to the widespread distribution of CTV to most citrus-growing areas in Italy, especially in Sicily which has the largest citrus growing area. The aim of this work was to characterize CTV isolates present in Italy by SSCP and sequence analysis.

MATERIALS AND METHODS

Nucleic acid extraction and SSCP analysis. Table 1 shows the different areas of Italy and the different citrus species and varieties affected by CTV. In each area fifty samples were randomly collected on the species reported in Table 1. Total RNA was extracted with RNeasy Plant Mini Kit (Qiagen Science, MD, USA) according to manufacturer's instructions, and used as templates for amplification by reverse transcription and polymerase chain reaction (RT-PCR) with primers p20F and p20R (encompassing the CTV p20 gene) (22). RT-PCR was performed in one-step in a 25 µl reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 µM of each primer, 4 U of RNaseOut (Invitrogen, Carlsbad, CA, USA), 20 U of SuperScript II reverse transcriptase-RNaseH (Invitrogen, Carlsbad, CA, USA) and 2 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out in a PTC 100 Peltier Celbio thermalcycler, using the following parameters: 42°C for 30 min, 94°C for 2 min, 35 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C and a final elongation of 4 min at 72°C. For SSCP analysis, 1 µl of RT-PCR product was mixed with 9 µl denaturing solution (95% deionized formamide, 20 mM EDTA and

TABLE 1
ORIGIN AND CITRUS SPECIES AND VARIETIES SAMPLED IN ITALY FOR GENETIC ANALYSIS OF CTV P20 GENE

Area	Plant
Apulia-Massafra	Comune Clementine
Apulia-Massafra	Navelina sweet orange
Sicily-Belpasso	Tarocco Comune sweet orange OL
Sicily-Belpasso	Tarocco Galici sweet orange
Sicily-Belpasso	Tarocco nucellar sweet orange
Sicily-Belpasso	Tarocco Scirè sweet orange
Sicily-Cassibile	Fortune mandarin
Sicily-Cassibile	Miyagawa Satsuma
Sicily-Cassibile	Navelina sweet orange OL
Sicily-Cassibile	New Hall sweet orange
Sicily-Cassibile	Nova mandarin
Sicily-Cassibile	Star Ruby grapefruit
Sicily-Cassibile	Valencia sweet orange
Sicily-Palermo	Tardivo di Ciaculli mandarin
Tuscany-Florence	Kumquat (*Fortunella margarita*)

bromophenol-blue), heated for 10 min at 94°C and chilled on ice. Electrophoresis was carried out in a non-denaturing 8% polyacrylamide gel using 1× TBE buffer, at 200 V for 3 h at 4°C. Gels were stained with silver nitrate according to the procedure of Beidler (5).

Molecular cloning and nucleotide sequence analysis. The sequence variants detected with SSCP were cloned in a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and plasmids for each molecular variant were extracted with the High Pure Plasmid Isolation Kit (Roche Diagnostics, Basel, Switzerland), following manufacturer's instructions. Two clones for each different SSCP profile were sequenced in both directions using an ABI PRISM DNA 377 sequencer (Perkin-Elmer). Sequences obtained were aligned with the program CLUSTAL W (24).

Phylogenetic relationships were inferred using Neighbor-joining analysis with 1000 bootstrap replicates (Fig. 2) with the program MEGA (15).

Nucleotide sequences of gene p20 from Italian isolates were compared to GenBank entries U16034 and AF260651 (Florida isolates T36 and T30), U56902 (Israel isolate VT), AF001623, AF203073, AF203075, AF356303, AF356305, AF356315 (California isolates SY568, 65, 107, 5, 59, 416), Y18420, AF356318, AF356323, AF356327 (Spanish isolates T385, T308, T346, T398), AB046398 (Japan isolate NUagA) and AY340979 (Egypt isolate Qaha).

RESULTS AND DISCUSSION

RT-PCR of all CTV-infected samples yielded the expected 549 bp DNA fragment, whereas no amplification was obtained from non-inoculated plants grown in the greenhouse. All CTV isolates collected in the same citrus area showed the same SSCP pattern, whereas patterns were different for the five geographic areas analyzed (Fig. 1).

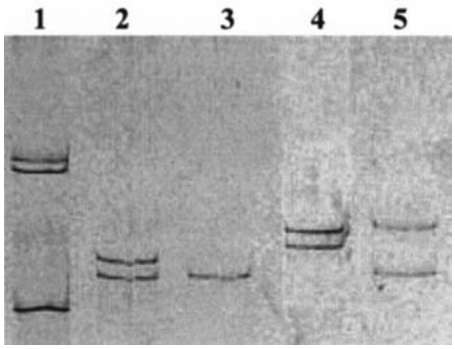

Fig. 1. Lanes 1-5 show SSCP patterns of CTV p20 gene amplified from CTV isolates collected from 1) Belpasso, 2) Massafra; 3) Cassibile, 4) Palermo, and 5) Florence.

Comparison with sequences retrieved from GenBank showed that Belpasso isolates were closely related to California isolates SY568 and 107 (nucleotide identities greater than 99%), to Japan isolates NUaga (nucleotide identities greater than 97%), and to the Israel isolate VT (nucleotide identities greater than 96%). Cassibile and Massafra isolates were closely related to Spanish isolates T385 and T398 (nucleotide identities greater than 99%), to California isolates 5 and 59 (nucleotide identities greater than 98%) and to the Florida isolate T30 (nucleotide identities greater than 98%).

Palermo isolates were related to Spanish isolates T385 and T398 (nucleotide identities greater than 98%), to Florida isolates T30 (nucleotide identities greater than 97%) and to Cassibile and Massafra isolates (nucleotide identities greater than 97%). Florence isolates were related to Spanish isolates T385 and T398 (nucleotide identities greater than 98%), to Florida isolates T30 (nucleotide identities greater than 97%), to Palermo isolates (nucleotide identities 98%) and to Cassibile and Massafra (nucleotide identities 98%).

Our observations of isolates genetically related but from geographically distant areas are simi-

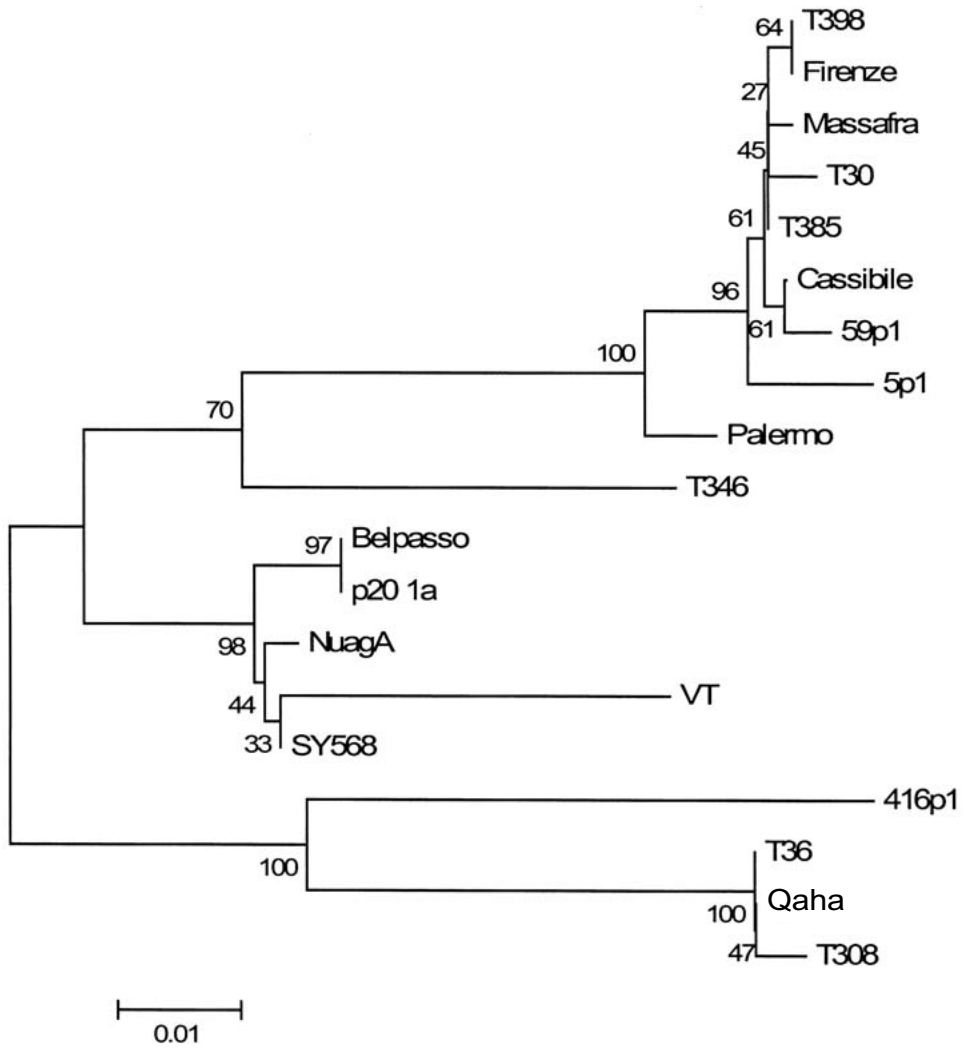

Fig. 2 Neighbor-joining tree of nucleotide sequences of the CTV p20 gene. Bootstrap replicate values (percentage) are indicated at the nodes. Scale bar indicates changes per nucleotide.

lar to earlier observations (1, 22, 23), and suggest migration of CTV isolates between distant citrus areas, probably by the frequent movement of CTV-infected plant material (21). Taken together, our data suggest five independent introductions of CTV in Italy, likely by illegal import of CTV-infected budwood. This supposition was supported by SSCP analysis that showed identical patterns in each area.

These results suggest that these five populations originated from independent introductions, as suggested by the following two arguments. First, extensive enzyme-linked-immunosorbent-assays (ELISA) (7) performed to detect CTV were negative in the area between Belpasso, Cassibile, Palermo, Massafra and Florence, suggesting that these are distinct disease foci. Second, the population in each area was homogeneous (only one haplotype was

detected) as supported by SSCP analysis. The results of the present work indicate that CTV is now established in Italy and is spreading by aphids. This will probably cause important damage to citrus cultivation in Southern Italy in the near future unless measures for eradicating CTV-infected plants and for avoiding new introductions are taken.

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