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Short communication

Molecular analysis suggests that recent *Citrus tristeza virus* outbreaks in Italy were originated by at least two independent introductions★

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Abstract

Citrus tristeza virus (CTV) is the causal agent of the most important virus disease of citrus. Numerous CTV isolates differing in biological and molecular characteristics have been reported worldwide. Recently, CTV was detected in Italy in several citrus crops from three separate areas: (1) Cassibile, province of Syracuse; (2) Massafra, province of Taranto; and (3) Belpasso, province of Catania. CTV isolates from Massafra and Cassibile were mild, whereas isolates from Belpasso induced severe symptoms. To study the genetic variation of CTV populations of these areas, 150 samples per area were examined by single strand conformation polymorphism (SSCP) and nucleotide sequence analysis of CTV gene p20. All isolates from the same area showed the same SSCP pattern whereas for each area a different SSCP pattern was obtained. The Massafra and the Cassibile isolates had a nucleotide identity higher than 99% with a mild isolate from Spain and about 92% with the Belpasso isolates, which were similar (identity higher than 99%) to severe isolates from California and Japan. These results suggest at least two independent introductions of CTV in Italy, probably by import of CTV-infected budwoods. Within each area, the virus population was homogeneous suggesting diffusion of CTV by aphid transmission.

Citrus tristeza virus (CTV), a member of the genus *Closterovirus*, is the most economically important virus affecting citrus production worldwide. The virus is phloem limited and transmitted by aphids in a semipersistent mode and by infected buds. CTV virions are flexuous filaments containing two capsid proteins of 25 and 27 kDa that cover about 95 and 5% of virion, respectively (Febres et al., 1996). CTV genome is a monopartite positive-sense RNA of ca. 20 kb containing 12 open reading frames (ORFs) encoding at least 19 proteins (Pappu et al., 1994; Karasev et al., 1995, Mawassi et al., 1996, Vives et al., 1999, Yang et al., 1999, Albiach-Martí et al., 2000). These include two papain-like proteases, replication-associated proteins (RNA polymerase, helicase, and methyltransferase), a homologue of the heat shock protein 70, two coat proteins (*p25* and *p27*), RNA binding protein *p23* (López et al., 2000) involved in regulation of RNA accumulation, protein p20 that accumulates in the amorphous inclusion bodies (Gowda et al., 2000), and other proteins of so far unknown function (p61, p13, and p18).

CTV isolates differing in the type and intensity of symptoms induced in different citrus species and cultivars, and in their aphid transmissibility have been reported worldwide (Roistacher and Moreno, 1991). Characterization of CTV isolates in each citrus area and estimation of their genetic

★The GenBank accession numbers of the sequences reported in this paper are: AY262000, AY263360 and AY263361 corresponding to gene ***p20*** of CTV isolates from Massafra, Cassibile and Belpasso (Italy), respectively.

variation are required to develop and apply effective disease control strategies and can provide epidemiological information. Italy has been a CTV-free area, but recently, CTV outbreaks (Davino et al., 2003b) appeared in three separate citrus areas (Figure 1): (a) Fortune mandarin (*Citrus reticulata* Blanco) crops in Cassibile, province of Syracuse (Davino et al., 2003a) (b) Navelina sweet orange (*Citrus sinensis* Osbeck) crops in Massafra, province of Taranto, and (c) Tarocco sweet orange crops in Belpasso, province of Catania, all grafted on sour orange (*Citrus aurantium* L.). CTV isolates from Cassibile and Massafra were mild, whereas isolates from Belpasso induced severe symptoms. These symptoms consisted in dwarfing and dieback of the Tarocco sweet orange trees, size reduction and interveinal chlorosis of leaves, size reduction and elongation of fruits, and root death. In this work, we studied the genetic structure and variation of the CTV populations from these three Italian areas, using single strand conformation polymorphism (SSCP) and nucleotide sequence analysis of gene p20.

One hundred fifty leaf samples were randomly collected in each area. Total RNAs were extracted with RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions, and used as template for reverse transcription and polymerase chain reaction (RT–PCR) with primers p20F and p20R (encompassing CTV p20 gene) as previously described (Rubio et al., 2001a). All samples yielded the expected 549 bp DNA fragment, whereas no amplification was obtained from non-CTV-infected plants of Tarocco and Navelina sweet orange and Fortune mandarin grown in greenhouse. To examine the genetic structure of the CTV populations, the RT–PCR amplified products were SSCP analyzed as previously described (Rubio et al., 2001a). All CTV isolates collected in the same citrus area showed the same SSCP pattern, whereas each area produced a distinct SSCP pattern (Figure 2). The difference between SSCP patterns of the Cassibile and the Massafra isolates was very small, but repeated SSCP analyses in adjacent gel wells showed that this difference was consistent. These data indicated that, within each area, the virus population is homogeneous while differences exist among the three areas. For each area, the nucleotide sequences of the amplified DNA from three CTV isolates were determined in both directions using an ABI PRISM DNA 377 sequencer (Perkin-Elmer). Sequences were aligned with the program CLUSTAL W (Thompson et al., 1994), and the program MEGA (Kumar et al., 2001) was used to estimate nucleotide distances between pairs of sequences (number of nucleotide differences per site) using the Kimura two-parameter correction (Kimura, 1980). All CTV isolates with the same SSCP pattern showed identical nucleotide sequence, confirming the great sensitivity of SSCP analysis for identification of nucleotide substitutions, previously observed in other studies of CTV populations (Kong et al., 2000; Rubio et al., 2001a, b). The nucleotide distance between the Cassibile and the Massafra isolates was 0.0038, whereas the distances of the Belpasso isolates between the Cassibile and the Massafra isolates were 0.0730 and 0.0773, respectively. To compare the Italian isolates with isolates from other countries, nucleotide sequences of gene p20 were retrieved from the GenBank entries: U16034 and AF260651 (Florida isolates T36 and T30); U56902 (Israel isolate VT); AF001623, AF203073, AF203075, AF356303, AF356305, and AF356315 (California isolates SY568, 65, 107, 5, 59 and 416); Y18420, AF356318, AF356323 and AF356327 (Spain isolates T385, T308, T346 and T398); AB046398 (Japan isolate Nuaga); and AY340979 (Egypt isolate Qaha). Phylogenetic relationships were

Figure 1. Citrus areas affected by CTV in Italy; (1) Cassibile, (2) Massafra, and (3) Belpasso.

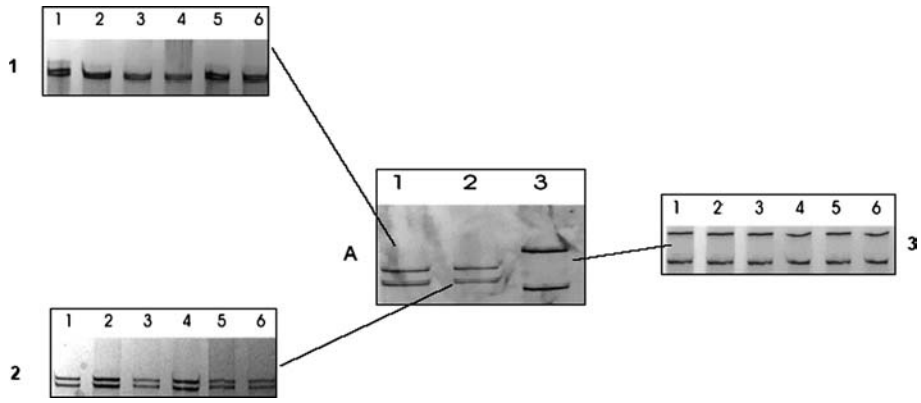

Figure 2. Single strand conformation polymorphism (SSCP) analysis of the RT–PCR products for *Citrus tristeza virus* (CTV) p20 gene. Panels 1, 2 and 3 correspond to different SSCP patterns obtained from CTV isolates collected in three Italian citrus areas: Cassibile(1), Massafra(2), and Belpasso(3), respectively. Panel (A) shows the comparison of the SSCP patterns of the three outbreaks.

inferred using the MEGA based neighbor-joining method with a 1000 replicate bootstrap value (Figure 3). According to this analysis, Belpasso isolates were closely related to California isolates SY568 and 107 (nucleotide identities greater than 99%), which induce similar symptoms (seedling yellows). Isolates Nuaga (Japan) and VT (Israel), belonging to the same cluster and showed nucleotide identities of 97 and 96% with the Belpasso isolates, respectively. The Cassibile and the Massafra isolates clustered very close in the phylogenetic tree (nucleotide identity around 99%) with Spain isolates T385 and T398, California isolates 5 and 59, and Florida isolate T30. Interestingly,

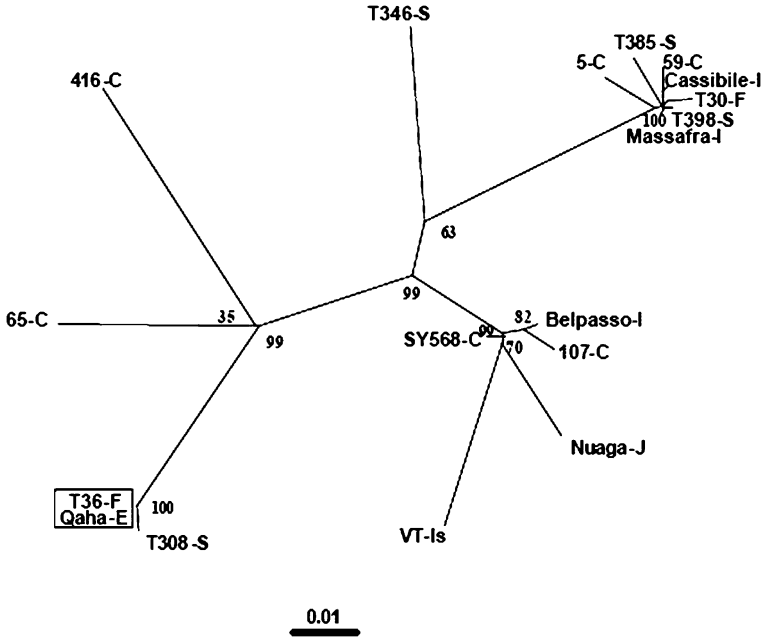

Figure 3. Unrooted phylogenetic tree of gene p20 of 18 CTV isolates, constructed with the program MEGA according the neighbor-joining method. Bootstrap values (percentage) are indicated in the nodes. CTV isolate names are followed by a hyphen and letters representing the origin country or USA state: C, California; E, Egypt; F, Florida; I, Italy; Is, Israel; J, Japan; and S, Spain.

most Spanish and California isolates also had a high nucleotide identity with T385 (Rubio et al., 2001a). The existence of isolates genetically related but geographically distant observed in this work, and in others (Albiach-Martí et al., 2000; Rubio et al., 2001a; Sambade et al., 2003), suggests migration of CTV isolates among distant citrus areas probably caused by the frequent movement of CTV-infected plant material between countries occurred since the beginning of 20th century (Roistacher and Moreno, 1991). Taken together, our data suggest at least two independent introductions of CTV in Italy, likely by illegal import of CTV-infected budwoods. In fact, the CTV population from Belpasso was clearly distinct from the other two populations, although we cannot rule out completely that the population from both, Cassibile and Massafra, have the same origin as they are genetically closely related.

We suppose that these two populations originated by independent introductions according to three arguments. First, extensive ELISA tests performed to detect CTV were negative in the area between Cassibile and Massafra (separated by more than 650 km) suggest that these are distinct disease foci. Second, the population in each area was homogeneous (only one haplotype was detected) while the two populations, although similar, were distinguished using both SSCP and nucleotide sequence analysis. Third, the Cassibile and Massafra CTV, were detected in two different citrus species. In spite of the small nucleotide distance between the Cassibile and Massafra isolates, we considered that, within each area, CTV was rapidly dispersed by aphids originating a homogeneous virus population.

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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