Biological, Serological and Molecular Characterization of Two *Citrus tristeza virus* Isolates from Corsica

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ABSTRACT. *Citrus tristeza virus* (CTV) is the most important viral pathogen for citriculture. CTV can infect almost all species, hybrids and varieties of the family *Rutaceae*. The symptoms are "quick decline" (QD), "vein clearing" (VC), "stem pitting" (SP) and/or "seedling yellows" (SY). A wide range of strains has been characterized. Symptom intensity depends on host, strain, rootstock and climate. Corsica, a Mediterranean island where CTV is not endemic, has one of the most important citrus collections free of graft-transmissible agents, and there is special interest and support for phytosanitary controls. This work compares two isolates of CTV: A new isolate (Cal-1) originating from illegally imported ornamental calamondins and the previously described K strain from kumquat. The two isolates were indexed by inoculation to citrus indicators plants. Serological profiles, dsRNA, molecular weight of coat protein (CP), and coat protein gene (CPG) sequence were determined. Isolate Cal-1 presented SP and VC symptoms, while the K strain remained asymptomatic in Mexican lime. The two isolates presented a 0.5 kDa ds RNA band, which has been related to severity, and the K strain also presented an additional 1.9 kDa band suggesting defective RNAs. Both isolates showed similar serological profiles when exposed to 11 McAbs. The CP was estimated at 26 kDa. The deduced amino acid sequences showed the MCA 13 antibody recognition epitope for both isolates. Isolate Cal-1 was determined as being a strain having moderate to severe characteristics. The K strain was reconfirmed as being an asymptomatic strain on Mexican lime, but could not be distinguished from severe strains by serological and/or molecular marker characteristics. These results demonstrate that ornamental citrus are high risk CTV-strain reservoirs due to their possible dispersion to surrounding commercial citrus orchards.

Index words. CTV, calamondins, kumquats, dsRNA, sequence, ELISA.

Citrus tristeza virus (CTV) is the most important viral pathogen for worldwide citriculture with losses reaching more than 50 million commercial citrus trees over the last 60 yr (4). CTV can infect all species, varieties and hybrids from the *Rutaceae* family, including genera *Citrus* and *Fortunella* (27). A wide range of strains has been characterized, from symptomless, mild to very severe strains, such as "stem pitting" (SP) strains detected in sweet orange or in grapefruit (7, 11, 20, 33). The symptoms produced are quick decline QD), vein clearing (VC), stem pitting (SP) seedling yellow (SY) and their manifestation and intensity depend on the host, the strain, rootstock and/or climate (26). Little work has been done on CTV in exotic citrus plants. The K strain from Marumi kumquat (clones K123 T22 and K123 T24) was characterized on the Mediterranean island of Corsica as being a symptomless strain in Mexican lime. The double-stranded RNA (dsRNA) profiles determined from this strain, including the 0.5 kDa band (1, 6) are similar to dsRNA patterns from severe strains capable of inducing severe symptoms (10, 15). However, the 0.5 kDa band has been detected in other mild strains (2, 13). Severe CTV strains from kumquat have been detected in Spain (21) and also a severe strain from calamondin capable of inducing SP and QD (3) which was used for obtaining 3CA5 monoclonal antibodies (31). Calamondins are susceptible to CTV (25). The present work was carried out on the island of Corsica, at the Station de Recherches Agronomiques (SRA INRA-CIRAD) in order to characterize a CTV isolate from an illegally introduced calamondin and to compare it with the previously described K strain (1, 6), using biological, serological and molecular markers.

MATERIALS AND METHODS

Viral isolates. The K strain from kumquat (K123 clone) was kept in Mexican lime since 1986 in the SRA greenhouse on Corsica. The calamondin isolate (Cal-1) from a Corsican nursery (introduced illegally) was moved and kept at SRA since 1994.

Indexing in indicator plants. The K strain and the Cal-1 isolate were inoculated into five indicator plants (12): Mexican lime, grapefruit, sour orange, sweet orange Hamlin grafted on sour orange and *Citrus excelsa*. The expression of symptoms was followed up each 15 days for a period of 18 mo.

Serological profiles. The ELISA DASI technique (8, 9) was used on Mexican lime extracts exposed to 11 monoclonal antibodies (MCAs): MCA13 (USA), 3DF1, 3CA5 (Spain), 3C1F10 (Cuba), 4F3, 1D12, 4E5, 4B1 (Morocco) and 4G12, 4H6, 10E3 (Taiwan). The plate was covered with 1 µg/ml INGENASA antibody (3DF1 and 3CA5) for 3 h at 37ºC. The antigen was diluted to 1:10 in extraction buffer (PBS Tween, 0.02 g/l PVP, MW: 40,000, pH 7.4) and kept at 4°C overnight. MCAs were used at 0.1 µg/ml in PBS buffer with 0.5% BSA for 3 h at 37°C. Goat anti-mouse-phosphatase alkaline (Sigma) was used as conjugate in 1:5,000 dilution.

dsRNA profiles. Seven grams of bark from Mexican lime inoculated with Cal-1 isolate and the K strain were taken and macerated in presence of liquid nitrogen. The dsRNA were extracted in a buffer containing 14 mg bentonite, 14 ml STE (2X), 2 ml 10% SDS and 20 ml phenol, pH 6.5. They were passed through gauze and centrifuged at 10,000 rpm for 20 min. The RNA were fixed in two consecutive CF11 cellulose columns (Whatman) calibrated in STE buffer (1X) with 16% ethanol and precipitated with 3M ammonium acetate (pH 5.5) and 2.5 volumes of 96% ethanol at -20°C. The dsRNA were eluted in STE (1X) and the profiles were detected by migration in 6% polyacrylamide gel in non denatured conditions (19, 30). The gels were stained with silver nitrate (5) and photographed on Kodak film.

Western Blots. Samples of Mexican lime bark inoculated with K strain or Cal-1 isolates, diluted in water (p/v) (1:1), were macerated in Laemmli extraction buffer with SDS and 10% mercaptoethanol. The polyacrylamide gel, in denatured conditions (SDS-PAGE) was run for 1.5 h at a constant 60 v in a discontinuous buffer system with a 15% separation gradient and 5% concentration, in a BIORAD Minigel vertical electrophoresis chamber. Total proteins were transferred (BIORAD chamber) to a 0.45 µm nitrocellulose membrane for 1 h and detection of the largest coat protein was done by exposure to INGENASA monoclonal antibody in 1:1,500 dilution.

GCP amplification and sequence. The coat protein gene (CPG) was amplified by IC-RT-PCR (22). K strain PCR product was ligated to PUC 118 plasmid, cloned in *E. coli* DH5α and sequenced with the Sequenase kit, version 2.0 (US Biochemical). The Cal-1 isolate PCR product was directly sequenced in a Perkin Elmer sequencer. The deduced amino acid sequences were analysed in the Plant Pathology Department, University of Florida, and UWGCG (University of Wisconsin) using the PILEUP programme and the CTV GCP sequence data banks.

RESULTS AND DISCUSSION

The Cal-1 isolate expressed VC and SP in Mexican lime, *C. excelsa* and expressed SP in grapefruit and sour orange (Fig. 1). The QD symptoms were not conclusive in the duplicates. The K strain induced no symptoms in Mexican lime and symptoms were not observed in other indicator plants. From the bio-

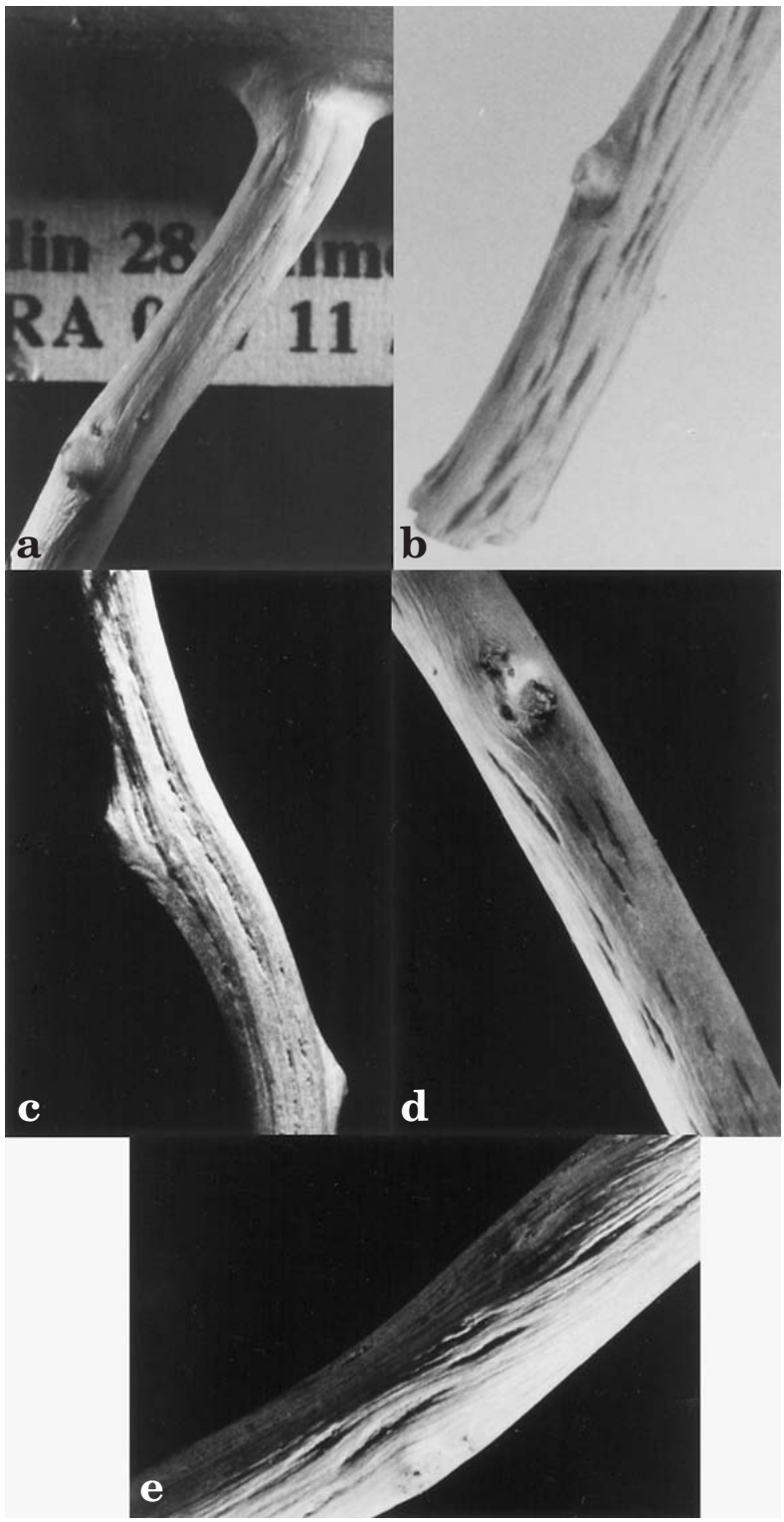

Fig. 1. Stem pitting symptoms following inoculation with Cal-1 isolate of *Citrus tristeza virus* in (a) Mexican lime after 5 mo; (b) *Citrus excelsa* after 8 mo; (c) sour orange after 12 mo; (d) grapefruit after 12 mo; and (d) calamondin after 12 mo.

logical point of view, these results lead to determination of the Cal-1 isolate as being a strain having moderate to severe SP characteristics. The K strain was reconfirmed as being a symptomless strain. The serologic patterns of the Cal-1 isolate and the K strain inoculated into Mexican lime exposed to the 11 MCAs, showed that both, K strain and the Cal-1 isolate, were similar in affinity and intensity to each one of the MCAs used with OD ranges between 0.5 to 1.6 (Fig. 2). However, K strain slightly surpassed Cal-1 isolate with MCA-13, 3DF1, 3CA5, 3CF10 1D12, 4F3, 4E5, and 4G12 MCAs, while presenting ODs similar to or slightly lesser than those for the Cal-1 isolate with the other MCA. In general, K strain and Cal-1 isolate serological patterns were similar to those reported for different severe strains (24, 28, 34). The MCA-13 antibody discriminating severe strains from mild ones from Florida with 95% reliability, was positive with the K strain (OD 0.5). Low positivity was also found (OD less than 0.6) with the Taiwanese 4G12, 4H6 and 10E3 antibodies reported to detect severe strains epitopes (29).

The K strain and the Cal-1 isolate presented the same dsRNA patterns from Mexican lime with the presence of bands 13 kDa, 2 kDa, 1.7 kDa, 1.5 kDa, 0.8 kDa and 0,5 kDa (Fig. 3). The 13 kDa band reflected the replicative form (RF). The presence of the 1.7 and 0.5 kDa bands has been associated with the SY and SP strains (10). An additional 1.9 kDa band was detected in the K strain, suggesting the presence of defective RNA, as reported for other CTV strains (17, 18, 32). The defective RNA may compete with the complete genome and have repercussions on the increase or decrease of symptom expression (14).

The coat protein weight was 26 kDa for K strain and Cal-1 isolate which is inside the range reported for other CTV strains (16). The K strain was 96% similar to T-30 and T-36 strains (mild and severe from Florida, respectively) for CP nucleotide sequences. According to the deduced aa sequence, the Cal-1 isolate as well as the K strain presented the MCA-13 antibody recognition

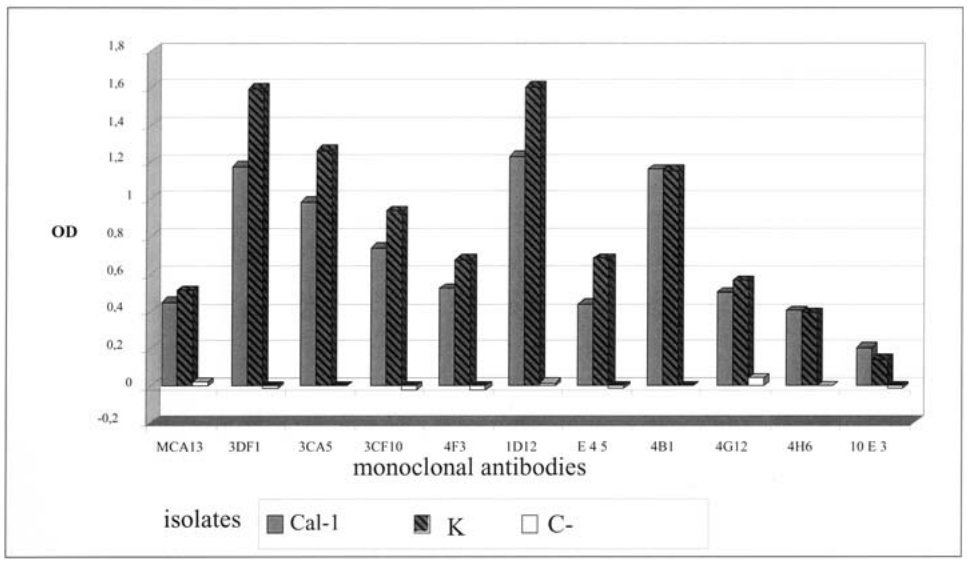

Fig. 2. ELISA patterns of *Citrus tristeza virus* Cal-1 isolate and K strain from Mexican lime exposed to 11 Mabs: MCA 13 (USA), 3DF1, 3CA5 (Spain), 3CF10 (Cuba), 1D12, 4E5, 4B1 (Morocco) and 4G12, 4H6, 10E3 (Taiwan).

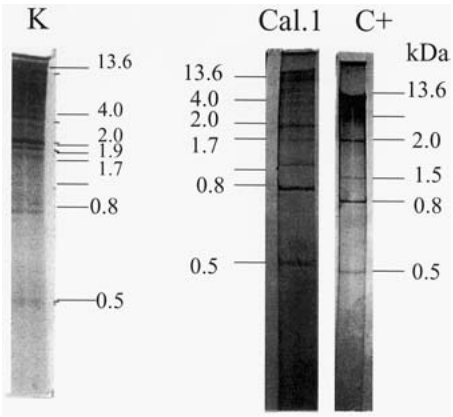

Fig. 3. Double-stranded RNA patterns from *Citrus tristeza virus* isolates K and Cal-1 inoculated into Mexican lime following 6% PAGE and silver nitrate staining. Positive control T388 from Spain. (lanes are from different gels).

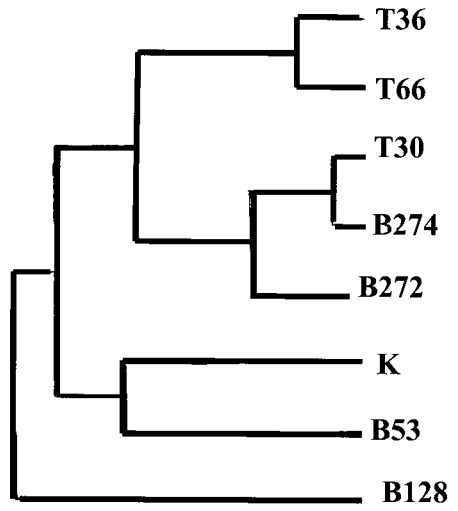

Fig. 4. Dendogram of amino acids deduced from the *Citrus tristeza virus* K strain major coat protein, and B272, B274 exotic isolates (mild, Colombia), B128 (severe, Colombia), B53 (severe, Japan), T30 (mild, Florida) and T36 (severe, Florida).

epitope (23). These results ratify those positive serological findings obtained with MCA 13 for the K strain and the Cal-1 isolate.

The alignment of deduced aa from K strain, a mild (B272 and B274) and severe (B128) Colombian isolates, a severe isolate from Japan (B53) and the T-36 and T-30 strains from Florida were compared in a dendrogram (Fig. 4). The K strain is grouped with the Japanese severe isolate and is far from the Colombian mild and severe isolates and those from the United States.

CONCLUSIONS

The calamondin isolate (Cal-1), which recently appeared in three neighboring nurseries, was characterized as being a moderate to severe stem pitting strain, which could affect a wide spectrum of hosts. Congruence was found between all biological, serological and molecular markers used with this strain. Serological and dsRNA profiles did not allow the Cal-1 isolate, with profiles similar to those reported for QD, SY and SP strains, to be distinguished from the K strain, even though their respective biological patterns were clearly different. No concordance was found between the asymptomatology of the K strain and the serological and molecular markers previously described to be associated with the severity of strains. The foregoing confirms the necessity of indexing to discriminate among mild and severe CTV strains having different epidemiological consequences. The serological and molecular tools used in this study did not allow us to separate our two different strains. Additional tools will have to be evaluated or investigated for this purpose. Finally, it is of importance to look the ornamental or exotic citrus as possible reservoirs of potentially aggressive CTV strains for neighboring commercial citrus crops. Exotic citrus must thus be included in phytosanitary programs in all citriculture regions and countries.

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