Discrimination Between Mild and Severe *Citrus tristeza virus* Isolates with a Rapid and Highly Specific Real-Time Reverse Transcription-Polymerase Chain Reaction Method Using TaqMan LNA Probes

S. Ruiz-Ruiz, P. Moreno, J. Guerri, and S. Ambrós

First, second, and third authors: Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, Moncada, 46113-Valencia, Spain; and fourth author: Fundación Agroalimed and Centro de Protección Vegetal y Biotecnología, IVIA, Moncada, 46113-Valencia, Spain.

Current address of fourth author: Fundación Agroalimed, IVIA Cra. Moncada-Náquera Km. 4.5, Moncada, 46113-Valencia, Spain.

Accepted for publication 24 November 2008.

ABSTRACT

Ruiz-Ruiz, S., Moreno, P., Guerri, J., and Ambrós, S. 2009. Discrimination between mild and severe *Citrus tristeza virus* isolates with a rapid and highly specific real-time reverse transcription-polymerase chain reaction method using TaqMan LNA probes. Phytopathology 99:307-315.

Severe isolates of *Citrus tristeza virus* (CTV) inducing seedling yellows (SY) and/or stem pitting (SP) in grapefruit or sweet orange are a major threat for the citrus industry worldwide. Identification of these CTV variants was achieved by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) using a general primer set and three TaqMan locked nucleic acids (LNA) probes targeting sequences characteristic of severe, mild (non-SY, non-SP), and T36-like isolates. Successful amplification was achieved from fresh or silica-desiccated CTV-infected samples and all isolates but one reacted with one or more probes. Standard curves using RNA transcripts homologous to the three probes allowed a reproducible quantitative assay, with a wide dynamic range of detection starting with 10^2 copies. RT-PCR assays with homologous and heterologous transcript RNA mixes demonstrated that each probe reacted only with its cognate sequence which was detected even at ratios below 2.5%. Analysis of 56 pathogenically distinct CTV isolates from 20 countries showed that mild isolates reacted only with the mild probe, whereas severe SP and SY isolates reacted with the severe-SP or the T36-like probes, respectively, and often with a second probe. This procedure can be useful to identify and control potentially dangerous CTV isolates in areas affected only by mild isolates.

Additional keywords: biological indexing, external standard curves, melting curve analysis, population of sequence variants, SYBR Green I detection.

Citrus tristeza virus (CTV) (genus *Closterovirus*, family *Closteroviridae*), is the causal agent of economically important citrus diseases that in the last 75 years have had devastating effects on citrus industries in several citrus growing countries worldwide. CTV is usually dispersed to new areas by propagation of infected buds and then it is locally spread in a semipersistent mode by several aphid species, with *Toxoptera citricida* being the most efficient vector (28). Although CTV isolates essentially asymptomatic have been reported (9,28,29,30), most isolates may incite (i) the tristeza syndrome (decline and death of scion varieties grafted on sour orange (*C. aurantium* L.) rootstock), (ii) the stem pitting syndrome (SP) (stunting, stem pitting, chronic low yield, and poor fruit quality of some citrus varieties regardless the rootstock used), and/or (iii) the seedling yellows syndrome (SY), rarely observed in the field, that is characterized by stunting and leaf yellowing of sour orange, lemon (*C. limon* [L.] Burn. f.) or grapefruit (*C. paradisi* Macf.) seedlings inoculated in the greenhouse. Damage caused by CTV is highly variable and it largely depends on the scion and rootstock varieties grown at each location and on the predominant virus strains. While common isolates in the Mediterranean basin are relatively mild (they cause tristeza decline, but sweet orange [*C. sinensis* (L.) Osb.] or grapefruit varieties propagated on decline-tolerant rootstocks are not affected), severe isolates inducing SY and/or SP in grapefruit or sweet orange varieties cause additional losses in other citrus areas (15,28,49).

Corresponding author: S. Ambrós; E-mail address: sambros@ivia.es

doi:10.1094/PHYTO-99-3-0307

CTV virions are flexuous filaments of 2,000 × 11 nm in size composed of two coat proteins and a single-stranded, positive-sense genomic RNA (gRNA) of ≈20 kb with 12 open reading frames (ORFs) and untranslated regions (UTRs) at the 5′ and 3′ termini (22,34). ORFs 1a and 1b, encoding proteins of the replicase complex, span the 5′ half of the genome and are directly translated from the gRNA, whereas the 10 3′-proximal ORFs, encoding proteins p33, p6, p65, p61, p27, p25, p18, p13, p20, and p23, are expressed via 3′ co-terminal subgenomic RNAs (sgRNAs) (20). Additional viral RNAs generated during CTV replication in infected cells include: negative strands of the 3′ co-terminal sgRNAs, two abundant positive-stranded 5′ co-terminal RNAs of about 800 nt (LMTs 1 and 2), a set of larger positive-stranded 5′ co-terminal sgRNAs which accumulate at lower concentrations than the other sgRNA species, and different defective RNA molecules (dRNAs) that contain both gRNA ends but lack internal regions of variable size, thus totalizing more than 30 different RNA species (14,19,26,28).

As with other RNA viruses, CTV isolates are composed of a population of sequence variants resulting from the error-prone nature of RNA polymerases, repeated inoculations of field trees by aphids carrying different CTV variants, frequent recombination events between diverged sequences and different selection pressures (5,28,39,51). While some CTV populations have a predominant sequence and a pool of closely related variants, others contain highly divergent variants and their ratio may affect biological properties of the isolates (5,42,43,51). The complete gRNA sequence of CTV isolates T36 and T30 from Florida (2,22,34; GenBank accession nos. U16034 and AF260651), VT from Israel (27; U56902), SY568 from California (52; AF001623, and an in silico restored version thereof, SY568R [51]), T385 and

T318A from Spain (40,50; Y18420 and DQ151548), NUagA from Japan (47; AB046398), Qaha from Egypt (AY340974) and a Mexican isolate (DQ272579) have been reported and their genetic relationships examined in a phylogenetic analysis (28,51). These sequences clustered in three main groups including those corresponding to (i) severe SY and SP isolates (NUagA, SY568R, T318A and VT), (ii) mild isolates (T30 and T385), and (iii) isolates T36, Qaha and Mexican with intermediate characteristics (at least T36 induces SY but not SP).

To set up adequate control measures quick procedures to identify CTV isolates of different pathogenicity characteristics are needed. For years this was done by biological indexing on several indicator species that allowed defining different biogroups (6, 17,38). After CTV purification and sequencing of its gRNA quick and specific detection procedures were developed based on serology, molecular hybridization, or reverse transcription and PCR amplification (RT-PCR) (13,31,33), with highest sensitivity being achieved using real-time RT-PCR protocols (8,41,44). Procedures to discriminate between CTV isolates were also developed based on markers like (i) reaction with monoclonal antibodies (35), (ii) hybridization with cDNA probes (31), (iii) restriction fragment length polymorphism (RFLP) pattern of the coat protein gene (18), (iv) single-strand conformation polymorphism (SSCPs) profiles of several gRNA regions (5,39,43), or (v) RT-PCR amplification with different genotype-specific primer sets (3,21,42). However, since the genetic determinants of CTV symptoms are presently unknown, the level of discrimination afforded by these procedures was usually limited by at least two factors: first, the degree of association between the molecular marker used and the actual pathogenicity determinants, and second, the sensitivity of the procedure to detect minor CTV variants that might affect symptom expression.

Recently we developed a real-time RT-PCR protocol for sensitive detection and absolute quantification of CTV virions in citrus tissues (41) using a general primer set targeting ORFs 1b and 2. By melting curve analysis of the PCR products synthesized, we found that Tm values allowed discrimination between the mild and the severe isolates tested, suggesting that this parameter might be useful for preliminary characterization of isolates. Here our objective was setting up a sensitive and specific method to discriminate between mild and severe SP and SY isolates by (i) further examining Tm values obtained with an international panel of biologically characterized CTV isolates (17), and (ii) developing a genotype-specific real-time RT-PCR protocol with TaqMan probes that yield fluorescence upon digestion of the annealed probe by the 5′ nuclease activity of the *Taq* DNA polymerase (12,25). The latter approach required selecting a general primer set to amplify all sequence variants in the viral population and designing efficient genotype-specific probes within the region encompassed by the general primer set. We used the general primer set selected in our previous protocol (41) and three genotype specific probes based on sequences conserved in each of the three CTV clusters detected previously (28,40). The discrimination capacity of the probes was improved by the use of locked nucleic acids (LNA) modified nucleotides whose special conformation improves their binding affinity, stability of the duplex, and specificity by increasing Tm 1 to 8°C per LNA nucleotide incorporated (10,23,24,36). These TaqMan LNA probes enabled specific and reliable detection of each of the three main CTV genotypes reported, even in RNA mixes where the homologous sequence was at low ratio. This assay, which was validated with a group of 56 CTV isolates of different origin and pathogenicity characteristics, may be useful for quick identification of potentially dangerous isolates containing sequence variants characteristic of severe SP and SY isolates.

MATERIALS AND METHODS

Virus isolates. A total of 56 biologically characterized CTV isolates, divided in two groups, were used in this study (Tables 1 and 2). The first group of 23 isolates was sampled from plants grown at the Instituto Valenciano de Investigaciones Agrarias

TABLE 1. Detection of sequence variants of *Citrus tristeza virus* in plants infected with pathogenically distinct isolates by real-time reverse transcription-polymerase chain reaction (RT-PCR) using three genotype-specific locked nucleic acid (LNA) probes

		Symptoms[x]								
Isolate	Origin	Lime	Decline SwO/SO	SY_{so}	SP_G	SP_{SwO}	Mild probe[y] (Ct ± SD)	Severe-SP probe[y] (Ct ± SD)	T36-like probe[y] (Ct ± SD)	Tm[z]
T11	Spain	–	–	–	–	–	19.1 ± 0.0	–	–	83.5
T32	Spain	+/-	-	–	–	–	18.1 ± 0.0	–	–	83.5
T300	Spain	+	+	–	–	–	16.6 ± 0.0	–	–	83.6
T305	Spain	+	+	+	+	+	18.3 ± 0.1	18.0 ± 0.1	–	84.8
T306	Spain	+	nd	–	nd	–	16.7 ± 0.0	–	–	83.6
T308	Spain	+	+/–	+/–	–	–	22.6 ± 0.0	–	22.4 ± 0.2	84.9
T309	Spain	+	+	–	–	–	18.3 ± 0.0	–	–	83.6
T311	Spain	+	+	–	–	–	16.6 ± 0.0	–	–	83.6
T312	Spain	+	+	–	–	–	18.0 ± 0.1	–	–	83.6
T318	Spain	+	+	+	+	+	19.5 ± 0.0	19.1 ± 0.0	–	84.9
T318A	Spain	+	+	+	+	+	–	18.0 ± 0.0	–	85.0
T340	Spain	+	+	–	–	–	17.7 ± 0.0	–	–	83.5
T346	Spain	+	+	–	–	–	23.9 ± 0.0	–	–	84.2
T315	Spain	+	+	–	–	–	18.5 ± 0.0	–	–	83.5
T362	Spain	+	+	–	–	–	17.1 ± 0.0	–	–	83.5
T373	Spain	+	+	–	–	–	23.8 ± 0.1	–	–	84.3
T385	Spain	+/–	–	–	–	–	17.1 ± 0.1	–	–	83.4
T388	Japan	+	+	+	+	+	–	21.2 ± 0.1	–	85.0
T390	Spain	+	+	–	–	–	16.1 ± 0.0	–	–	83.7
T405	Spain	+	+	–	–	–	22.1 ± 0.0	–	–	83.3
5.14	Spain	+	+	–	–	–	21.5 ± 0.1	–	–	83.4
6.10	Spain	+	+	+	+/–	–	21.6 ± 0.0	21.6 ± 0.2	–	83.6
T36	Florida	+	+	+	–	–	–	–	22.9 ± 0.0	85.0

[x] Symptoms included vein clearing, leaf cupping, stem pitting, and stunting in Mexican lime, decline of sweet orange (SwO) propagated on sour orange (SO) rootstock, seedling yellows in sour orange (SY_{SO}), and stem pitting in Duncan grapefruit (SP_G) or sweet orange (SP_{SwO}): symptoms consistently observed (+), not observed (–), sporadically observed or very faint (+/–), or test not done (nd) (3,6,7,29,30,39, *unpublished data*).

[y] Average threshold cycle (Ct) and standard deviation (SD) values obtained for each LNA probe from two different assays using two replicates.

[z] Melting temperature (°C) of the RT-PCR product obtained with primers PM197F-PM198R (see Methods) as detected with SYBR Green I.

(IVIA) and included (i) 19 isolates from different citrus growing areas in Spain (isolates T32, T11, T300, T305, T306, T308, T309, T311, T312, T315, T318, T318A, T340, T346, T362, T373, T385, T390, and T405) and one from Japan (isolate T388) that are part of a collection maintained in sweet orange propagated on Carrizo citrange (*C. sinensis* × *P. trifoliata* [L.] Raf.) rootstock in an insect-proof screen house (6,7,29,30, and *unpublished data*), (ii) isolate T36 from Florida (provided by W. O. Dawson, University of Florida-CREC, Lake Alfred, FL) (45) that is maintained in an insect-proof, temperature-controlled (18/26°C night/day) greenhouse, and (iii) isolates 5.14, and 6.10 from a field cross protection experiment (*unpublished data*). The second group of 33 isolates, analyzed on desiccated leaf tissue, was sampled at the international collection of exotic citrus pathogens maintained at the quarantine facilities of the USDA in the Beltsville Agricultural Research Center (BARC, Maryland) and comprises CTV isolates with different pathogenicity characteristics from 19 different countries (17).

Biological indexing on Mexican lime (*C. aurantifolia* [Christm.] Swing.), Duncan grapefruit, sour orange, and sweet orange seedlings was performed according to Roistacher (38), and decline of the sweet/sour orange combination was observed in the source trees of these isolates or in plants of Valencia sweet orange propagated on sour orange or of sour orange propagated on Pineapple sweet orange inoculated in the greenhouse (6,17; *unpublished data*). The characteristics presented in Tables 1 and 2 are data from two different research centers and might be subjected to some variability due to differences in cultivation and/or environmental conditions.

Primers and TaqMan LNA probes. The general primer set (PM197F-PM198R) targeting CTV ORFs 1b and 2 was designed previously (41). The three TaqMan LNA probes (Roche Diagnostics) were directed to a variable domain within the region encompassed by the general primer set that discriminates the three main CTV genotypes (mild, severe SP, and T36-like) found in previous phylogenetic analyses (28,40). The probes, 18 to 19 nucleotides in length comprising 3 to 4 LNA residues with a 3 to 6 nt separation, were 5′-labeled with the reporter dye FAM (6-carboxyfluorescein) and 3′-labeled with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine). Their predicted Tm values (69 to 71°C) were calculated with the prediction tool for LNA oligonucleotides provided by Exiqon.

The probe specific for mild isolates (AATGGACGACTTGATGACT, with LNA positions underlined), based on the sequence of isolates T385 and T30, has 7 to 8 mismatches with the sequence of severe SP isolates T318A, SY568, VT, and NUagA, three of which are LNA nucleotides, and 10 mismatches with that of isolates T36, Qaha, and Mexican, including the four LNAs. The probe specific for severe SP isolates (AGCGGATGACTTAGCGAC, with LNA positions underlined), based on the sequence of isolates T318A and SY568, has one mismatch with the sequence of the severe isolates VT and NUagA, six mismatches with the sequence of mild isolates T385 and T30, one of which is an LNA nucleotide, and three mismatches with that of isolates T36, Qaha

TABLE 2. Detection of sequence variants of *Citrus tristeza virus* in silica-desiccated tissue infected with isolates of different origin and pathogenicity characteristics by real-time reverse transcription-polymerase chain reaction (RT-PCR) using three genotype-specific locked nucleic acid (LNA) probes[y]

		Symptoms[x]								
Isolate	Origin	Lime	Decline SwO/SO	SY_{so}	SP_G	SP_{SwO}	Mild probe[y] (Ct ± SD)	Severe-SP probe[y] (Ct ± SD)	T36-like probe[y] (Ct ± SD)	Tm[z]
B5	California	+	–	–	–	–	22.4 ± 0.0	–	–	83.4
B12	Brazil	+	+	+	+	+	24.4 ± 0.0	24.0 ± 0.2	–	84.4
B12-1	Brazil	+	+	+	–	–	25.5 ± 0.0	24.0 ± 0.1	–	84.8
B14	Brazil	+	+	+	–	+	22.7 ± 0.0	23.2 ± 0.1	–	84.6
B29	Japan	+/–	–	–	–	–	21.8 ± 0.0	–	–	82.4
B55	Hawaii	+	+	+	+/–	+	24.5 ± 0.1	24.6 ± 0.1	–	84.7
B65	China	+	+	+	+/–	+	22.5 ± 0.3	22.7 ± 0.0	–	84.9
B83	China	+	+/–	–	+/–	–	–	–	26.2 ± 0.1	85.1
B105	California	+	+/–	–	–	–	23.4 ± 0.1	–	–	83.9
B128	Colombia	+	+	+/–	+	–	19.1 ± 0.1	19.7 ± 0.0	–	84.4
B135	Peru	+	+	+	–	+	–	22.8 ± 0.1	–	84.5
B151	California	+	+	–	–	–	–	–	–	85.0
B156	Australia	+	+	+	+/–	+	24.7 ± 0.1	24.2 ± 0.1	–	83.8
B181	C. Rica	+	+	+/–	–	–	28.8 ± 0.1	–	27.9 ±0.0	84.3
B185	Japan	+	+	+	–	+	21.9 ± 0.1	21.1 ± 0.2	–	84.7
B227	India	+	+	+	–	+	–	19.8 ± 0.2	–	84.5
B228	Colombia	+	+	+	–	+/–	–	24.2 ± 0.1	–	84.2
B252	Taiwan	+/–	+/–	–	–	–	24.5 ± 0.2	–	–	83.6
B253	Taiwan	+	+	+	–	+	–	22.8 ± 0.2	–	84.7
B255	Indonesia	+	+	+	–	+	21.5 ± 0.2	21.4 ± 0.3	–	85.1
B272	Colombia	–	–	–	–	–	22.1 ± 0.1	–	–	83.0
B296	D.R.	+/–	+/–	–	–	–	24.2 ± 0.2	–	–	83.8
B302	Jamaica	+	–	–	–	–	27.2 ± 0.2	–	–	84.3
B339	Trinidad	+	+	–	–	–	25.9 ± 0.1	–	–	83.8
B352	California	+	+	+	+/–	–	–	25.9 ± 0.2	–	85.1
B358	Corsica	+	–	+/–	+/–	–	28.8 ± 0.2	–	26.8 ± 0.2	84.5
B400	Venezuela	+	nd	–	–	nd	21.1 ± 0.1	–	–	83.8
T3	Florida	+	+	+	+	+	–	26.4 ± 0.2	–	84.5
T4	Brazil	+	–	–	–	–	24.8 ± 0.1	–	–	83.5
T66-1	Florida	+	+	+	–	–	27.7 ± 0.0	–	27.7 ± 0.0	84.1
B28-T68	Florida	+	+	+	+	+	–	21.6 ± 0.1	–	84.7
VT	Israel	+	+	+	+	+/–	–	27.3 ± 0.1	30.7 ± 0.3	84.9
B192-K	Corsica	–	–	–	–	–	23.1 ± 0.0	–	–	81.2

[x] Symptoms included vein clearing, leaf cupping, stem pitting and stunting in Mexican lime, decline of sweet orange (SwO) propagated on sour orange (SO) rootstock, seedling yellows in sour orange (SY_{SO}), and stem pitting in Duncan grapefruit (SP_G) or sweet orange (SP_{SwO}): symptoms consistently observed (+), not observed (–), sporadically observed or very faint (+/–), or test not done (nd) (9,17,21; S. M. Garnsey, *personal communication*).

[y] Average threshold cycle (Ct) and standard deviation (SD) values obtained for each LNA probe from two different assays using two replicates.

[z] Melting temperature (°C) of the RT-PCR product obtained with primers PM197F-PM198R (see Methods) as detected with SYBR Green I.

and Mexican. The probe specific for T36-like isolates (CGAT-AACGTGGACGACCT, with LNA positions underlined), based on the sequence of isolates T36, Qaha and Mexican, overlaps in 13 nt an insertion sequence present only in T36-like genotypes. The overlapping segment includes the three LNAs, whereas the remaining nucleotides that are present in other isolates contain two mismatches with the sequence of the mild and the severe SP isolates.

RNA extraction. For isolates kept at the IVIA, total RNA (RNAt) was prepared from 3 g of fresh young bark using a standard protocol with two phenol/chloroform/isoamyl alcohol extractions followed by precipitation with 12 M lithium chloride (3). For isolates from the BARC collection, 100 mg of desiccated tissue were re-hydrated with extraction buffer and then RNAt was prepared using the standard protocol (3) followed by a clean up step with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA extracts were re-suspended in 25 µl of RNase-free water and treated with RNase-free DNase (Turbo DNA-free, Ambion, Austin, TX). RNA concentrations were adjusted to approximately 10 ng/µl and then measured in duplicate with a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE). Aliquots were stored at –80°C until use.

Standard curves. To determine sensitivity of the real-time RT-PCR protocol with the different LNA TaqMan probes, serial dilutions of in vitro-synthesized, positive sense RNA transcripts of the selected gRNA region, were amplified using the real-time RT-PCR TaqMan-LNA assay to generate three external standard curves. The cDNA used as template for the in vitro transcription was obtained by conventional RT-PCR amplification using RNAt extracts from citrus bark infected with CTV isolates T385 (for the mild probe), T318A (for the severe-SP probe) and T36 (for the T36-like probe) and the primer set PM261F-PM198R (PM261F is a modified version of the forward primer PM197F that includes the T7-promoter sequence at its 5′ end) (41). PCR amplification products were transcribed in vitro with the T7 RNA polymerase (New England BioLabs, Ipswich, MA) following the manufacturer instructions. Transcripts were purified with the RNaid w/Spin Kit (Q-BIO gene, Irvine, CA), treated twice with RNase-free DNase (Turbo DNA-free from Ambion) and their concentration determined in duplicate with a NanoDrop UV-Vis Spectrophotometer. Tenfold serial dilutions of each transcript species in healthy citrus RNAt extracts (10 ng/µl) containing 10^{10} to 10^{1} copies were used in real-time RT-PCR assays with the cognate TaqMan LNA probe, with and without reverse transcriptase, to ensure the absence of DNA template in transcript preparations. The transcript RNA concentration (pmol) in each dilution was calculated with the formula: micrograms of transcript RNA × (10^6 pg/1 µg) × (1 pmol/340 pg) × (1/number of bases of the transcript), and the number of RNA copies using this concentration value and Avogadro's constant. Standard curves for T385, T318A, and T36 were constructed plotting the threshold cycle (Ct) values from two independent assays with four replicates per standard dilution versus the logarithm of the RNA dilution. The amplification efficiency was calculated from the slope of the corresponding curve using the formula $10^{(-1/\text{slope of the standard curve})}$, or the same formula × 100 (when given as a percentage value). The specificity of the three probes was tested with serial dilutions of homologous and heterologous RNA transcripts.

General detection of CTV by real-time RT-PCR and melting curve analysis. The 56 citrus samples included in this study were first tested for CTV infection in the LightCycler platform (Roche Molecular Diagnostics, Indianapolis, IN) following the real-time RT-PCR protocol previously developed that uses the general primer set PM197F-PM198R and SYBR Green I detection (41). The melting curve analysis was done with the software provided by the LightCycler platform that displays the first derivative of the fluorescence intensity versus the temperature. Synthesis of a DNA product of the expected size was confirmed by electrophoresis analysis in a 2% agarose gel and ethidium bromide staining.

Genotype-specific RT-PCR assays with TaqMan LNA probes. The real-time RT-PCR analyses with TaqMan LNA probes were also performed in a LightCycler platform using 20-µl glass capillaries. The probes annealed specifically in an internal region of the PCR product amplified with primers PM197F-PM198R and then they were cleaved by the 5′ exonuclease activity of the DNA polymerase, which released the reporter molecule away from the quencher, thus allowing the reporter dye to emit its characteristic fluorescence. In each run, the LightCycler software plotted the fluorescence intensity against the number of cycles and provided the Ct value using the automatic method. Amplification was performed in a reaction mix (10 µl) containing 2 µl of RNAt extract (≈10 ng RNA/µl), 2 µl of LightCycler TaqMan Master mix (Roche Diagnostics), 3.65 µl of RNase-free water, 0.05 µl of RT MultiScribe reverse transcriptase (Applied Biosystems, Branchburg, NJ), 0.05 µl of RNase Inhibitor (Applied Biosystems), 0.5 µM of primers PM197F and PM198R, and 0.25 µM of TaqMan LNA probe. Control samples included: (i) RNAt from healthy citrus, from citrus infected with CTV but using no reverse transcriptase or from citrus infected with *Citrus psorosis virus* (CPsV) or *Citrus variegation virus* (CVV), (ii) water instead of RNAt extract, and (iii) at least one RNA transcript dilution of the corresponding standard curve in each run. Each plant sample was analyzed in duplicate in two independent real-time RT-PCR assays. The cycling conditions consisted of reverse transcription at 48°C for 30 min, enzyme denaturation at 95°C for 10 min, and 45 cycles of 95°C for 2 s, 60°C for 10 s and 72°C for 15 s. The mean (*X*) Ct value and the standard deviation (SD) for each plant sample were calculated from the four Ct values obtained. The variation coefficient (CV%) within or between assays was calculated as percentage of the SD in comparison with the (*X*) Ct value.

RESULTS

General detection of CTV isolates and melting curve analysis. Previously we developed a sensitive real-time RT-PCR protocol that enabled quantitative CTV detection in several hosts and citrus tissues using eight biologically characterized isolates (41). Here we tested 48 additional isolates of different origin and pathogenicity characteristics, including (i) 15 new isolates from Spain that were analyzed using fresh tissue (Table 1), and (ii) 33 isolates from 19 different countries that were sampled 7 to 9 years before from the BARC collection and conserved as silica-desiccated leaf tissue (Table 2). Positive CTV detection was achieved with all samples, thus supporting the general validity of our protocol, albeit detection in fresh tissue was generally more sensitive than in desiccated tissue (Ct values between 14.06 and 22.11, with an average of 17.68, for the first group versus Ct values ranging from 18.55 to 29.12, with an average of 22.78, for the second) (Tables 1 and 2), as reported by Saponari et al. (44). Specificity of the product synthesized was checked by melting curve analysis and by agarose gel electrophoresis. For each sample the melting curve showed a single peak indicating a specific PCR product with no significant primer-dimer or non-specific amplification products. The electrophoresis analysis confirmed amplification of a single DNA species of 186-bp for most isolates, or 204-bp for T36-like isolates, due to the presence of an 18-bp insertion in the region encompassed by the primers in these latter isolates (41).

The Tm value of CTV isolates ranged from 81.2 to 85.1°C (Tables 1 and 2). However, while the Tm values of most severe SY and/or SP isolates (26 out 28) varied between 84.1 (isolate T66-1) and 85.1°C (isolates B83, B255, and B352), the Tm values of most mild isolates (24 out of 28) spanned from 81.2 (isolate B192) to 83.9°C (isolate B105) (Tables 1 and 2), thus

confirming our previous results (41). Exceptions to this rule were the severe isolates 6.10 and B156, with Tm values in the range of mild isolates (83.6 and 83.8°C, respectively), and the mild isolates T346, T373, B302, and B151, with Tm values within the range of the severe group (84.2, 84.3, 84.3, and 85°C, respectively). Variation in the Tm value between isolates could be due to different GC content in the amplified segment of their gRNA, or to the presence of a mixed population of different sequence variants (32,37). To examine this possibility, we sequenced the fragments amplified from mild isolates B192, B29, T385, B105, T346, T373, B302, and B151 that showed widely different Tm values. In comparison with T385 (Tm 83.4°C), isolates B192 and B29 had lower Tm values (81.2 and 82.2°C) and 8 and 3 GC pairs less, respectively, whereas isolates B105, T346, T373, B302, and B151, with higher Tm values (83.9, 84.2, 84.3, 84.3, and 85°C) had 3, 2, 2, 2, and 6 additional GC pairs, respectively. On the other hand, sequencing the product amplified from isolate 6.10, with the lowest Tm among severe isolates (83.6°C), revealed sequence polymorphisms characteristics of the mild and the severe genotypes, suggesting a mixed population with both type of variants. Interestingly, the source plant of isolate 6.10 was part of a cross protection experiment in which this plant had been preinoculated with the mild isolate T385, but protection failed and, after 7 years in the field, it induced SY in sour orange and grapefruit and mild SP in the latter host.

Although 90% of the isolates were correctly identified as mild or severe by their Tm value, overlapping between the Tm ranges of both groups limits reliability of melting curve analysis to predict the pathogenicity characteristics of unknown isolates. Moreover, isolates with a mixed population of diverged sequence variants are common (5,39) and might yield variable Tm values depending on the ratio of sequence variants in the population. To overcome this limitation we developed a new procedure to detect and quantify specific sequence variants present in CTV isolates.

Detection of specific CTV variants by real-time RT-PCR using TaqMan LNA probes. The real-time RT-PCR procedure developed for detection of specific sequence variants used the general primer set PM197F-PM198R designed previously (41) and TaqMan LNA probes directed to a variable domain within the region encompassed by these primers. The probes, 18 to 19 nt in length and about 70°C Tm, were designed on sequences characteristic of each of the three main CTV groups observed (28,40), that include mild, severe SP, and T36-like isolates, respectively. After checking several primer/probe ratios for the reaction (0.3/0.3, 0.4/0.2, and 0.5/0.25 μM) it was found that concentrations of 0.5 μM for each primer and 0.25 μM for the probe yielded the lowest Ct values with the three probes. Therefore these concentrations were used in subsequent experiments.

The three TaqMan LNA probes yielded no fluorescent signal with negative controls using water or RNAt from citrus plants healthy or infected with other citrus viruses as template, or using RNAt from CTV-infected tissue and no reverse transcriptase. No fluorescence was either observed when the probes were tested with RNA transcripts of the heterologous isolates, even at concentrations as high as 10^9 copies (Fig. 1), thus confirming the absence of false priming sites or cross-reactivity of the probes with nonhomologous CTV sequence variants. The assay was highly reproducible both within and between experiments, with coefficients of variation being always below 1.5% and normally ranging from 0 to 0.7% (data not shown). To determine the sensitivity of detection with each individual probe (mild, severe-SP, and T36-like), three standard curves were prepared using 10-fold serial dilutions of the cognate RNA transcript (T385, T318A, and T36, respectively), containing 10^{10} to 10^1 copies (Fig. 2). The three curves provided a wide dynamic range (6 to 7 log units of concentration) of target amplification, and regression analyses demonstrated a strong linear relationship with correlation coefficients of 0.9984 (T385), 0.9894 (T318A), and 0.9822 (T36), and amplification efficiencies between 94 and 100% (Fig. 2A, C, and D). This new real-time assay enabled detection of as few as 10^2 copies of the CTV gRNA in plant extracts using any of the three TaqMan LNA probes (Fig. 2B), albeit quantification of such a low copy number was not reliable because this concentration fell out of the linear range of the standard curves.

To assess the ability of the probes to detect small amounts of their homologous sequence in mixed populations of sequence variants, each probe was tested with mixes containing a variable ratio (1 to 99%) of the homologous and either of the non-homologous RNA transcripts (T385+T318A, T385+T36, or T318A+T36). The specificity of the TaqMan LNA probes was not affected by mixed populations, and the presence of each type of RNA transcript was exclusively detected by its homologous probe. The severe-SP and the T36-like probes yielded positive detection in mixes containing 2.5% of homologous and 97.5% of heterologous transcripts, whereas the mild probe was able to detect 1% of the homologous transcript mixed with 99% of heterologous variants.

Quantitative detection of the three types of sequence variants in CTV isolates. A total of 56 biologically characterized CTV isolates were tested for the presence of CTV variants characteristic of mild, severe SP or T36-like isolates. Each citrus

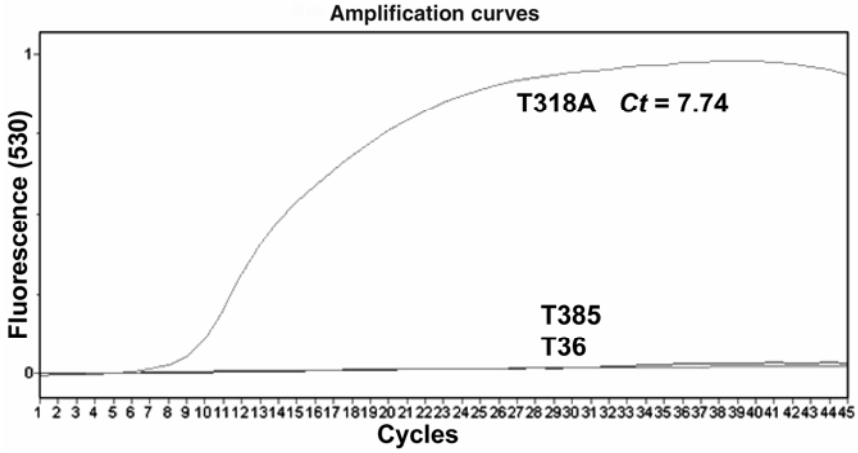

Fig. 1. Amplification curves obtained by real-time reverse transcription-polymerase chain reaction using the severe-SP probe (based on the sequence of *Citrus tristeza virus* (CTV) isolate T318A) and 10^9 copies of in vitro synthesized RNA transcripts from representative isolates of the severe SP (T318A), the mild (T385), and the T36-like (T36) groups (28,40). A cycle threshold (*C*t) value of 7.74 was obtained with the T318A transcripts, but no fluorescence was observed with T385 and T36 transcripts.

sample was analyzed in duplicate in two independent real-time RT-PCR assays for each of the three probes. All CTV isolates from both collections reacted with at least one of the probes, with the only exception of isolate B151 from California that was PCR amplified with the general primers and detected by the SYBR Green I assay but not with the probes.

The 27 isolates characterized as mild (excluding B151) because they did not induce SY or SP in grapefruit or sweet orange, were exclusively detected with the mild probe. Generally detection was more sensitive for isolates of the Spanish group, with Ct values that ranged from 16.1 to 23.9, than for isolates of the BARC collection, with Ct values between 21.1 and 27.2 (Tables 1 and 2), as expected from the use of fresh tissue for the first group and desiccated tissue for the second. The reaction pattern of the 28 isolates characterized as severe (inducing SY or SP in grapefruit or sweet orange) was more variable, but it was observed that (i) all of them reacted with the severe-SP probe, with the T36-like probe or with both, and (ii) all isolates inducing SP in sweet orange reacted with the severe-SP probe (Tables 1 and 2). Many isolates reacted with one of these probes and with the mild probe, but none was found reacting with the three probes. Nine of these 28 isolates (seven from the BARC and two from the IVIA collection) reacted exclusively with the severe-SP probe and two (T36 and B83) reacted only with the T36-like probe. The remaining 17 isolates were detected with two probes: 12 of them with the mild and the severe-SP probes, four with the mild and the T36-like probes, and one with the severe-SP and the T36-like probes. Although reaction with the severe-SP probe (alone or in combination with other probes) was essentially associated with isolates inducing SP in grapefruit or sweet orange, the Brazilian isolate B12-1 (an aphid-transmitted subculture of the SP isolate B12) induced severe SY but no detectable SP (Table 2). Similarly, reaction with the T36-like probe (alone or in combination with the mild probe) was mostly associated with SY non-SP isolates, but mild grapefruit SP was occasionally observed with isolates B83 and B358.

Specificity of the three TaqMan LNA probes was further validated by sequencing the end-point RT-PCR products of a representative group of isolates. As expected, the sequence of the DNA product obtained from the mild isolates B5, B29, B105, B272, B302, and B192 (from the BARC collection) and T340, T346, T373, and T405 (from the IVIA collection), reacting only with the mild probe, was genetically close to the sequence of the mild isolate T385. Similarly, the sequence of the products amplified from isolates B83 and T36, which reacted only with the T36-like probe, corresponded to that of the latter isolate and presented its characteristic 18-bp insertion. Most isolates detected with two probes showed similar Ct values for both probes using the same RNAt preparation (Tables 1 and 2), suggesting that the cognate sequence variants were at similar frequency in the viral population. Sequencing the PCR products amplified from isolates T318, 6.10, and B12, that reacted with the mild and the severe-SP probes, or the products from isolates T308 and T66-1, that reacted with the mild and the T36-like probes with similar Ct values, showed polymorphic positions specific of the mild and the severe-SP genotypes in the first case, and of the mild and the T36-like genotypes in the second. In contrast, reaction of some isolates with the two probes showed distinct Ct values, suggesting predominance of one of the sequence variants. For example, the Ct value obtained for isolates B181 and B358 with the mild probe was slightly higher than that obtained with the T36-like probe (Table 2), and for both isolates, the sequence obtained from the PCR product was related to the T36 genotype. Similarly, the VT isolate reacted with the severe-SP and the T36-like probes with Ct values of 27.3 and 30.7, respectively (Table 2), and the only sequence variant detected in the PCR product was related to the

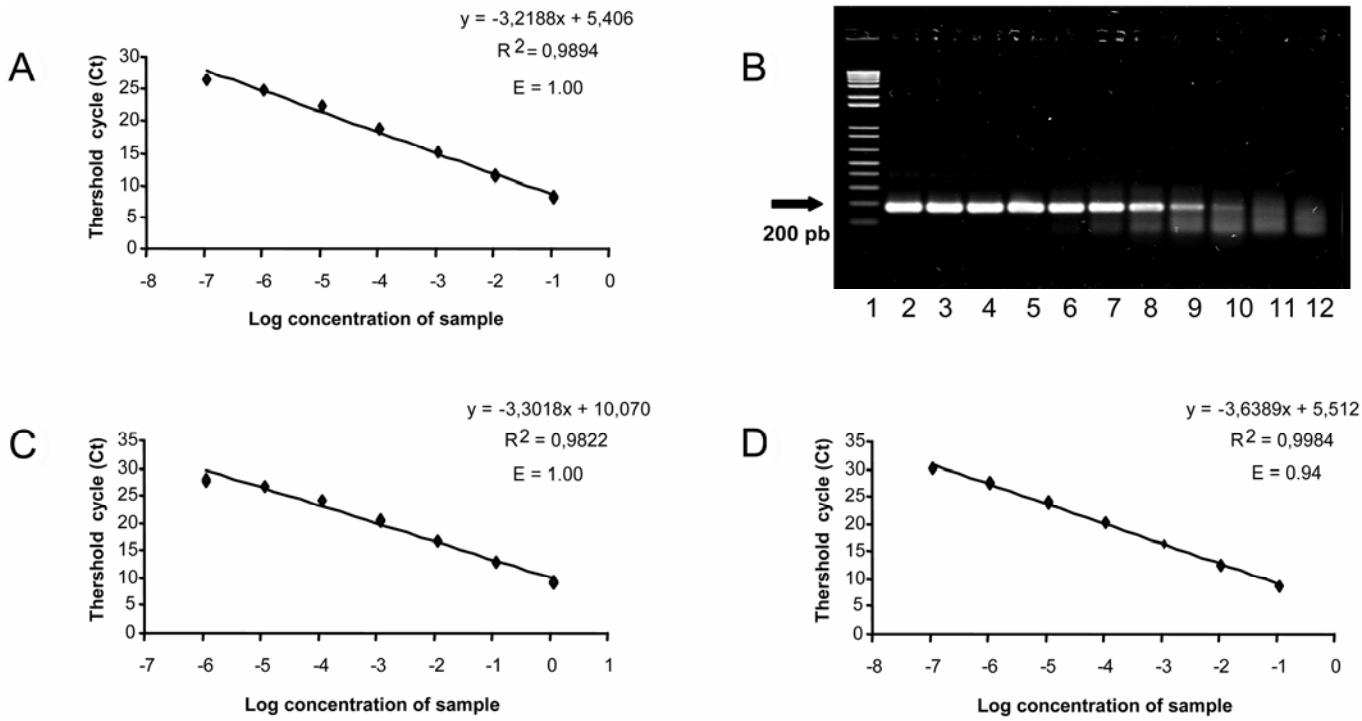

Fig. 2. Standard curves prepared with 10-fold serial dilutions of in vitro-synthesized RNA transcripts from *Citrus tristeza virus* (CTV) isolates **A,** T318A, **C,** T36, and **D,** T385 using real-time reverse transcription-polymerase chain reaction (RT-PCR) with TaqMan LNA probes specific for severe SP, T36-like, or mild genotypes, respectively. Curves were generated by linear regression analysis plotting the threshold cycle values (*C*t) in the Y axis versus the logarithm of the starting RNA dilutions (containing 10^3 to 10^9 copies) in the X axis. Each plotted point represents the mean Ct value calculated from four different experiments with two replicates. Calculated correlation coefficient (R^2) and amplification efficiency (E) values (see Materials and Methods) are indicated in each curve. **B,** Electrophoretic migration in a 2% agarose gel of the amplification products obtained from 10-fold serial dilutions of T318A RNA transcripts (**A**), containing about 10^{10} to 10^1 copies (lanes 2 to 11, respectively). Lane 1: 1-kb plus ladder DNA marker. Lane 12: Control with no template. The gel was stained with ethidium bromide.

severe SP genotypes, indicating that the T36-like variant may be a minor component in the viral population.

Isolates B12-1 from Brazil and B151 from California showed abnormal reaction patterns. The first was the only isolate reacting with the severe-SP probe that did not induce SP in grapefruit or sweet orange, and the second had mild pathogenicity characteristics but it did not react with any probe. Sequence of the final PCR product from B12-1 was characteristic of the severe SP genotype, whereas the mild type variant expected from its reaction with the mild probe was not detected, likely due to its low titer (Ct value was 25.5 with the mild probe, but only 24 with the SP-probe). Sequence analysis of the PCR product from B151 revealed that (i) it lacked the 18-bp insertion characteristic of T36-like isolates, and (ii) the region annealing with the probes was a recombinant between the mild and the severe SP genotypes that had four mismatches with the first and three with the second, thus preventing hybridization with any probe.

DISCUSSION

In many countries CTV-induced decline and death of citrus trees propagated on sour orange rootstock forced to replant citrus using decline-tolerant rootstocks; however, dispersal of severe isolates causing stunting and stem pitting in grapefruit or sweet orange varieties, regardless the rootstock used, posed a new threat to the citrus industry that in the long term may be economically as important as tristeza decline (15,28,49). Control of this CTV disease requires (i) quarantine measures, (ii) eradication of new foci, (iii) nursery propagation of buds free from severe SP isolates, and eventually (iv) using cross protection with mild CTV isolates (15,28,49), a set of measures that needs efficient procedures to identify potentially dangerous isolates. Identification of these isolates is currently done by biological indexing on selected indicator plants (17,38), although several serological and molecular markers have been used for isolate characterization (4,18,21,31,35,43). Some of these markers tend to separate mild and severe SP isolates but lack of knowledge on the actual genetic determinants of CTV pathogenicity, the presence of mixed populations in field trees, and limited sensitivity or specificity of the detection procedures, have jeopardized their practical use to predict the pathogenic behavior of unknown isolates (46).

Real-time RT-PCR amplification of 56 biologically characterized CTV isolates from 20 countries and melting curve analysis of the amplified DNA products enabled to confirm that (i) the procedure previously developed (41) was sensitive enough to detect CTV in citrus tissues silica-desiccated 7 to 9 years before, (ii) CTV isolates of different origin and pathogenicity characteristics can be reliably detected with the primers selected, and (iii) the Tm values of severe isolates spanned a narrow range of about 1°C and they were usually higher than those of mild isolates. However, some isolates with mixed populations of mild and severe sequence variants showed intermediate Tm values that made difficult their assignment to one or the other group. Although 90% of the isolates were properly classified, frequent occurrence of mixed populations in field CTV isolates (4,5,39,42) may limit accuracy of Tm values for characterization of unknown isolates.

To overcome this limitation we developed a real-time RT-PCR protocol, that used a general primer set (41) to amplify all sequence variants and three TaqMan LNA probes specific for sequence variants characteristic of mild, severe SP and T36-like isolates (28,40). A limitation of standard TaqMan probes is that frequently they are not specific enough to discriminate between sequence variants containing one or a few mismatches (24). Since the target region selected in the CTV genome is very variable we improved sensitivity and specificity by (i) using short probes (less than 20 nt) to reduce the number of potential polymorphic positions, and (ii) introducing three to four LNA modified residues in each probe to increase their Tm to about 70°C, so that they would strongly hybridize with homologous and discriminate between heterologous sequence variants. Enhanced specificity provided by incorporation of LNAs in TaqMan probes has been reported for other viruses (16). Specificity of the probes was first tested with different mixes of homologous and heterologous RNA transcripts. Our TaqMan LNA probes enabled detection of their specific target in mixes containing less than 2.5% of the homologous sequence. The absence of nonspecific fluorescence signal or cross-reactivity with nonhomologous sequence variants confirmed that the probes were genotype-specific and that they could be used for isolate discrimination. The low CV values within and between assays (<1.5%) confirmed the high reproducibility of this protocol, while the strong linear correlation, high amplification efficiency and wide dynamic range of the three standard curves (10^3 to 10^9 copies), supported its validity for quantitative assays. The detection limit for the three probes was about 100 gRNA copies, that is in the range of sensitivity obtained previously for detection of CTV and other plant viruses using SYBR Green I or conventional TaqMan assays (1,8,41), albeit quantification of such a low copy number would be inaccurate. The first step of CTV amplification and SYBR Green I detection provided some clues on the quality of RNAt preparations and enabled CTV detection in sample B151, even if this isolate failed to react with the three probes due to the presence of a recombination event in the region selected for probe annealing.

Detection of CTV isolates of different geographic origin and pathogenicity characteristics with one or two probes (except for isolate B151) further confirmed their sensitivity, specificity, and usefulness for isolate characterization. The variable reaction patterns observed reflected genetic diversity of CTV populations within and between isolates. Association between the reaction pattern and the actual population structure was confirmed by sequencing the RT-PCR products amplified from 21 isolates (ten mild, two T36-like, and nine severe SP). Thus while sequencing the RT-PCR products from isolates T318A and T388 of the IVIA collection, reacting only with the severe-SP probe, showed a single variant closely related to the sequence of other severe SP isolates, the products from isolates T318 and T305, reacting with both the mild and the severe-SP probes, showed two sequence variants genetically close to those of mild and severe SP isolates, respectively.

Interestingly, while all mild isolates reacted only with the mild LNA probe, suggesting a genetically homogeneous population, most severe isolates (about 61%) reacted with two probes, indicating a more complex population, as previously suggested by analyses of other gRNA regions (4,42,43). It should be emphasized that lack of reaction of mild isolates with the severe-SP or the T36-like probes does not necessarily mean absence of severe CTV sequence variants, but just that those variants represent less than 2.5% of their viral population. Indeed, SP and SY subisolates were obtained after experimental aphid or graft transmission of B192 and T385 to different receptor plants (9,11,29,30), even if those isolates are essentially asymptomatic, they reacted only with the mild probe, and at least the T385 population showed low genetic diversity (5). About 78.6% of the severe isolates reacted with the severe-SP probe (32% only with this probe, 43% with the mild and the severe-SP probes, and 3.6% with the severe-SP and the T36-like probes) but only 25% was detected by the T36-like probe (7.1% only with this probe, 14.3% with the mild and the T36-like probes, and 3.6% with the T36-like and the severe-SP probes). Reaction with the severe-SP probe was essentially associated with SP inducing isolates while reaction with the T36-like probe mainly identified isolates inducing SY but not SP. We only found two SP isolates (B83 and B358) that failed to react with the severe-SP probe, although they were identified as severe by their reaction with the T36-like probe, and one isolate (B12-1) reacting with the severe-SP probe that did not induce SP but

caused strong SY. Isolates B83 and B358 were found associated to T36 using the POL marker, and B358 also using the K17 marker, proposed by Hilf et al. (21). Isolate B12-1 is an aphid-transmitted subisolate of the severe SP-inducing isolate B12. Overall, mild sequence variants were detected in about 77% of the isolates, severe-SP variants in about 39%, and T-36-like variants in about 12.5%, further confirming previous findings that sequence variants characteristic of the mild isolates are the most widespread (4,42).

Previous attempts to predict pathogenicity characteristics of CTV isolates by their association with serological or molecular markers had two main drawbacks: (i) a limited number of isolates was used or only partial characterization was performed, and (ii) exceptions to the claimed association were easily found and several tests were necessary to reliably predict the potential threat from unknown isolates. Sieburth et al. (46) used several serological and nucleic acid-based procedures to discriminate between SP and non-SP CTV isolates and they classified correctly 75% of the isolates, considering combined results from all tests. Here we used 56 well characterized isolates from 20 different countries and found that reaction with our severe-SP probe identified 96% of the SP isolates, whereas reaction with only the mild probe identified 100% of the mild isolates. Moreover, reaction with the severe-SP or the T36-like probes, alone or in combination with other probes, enabled identification of all potentially severe (SY and/or SP) isolates. Although this protocol includes three separate real-time RT-PCR reactions (one with each TaqMan LNA probe), it is more rapid, sensitive, and specific than previous methods. Additionally, it enables estimating the ratio of different genotypes present in the viral population. Finding that CTV isolates from geographically distant origins but sharing similar pathogenicity characteristics yielded a similar reaction pattern supports the validity of this procedure to discriminate between mild and severe isolates. Our TaqMan LNA probe-based protocol can be a useful tool for (i) quarantine and certification programs to avoid introduction or nursery propagation of potentially dangerous isolates in areas affected only by mild isolates, (ii) examining incidence and epidemiology of severe isolates in CTV endemic areas, (iii) assessing citrus genotypes or transgenic plants for strain-specific resistance, (iv) evaluating cross protection stability, or (v) analyses of fitness or competition experiments with CTV genotypes obtained from engineered infectious cDNA clones (45,48).

ACKNOWLEDGMENTS

We thank S. M. Garnsey (University of Florida-C.R.E.C., Lake Alfred, FL) for providing CTV isolates from the BARC collection with detailed pathogenicity characteristics. S. Ruiz-Ruiz and S. Ambrós were recipients of a predoctoral fellowship and a postdoctoral "Ramón y Cajal" contract from the Ministerio de Educación y Ciencia of Spain (MEC), respectively, and presently S. Ambrós has a research contract from Agroalimed "Fundación de la Comunidad Valenciana para la Investigación Agroalimentaria". This research work was partially supported by grant AGL2007-61885/AGR from the MEC.

LITERATURE CITED

1. Agindotan, B. O., Shiel, P. J., and Berger, P. H. 2007. Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan real-time RT-PCR. J. Virol. Methods 142:1-9.
2. Albiach-Martí, M. R., Mawassi, M., Gowda, S., Satyanayanana, T., Hilf, M. E., Shanker, S., Almira, E. C., Vives, M. C., López, C., Guerri, J., Flores, R., Moreno, P., Garnsey, S. M., and Dawson, W. O. 2000. Sequences of C*itrus tristeza virus* separated in time and space are essentially identical. J. Virol. 74:6856-6865.
3. Ancillo, G., Gadea, J., Forment, J., Guerri, J., and Navarro, L. 2007. Class prediction of closely related plant varieties using gene expression profiling. J. Exp. Bot. 58:1927-1933.
4. Ayllón, M. A., López, C., Navas Castillo, J., Garnsey, S. M., Guerri, J., Flores, R., and Moreno, P. 2001. Polymorphism of the 5′ terminal region of *Citrus tristeza virus* (CTV) RNA: Incidence of three sequence types in isolates of different origin and pathogenicity. Arch. Virol. 146:27-40.
5. Ayllón, M. A., Rubio, L., Sentandreu, V., Moya, A., Guerri, J., and Moreno, P. 2006. Variations in two gene sequences of *Citrus tristeza virus* after host passage. Virus Genes 32:119-128.
6. Ballester-Olmos, J. F., Pina, J. A., Carbonell, E. A., Moreno, P., Hermoso de Mendoza, A., Cambra, M., and Navarro, L. 1993. Biological diversity of *Citrus tristeza virus* (CTV) isolates in Spain. Plant Pathol. 42:219-229.
7. Ballester-Olmos, J. F., Pina, J. A., and Navarro, L. 1988. Detection of a tristeza-seedling yellows strain in Spain. Pages 28-32 in: Proc. 10th Conf. Int. Organ. Citrus Virol. L. W. Timmer, S. M. Garnsey, and L. Navarro, eds. IOCV, Riverside, CA.
8. Bertolini, E., Moreno, A., Capote, N., Olmos, A., De Luis, A., Vidal, E., Pérez-Panadés, J., and Cambra, M. 2008. Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time RT-PCR. Eur. J. Plant Pathol. 120:177-188.
9. Bové, C., Vogel, R., Albertini, D., and Bové, J. M. 1988. Discovery of a strain of tristeza virus (K) inducing no symptoms in Mexican lime. Pages 14-16 in: Proc. 10th Conf. Int. Organ. Citrus Virol. L. W. Timmer, S. M. Garnsey, and L. Navarro, eds. IOCV, Riverside, CA.
10. Braasch, D. A., and Corey, D. R. 2001. Locked nucleic acid (LNA): Fine-tuning the recognition of DNA and RNA. Chem. Biol 8:1-7.
11. Brlansky, R. H., Damsteegt, V. D., Howd, D. S., and Roy, A. 2003. Molecular analyses of *Citrus tristeza virus* subisolates separated by aphid transmission. Plant Dis. 87:397-401.
12. Bustin, S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. J. Mol. Endocrinol. 29:23-39.
13. Cambra, M., Camarasa, E., Gorris, M. T., Garnsey, S. M., and Carbonell, E. 1991. Comparison of different immunosorbent assays for *Citrus tristeza virus* (CTV) using CTV specific monoclonal and polyclonal antibodies. Pages 38-45 in: Proc. 11th Conf. Int. Organ. Citrus Virol. R. H. Brlansky, R. F. Lee, and L. W. Timmer, eds. IOCV, Riverside, CA.
14. Che, X., Piestum, D., Mawassi, M., Yang, G., Satyanarayana, T., Gowda, S., Dawson, W. O., and Bar-Joseph, M. 2001. 5′-coterminal subgenomic RNAs in *Citrus tristeza virus*-infected cells. Virology 283:374-381.
15. Costa, A. S., and Müller, G. W. 1980. Tristeza control by cross protection: A U.S.-Brazil cooperative success. Plant Dis. 64:538-541.
16. Dreier, J., Störmer, M., Mäde, D., Burkhardt, S., and Kleesiek, K. 2006. Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. J. Clin. Microbiol. 44:2714-2720.
17. Garnsey, S. M., Civerolo, E. L., Gumpf, D. J., Paul, C., Hilf, M. E., Lee, R. F., Brlansky, R. H., Yokomi, R. K., and Hartung, J. S. 2005. Biological characterization of an international collection of *Citrus tristeza virus* (CTV) isolates. Pages 75-93 in: Proc. 16th Conf. Int. Organ. Citrus Virol. M. E. Hilf, N. Duran-Vila, and M. A. Rocha-Peña, eds. IOCV, Riverside, CA.
18. Gillings, M., Broadbent, P., Indsto, J., and Lee, R. 1993. Characterization of isolates and strains of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by the polymerase chain reaction. J. Virol. Methods 44:305-317.
19. Gowda, S., Ayllón, M. A., Satyanarayana, T., Bar-Joseph, M., and Dawson, W. O. 2003. Transcription strategy in a Closterovirus: a novel 5′-proximal controller element of *Citrus tristeza virus* produces 5′- and 3′-terminal subgenomic RNAs and differs from 3′ open reading frame controller elements. J. Virol. 77:340-352.
20. Hilf, M. E., Karasev, A. V., Pappu, H. R., Gumpf, D. J., Niblett, C. L., and Garnsey, S. M. 1995. Characterization of citrus tristeza virus subgenomic RNAs in infected tissue. Virology 208:576-582.
21. Hilf, M. E., Mavrodieva, V. A., and Garnsey, S. M. 2005. Genetic marker analysis of a global collection of isolates of *Citrus tristeza virus*: Characterization and distribution of CTV genotypes and association with symptoms. Phytopathology 95:909-917.
22. Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O. V., Hilf, M. E., Koonin, E. V., Niblett, C. L., Cline, K., Gumpf, D. J., Lee, R. F., Garnsey, S. M., Lewandowsky, D. J., and Dawson, W. O. 1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208:511-520.
23. Kumar, R., Singh, S. K., Koshkin, A. A., Rajwanshi, V. K., Meldgaard, M., and Wengel, J. 1998. The first analogues of LNA (Locked Nucleic Acids): Phosphorothioate-LNA and 2′-thio-LNA. Bioorg. Med. Chem. Lett. 8:2219-2222.
24. Latorra, D., Arar, K., and Hurley, J. M. 2003. Design considerations and effects of LNA in PCR primers. Mol. Cell. Probes 17:253-259.
25. Mackay, I. M., Arden, K. E., and Nitsche, A. 2002. Real-time PCR in Virology. Nucleic Acids Res. 30:1292-1305.
26. Mawassi, M., Karasev, A. V., Mietkiewska, E., Gafny, R., Lee, R. F., Dawson, W. O., and Bar-Joseph, M. 1995. Defective RNA molecules associated with citrus tristeza virus. Virology 208:383-387.
27. Mawassi, M., Mietkiewska, E., Gofman, R., Yang, G., and Bar-Joseph, M. 1996. Unusual sequence relationships between two isolates of citrus tristeza virus. J. Gen. Virol. 77:2359-2364.

28. Moreno, P., Ambrós, S., Albiach-Martí, M. R., Guerri, J., and Peña L. 2008. Plant diseases that changed the world - *Citrus tristeza virus*: A pathogen that changed the course of the citrus industry. Mol. Plant Pathol. 9:251-268.
29. Moreno, P., Guerri, J., Ballester-Olmos, J. F., Albiach, R., and Martínez, M. E. 1993. Separation and interference of strains from a *Citrus tristeza virus* isolate evidenced by biological activity and double-stranded RNA (dsRNA) analysis. Plant Pathol. 42:35-41.
30. Moreno, P., Guerri, J., Ballester-Olmos, J. F., Fuertes-Polo, C., Albiach, R., and Martínez, M. E. 1993. Variations in pathogenicity and double-stranded RNA (dsRNA) patterns of *Citrus tristeza virus* isolates induced by host passage. Pages 8-15 in: Proc. 12th Conf. Int. Organ. Citrus Virol. P. Moreno, J. V. da Graça, and L. W. Timmer, eds. IOCV, Riverside, CA.
31. Narváez, G., Skander, B. S., Ayllón, M. A., Rubio, L., Guerri, J., and Moreno, P. 2000. A new procedure to differentiate *Citrus tristeza virus* isolates by hybridisation with digoxigenin-labelled cDNA probes. J. Virol. Methods 85:83-92.
32. Nicolas, L., Milon, G., and Prina, E. 2002. Rapid differentiation of Old World Leishmania species by LightCycler polymerase chain reaction and melting curve analysis. J. Microbiol. Methods 51:295-299.
33. Olmos, A., Cambra, M., Esteban, O., Gorris, M. T., and Terrada, E. 1999. New device and method for capture, reverse transcription and nested PCR in a single closed tube. Nucleic Acids Res. 27:1564-1565.
34. Pappu, H. R., Karasev, A. V., Anderson, E. J., Pappu, S. S., Hilf, M. E., Febres, V. J., Eckloff, R. M. G., McCaffery, M., Boyko, V., Gowda, S., Dolja, V. V., Koonin, E. V., Gumpf, D. J., Cline, K. C., Garnsey, S. M., Dawson, W. O., Lee, R. F., and Niblett, C. L. 1994. Nucleotide sequence and organization of eight 3′ open reading frames of the citrus tristeza closterovirus genome. Virology 199:35-46.
35. Permar, T. A., Garnsey, S. M., Gumpf, D. J., and Lee, R. F. 1990. A monoclonal-antibody that discriminates strains of *Citrus tristeza virus*. Phytopathology 80:224-228.
36. Petersen, M., and Wengel, J. 2003. LNA: A versatile tool for therapeutics and genomics. Trends Biotechnol. 21:74-81.
37. Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. 245:154-160.
38. Roistacher, C. N. 1991. Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. FAO, Rome.
39. Rubio, L., Ayllón, M. A., Kong, P., Fernández, A., Polek, M., Guerri, J., Moreno, P., and Falk, B. W. 2001. Genetic variation of *Citrus tristeza virus* isolates from California and Spain: Evidence for mixed infections and recombination. J. Virol. 75:8054-8062.
40. Ruiz-Ruiz, S., Moreno, P., Guerri, J., and Ambrós, S. 2006. The complete nucleotide sequence of a severe stem pitting isolate of *Citrus tristeza virus* from Spain: Comparison with isolates from different origins. Arch. Virol. 151:387-398.
41. Ruiz-Ruiz, S., Moreno, P., Guerri, J., and Ambrós, S. 2007. A real-time RT-PCR assay for detection and absolute quantitation of *Citrus tristeza virus* in different plant tissues. J. Virol. Methods 145:96-105.
42. Sambade, A., López, C., Rubio, L., Flores, R., Guerri, J., and Moreno, P. 2003. Polymorphism of a specific region in gene p23 of *Citrus tristeza virus* allows discrimination between mild and severe isolates. Arch. Virol. 148:2325-2340.
43. Sambade, A., Rubio, L., Garnsey, S. M., Costa, N., Müller, G. W., Peyrou, M., Guerri, J., and Moreno, P. 2002. Comparison of viral RNA populations of pathogenically distinct isolates of *Citrus tristeza virus*: Application to monitoring cross-protection. Plant Pathol. 51:257-265.
44. Saponari, M., Manjunath, K., and Yokomi, R. K. 2008. Quantitative detection of *Citrus tristeza virus* in citrus and aphids by real-time reverse transcription-PCR (TaqMan [R]). J. Virol. Methods 147:43-53.
45. Satyanarayana, T., Bar-Joseph, M., Mawassi, M., Albiach-Martí, M. R., Ayllón, M. A., Gowda, S., Hilf, M. E., Moreno, P., Garnsey, S. M., and Dawson, W. O. 2001. Amplification of *Citrus tristeza virus* from a cDNA clone and infection of citrus trees. Virology 280:87-96.
46. Sieburth, P. J., Nolan, K. G., Hilf, M. E., Lee, R. F, Moreno, P., and Garnsey, S. M. 2005. Discrimination of stem-pitting from other isolates of *Citrus tristeza virus*. Pages 1-10 in: Proc. 16th Conf. Int. Organ. Citrus Virol. M. E. Hilf, N. Duran-Vila, and M. A. Rocha-Peña, eds. IOCV, Riverside, CA.
47. Suastika, G., Natsuaki, T., Terui, H., Kano, T., Ieki, H., and Okuda, S. 2001. Nucleotide sequence of *Citrus tristeza virus* seedling yellows isolate. J. Gen. Plant Pathol. 67:73-77.
48. Tatineni, S., Robertson, C. J., Garnsey, S. M., Bar-Joseph, M., Gowda, S., and Dawson, W. O. 2008. Three genes of *Citrus tristeza virus* are dispensable for infection and movement throughout some varieties of citrus trees. Virology 376:297-307.
49. van Vuuren, S. P., Collins, R. P., and da Graça, J. V. 1993. Evaluation of citrus tristeza virus isolates for cross protection of grapefruit in South Africa. Plant Dis. 77:24-28.
50. Vives, M. C., Rubio, L., López, C., Navas-Castillo, J., Albiach-Martí, M. R., Dawson, W. O., Guerri, J., Flores, R., and Moreno, P. 1999. The complete genome sequence of the major component of a mild citrus tristeza virus isolate. J. Gen. Virol. 80:811-816.
51. Vives, M. C., Rubio, L., Sambade, A., Mirkov, T. E., Moreno, P., and Guerri, J. 2005. Evidence of multiple recombination events between two RNA sequence variants within a *Citrus tristeza virus* isolate. Virology 331:232-237.
52. Yang, Z. N., Mathews, D. M., Dodds, J. A., and Mirkov, T. E. 1999. Molecular characterization of an isolate of citrus tristeza virus that causes severe symptoms in sweet orange. Virus Genes 19:131-142.