Eur J Plant Pathol (2008) 120:177–188
DOI 10.1007/s10658-007-9206-9

FULL RESEARCH PAPER

Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time RT-PCR

Edson Bertolini · Aranzazu Moreno · Nieves Capote · Antonio Olmos · Ana de Luis · Eduardo Vidal · Jordi Pérez-Panadés · Mariano Cambra

Received: 17 April 2007 / Accepted: 31 July 2007 / Published online: 11 October 2007

Abstract TaqMan real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) using purified RNA targets or coupled to tissue-print and squash procedures was developed to detect and quantify *Citrus tristeza virus* (CTV) RNA-targets in plant tissues and in single aphids. With this method all CTV isolates tested from different hosts and origins were detected. The sensitivity of conventional real-time RT-PCR was 1,000 times higher than immunocapture (IC)-RT-nested PCR and 10^6 times higher than enzyme linked immunosorbent assay (ELISA). The quantitation limit ranged from 1.7×10^2 to 1.7×10^9 transcript copies. The estimated number of CTV RNA-targets detected in different organs of a CTV-infected tree ranged from 4.5×10^5 to 6.5×10^8 copies when purified RNA was used as template and from 1.9×10^4 to 3.7×10^6 when tissue-printed material was used. In single squashed aphids the number of copies ranged from 4.73×10^3 to 1.23×10^5. Reliable quantitation of CTV targets present in infected plant material or acquired by single aphid species, was achieved with tissue-print and squash procedures combined with real-time RT-PCR, both of which do not require extraction procedures or nucleic acid purification.

Keywords Acquisition period · CTV · Tissue print-ELISA · Tissue-print and squash real-time RT-PCR · Viral titre

E. Bertolini · A. Moreno · N. Capote · A. Olmos · A. de Luis · E. Vidal · J. Pérez-Panadés · M. Cambra (✉)
Virología e Inmunología, Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA),
Carretera Moncada-Náquera Km 5,
46113 Moncada, Valencia, Spain
e-mail: mcambra@ivia.es

Introduction

Citrus tristeza virus (CTV), a member of the genus *Closterovirus*, causes one of the most harmful diseases affecting citrus and it is one of the most economically important pathogens of the crop (Lee and Bar-Joseph 2000; Román et al. 2004). The term tristeza refers to the decline of many CTV-infected citrus species when grafted onto sour orange rootstock (*Citrus aurantium*). Furthermore, CTV causes stem pitting in some cultivars regardless of the rootstock used, leading to significant losses in fruit quality and yield in several million trees infected with aggressive isolates affecting most citrus industries worldwide (Lee and Bar-Joseph 2000). CTV has been disseminated to almost all citrus-growing countries through the exchange of infected budwood. Subsequent spread by aphid vectors, in a semi-persistent manner, caused and is still causing major epidemics (Cambra et al. 2000a). Closterovirus transmission has no latent period and acquisition and inoculation

periods vary from at least 30 min to seconds (Bar-Joseph et al. 1989; Limburg et al. 1997). The most efficient vector of CTV is *Toxoptera citricida* (Yokomi et al. 1989). However, *Aphis gossypii* is the main vector in Spain, Israel, California (USA) and in all other important citrus-growing areas where *T. citricida* is absent (Gottwald et al. 1996; Marroquín et al. 2004).

Complete protocols for CTV diagnosis, including sampling, detailed techniques, procedures and validated reagents are available (EPPO 2004; http://www.eppo.org/QUARANTINE/virus/Citrus_tristeza/pm7-31(1)%20CTV000%20web.pdf). Traditionally, CTV has been detected by biological indexing using graft-inoculated Mexican lime seedlings (*Citrus aurantifolia*). However, the major limitations of this technique are its high cost and the long time required (weeks to months) to confirm detection, as well as the inherent difficulties for large-scale analysis. Serological tests overcome these disadvantages. With enzyme-linked immunosorbent assay (ELISA) (Garnsey and Cambra 1993) and tissue print-ELISA (Cambra et al. 2000b) using a mixture of 3DF1 and 3CA5 CTV specific monoclonal antibodies (Vela et al. 1986), universal and specific detection of any CTV isolate (Cambra et al. 1990) can be achieved. The main drawbacks of these techniques are their sensitivity, which is important when the viral titre is very low. In addition, the information provided by serological techniques is qualitative or only semi-quantitative (Cambra et al. 1989). Polymerase chain-reaction (PCR)-based methods, such as reverse transcriptase (RT)-nested PCR in a single closed tube have also been developed (Olmos et al. 1999). With this method that includes a previous immunocapture (IC) phase (Nolasco et al. 1993), a highly sensitive detection of CTV can be achieved without a nucleic acid purification step. However, plant material homogenisation, that is laborious, time-consuming and increases the risk of contamination, is still required before the IC step. The latter can be solved by using printed sections of plant tissues or squashed aphid species on paper (Olmos et al. 1996, 1997; Cambra et al. 1996) with or without an IC phase. Sample preparation by tissue-print or squash were successfully used for CTV detection by hemi-nested RT-PCR and RT-nested PCR in a single closed tube (Cambra et al. 2000c), but was unsuccessful in conventional RT-PCR probably due to its lower sensitivity. Application of these fast and friendly sample preparation methods has overcome the sensitivity drawback. The usefulness of real-time RT-PCR has previously been reported to detect *Plum pox virus* (PPV) from squashed aphids (Olmos et al. 2005) and from plant extracts spotted on paper for the analysis of several viruses affecting woody plants (Osman and Rowhani 2006). However, the direct use of imprints for virus quantitation has never been reported.

CTV was first detected in aphids by ELISA (Cambra et al. 1981). Only a few reports demonstrate the detection of viruses in aphid vectors using the most sensitive PCR-based assays (Cambra et al. 2006) either by using RNA purification and RT-PCR (Mehta et al. 1997) or by RT-nested PCR in a single closed tube using squashed aphids on Whatman 3MM paper (Olmos et al. 1999). Few studies quantitatively estimate the number of viral targets in single aphids (Fabre et al. 2003; Olmos et al. 2005), but none of them refer to semi-persistently transmitted viruses and consequently, there are no estimations of the number of CTV targets that a single aphid might acquire and transmit.

In the present study, different approaches for sample preparation prior to real-time RT-PCR based on the TaqMan chemistry for a simple, rapid, sensitive and universal detection and quantification of CTV, have been developed. The methods were applied to plant samples and aphids after different access periods, in an attempt to quantitatively establish relationships between the duration of the feeding period and the number of viral targets acquired by a single aphid.

Materials and methods

Virus isolates and sample preparation

The following isolates were employed: 48 CTV isolates (Ballester-Olmos et al. 1993; Narváez et al. 2000) from the IVIA collection, including the aggressive T-397 and T-388 CTV isolates mentioned below, maintained on sweet orange in an insect-proof screenhouse; 100 characterized isolates from Chile (kindly provided by Dr. X. Besoain); 30 CTV isolates from Egypt (kindly provided by Dr. H. M. Abdelmaksoud), and T-3, T-30, T-36 and VT reference CTV isolates (Hilf and Garnsey 2000). The non-Spanish CTV isolates were introduced and handled as freeze-dried tissues. In addition,

specificity analyses were performed with other citrus viruses (*Citrus psorosis virus*-CPsV, *Citrus vein enation virus*-CVEV, *Citrus variegation virus*-CVV) and viroids (*Citrus exocortis viroid*-CEVd, *Hop stunt viroid*-HSVd, *Citrus bent leaf viroid*-CBLVd, *Citrus viroid III*-CVd-III and *Citrus viroid IV*-CVd-IV, kindly provided by Dr. N. Duran-Vila). Virus and viroids were maintained in the IVIA collection on sweet orange (*Citrus sinensis*) and citron (*Citrus medica*), respectively.

Plant samples were collected in winter from a CTV infected adult 'Clemenules' mandarin (*Citrus reticulata*) tree, according to EPPO (2004). Five young and mature shoots, mature fruits (including peduncle and columella), ten young leaves and fully expanded mature leaves including petioles, were collected from around the canopy of the tree. For validation purposes, samples (young shoots) were collected from 100 alemow (*C. macrophylla*), 137 Mexican lime, and 195 sweet orange field trees.

Extracts from infected and healthy plant samples were prepared by grinding plant material 1/20 (*w*/*v*) in extraction buffer (PBS buffer, pH 7.2, supplemented with 0.2% (*w*/*v*) sodium diethyl dithiocarbamate) (EPPO 2004). Serial dilutions of a CTV-infected 'Pineapple' sweet orange (T-388 isolate from IVIA collection) extract were diluted in a CTV-free Mexican lime extract (1:20 *v*/*v* to $1:10^{10}$ *v*/*v*). RNA was purified from plant extracts using RNeasy plant mini kit (Qiagen). A Mexican lime plant infected with CTV T-397 isolate was used as source for the aphid acquisition-detection tests. Different citrus and citrus relatives such as Mexican lime, sweet and sour oranges, lemon (*C. limon*), mandarin and *Poncirus trifoliata*, were used as CTV-free controls in different experiments.

Tissue-print and squash procedures

Samples were immobilized on membranes (Olmos et al. 1996) using the tissue-print procedure by pressing fresh sections of citrus tissues on 0.45 μ nitrocellulose (Millipore), nylon (Roche), or paper 3 MM (Whatman) membranes. Several partially overlapping imprints were made on a 1 cm^2 nylon membrane as follows: (1) one imprint from a young shoot of a CTV-infected tree followed by a one imprint from a young shoot of a CTV-free tree (1:1); (2) one imprint from a CTV-infected followed by two imprints from a CTV-free tree (1:2); (3) the same strategy followed by three to ten imprints from a healthy three (1:3–1:10); (4) imprints of one infected and 10 CTV-free was repeated by imprinting the infected tissues first, fifth and last; (5) a single imprint and ten overlapping imprints from the CTV-infected tree were included as positive controls.

Imprints that had been previously processed by serological tissue print-ELISA procedure using nitrocellulose membranes were also tested by real-time RT-PCR. Twelve consecutive sections of the same shoot were imprinted alternatively on nitrocellulose and nylon membranes (six imprints on each membrane). This operation was performed using four different CTV-infected adult sweet orange trees. The negative control consisted of imprints from a CTV-free sweet orange tree. The sections imprinted on nitrocellulose were then developed using the tissue print-ELISA procedure, as described below, and subsequently used for tissue-print real-time RT-PCR. The fresh imprints on nylon membranes were directly analysed by tissue-print real-time RT-PCR. The Ct values and estimated number of targets were compared.

Single aphid species were squashed on paper or nylon membranes with the rounded end of an Eppendorf tube (Olmos et al. 1999). Pieces of membranes harbouring the printed and/or squashed samples were inserted into Eppendorf tubes or placed inside ELISA plate wells for large scale assays. One hundred microliters of Triton X-100 0.5% (Olmos et al. 1996) or 100 μl buffer (0.1 M Glycine, 0.05 M NaCl, 1 mM EDTA) (Osman and Rowhani 2006) were added, incubated at 95°C for 10 min, vortexed and placed on ice. Five microliters of this extract were directly used as template for real-time RT-PCR assays.

DAS-ELISA and tissue print-ELISA

Plant extracts were subjected to the double antibody sandwich (DAS)-ELISA biotin/streptavidin system, with a mixture of 3CA5 and 3DF1 CTV specific monoclonal antibodies, using a commercial kit (Ingenasa) and following EPPO (2004) protocol. Tissue print-ELISA (Cambra et al. 2000b; EPPO 2004), using the same mixture of monoclonal antibodies, was performed with tissue-prints on nitrocellulose membranes using a commercial kit (PlantPrint Diagnostics) as described by the manufacturer.

IC-RT-nested PCR

IC-RT-nested PCR in a single closed tube was performed with crude plant extracts according to EPPO (2004) using the device and primers Pex1 (5′TAA ACA ACA CAC ACT CTA AGG 3′) and Pex2 (5′ CAT CTG ATT GAA GTG GAC 3′) for the first amplification, and Pin1 (5′GGT TCA CGC ATA CGT TAA GCC TCA CTT 3′) and Pin2 (5′TAT CAC TAG ACA ATA ACC GGA TGG GTA 3′) for the second amplification, as described by Olmos et al. (1999). PCR products (10 μl) were analysed by electrophoresis in 3% agarose gels and stained with ethidium bromide.

Transcripts preparation for generation of real-time RT-PCR standard curves

A specific CTV nucleotide sequence (241 bp) amplified by RT-PCR using Pex1/Pex2 primers, consisting of the target region for the real-time primers, was inserted into the vector pGem-T (Promega) and cloned into *E. coli* JM-109. Transformants were selected by ampicilin resistance and the orientation of the fragments verified by PCR using forward T7 and Pex1 primers. The plasmid was linearized at the *Not* I site and used as target in an *in vitro* transcription reaction performed with Megascript T7 kit (Ambion, TX) followed by DNase I digestion at 37°C for 30 min. The synthesis of the 302 nucleotide RNA (241 nucleotide bases of the CTV target +61 nucleotide bases of the pGem-T vector) was confirmed by electrophoretical analysis in a 2% agarose gel. RNA was purified by phenol-chloroform extraction, ethanol precipitation and the amount of RNA (μg) was quantified by UV densitometry. Conversion of μg of single stranded RNA to pmol was performed considering the average molecular weight of a ribonucleotide (340 Da) and the number of bases of the transcript (N_b). The following mathematical formula was applied: pmol of ssRNA=μg (of ssRNA)×(10^6 pg/1 μg)×(1 pmol/340 pg)×($1/N_b$). Avogadro constant (6.023×10^{23}) was used to estimate the number of transcripts. Tenfold serial dilutions of the transcripts were prepared from 1.7×10^{11} to 1.7×10^{0}, aliquoted and stored at −80°C until use. Dilutions from 1.7×10^{9} to 1.7×10^{2} were employed to generate the standard curve.

To confirm the suitability of the standard curve generated with the transcripts for an accurate quantitation of CTV targets, amplification efficiencies of standards and field sample reactions were compared according to Rasmussen (2001) and Olmos et al. (2005). The slopes of the calibration curves of the standards and field samples were used to calculate efficiencies using the mathematical formula: slope= −1/log (efficiency).

Real-time RT-PCR

To design appropriate primers and probe, the nucleotide sequence flanked by the universal primers Pin1 and Pin2 was selected. Alignment of nucleotide CTV sequences recovered from databases GenBank, EMBL and DDBJ was carried out. Primer Express software (Applied Biosystems) was used to obtain the optimal oligo and probe sequences. TaqMan assays for real-time RT-PCR were performed in ABI Prism 7000 Sequence Detection System software (Applied Biosystems). The reaction cocktail contained 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 1X MultiScribe and RNase Inhibitor Mix (Applied Biosystems), 1 μM primer 3′UTR1 (5′ CGT ATC CTC TCG TTG GTC TAA GC 3′), 1 μM primer 3′UTR2 (5′ ACA ACA CAC ACT CTA AGG AGA ACT TCT T 3′), 150 nM TaqMan probe 181T (5′ TGG TTC ACG CAT ACG TTA AGC CTC ACT TG 3′) and 5 μl of purified RNA or extracted RNA targets from the immobilized samples.

Real-time RT-PCR protocol consisted of one step at 48°C for 30 min and 95°C for 10 min followed by 45 cycles of amplification (95°C for 15 s and 60°C for 1 min). Data acquisition and analysis were performed with the ABI Prism 7000 software. The default threshold set by the machine was slightly adjusted above the noise to the linear part of the growth curve, at its narrowest point according to the ABI Prism 7000 manufacturers. To determine the theoretical sensitivity and the reliability of the real-time RT-PCR, six repetitions of the assay were undertaken using the 10-fold serial dilution of the previously prepared transcripts.

Aphid species and CTV acquisition assay

Colonies of *A. gossypii* were reared on ‘Celia’ cotton (*Gossypium hirsutum*) plants in environmental growth chambers under controlled conditions [23:16° C (day/night) and a photoperiod of 16/8 h (light/dark)].

To compare CTV detection levels in single aphids by RT-nested PCR and squash real-time RT-PCR methods, both methods were used to analyse fifty aphids feeding on CTV-infected Mexican lime seedlings for 72 h. Furthermore, to evaluate the squash real-time RT-PCR method for detection of CTV-targets present in single aphids, 280 winged individuals from different species captured in the field were tested. These aphids were captured by the sticky shoot method and preserved in 70% alcohol until analysis (Marroquín et al. 2004).

To assess relationships between the duration of the acquisition period and the number of acquired CTV-targets, groups of adult aphids were released on detached CTV-infected leaves after different acquisition access periods: 1, 24, and 48 h. Following the feeding period, a total of 129 aphids were individually squashed on nylon membranes and analysed by squash real-time RT-PCR as previously described. As controls, aphid species were similarly managed using CTV-free plants. Detection rates in each period were statistically analysed using the generalized linear model (McCullagh and Nelder 1989) assuming a binomial distribution. Differences between quantitation levels of acquired CTV-targets obtained in each time-period treatment were analysed using a one-way ANOVA after transforming the response variable by the natural logarithm. For both analyses, planned contrasts were used to compare specific period levels.

Results

Specificity analysis

The designed primers and TaqMan probe were able to recognize all tested CTV sources that included several reference isolates as well as a large number of isolates from Spain, Chile and Egypt. No amplification was obtained from healthy plants used as controls or from plant tissues infected with other citrus virus and viroid agents. *In vitro* assay of available CTV isolates confirmed previous *in silico* specificity analysis performed with CTV sequences from databases.

Sensitivity analysis

Although it was possible to detect as few as 17 transcript copies in three out of the six performed assays, the quantitation range was established from 1.7×10^2 copies up to 1.7×10^9 due to of the reliability of six repetitions (Fig. 1). Amplification efficiencies were calculated for transcripts (1.91) and for field samples (1.89). Comparison of the sensitivities achieved by DAS-ELISA, IC-RT-nested PCR, and conventional (using purified RNA) real-time RT-PCR are shown in Table 1. The serological test was able to detect up to $1{:}10^3$ dilution, whilst IC-RT-nested PCR detected CTV-targets up to $1{:}5\times10^6$ and real-time RT-

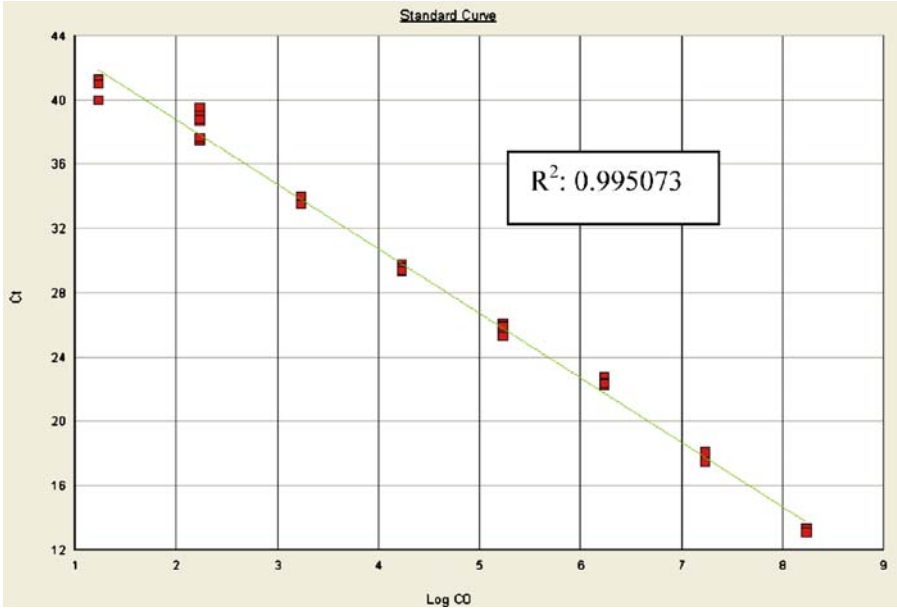

Fig. 1 Quantitation range of the real-time assay based on the standard curve obtained with six repetitions of 10-fold serial dilutions of *Citrus tristeza virus* control transcripts

Table 1 Comparison of DAS-ELISA, immunocapture (IC)-RT-nested PCR in a single closed tube and conventional real-time RT-PCR sensitivities for the detection of *Citrus tristeza virus* (T-388 isolate) in serial dilutions of an infected sweet orange extract in a healthy plant extract

Dilution level	DAS-ELISA values *X*±SE[a]	IC-RT-nested PCR[b]	Real-time RT-PCR (Ct)[e] values *X*±SE[a]
1:20	2,677±49 (v.g.)	++[c]	16.30±0.53
1:100	1,752±64	++	18.30±0.53
1:500	664±20	++	19.00±1.15
1:1,000	371±15	++	21.70±0.53
1:5,000	173±29	++	23.00±1.15
1:10,000	124±26	++	25.00±0.00
1:50,000	129±07	++	26.70±0.53
1:100,000	120±27	+	28.30±0.53
1:500,000	180±22	+	30.00±0.00
1:1,000,000	84±28	+	31.30±0.53
1:5,000,000	131±23	+	33.70±0.53
1:10,000,000	120±15	–[d]	35.00±1.15
1:50,000,000	187±26	–	37.30±0.53
1:100,000,000	116±19	–	39.30±0.53
1:1,000,000,000	134±26	–	39.41, Undet [f]
1:5,000,000,000	154±08	–	Undet.
Healthy Mexican lime	118±04	–	Undet.

[a] *X*: Average of three repetitions using two ELISA wells per assay; *SE*: Standard error. A sample was considered positive if the O.D. (405 nm) measured after 1 h substrate incubation, was double that of the healthy control O.D.

[b] Three repetitions with coincidental results

[c] Number of crosses from ++ to + indicate the relative intensity of electrophoretical band of the amplified products

[d] No amplification

[e] *(Ct)*: Threshold cycle

[f] *Undet*, no fluorescent signal from the lysis of the TaqMan probe was detected by ABI PRISM 7000

PCR up to $1{:}10^9$. CTV-free plant and cocktail controls tested negative by all assayed techniques.

Detection and quantitation of CTV-targets in citrus tissues

Out of the 432 analysed field trees, 235 were found to be CTV infected by tissue print-ELISA, and this status was confirmed by conventional and tissue-print real-time RT-PCR. All CTV control plants tested positive by all assayed techniques. Differences in the number and intensity of immunoprecipitates localized in the vascular area of the imprints, revealed by tissue print-ELISA, were observed among the different plant materials tested. For example, Table 2 shows a selection of imprints ranging from high levels of immunoprecipitates to an absence of them. A comparison was made between the intensity of tissue print-ELISA reaction and the number of CTV copies. The same shoot that had previously been tested by tissue print-ELISA was used to prepare fresh imprints on nylon membranes. The imprints were analysed by tissue-print real-time RT-PCR and the rest of the shoot by conventional real-time RT-PCR. A 'Pineapple' sweet orange control plant inoculated with the T-388 CTV isolate showed the highest accumulation of immunoprecipitates and the lowest Ct values (highest estimation of CTV copies) in both tissue-print and conventional real-time RT-PCR versions. All techniques detected a progressive decrease in the level of CTV infection in the remaining CTV samples analysed. The C-36 'Washington Navel' sweet orange sample showed the lightest (doubtful) presence of immunoprecipitates and the highest Ct values (lowest estimated number of CTV copies). No immunoprecipitates or RNA amplification was observed in the CTV-free Mexican lime control (Table 2).

Conventional real-time RT-PCR was the most sensitive method for CTV detection and quantitation showing lower Ct values and higher quantitation

yields than tissue-print real-time RT-PCR. Conventional real-time RT-PCR estimated about one hundred more CTV copies than tissue-print real-time RT-PCR in the same sample (Table 2).

Sixty analyses were performed by tissue-print real-time RT-PCR in different organs of the same CTV-infected mandarin tree. The lowest average Ct value was obtained from fruit peduncles (24), followed by the columella (25) and young and mature shoots (26). The highest Ct values were obtained from leaves (young and mature fully expanded; 28).

The analysis of one imprint from a sweet orange tree infected with CTV for 15 years, overlapping with a variable number (from 0 to 10) of imprints from a CTV-free tree, gave similar Ct values (average 28). Similarly, when one to 10 CTV-infected imprints from the same shoot of the above-mentioned sweet orange tree were analysed, no remarkable differences in Ct values were observed (data not shown). When positive individually printed sections of plant material developed by tissue print-ELISA were re-tested by tissue-print real-time RT-PCR, it was possible to detect and quantify CTV in 19 out of 24 tested imprints. A decrease in CTV titre, ranging from 10^2 to 10^3 copies, was observed in developed imprints compared with fresh ones from the same stem (data not shown).

Table 2 Comparison between tissue print-ELISA results and quantitation of CTV targets by real-time RT-PCR (tissue-print and conventional)-based methods in sweet orange trees

Sample	Tissue print-ELISA[a]	Real-time RT-PCR			
		Tissue-print (Ct)[b]	Tissue-print (Qty)[d]	Conventional (Ct)	Conventional (Qty)
Sweet orange T-388		27.5 ± 1.9	$3.7 \times 10^6 \pm 1.2 \times 10^6$	22.7 ± 1.0	$6.5 \times 10^8 \pm 1.8 \times 10^8$
Sweet orange E-69		27.9 ± 0.3	$1.4 \times 10^6 \pm 1.0 \times 10^6$	21.4 ± 0.8	$5.0 \times 10^8 \pm 1.6 \times 10^8$
Sweet orange T-397		29.8 ± 0.5	$9.5 \times 10^5 \pm 2.1 \times 10^5$	22.2 ± 0.7	$4.1 \times 10^8 \pm 1.8 \times 10^8$
Sweet orange A-32		34.6 ± 0.3	$4.1 \times 10^4 \pm 7.2 \times 10^3$	28.4 ± 0.8	$9.5 \times 10^6 \pm 2.8 \times 10^6$
Sweet orange C-36		35.7 ± 0.9	$1.9 \times 10^4 \pm 8.7 \times 10^3$	30.6 ± 0.9	$4.5 \times 10^5 \pm 1.3 \times 10^5$
Mexican lime. CTV-free control		Undet.[c]	Undet.	Undet.	Undet.

[a] Progressive decrease of the number and intensity of immunoprecipitates located in the vascular area of the imprinted sections of shoots. Pictures at ×6 magnification

[b] *Ct*, threshold cycle

[c] *Undet*, no fluorescent signal from the lyses of the TaqMan probe was detected by ABI PRISM 7000

[d] *Qty*, number of estimated target copies

CTV-target detection in individual squashed aphids

IC-RT-nested PCR in a single closed tube was able to detect CTV-targets in 12 out of 50 (24%) *A. gossypii* individually squashed on paper, and squash real-time RT-PCR detected CTV-targets in 20 out of 50 (40%) single aphids tested. Fifty-four out of 280 (19.3%) tested field aphids gave positive amplification by squash real-time RT-PCR.

Access period and quantitation of CTV targets in individual aphids

Comparison of the detection rates obtained after different acquisition periods is shown in Table 3. After the shortest access period (1 h) on the infected plant, CTV-targets were detected in 4 out of 40 *A. gossypii* analysed (10.0%). After longer access periods (24 h) CTV-targets were detected in 8 out of 40 analysed aphids (20.0%) and increased to 17 out of 49 analysed aphids (34.7%) after 48 h of acquisition. Significant differences were found between detection rates corresponding to 1 and 48 h of acquisition period. However, no significant differences were observed between 1 and 24 h or between 24 and 48 h of acquisition (Table 3).

Differences in detection rates between treatments were also reflected in the quantitation analysis when short and long acquisition periods were compared (Table 3). At the shortest period the mean value was estimated to be 8,379 copies of acquired CTV-targets. At longer periods the mean values were estimated to

Table 3 Detection and quantitation by squash real-time RT-PCR of CTV targets in *Aphis gossypii* after different acquisition periods

Acquisition periods	Number of analysed aphids	Number of aphids positive/%[a]	Number of CTV targets	Mean±SE[b]
1 h	40	4/10.0 a	4,728	8,379±2,806 a
			5,892	
			6,149	
			16,748	
24 h	40	8/20.0 ab	11,285	35,922±11,146 b
			11,305	
			11,865	
			14,182	
			39,883	
			45,047	
			52,061	
			101,753	
48 h	49	17/34.7 b	3,028	38,280±8,035 b
			4,986	
			5,755	
			7,494	
			12,854	
			17,120	
			17,536	
			19,098	
			41,121	
			43,453	
			45,379	
			48,095	
			49,914	
			54,271	
			68,293	
			88,574	
			123,791	

[a] Means followed by different letters are significantly different using the generalized linear mode statistical analysis

[b] Means followed by different letters are significantly different using a one-way ANOVA after transforming the response variable by the natural logarithm statistical analysis

be 35,922 copies at 24 h and 38,280 copies at 48 h. According to the mean number of CTV-targets quantified, significant differences were observed when 1 h treatment was compared to 24 h treatment (P=0.0457) and to 48 h treatment (P=0.0369). However, when longer treatments (24 and 48 h) were compared, no significance difference was observed (P=0.885) in the number of acquired CTV-targets.

Discussion

This is the first report of sensitive and universal detection and quantitation of CTV targets in plant tissues and individual aphids by TaqMan real-time RT-PCR using conventional and improved versions of sample preparation. TaqMan chemistry was reported as very sensitive, specific, reliable, easy-to-perform and applicable to high throughput testing (Olmos et al. 2005; Osman et al. 2007). Furthermore, Gunson and Carman (2005) and Olmos et al. (2004) compared TaqMan with SYBR Green chemistries and reported that it had the highest detection limits of the target whilst providing the highest specificity. The successful recognition of all CTV isolates tested from different origins by the newly designed primers (3′ UTR1/3′UTR2) and TaqMan probe (T181) designed according to the sequence of the most conserved region of the CTV genome, demonstrated that real-time RT-PCR is capable of universal CTV detection. No cross-reaction was obtained with other graft-transmitted citrus pathogens or with healthy plant material.

Real-time RT-PCR consistently detected as few as 17 targets (after 40–41 cycles) of purified CTV transcripts (R^2=0.995) (Fig. 1). These results are in agreement with the data obtained with purified total RNA from serial dilutions of a plant extract. Moreover, this is supported by similar efficiencies of amplification obtained from both transcripts and plant extracts. Real-time RT-PCR using purified RNA was the most sensitive technique when compared with other techniques that directly use crude plant extracts. Conventional real-time RT-PCR was 100 to 500 times more sensitive than IC-RT-nested PCR and 10^6 times more sensitive than DAS-ELISA. Due to this high sensitivity, real-time RT-PCR could detect the virus and other viral targets in samples that tested CTV negative by other techniques (Tables 1 and 2).

Extraction buffer containing Triton X-100 proved efficient with good reproducibility in detecting PPV and other viruses; however, it did not give consistent results in the case of CTV. The Osman and Rowhani (2006) buffer solution, gave the best yield, efficiency and reproducibility for CTV detection and quantitation probably because it preserves the integrity of the nucleic acids and removes more efficiently PCR inhibitors present in the citrus plant extracts.

When comparing tissue print-ELISA (number and intensity of observed immunoprecipitates) and tissue-print real-time RT-PCR (Ct values) for the quantitative detection of CTV in plant material (Table 2), a good correlation was observed between the two techniques, although tissue-print real-time RT-PCR was more consistent than tissue print-ELISA. The serological method detects only CTV coat proteins, whilst the molecular method detects RNA targets. In fact, in spite of the doubtful presence of CTV immunoprecipitates in sample C-36 (Table 2), real-time RT-PCR gives unquestionable Ct values. This demonstrates the usefulness of tissue-print real-time RT-PCR for the detection and/or quantitation of CTV targets. Nucleic acid extraction and crude extract preparation steps are not required providing a rapid and friendly analysis and saving costs. In addition, prints can be stored at room temperature without decreasing detection sensitivity, thus facilitating detection/quantitation of the CTV targets immobilized on paper for a long time, and reducing risks in quarantine sample management.

A reliable detection of CTV is achieved by tissue print-ELISA using 5 shoots or ten leaves collected around the canopy of an adult tree. This has been demonstrated even in recently infected trees with an uneven distribution of the virus (Cambra et al. 2002; EPPO 2004). Consequently, a good estimation of the number of CTV targets should be possible analysing up to ten imprints of an adult tree by tissue-print real-time RT-PCR. This is supported by the results of the assays performed using one or up to ten shoot sections imprinted on the same piece of membrane. In fact CTV was detected in all instances and gave similar Ct values when one to 10 imprints from the same stem were analysed. In the same way, the number of CTV-free imprints did not affect the Ct values obtained: similar results were achieved when analysing one CTV positive imprint alone or together with 10 imprints from a CTV-free plant, independent-

ly of the order of printing of the positive. In an adult CTV-infected tree, the lowest average Ct value was obtained from fruit peduncles as previously reported using ELISA techniques (Garnsey and Cambra 1993).

The detection of RNA from CTV in previously processed membranes by tissue print-ELISA suggests that quantification of CTV targets that have been immobilized on paper for a long time, is feasible. The detected targets are probably encapsidated RNA from immobilized CTV virions previously detected by the antibodies used in tissue print-ELISA. However, the Ct values from the developed membranes were always higher than those obtained from fresh imprints, probably due to inhibition and RNA degradation. With this system is possible to confirm serological results by molecular and quantitative techniques when fresh imprints are not available.

Plants infected with CTV often contain one or several populations of dRNAs and approximately 30 different subgenomic RNAs (Gowda et al. 2001). The molecular size of the dRNAs ranges from about 2 to 12 kb (Che et al. 2002). These large dRNAs can be encapsidated and are able to replicate independently in protoplasts, and presumably in plant cells, and are transmitted by mechanical and graft-inoculation (Che et al. 2002; 2003). Conventional real-time RT-PCR detects and quantifies total CTV RNA, including genomic RNA, dRNAs, subgenomic RNAs and double-stranded RNA (dsRNA). This is an advantage for detection purposes but a drawback for 'absolute' quantitation of encapsidated virions. However, print or squash real-time RT-PCR probably only detects genomic RNA from virions, encapsidated large dRNA and some stable dsRNAs. The remaining of the RNA population is probably degraded by RNases present in the plant extract, in the sections of plant material or in squashes when immobilized in porous membranes.

Studies ranging from virus replication processes to epidemiological studies (Fabre et al. 2003; Olmos et al. 2005) can benefit from the simultaneous detection and quantitative determination of targets carried in a single aphid presented here. Squash real-time RT-PCR using TaqMan probes is gaining acceptance as a sensitive method because it allows a large range of target quantitation in aphids (Fabre et al. 2003; Olmos et al. 2005; Cambra et al. 2006). Comparison between RT-nested PCR and real-time RT-PCR for the detection of CTV in single squashed *A. gossypii* confirmed that squash real-time RT-PCR is more sensitive than RT-nested PCR and it is a suitable and valuable tool for monitoring CTV in aphids.

The percentage of detection (34.7%) of CTV targets in *A. gossypii* by squash real-time RT-PCR after long acquisition periods under controlled feeding conditions is similar to the percentage (34%) reported by Marroquín (2004). Obviously, the percentage of CTV detection in field samples depends on the CTV prevalence in the monitored area. Consequently, in order to use this methodology to estimate viral load of aphids under field conditions, a large number of aphids should be caught and analysed over several years to obtain an accurate estimation.

Comparison of different periods of virus acquisition revealed that at long acquisition periods (24 to 48 h), high numbers of CTV-viruliferous *A. gossypii* are detected (34.7%). Likewise, this trend is found in terms of the titre or quantity of CTV targets acquired by a single aphid (as high as 123,791 in a single case, average of 38,280±8,035 after 48 h). Differences in CTV target detection observed at different access periods could contribute to understanding the CTV transmission mechanism. CTV can be acquired after a short accession period on an infected plant, but high rates of acquisition are only achieved when aphids feed for a long time, as it is more likely that the aphid reaches the phloem. According to this hypothesis, Limburg et al. (1997) suggested that *Beet yellows virus* (BYV), another *Closterovirus*, is acquired from sieve elements by aphids during phloem ingestion phase, correlating with the average time needed to reach the sieve elements with the minimum time needed for virus acquisition (20 min). However, the possibility cannot be excluded that the virus may also be acquired from non-phloem tissues. In this way, CTV acquisition could be similar to the acquisition of *Cauliflower mosaic virus* (CaMV), another semi-persistently transmitted virus. CaMV can be acquired from non-phloem tissues, but the probability of acquisition is significantly higher when aphids reach the committed phloem ingestion (Palacios et al. 2002).

CTV spread depends on vector activity, aphid species behaviour, inoculum pressure (CTV prevalence) and the predominant citrus species present in a given area. To determine the major aphid species involved in spreading viruses, both vector propensity (rate of transmission under field conditions) and vector activity (number of aphids landing on the crop) should be taken into consideration (Irwin and

Ruesink 1986). If CTV targets can be detected and quantified in its vectors, then relationships can be established between the presence of an aphid species in the field and the spread of the virus. The high levels of CTV detected by real-time RT-PCR in *A. gossypii*, together with its population dynamics in the field, confirmed that *A. gossypii* is primarily responsible for spreading the virus in citrus orchards in the Mediterranean area (Marroquín et al. 2004).

The sensitivity, specificity, feasibility and reliability of tissue-print and squash real-time RT-PCR for detection and quantitation of CTV in immobilized plant materials and in single aphid species without the need of an immunocapture phase have been demonstrated. In addition, this technology has been validated and could be used to prepare and process large numbers of samples using microtiter plates. Immobilized material can be used when dealing with quarantine pathogens without risks and it is a direct sample preparation system without extract preparation or RNA purification. The reliable and sensitive universal detection and quantitation of CTV are also critical in applied research, such as epidemiological studies and quarantine stations and citrus nurseries, which must certify the virus-free status of plant materials.

Acknowledgements This work was supported by the INIA RTA2005-00190 (IVIA 1606) project. E. Bertolini and A. Moreno are recipients of a Juan de la Cierva contract from Ministerio de Educación y Ciencia of Spain. A. de Luis was the recipient of a fellowship grant from IVIA (Generalidad Valenciana). Dr. E. Carbonell assisted in statistical analysis. Dr. N. Duran-Vila critically read the manuscript. English text was revised by F. Barraclough.

References

Ballester-Olmos, J. F., Pina, J. A., Carbonell, A., Moreno, P., Hermoso de Mendoza, A., Cambra, M., & Navarro, L. (1993). Biological diversity of citrus tristeza virus (CTV) isolates in Spain. *Plant Pathology, 42*, 219–229.

Bar-Joseph, M., Marcus, R., & Lee, R. F. (1989). The continuous challenge of citrus tristeza virus control. *Annual Review of Phytopathology, 27*, 291–316.

Cambra, M., Ballester-Olmos, J. F., Pina, J. A., Laviña, A., & Camarasa, E. (1989). Distinction of populations infected with severe and common strains of *Citrus tristeza virus* in Spain, by ELISA-DASc (quantitative). *Fruits, 44*, 335–341.

Cambra, M., Bertolini, E., Olmos, A., & Capote, N. (2006). Molecular methods for detection and quantitation of virus in aphids. In I. Cooper, T. Kuhne, & V. Polischuk (Eds.), *Virus diseases and crop biosecurity* (pp. 81–88). Dordrecht: Springer.

Cambra, M., Garnsey, S. M., Permar, T. A., Henderson, C. T., Gumpf, D. J., & Vela, C. (1990). Detection of *Citrus tristeza virus* (CTV) with a mixture of monoclonal antibodies. *Phytopathology, 80*, 1034.

Cambra, M., Gorris, M. T., Marroquín, C., Román, M. P., Olmos, A. Martínez, M. C., et al. (2000a). Incidence and epidemiology of *Citrus tristeza virus* in the Valencian Community of Spain. *Virus Research, 71*, 85–95.

Cambra, M., Gorris, M. T., Olmos, A., Martínez, M. C., Román, M. P., Bertolini, E., et al. (2002). European Diagnostic Protocols (DIAGPRO) for *Citrus tristeza virus* in adult trees. In N. Duran-Vila, R. G. Milne, & J. V. da Graça (Eds), *Proceedings of 15th Conference of the International Organization of Citrus Virologists, IOCV* (pp. 69–77), Riverside.

Cambra, M., Gorris, M. T., Román, M. P., Terrada, E., Garnsey, S. M., Camarasa, E., et al. (2000b). Routine detection of citrus tristeza virus by direct Immunoprinting–ELISA method using specific monoclonal and recombinant antibodies. In J. V. da Graça, R. F. Lee, & R. K. Yokomi (Eds), Proceedings of 14th Conference of the International Organization of Citrus Virologists, IOCV (pp. 34–41). Riverside.

Cambra, M., Hermoso de Mendoza, A., Moreno, P., & Navarro, L. (1981). Use of enzyme linked immunosorbent assay (ELISA) for detection of citrus tristeza virus (CTV) in different aphid species. Proceedings of 4th International Society of Citriculture, pp. 444–448, Japan.

Cambra, M., Olmos, A., & Dasi, M. A. (1996). Procedimiento para la preparación de dianas para la reacción en cadena de la polimerasa (PCR). Spanish Patent. Registro de la Propiedad Industrial P9601155/6 del 24 de mayo de 1996.

Cambra, M., Olmos, A., Gorris, M. T., Marroquín, C., Esteban, O., Garnsey, S. M., et al. (2000c). Detection of *Citrus tristeza virus* by print-capture and squash capture-PCR in plant tissues and single aphids. In J. V. da Graça, R. F. Lee, & R. K. Yokomi (Eds), Proceedings of 14th Conference of the International Organization of Citrus Virologists, IOCV (pp. 42–49). Riverside.

Che, X., Dawson, W. O., & Bar-Joseph, M. (2003). Defective RNAs of *Citrus tristeza virus* analogous to *Crinivirus* genomic RNAs. *Virology, 310*, 298–309.

Che, X., Mawassi, M., & Bar-Joseph, M. (2002). A novel class of large and infectious defective RNAs of citrus tristeza virus. *Virology, 298*, 133–145.

EPPO (2004). Diagnostic protocol for regulated pests. *Citrus tristeza closterovirus*. *Bulletin OEPP/EPPO Bulletin, 34*, 155–157. http://www.eppo.org/QUARANTINE/virus/Citrus_tristeza/pm7-31(1)%20CTV000%20web.pdf)

Fabre, F., Kervarrec, C., Mieuzet, L., Riault, G., Vialatte, A., & Jacquot, E. (2003). Improvement of Barley yellow dwarf virus-PAV detection in single aphids using a fluorescent real time RT-PCR. *Journal of Virological Methods, 110*, 51–60.

Garnsey, S. M., & Cambra, M. (1993). Enzyme-linked immunosorbent assay (ELISA). In G. P. Martelli (Ed.), *Graft transmissible diseases of grapevines* (pp. 169–192). Roma: FAO, Distribution and Sales Section.

Gottwald, T. R., Cambra, M., Moreno, P., Camarasa, E., & Piquer, J. (1996). Spatial and temporal analyses of citrus tristeza virus in Eastern Spain. *Phytopathology, 86*, 45–55.

Gowda, S., Satyanarayana, S., Ayllón, M. A., Albiach-Marti, M. R., Mawassi, M., Rabindran, S., et al. (2001). Characterization of the cis-acting elements controlling subgenomic mRNAs of citrus tristeza virus: Production of positive- and negative-stranded 3′-terminal and positive-stranded 5′-terminal RNAs. *Virology, 286*, 134–151.

Gunson, R. N., & Carman, W. F. (2005). Comparison of two real-time PCR methods for diagnosis of *Norovirus* infection in outbreak and community settings. *Journal of Clinical Microbiology, 43*, 2030–2031.

Hilf, M. E., & Garnsey, S. M. (2000). Characterization and classification of citrus tristeza virus isolates by amplification of multiple molecular markers. In J. V. da Graça, R. F. Lee, & R. K. Yokomi (Eds.), Proceedings of 14th Conference of the International Organization of Citrus Virologists, IOCV (pp. 18–27). Riverside.

Irwin, M. E., & Ruesink, W. G. (1986). Vector intensity: a product of propensity and activity. In G. D. McLean, R. G. Garrett, & W. G. Ruesink (Eds.), *Plant virus epidemics: Monitoring, modelling and predictions outbreaks* (pp. 13–33). Sydney.

Lee, R. F., & Bar-Joseph, M. (2000). Tristeza. In L. W. Timmer, S. M. Garnsey, & J. H. Graham (Eds.), *Compendium of citrus diseases* (pp. 61–63). Saint Paul, MN: APS.

Limburg, D. D., Mauk, P. A., & Godfrey, L. D. (1997). Characteristics of beet yellows closterovirus transmission to sugar beets by *Aphis fabae*. *Phytopathology, 87*, 766–771.

Marroquín, C. (2004). Evaluación de la fauna de vectores potenciales del virus de la tristeza de los cítricos (CTV), detección en pulgones individuales (Hemiptera, Aphididae), comparación con la transmisión e importancia epidemiológica. Ph.D. Thesis, Universidad Politécnica de Valencia. 87 P.

Marroquín, C., Olmos, A., Gorris, M. T., Bertolini, E., Martínez, M. C., Carbonell, E., et al. (2004). Estimation of the number of aphids carrying *Citrus tristeza virus* that visit adult citrus trees. *Virus Research, 100*, 101–108.

McCullagh, P., & Nelder, J. A. (1989). *Generalized linear models*. London. Chapman & Hall.

Mehta, P., Brlansky, R. H., Gowda, S., & Yokomi, R. K. (1997). Reverse transcription polymerase chain reaction detection of citrus tristeza virus in aphids. *Plant Disease, 81*, 1066–1069.

Narváez, G., Ben Slimane, S., Ayllón, M. A., Rubio, L., Guerri, J., & Moreno, P. (2000). A new procedure to differentiate *Citrus tristeza virus* isolates by hybridisation with digoxigenin-labelled cDNA probes. *Journal of Virological Methods, 85*, 83–92.

Nolasco, G., de Blas, C., Torres, V., & Ponz, F. (1993). A method combining immunocapture and PCR amplification in a microtiter plate for the routine diagnosis of plant viruses and subviral pathogens. *Journal of Virological Methods, 45*, 201–218.

Olmos, A., Bertolini, E., Gil, M., & Cambra, M. (2004). Real-time RT-PCR for quantitative detection of *Plum pox virus*. *Acta Horticulturae, 657*, 149–153.

Olmos, A., Bertolini, E., Gil, M., & Cambra, M. (2005). Real-time assay for quantitative detection of non persistently transmitted *Plum pox virus* RNA targets in a single aphid. *Journal of Virological Methods, 128*, 151–155.

Olmos, A., Cambra, M., Dasí, M. A., Candresse, T., Esteban, O., Gorris, M. T., et al. (1997). Simultaneous detection and typing of plum pox potyvirus (PPV) isolates by hemi-nested-PCR and PCR-ELISA. *Journal of Virological Methods, 68*, 127–137.

Olmos, A., Cambra, M., Esteban, O., Gorris, M. T., & Terrada, E. (1999). New device and method for capture, reverse transcription and nested PCR in a single closed tube. *Nucleic Acids Research, 27*, 1564–1565.

Olmos, A., Dasí, M. A., Candresse, T., & Cambra, M. (1996). Print capture PCR: a simple and highly sensitive method for the detection of plum pox virus (PPV) in plant tissues. *Nucleic Acids Research, 24*, 2192–2193.

Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2007). Real-time (TaqMan) assays for the detection of *Grapevine leafroll associated viruses* 1–5 and 9. *Journal of Virological Methods, 141*, 22–29.

Osman, F., & Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods, 133*, 130–136.

Palacios, I., Drucker, M., Blanc, S., Leite, S., Moreno, A., & Fereres, A. (2002). *Cauliflower mosaic virus* (CaMV) is preferentially acquired from the phloem by its aphid vectors. *Journal of General Virology, 83*, 3163–3171.

Rasmussen, R. (2001). Quantification on the LightCycler instrument. In S. Meuer, C. Wittwer, & K. Nakagawara (Eds.), *Rapid cycle real-time PCR: Methods and applications* (pp.21–34). Heidelberg: Springer.

Román, M. P., Cambra, M., Juárez, J., Moreno, P., Durán-Vila, N., Tanaka, F. A. O., et al. (2004). Sudden death of citrus in Brazil: A graft transmissible, bud union disease. *Plant Disease, 88*, 453–467.

Vela, C., Cambra, M., Cortés, E., Moreno, P., Miguet, J., Pérez de Román, C., et al. (1986). Production and characterization of monoclonal antibodies specific for the tristeza virus and their use for diagnosis. *Journal General Virology, 67*, 91–96.

Yokomi, R. K., Garnsey, S. M., Civerolo, E. L., & Gumpf, D. (1989). Transmission of exotic citrus tristeza isolates by a Florida colony of *Aphis gossypii*. *Plant Disease, 73*, 552–556.