ORIGINAL ARTICLE

Improved Detection of *Citrus psorosis virus* and Coat Protein-Derived Transgenes in Citrus Plants: Comparison Between RT-qPCR and TAS-ELISA

Agustina De Francesco[1], Norma Costa[2], María I. Plata[2] and María L. García[1]

1 Instituto de Biotecnología y Biología Molecular, CCT-La Plata, CONICET – UNLP, La Plata, Argentina
2 Estación Experimental Agropecuaria Concordia (EEA-Concordia), INTA, Entre Ríos, Argentina

Keywords
Citrus psorosis virus, diagnosis, RT-qPCR, TAS-ELISA, transgenic citrus

Correspondence
María L. García, Instituto de Biotecnología y Biología Molecular, CCT-La Plata, CONICET – UNLP, La Plata, Buenos Aires, Argentina.
E-mail: garcia_m@biol.unlp.edu.ar

Received: December 10, 2014; accepted: March 15, 2015.

doi: 10.1111/jph.12392

Abstract

Citrus is one of the most economically important fruit crops in the world. Citrus psorosis is a serious disease affecting mainly oranges and mandarins in Argentina and Uruguay. The causal agent is *Citrus psorosis virus* (CPsV), an ophiovirus with a tripartite ssRNA genome of negative polarity. The coat protein (CP), the most abundant viral protein in infected plants, has been used to detect CPsV by TAS-ELISA, but only biological indexing, requiring 1 year, is the current and validated technique for diagnosis of citrus psorosis. In this study, a SYBR Green RT-qPCR protocol was developed, with primers designed to the most conserved region of the cp gene. We tested their specificity and sensitivity in comparison with TAS-ELISA. This RT-qPCR was applied successfully to field samples from Argentina, to a variety of isolates from different countries maintained in the greenhouse, to young seedlings and old trees from a psorosis natural transmission plot, and to transgenic citrus expressing the *cp* gene of CPsV or a fragment thereof. This method allowed accurate quantification of viral titer and *cp* gene expression in transgenic plants, which could not be detected previously. The sensitivity and reliability of quantitative CPsV detection were improved with greater speed using commercial reagents, and the sensitivity was three orders of magnitude higher than that of TAS-ELISA. All these data encourage its validation.

Introduction

Citrus is one of the most important fruit crops in Argentina, and this country is one of the leaders in citrus exports, mainly lemons. Citrus plants are produced in two regions located in the north-east (NE) and north-west (NW) of Argentina, each with different climates and cultivars, with oranges and mandarins being the most important cultivars in the NE and grapefruit and lemon in the NW. All citrus species and citrus relatives used as rootstocks are affected by citrus psorosis, one of the oldest viral and graft-transmissible diseases (Fawcett 1932). Psorosis was a serious disease in many countries for several decades (Roistacher 1993). Although eradicated from Europe, it is still important in Argentina and Uruguay, due to its natural spread by an unidentified vector (Beñatena and Portillo 1984), in Texas, USA (Gottwald et al. 2005), and more recently, the disease has been reported in countries where psorosis has never been detected before such as Cuba, Egypt, Turkey and Japan (Velazquez et al. 2005; Ghazal et al. 2008; Kayim 2010; Ito et al. 2011).

Psorosis has been endemic for years in the NE of Argentina, reducing fruit production and causing economic losses (Diamante de Zubrzycki et al. 1984). Despite a successful certification programme implemented in the NE region, psorosis still occurs, and more recently, CPsV has been detected in NW Argentina (Zanek et al. 2006).

In the field, psorosis symptoms can be observed in 10- to 15-year-old trees, which could have been infected at an early age but not detected. Characteristic psorosis symptoms are bark scaling and gum accumulation in the trunk, flecking, spots and shock in young shoots.

The progress of the infection and the onset of these symptoms are very slow, but after several years, gummosis on the trunk causes obstruction of the vascular bundles and general decay, and triggers death. This fact emphasizes the need for a rapid method of diagnosis, before the disease has progressed.

Once psorosis had been graft-transmitted, a biological index, using young sweet orange seedlings as indicator plants, was developed (Roistacher 1991). Essentially, this index involved grafting of sweet orange seedlings with a bark segment of the plant and an observation period of 1–6 months to evaluate the symptoms (Roistacher 1991). Two kinds of psorosis have been described by graft transmission: A and B. Psorosis A shows leaf symptoms as flecking and spots, and shock reaction in very young shoots, in some isolates. Psorosis B, more aggressive than psorosis A, is characterized by bark scaling even on twigs, and gummosis and pustules on the underside of leaves (Roistacher 1993). Biological indexing is very efficient and sensitive, and therefore, it has been used for decades in certification programmes. However, it is costly, takes a long time and requires specialized staff. For those reasons, alternative methods of detection and diagnosis have arisen, which were developed after the causal agent was characterized (García et al. 1991, 1997).

Citrus psorosis is caused by *Citrus psorosis virus* (CPsV), the type member of the genus *Ophiovirus*, *Ophioviridae* family. The particles are filamentous and circular (García et al. 1994; Milne et al. 2000), and its genome has three single-stranded RNAs of negative polarity, encapsidated by a coat protein (CP) (Sánchez de la Torre et al. 1998). RNA 1 encodes the replicase and the 24K protein (Naum-Onganía et al. 2003). RNA 2 encodes the movement protein (Robles Luna et al. 2013), and RNA 3 encodes the CP (Sánchez de la Torre et al. 1998). The sequence of several isolates has been obtained and used for molecular studies and detection of CPsV (Legarreta et al. 2000; Martín et al. 2006).

The CP is the most abundant viral protein in infected tissue (García et al. 1991), and based on this fact, serological methods were developed for diagnosis. DAS-ELISA (García et al. 1997) and TAS-ELISA (Alioto et al. 1999) were applied with polyclonal and monoclonal antibodies obtained against the CP of CPsV, detecting the virus in field samples. Using the same antibodies, a modified TAS-ELISA protocol (TAS-ELISA-HRP) was optimized, and samples from different geographic locations of Argentina and transgenic citrus transformed with the coat protein gene were analysed, resulting in improved sensitivity and faster analysis (Zanek et al. 2006, 2008). RT-PCR has been an alternative method to detect CPsV. Specific primers designed from the RNA1 (García et al. 1997) and from the CP (Barthe et al. 1998) were applied, and later, heminested RT-PCR assay targeted to a conserved region of RNA1 was used to detect Argentinian isolates (Legarreta et al. 2000). All these methods are less expensive, faster and more reliable than biological indexing. In this sense, Martín et al. (2002) reported a comparison among biological indexing, hybridization probes, RT-PCR and serological techniques, concluding that ELISA was the most sensitive and universal method.

We have generated transgenic sweet orange plants expressing the *cp* gene of CPsV (Zanek et al. 2008). In these plants, the CP is expressed at very low levels, which has been difficult to detect by TAS-ELISA (Zanek et al. 2008). Currently, the CP in these transgenic plants is not detectable, probably due to the lower polyclonal antibody titer obtained by Alioto et al. (1999) or to lower *cp* expression in adult than in young citrus, as they were assayed in 2008. Citrus transgenic lines expressing hairpin transcript-derived CPsV *cp* gene, which could not be analysed by TAS-ELISA, were also generated in our laboratory requiring an alternative detection method.

RT-qPCR is a powerful diagnostic method widely applied to many pathogens and crops, including woody plants such as citrus and citrus relatives. Recently, many studies have shown the reliability of this tool even for the simultaneous detection of several viruses in citrus (Loconsole et al. 2010; Lin et al. 2013; Saponari et al. 2013). However, in the case of psorosis, this technique has not been compared to TAS-ELISA, which is the most universal method used to date.

In this study, a new RT-qPCR protocol was developed using SYBR Green and specific primers designed from the *cp* gene sequence conserved in all available isolates deposited in databases. Moreover, we compared TAS-ELISA and RT-qPCR applied to field samples from Argentina, isolates maintained in the greenhouse, seedlings from a psorosis natural transmission plot and transgenic plants. We have improved CPsV detection and performed virus quantification, contributing to disease control, evaluation of transgenic plants, and as a diagnostic test in citrus certification programmes.

Materials and Methods

Samples

Samples of CPsV isolates were taken from the collection of citrus pathogens from the National Institute of Agriculture Technology (INTA), Experimental Station of Concordia (EEA-Concordia), Entre Ríos, Argentina, maintained under greenhouse conditions. All available information about the CPsV isolates is detailed in Table 1 and previous publications (Roistacher et al. 2000; Martín et al. 2006).

Samples from 44 trees from a psorosis natural transmission plot (L504) located at the EEA-Concordia were collected during the spring of 2012 and 2013. This plot, planted 35 years ago, contained 504 healthy Pineapple and common sweet oranges, and a row of citrus affected by psorosis was adjacent, as an inoculum, to evaluate natural spread and aerial transmission of psorosis (Beñatena and Portillo 1984; Portillo and Beñatena 1989). Samples from ten additional CPsV-free seedlings recently located near infected adult trees were analysed. Transgenic citrus plants maintained at the EEA-Concordia were included in this work. Leaf samples from transgenic Pineapple sweet orange CP-18 line (Zanek et al. 2008), expressing the complete *cp* gene of CPV4, and ihpCP-10 and ihpCP-15 lines, expressing a hairpin construct with a fragment of the *cp* gene of CPsV 90-1-1 isolate, (Reyes et al. 2011) were tested. Leaf samples from CPsV 90-1-1-infected Pineapple sweet orange and non-transgenic, non-inoculated plants were used as positive and negative controls, respectively. For each sample, four to five representative leaves of each plant were collected.

Samples of lettuce (*Lactuca sativa*) infected with *Mirafiori lettuce big-vein virus* (MiLBVV), another member of the *Ophioviridae* family, citrus samples infected with other viruses such as *Citrus tristeza virus* (CTV) or the unidentified virus that causes concave gum, and citrus infected with bacteria, *Xanthomonas citri* subsp. *citri* that causes citrus canker and *Xylella fastidiosa* that causes citrus variegated chlorosis, were included to determine specificity of the assay (Table 2).

Sequence analysis and primer design

To design specific primers for the *cp* gene of CPsV, a multiple alignment of all available RNA3-CPsV sequences was performed with the algorithm ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2) (Fig. 1). Sequences included were as follows: CPsV 90-1-1 (Accession number FJ495195) from Argentina, CPV4 (AF060855) from the USA, NA63 (AY194898) from Italy and P-121 (AY654894.1) from Spain. From several primers designed in the 5′ conserved region of the *cp* gene, according to the optimization of the RT-qPCR parameters, primers selected for retro-transcription and amplification were CP1c (forward): 5′ GTTCAA GATGGAGCAAGTTGATGG 3′, and CP3 (reverse): 5′ GAGACCCTTGTGTAAAAACCAGCAC 3′ amplifying a fragment of 113 nucleotides (nt). As a reference gene, the ubiquitin transcript GU362416.1 from *Citrus sinensis* was selected, and new primers were designed: ubqtL

Table 1 Plant samples infected with *Citrus psorosis virus* (CPsV) isolates used in this study

CPsV isolate	Isolated from	Maintained in	Origin	Psorosis type	Symptoms
90-1-1	Trifoliate orange (*Poncirus trifoliata* L. Raf.)	Pineapple sweet orange (PSwO) (*Citrus sinensis* L. Osb.) and rough lemon (*C. lemon jambhiri* Lush.)	Argentina	A	F, S, Sh
112-1	Willowleaf mandarin (*Citrus deliciosa* Tenore)	PSwO	Argentina	A	F, S, Sh
CPV4	PSwO	PSwO	USA	A	F, S
P-121	PSwO	PSwO	Spain	A	F, S
173-22	Ellendale mandarin (*Citrus reticulata* Blanco)	PSwO	Argentina	–	Sh, F, M, V
189-34	PSwO	PSwO	Argentina	B	G
P-250	PSwO	PSwO	USA	B	BS, B
504-3	PSwO	PSwO	Argentina	A	Sh, F, BS
248-2	Satsuma mandarin (*Citrus unshiu* Marcovitch)	PSwO	Argentina	A	F, S, Sh

Symptoms found upon characterization of isolates. F, flecking; S, spots on young leaves; Sh, shock in young shoots; M, mottle; V, variegation; G, gum in mature leaves and twigs; BS, bark scaling on trunk; B, blisters.

Table 2 Specificity. Detection of CPsV by RT-qPCR in citrus samples infected with other pathogens

Plant host	Origin	Disease/causal agent	Psorosis diagnosis by RT-qPCR
Lettuce	La Plata, Argentina (field)	Big-vein/MiLBVV[a]	–
Pineapple sweet orange	Concordia, Argentina (greenhouse)	Tristeza/CTV (126-13 isolate)[b]	+
Key lime	Concordia, Argentina (greenhouse)	Tristeza/CTV (268-2 isolate)[c]	–
Salustiana orange	Concordia, Argentina (field)	Citrus variegated chlorosis (CVC)/*Xylella fastidiosa*[d]	–
Pineapple sweet orange	USA (greenhouse)	Concave gum (CG-301)[e]	–
Valencia orange	Concordia, Argentina (field)	Citrus canker/*Xanthomonas citri* subsp. *citri* (1)[f]	+
Valencia orange	Concordia, Argentina (field)	Citrus canker/*X. citri* subsp. *citri* (2)[f]	+
Valencia orange	Concordia, Argentina (field)	Citrus canker/*X. citri* subsp. *citri*[f]	–
Ray Ruby grapefruit	Concordia, Argentina (field)	Citrus canker/*X. citri* subsp. *citri* (3)[f]	–

RT-qPCR (+) when Ct ≤ 30.

[a]Leaf sample with symptoms of big-vein and MiLBVV positive by RT-PCR.

[b]Leaf sample extracted from a Pineapple sweet orange field tree biologically tested positive for CTV.

[c]Leaf sample extracted from a field tree biologically tested positive for CTV, and by molecular analysis performed by Iglesias et al. (2008).

[d]Leaf sample extracted from a field tree tested positive for CVC by DAS-ELISA (Agdia®).

[e]Leaf sample extracted from a field tree biologically tested positive for concave gum and maintained in greenhouse by inoculation in indicator citrus, which currently used as positive control for concave gum (Roistacher et al. 2000).

[f]Leaf sample extracted from field trees defined positive for canker by symptomatology. (1), (2) and (3) refer to three different field trees.

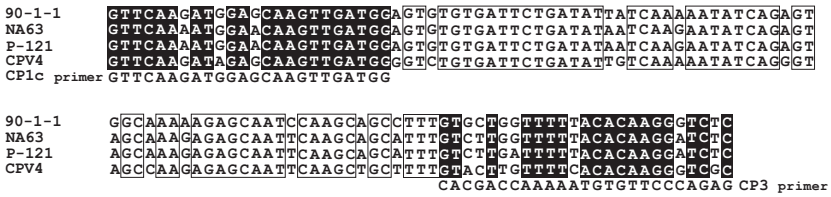

Fig. 1 Alignment of GenBank sequences of CPsV isolates from Argentina, Italy, Spain and USA in the region amplified by the CP1c/CP3 primers. Homology in the primer region is indicated by black boxes. White boxes indicate homology in the remaining sequence.

(forward): 5′ TCTTCACCTCGTGCTTCGTCTCCGT 3′, and ubqtR (reverse): 5′ GTCCTGGATCTTGGCCTT GACGTTG 3′ amplifying a fragment of 127 nt.

RNA isolation and cDNA synthesis

Total RNA was prepared from 200 mg of fresh leaf tissue ground in liquid nitrogen, mixed with 1 ml of TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and processed according to the manufacturer's instructions. RNA extracts were resuspended in 25 μl of RNase-free water and quantified by absorbance at 260 nm. Two micrograms (μg) of total RNA in a volume approximately 0.5–5 μl of RNA preparation were digested with RQ1 RNase-free DNase (PROMEGA, Madison, WI, USA) for 1 h at 37°C in a final volume of 10 μl, following the manufacturer's specifications. After DNase treatment, 5 μl of the preparations (1 μg of total RNA) were subjected to retrotranscription (RT) reaction with MMLV Reverse Transcriptase (PROMEGA) in a final volume of 25 μl, using specific or reference primers, dNTPs, buffer and enzyme at the concentrations specified by the manufacturer. Then, 0.3 μl (12 units) of RiboLock RNase Inhibitor (Thermo Scientific, Waltham, MA, USA) per reaction was added and incubated 1 h at 42°C. Aliquots of 1 μl of cDNA were used as the RT-qPCR template. To confirm the efficiency of DNase treatment, qPCR was performed without previous cDNA synthesis and considered as a negative control of RT-qPCR assays.

RT-qPCR assay development

SYBR Green RT-qPCR was performed in the Fluorescent Quantitative Detection System FQD-48A (BIOER, Hangzhou, China). The reactions were performed in a total volume of 20 μl using 5 × HOT FIREPol® EvaGreen® qPCR Mix (SOLIS Biodyne, Tartu, Estonia). The amplification conditions were as

follows: one cycle of 5 min at 95°C followed by 44 amplification cycles of 95°C for 20 s, 56°C for 30 s and 72°C for 20 s, and one final elongation step of 72°C for 5 min. The melting curve was performed from 72 to 90°C. The optimal concentration for both cp and ubqt primers was 250 ηM. Except for the calibration and serial dilution curves (samples assayed in triplicate), all samples were conducted in duplicate and the fluorescence values averaged. Data were analysed with the software built into the device, Linegene k FQD-48A (BIOER). Final products were checked by electrophoresis on 2.0% agarose gel stained with ethidium bromide. The amplified DNA fragments (CP1c/CP3 amplicon) obtained from different Argentine isolates were confirmed by sequencing.

To normalize the quantification of the cp-CPsV gene product (cp product), *C. sinensis* ubiquitin transcript was selected as a reference gene and the fragment (ubqt product) quantified. Amplification conditions for both the cp and ubqt products were the same. For calibration curves, the CP1c/CP3 and ubqtL/ubqtR DNA fragments were purified and quantified by $OD^{260\ nm}$. Copy numbers of the gene products were calculated referred as: 10^3, 10^4, 10^5, 10^6 and 10^7 copies.

TAS-ELISA-HRP assay

Triple sandwich immunoassay–horseradish peroxidase (TAS-ELISA-HRP, hereinafter TAS-ELISA) was carried out essentially as described by Zanek et al. (2006), with modifications in the antibody dilutions and number of washes. Total protein was extracted from 200 mg of leaf tissue ground in liquid nitrogen with 1 ml of PBS buffer containing 0.1% (v/v) Tween-20 (PBS-T), 2.5% w/v non-fat milk powder and 2% (w/v) polyvinylpyrrolidone (PVP – 40 000) (extraction buffer). Wells were first coated with 100 μl of A376 polyclonal antiserum 1/7500 dilution and the plates incubated for 4 h at 37°C. After three washes with PBS-T, 100-μl aliquots of total protein preparations were incubated with gentle stirring overnight at 4°C. After washes, the plates were incubated for 90 min at 37°C with 100 μl of monoclonal antibody MAbs 13C5 (IgG) 1/10 000 dilution and later, washed five times. The procedure was repeated with the conjugated antibody, goat anti-mouse-HRP IgG (H+L)–peroxidase (Jackson Immuno Research Laboratories, Inc, West Grove, PA, USA) at a dilution of 1/2000. The peroxidase substrate o-phenylenediamine (OPD) was incubated at room temperature for 15–45 min. The reaction was stopped with 50 μl 2 M H_2SO_4. Optical density was measured at 492 nm. Samples were analysed in triplicate. Samples of infected plants, healthy, non-transgenic plants and extraction buffer were taken as positive and negative controls, respectively. The data analysis was performed by calculating the ratio between the average $OD^{492\ nm}$ of the sample and the average $OD^{492\ nm}$ of the healthy control (I/H).

Results

Primer design, RT-qPCR development and test specificity

The RT-qPCR protocol has been designed to detect the RNA3 of CPsV coding the CP. Considering that the 5′ region of the *cp* gene is more conserved than the 3′ region (Alioto et al. 2003), a multiple alignment of the 5′ region sequences among all available sequences in the GenBank, including representative CPsV isolates from Argentina, Italy, Spain and USA (Martín et al. 2006), was performed. Figure 1 shows a conserved fragment of 113 nt located between positions 738 and 850 of the viral complementary RNA 3 of CPsV. CP1c and CP3 primers were designed on the most conserved region, which is 100% homologous to the CPsV 90-1-1 sequence. Percentages of identity between CPsV 90-1-1 and tested isolates, with available sequences, in the amplified region were as follows: 90% with NA63, 89% with P-121 and 87% with CPV4, the latter being the most distant isolate according to previous studies (Martín et al. 2006).

To optimize qPCR conditions, the hybridization temperature was evaluated using a 52–58°C gradient temperature, with 56°C being the optimal condition for primers CP1c/CP3.

To test the specificity of the measured SYBR Green fluorescence, the melting curves of the amplified fragments were analysed. Figure 2a shows a characteristic melting peak at 79.5 ± 0.5°C for cp product, CPsV-positive samples such as S1 and S2, but not in healthy citrus samples as S3. Non-specific melting peaks were not observed. Ubiquitin was selected as internal control, as it is highly conserved in all citrus species and shows stable expression under different growth conditions (Boava et al. 2011). A melting peak at 84 ± 0.5°C for the ubqt product is presented in all samples (Fig. 2a).

Electrophoretic analysis of the qPCR products for both pairs of primers shows single bands corresponding to the expected sizes and did not reveal non-specific bands nor marked presence of primer dimers (Fig. 2b).

qPCR products (CP1c/CP3 amplicon) amplified from CPsV isolates 90-1-1, 173-22 and 504-3

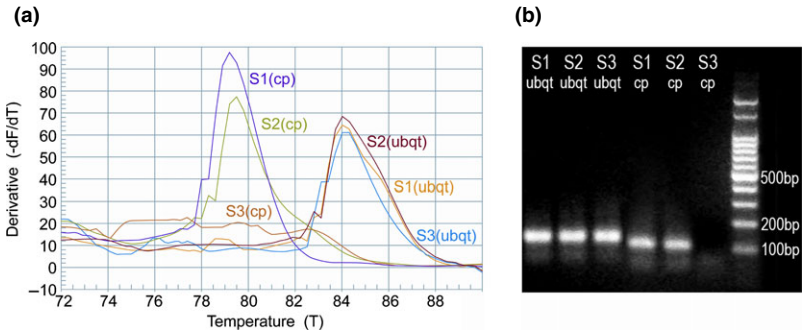

Fig. 2 Analysis of the RT-qPCR products of CPsV-positive samples (S1, S2) and CPsV-negative sample (S3). (a) Melting curve analysis for cp and ubqt products; (b) electrophoresis analysis in 2% agarose gel stained with ethidium bromide. Bands corresponding to 127 nt and 113 nt for ubqt and cp, respectively, are shown. DNA ladder of 100 bp is indicated.

(Table 1) were confirmed by cloning and sequencing. BLASTn was used to compare the sequences of this amplicon against the GenBank database, and only similarity with the CPsV sequences was found.

To challenge the specificity of the RT-qPCR, in addition to the healthy citrus, leaf samples infected with other pathogens were included. Firstly, we tested lettuce infected with MiLBVV, another member of the *Ophioviridae* family, and the result was negative for CP1c/CP3 amplicon by RT-qPCR (Table 2). Citrus samples infected with pathogens present in the citrus regions of Argentina, previously tested at the Experimental Station INTA Concordia, were also assayed for CPsV. As shown in Table 2, one sample with CVC and one with CG gave negative results for CPsV. Two samples that tested positive for CTV were subjected to CPsV RT-qPCR, one was positive (CTV 126-13) and one negative (CTV 268-1). In the case of citrus canker samples, two of them were positive (Valencia orange 1 and 2) and two negative (Valencia orange 3 and Ray Ruby grapefruit).

These assays showed that the RT-qPCR developed with primers CP1c/CP3 is specific for CPsV detection, even in field samples with mixed infections.

Sensitivity analysis of RT-qPCR and comparison with TAS-ELISA

To test the sensitivity in the quantitative detection of CPsV, a correlation between the Ct value and the number of cp copies was performed. Calibration curves for cp and the ubqt products were done by serial dilutions of purified and quantified CP1c/CP3 and ubqtL/ubqtR DNA fragments. For CP1c/CP3 amplicon, dilutions were adjusted to encompass the Ct values usually obtained in infected plants. Linear regression coefficients (R^2) were 0.9913 for cp and 0.9904 for ubqt (Fig. 3a and b, respectively), indicating a high degree of accuracy for the assays.

The minimum number of cp copies detected in positive samples was 10^3 with a maximum Ct value of 30, as 10^2 copies did not show amplification bands in electrophoresis, nor the characteristic melting peak, and therefore were considered negative. In addition, Ct values above 30 overlapped with the negative controls. In the case of ubqt, 10^5 to 10^8 copies were included in the calibration curve, as samples typically contain approximately 10^7 copies. With the aim to determine whether the expression of ubqt gene is

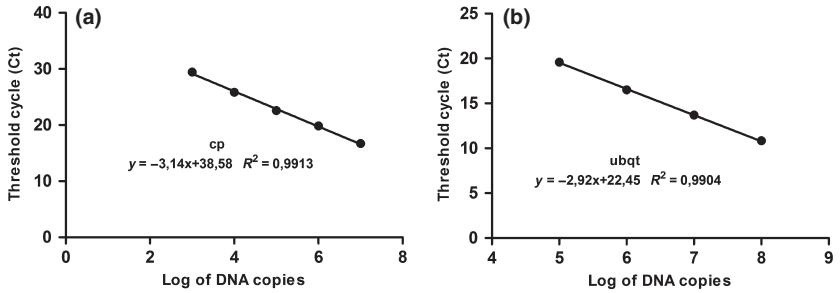

Fig. 3 Calibration curves for RT-qPCR. Threshold cycle (Ct) values vs. the logarithm of DNA copies of (a) CP1c/CP3 amplification products (cp), from 10^3 to 10^7 copies; (b) ubqtL/ubqtR products (ubqt), from 10^5 to 10^8 copies. Linear regression parameters and coefficients are indicated in each graph.

altered by the infection of CPsV, we analysed ubq Ct values in 50 samples, 25 positives for CPsV and 25 negatives. Their ubqt Ct values were listed and compared statistically, and the Ct averages with standard deviations were 14.1 ± 0.7 and 13.8 ± 0.9 for CPsV-positive samples and CPsV-negative samples, respectively. Furthermore, Student's *t*-test was applied to compare both groups of Ct values, yielding a P value of 0.5149 ($P > 0.05$), indicating no significant differences between them. This fact provides experimental evidence for the stability of this internal control in this context. Thus, if quantification is desired, RT-qPCR values can be calculated and referred as the number of cp copies with respect to ubqt copies as shown in Tables 3 and 5.

For comparing the sensitivity of RT-qPCR and TAS-ELISA, the same sample preparations and dilutions were analysed in parallel by both methods (Fig. 4). Positive and negative samples were taken from CPsV 90-1-1-infected and healthy rough lemon, respectively. To prepare comparable samples, leaf tissue was ground and two identical aliquots of 200 mg each were taken either for RNA or protein extraction. After RNA or protein extraction, serial dilutions of positive samples were prepared using the same extract from healthy plants as the diluent, instead of water or buffer. All samples were tested in triplicate.

For TAS-ELISA, a sample was considered positive for CPsV when the ratio between the OD of the sample and the healthy control (OD^I/OD^H) is 3 : 1 or greater (Alioto et al. 1999; Zanek et al. 2006). Only samples diluted 1/10 (10^{-1}) or undiluted (1) were considered positive by TAS-ELISA (Fig. 4a). In contrast, RT-qPCR was positive up to the 10^{-4} dilution (Fig. 4b). From these results, it can be concluded that detecting CPsV by RT-qPCR provides a test three orders of magnitude more sensitive than the TAS-ELISA.

RT-qPCR is able to detect all tested CPsV isolates

A test was performed with 14 samples of citrus plants kept in the greenhouse, which included five healthy plants and nine plants infected with different isolates of CPsV including psorosis A and B, covering different symptoms and geographic distributions of psorosis. Samples were analysed by TAS-ELISA and RT-qPCR (Table 3). Both methods agree on the results for all isolates assayed, including the CPV4, the most distant isolate studied so far. As the TAS-ELISA values correspond to coat protein concentration, there is not necessarily a correlation with the RT-qPCR values detecting RNA, and thus, values by the two methods are not comparable.

RT-qPCR is more sensitive than TAS-ELISA in naturally infected citrus

For field sampling, we chose the L504 plot (Beñatena and Portillo 1984), which had a high incidence of psorosis. In this plot, samples were selected according to a random spatial distribution and analysed by TAS-ELISA and RT-qPCR. Table 4 shows the results for 44 plants. Thirty-six diagnoses were coincident by both methods, 20 samples were CPsV positive and 16 were negative. Within the group of 20 positive samples, six were symptomatic, whereas in the group of the 16 negative samples, none of them was symptomatic. The eight remaining samples resulted positive by RT-qPCR but negative by TAS-ELISA, two of them were symptomatic supporting the RT-qPCR. Moreover, there were no positive samples by TAS-ELISA and negative by RT-qPCR, and none of them showed any symptoms of psorosis.

In the same plot, trays of 100 healthy young sweet oranges were located near the infected trees. Months later, they were analysed by TAS-ELISA as pools of 10

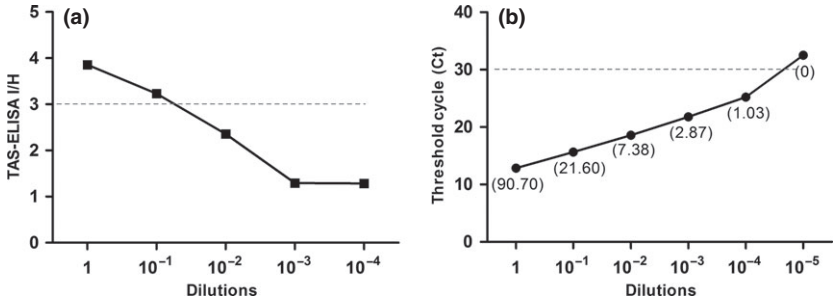

Fig. 4 Ten-fold serial dilution of total RNA and proteins extracted from the same CPsV 90-1-1-infected tissues diluted in healthy extract, analysed by (a) TAS-ELISA and (b) RT-qPCR. (a) I/H ratio calculated as $OD^{492\ nm}$ average of the individual triplicates by the same average of healthy control; (b) threshold cycle (Ct) average of triplicates. The values shown in parentheses show the number of cp copies divided by 100 ubqt copies. Dashed lines indicate the cut-off of TAS-ELISA, I/H = 3, and Ct = 30 for RT-qPCR.

Table 3 Comparison of TAS-ELISA and RT-qPCR results for greenhouse trial with different CPsV isolates

Sample	Symptoms	TAS-ELISA diagnosis	RT-qPCR Ct average ±SD	RT-qPCR (cp copies/100 ubqt copies	RT-qPCR diagnosis
CPsV 189-34	Sh, V, F, S, G	+	17.9 ± 0.2	3.6	+
CPsV P-250	F, S, G, B	+	15.8 ± 0.6	7.6	+
CPsV 504-3	Sh, F, M, V	+	19.2 ± 0.8	8.0	+
CPsV 293-24	M	+	19.8 ± 0.9	5.2	+
CPsV 173-22	Sh, F, M, V	+	21.1 ± 0.2	2.3	+
Healthy 1	None	−	31.3 ± 0.2	0	−
Healthy 2	None	−	34.5 ± 0.4	0	−
Healthy 3	None	−	32.0 ± 0.1	0	−
Healthy 4	None	−	30.5 ± 0.9	0	−
Healthy 5	None	−	29.8 ± 0.7	0	−
90-1-1 isolate	Sh, F, M, V	+	15.3 ± 0.4	18.2	+
CPsV 112-1	Sh, F, M, V	+	19.0 ± 0.5	11.5	+
CPsV 248-2	Sh, F, M, V	+	15.3 ± 0.6	5.6	+
CPV4 isolate	S	+	18.5 ± 0.3	1.4	+

Symptoms at the time of sampling: Sh, shock; V, variegation; F, flecking; S, spots; G, Gum accumulation; M, mottle; B, blisters.
TAS-ELISA (+) when $OD^I/OD^H \geq 3$. RT-qPCR (+) when Ct ≤ 30.
Ct average, threshold cycle average of duplicates; SD, standard deviation.

Table 4 Comparison of diagnosis by TAS-ELISA and qRT-PCR in field samples

TAS-ELISA	RT-qPCR	Number of samples/total samples	Symptomatic samples/total samples analysed in the group
+	+	20/44	6/20
−	−	16/44	0/16
−	+	8/44	2/8
+	−	0/44	0

TAS-ELISA is (+) when $OD^I/OD^H \geq 3$.
RT-qPCR is (+) when Ct ≤ 30.

individuals. Nine of those pools were undoubtedly negative. One pool yielded a TAS-ELISA value close to the cut-off but below it, suggesting there may be positive samples, but it was clearly CPsV positive by RT-qPCR. Then, the 10 plants of this pool were analysed individually by RT-qPCR with five being positive and five negative for CPsV. This assay provided preliminary evidence for the natural spread of psorosis, strongly indicating that the RT-qPCR could detect very low concentrations of virus at the early stages of natural infection.

RT-qPCR is useful for measuring transgene expression in cp transgenic plants

Previously in our laboratory, TAS-ELISA was used to analyse the level of the CP expression in transgenic lines (Zanek et al. 2008). Although with very low value, several of them were positive, including the CP-18 line, demonstrating CP expression. After 7 years, these plants matured and CP protein expression could not be detected by this method, yielding values below the cut-off (data not shown). Later, Reyes et al. (2011) generated transgenic lines ihpCP-10 and 15, expressing a hairpin transcript of the *cp* gene, which was not detected by northern blot. This was not surprising, as it is expected that the amount of this transcript would be very low due to the triggering of the post-transcriptional gene silencing mechanism induced by its hairpin structure (Reyes et al. 2011). In this work, by RT-qPCR using the same primers, CP1c/CP3, the RNA synthesized from the transgenes in lines CP-18, ihpCP-10 and 15 was detected in three individual samples in duplicate (Table 5). Measuring the expression level as cp copies/100 ubqt copies, these lines showed different expression level, whereas non-transgenic citrus were negative. To discard detection of the DNA transgenes, RNA without cDNA synthesis was assayed by RT-qPCR after DNase treatment and showed no amplification (data not shown).

Discussion

RT-qPCR is currently the most widely used method to quantify changes in gene expression and diagnosis (Bustin et al. 2009). CPsV has been previously detected by RT-qPCR using Taqman probes applied

Table 5 Detection of cp product by RT-qPCR in transgenic Pineapple sweet orange

Transgenic citrus line	Sample	Ct average ±SD	cp copies/100 ubqt copies
CP-18	1	20.4 ± 0.9	20.2
	2	19.62 ± 0.06	17.5
	3	20.2 ± 0.5	15.6
ihpCP-10	1	19.19 ± 0.07	6.14
	2	18.32 ± 0.01	12.7
	3	24 ± 1	12.0
ihpCP-15	1	20.2 ± 0.9	10.9
	2	22 ± 1	10.0
	3	18.31 ± 0.02	14.7
Non-tg	1	34.1 ± 0.4	0
	2	31 ± 1	0
	3	29.8 ± 0.9	0

For each transgenic line, three independent samples were analysed (1, 2 and 3).
Ct average, threshold cycle average of duplicates; SD, standard deviation.

successfully for detection of Mexican and European CPsV samples (Barragán-Valencia et al. 2008; Loconsole et al. 2010), but no comparison has been done with TAS-ELISA or biological indexing. Here, we report for the first time the use of a RT-qPCR as a diagnostic tool in Argentina, applying a new and less-expensive protocol using SYBR Green. Extensive detection was demonstrated with isolates from different geographic regions, showing a variety of symptoms, in different types of psorosis (A and B), asymptomatic tissue, field samples from old trees, and in early naturally infected young seedlings. Despite the low levels of expression of the transgenes that transgenic citrus could have, the cp product was also successfully detected in such plants.

In comparison with current methods used to quantify virus, most of them being serological methods, the main advantage of RT-qPCR is its higher sensitivity, even when limited amounts of RNA is available. In this respect, we compared the new RT-qPCR protocol with TAS-ELISA (Zanek et al. 2006), used as the most sensitive method for quantitative CPsV detection in many countries. This RT-qPCR protocol is faster and reliable and can be performed with commercially available reagents, whereas TAS-ELISA depends on the availability of the serum, which becomes depleted. Furthermore, even if a new antiserum was available, it could not necessarily detect the virus at the same level as the current TAS-ELISA protocol.

RT-qPCR allowed us to show that CPsV-infected samples can have a wide range of viral titres, from 1 to 100 copies of cp/100 ubqt copies. We also quantified the cp-RNA and ihpCP-RNA in transgenic citrus plants by RT-qPCR that had not been detected before, indicating that the new RT-qPCR assay will serve for biological studies and to quantify viral RNA 3 species, which are expressed at very low levels (Sánchez de la Torre et al. 1998).

The detection limit of our RT-qPCR protocol is clearly established (Ct ≤ 30 or 1000 cp copies). In contrast, by TAS-ELISA a cut-off for positive samples needed clarification, as TAS-ELISA values frequently give ambiguous results, with I/H near the cut-off, as shown by Alioto et al. (1999) testing 119 samples. Neither this publication nor Zanek et al. (2006) detailed the treatment of those samples giving I/H values between 2 and 3, and for this reason, a value of 2 is sometimes considered the cut-off point (M. I. Plata and M. Cambra, personal communication). In this work, although TAS-ELISA and RT-qPCR gave a matching diagnosis in all greenhouse samples and in most field samples tested (36/44), we found that RT-qPCR can detect the CPsV in samples that were negative by TAS-ELISA. We could not confirm whether these results were false positives by RT-qPCR or false negative by TAS-ELISA, but the appearance of typical symptoms of psorosis in some of these cases supports the RT-qPCR results. While observation of symptoms strongly indicates the disease, we found asymptomatic samples that both TAS-ELISA and RT-qPCR revealed the presence of CPsV. Therefore, it could be that biological indexing, as a third method, supports one or the other method, but also could not be sensitive enough.

Our goal in the future is the validation of the RT-qPCR to have a fast, reliable and accurate diagnosis method for psorosis, including at early stages of the natural infection as shown in this work.

Acknowledgements

We thank Fabián Ramos and Patricia Fernández (EEA-Concordia) for their observations in the field and plant maintenance in the greenhouse. We thank Carina A. Reyes, Eduardo J. Peña and Gabriel Robles Luna for helpful comments on the manuscript. This work has been supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) PICT 2008-1094, PIP 445 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de La Plata (UNLP) X562 and Instituto Nacional de Tecnología Agropecuaria (INTA) (PNFRU-1172; 11721; ERIOS-630081).

References

Alioto D, Gangemi M, Deaglio S, Sposato S, Noris E, Luisoni E, Milne RG. (1999) Improved detection of citrus psorosis virus using polyclonal and monoclonal antibodies. Plant Pathol 48:735–741.

Alioto D, Malfitano M, Troisi A, Peluso A, Martín S, Milne G, Guerri J, Moreno P. (2003) Variability of the coat protein gene of Citrus psorosis virus in Campania, southern Italy. Arch Virol 148:2155–2166.

Barragán-Valencia G, Morales-Loredo A, Álvarez-Ojeda MG, Peña-del-Río MA, Quintero-Zapata I. (2008) Detección cuantitativa del virus psorosis de cítricos mediante RT-PCR Tiempo Real. Agrociencia 42:225–232.

Barthe GA, Ceccardi TL, Manjunath KL, Derrick KS. (1998) Citrus psorosis virus: nucleotide sequencing of the coat protein gene and detection by hybridization and RT-PCR. J Gen Virol 79:1531–1537.

Beñatena HN, Portillo MM. (1984) Natural spread of Psorosis in sweet orange seedlings. In: Garnsey SM, Timmer LW, Dodds JA. (eds) Proc 9th Conference of the International Organization of Citrus Virologists, 9–13 May 1983. Riverside, CA, USA, IOCV, pp 159–164.

Boava LP, Cristofani-Yaly M, Mafra VS, Kubo K, Kishi LT, Takita MA, Ribeiro-Alves M, Machado MA. (2011) Global gene expression of *Poncirus trifoliata, Citrus sunki* and their hybrids under infection of *Phytophthora parasitica*. BMC Genom 12:39.

Bustin SA, Benes V, Garson JA et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:4 (Reviews).

Diamante de Zubrzycki A, Zubrzycki HM, Correa M. (1984) Determination of the distribution of psorosis in commercial plantings. In: Garnsey SM, Timmer LW, Dodds JA. (eds) Proc 9th Conference of the International Organization of Citrus Virologists, 9–13 May 1983. Riverside, CA, USA, IOCV, pp 165–170.

Fawcett HS. (1932) New angles on treatment of bark diseases of citrus. Calif Citograph 17:406–408.

García ML, Grau O, Sarachu AN. (1991) Citrus psorosis is probably caused by a bipartite ssRNA virus. Res Virol 142:303–311.

García ML, Dal Bó E, Grau O, Milne RG. (1994) The closely related citrus ringspot and citrus psorosis viruses have particles of novel filamentous morphology. J Gen Virol 75:3585–3590.

García ML, Sánchez de la Torre ME, Dal Bo E, Djelouah K, Rouag N, Luisoni E, Milne RG, Grau O. (1997) Detection of citrus psorosis – ringspot virus using RT-PCR and DAS-ELISA. Plant Pathol 46:830–836.

Ghazal SA, El-Dougdoug KA, Mousa AA, Fahmy H, Sofy AR. (2008) Isolation and identification of citrus psorosis virus Egyptian isolate (CPsV-EG). Commun Agric Appl Biol Sci 73:285–295.

Gottwald TR, Palle SR, Miao H, Seyran M, Skaria M, da Graca JV. (2005) Assessment of the possibility of natural spread of Citrus Psorosis disease. In: Hilf ME, Duran-Vila M, Rocha-Peña MA. (eds) Proc 16th Conference of the International Organization of Citrus Virologists, 3–6 November 2004. Riverside, CA, USA, IOCV, pp 240–250.

Iglesias NJ, Gago-Zachert SP, Robledo G, Costa N, Plata MI, Vera O, Grau O, Semorile LC. (2008) Population structure of Citrus tristeza virus from field Argentinian isolates. Virus Genes 36:199–207.

Ito T, Furuta T, Namba N. (2011) First report of Citrus psorosis virus in Japan. J Gen Plant Pathol 77:257–259.

Kayim M. (2010) Biological and molecular detection of Citrus psorosis virus in Citrus in the Eastern Mediterranean Region of Turkey. J Plant Biochem Biotechnol 19:259–262.

Legarreta GG, García ML, Costa N, Grau O. (2000) A highly sensitive heminested RT-PCR assay for the detection of citrus psorosis virus targeted to a conserved region of the genome. J Virol Methods 84:15–22.

Lin L, Li R, Bateman M, Mock R, Kinard G. (2013) Development of a multiplex TaqMan real-time RT-PCR assay for simultaneous detection of Asian prunus viruses, plum bark necrosis stem pitting associated virus, and peach latent mosaic viroid. Eur J Plant Pathol 137:797–804.

Loconsole G, Saponari M, Savino V. (2010) Development of real-time PCR based assays for simultaneous and improved detection of citrus viruses. Eur J Plant Pathol 128:251–259.

Martín S, Alioto D, Milne RG, Guerri J, Moreno P. (2002) Detection of citrus psorosis virus in field trees by direct tissue blot immunoassay in comparison with ELISA, symptomatology, biological indexing and cross-protection tests. Plant Pathol 51:134–141.

Martín S, García ML, Troisi A, Rubio L, Legarreta GG, Grau O, Alioto D, Moreno P, Guerri J. (2006) Genetic variation of populations of Citrus psorosis virus. J Gen Virol 87:3097–3102.

Milne RG, García ML, Grau O. (2000) Genus ophiovirus. In: van Regenmortel MVH, Fauquet CM, Bishop DHL, Carstens EB, Estes MK (eds) Virus Taxonomy, 7th Report of the International Committee on Taxonomy of Viruses. London, UK, Elsevier/Academic, pp 626–631.

Naum-Onganía G, Gago-Zachert S, Peña E, Grau O, García ML. (2003) Citrus psorosis virus RNA 1 is of negative polarity and potentially encodes in its complementary strand a 24K protein of unknown function and 280K putative RNA dependent RNA polymerase. Virus Res 96:49–61.

Portillo MM, Beñatena HN. (1989) Transmisión de psorosis de citrus a citrus por áfidos. Rev Soc Entomol Argent 45:299–305.

Reyes CA, De Francesco A, Peña EJ, Costa N, Plata MI, Sendin L, Castagnaro AP, García ML. (2011) Resistance

to Citrus psorosis virus in transgenic sweet orange plants is triggered by coat protein–RNA silencing. J Biotechnol 151:151–158.

Robles Luna G, Peña EJ, Borniego MB, Heinlein M, García ML. (2013) Ophioviruses CPsV and MiLBVV movement protein is encoded in RNA 2 and interacts with the coat protein. Virology 441:152–161.

Roistacher CN. (1991) Psorosis complex: psorosis-A, psorosis-B and ringspot. In: International Organization of Citrus Virologists (ed) Graft-Transmissible Diseases of Citrus, Handbook for Detection and Diagnosis. Rome, Italy, Food and Agriculture Organization of the United Nations, pp 115–126.

Roistacher CN. (1993) Psorosis- a review. In: Moreno P, da Graca JV, Timmer LW. (eds) Proc 12th Conference of the International Organization of Citrus Virologists, 23–27 November 1992. Riverside, CA, USA, IOCV, pp 139–154.

Roistacher CN, D'Onghia AM, Djelouah K. (2000) Other viruses: Defining psorosis by biological indexing and ELISA. In: da Graca JV, Lee RF, Yokomi RK. (eds) Proc 14th Conference of the International Organization of Citrus Virologists, 13–18 September 1998. Riverside, CA, USA, IOCV, pp 144–151.

Sánchez de la Torre ME, Riva O, Zandomeni R, Grau O, García ML. (1998) The top component of citrus psorosis virus contains two ssRNAs, the smaller encodes the coat protein. Molecular Plant Pathology Online [http://www.bspp.org.uk/mppol] 1998/1019sanchez.

Saponari M, Loconsole G, Liao HH, Jiang B, Savino V, Yokomi RK. (2013) Validation of high-throughput real time polymerase chain reaction assays for simultaneous detection of invasive citrus pathogens. J Virol Methods 193:478–486.

Velazquez K, Perez JM, Alonso M, Batista L, Rodriguez J, Legarreta G, Grau O, García ML. (2005) Detection of Citrus psorosis virus in Cuba. In: Hilf ME, Duran-Vila M, Rocha-Peña MA. (eds) Proc 16th Conference of the International Organization of Citrus Virologists, 3–6 November 2004. Riverside, CA, USA, IOCV (Short Communications).

Zanek MC, Peña E, Reyes CA, Figueroa J, Stein B, Grau O, García ML. (2006) Detection of Citrus psorosis virus in the northwestern citrus production area of Argentina by using an improved TAS - ELISA. J Virol Methods 137:245–251.

Zanek MC, Reyes CA, Cervera M, Peña EJ, Velazquez K, Costa N, Plata MI, Grau O, Peña L, García ML. (2008) Genetic transformation of sweet orange with the coat protein gene of Citrus psorosis virus and evaluation of resistance against the virus. Plant Cell Rep 27:57–66.

