One-step multiplex RT-qPCR detects three citrus viroids from different genera in a wide range of hosts

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Abstract

A one-step multiplex reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) based on species-specific minor groove binding (MGB) probes, was developed for the simultaneous detection, identification, and quantification of three citrus viroids belonging to different genera. *Citrus exocortis viroid* (*Pospiviroid*), *Hop stunt viroid* (*Hostuviroid*), and *Citrus bark cracking viroid* (*Cocadviroid*) cause a variety of maladies in agriculturally significant crops. Therefore, reliable assays for their detection are essential tools for various government and industry organizations implementing disease management programs. Singleplex qPCR primers and MGB probes were designed individually for the detection of the three targeted viroids, and subsequently combined in a one-step multiplex RT-qPCR reaction. A wide host range of woody plants, including citrus, grapevines, apricots, plums and herbaceous plants such as tomato, cucumber, eggplant and chrysanthemum different world regions were used to validate the assay. Single, double and triple viroid infections were identified in the tested samples. The developed multiplex RT-qPCR assay was compared with a previously reported SYBR Green I RT-qPCR for the universal detection of citrus viroids. Both assays accurately identified all citrus viroid infected samples. The multiplex assay complemented the SYBR Green I universal detection assay by

differentiating among citrus viroid species in the positive samples. The developed multiplex RT-qPCR assay has the potential to simultaneously detect each targeted viroid and could be used in high throughput screenings for citrus viroids in field surveys, germplasm banks, nurseries and other viroid disease management programs.

Keywords: Citrus Nursery Stock Pest Cleanliness Program; Diagnostics; Germplasm; Graft-transmissible pathogens; National Clean Plant Network; Propagative Materials

1. Introduction

Citrus exocortis viroid (CEVd, *Pospiviroid*) is the causal agent of citrus exocortis, a bark scaling, stunting, and yield reducing disease of citrus trees propagated on trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) and trifoliolate orange hybrid rootstocks (Duran-Vila et al., 1988; Hashemian et al., 2009; Murcia et al., 2015; Timmer et al., 2000; Vernière et al., 2004, 2006). CEVd has a wide range of herbaceous hosts including but not limited to tomato, gynura, cucumber, eggplant, datura and chrysanthemum (Bernad et al., 2009; Duran-Vila and Semancik, 2003; Gandía et al., 2007, Semancik and Vidalakis, 2005; Verhoeven et al., 2004). *Citrus bark cracking viroid* (CBCVd, *Cocadviroid*) has been associated with severe bark cracking of trifoliolate and trifoliolate citrus hybrids, and has been identified as a critical component of citrus viroid mixtures that can induce severe exocortis-like bark scaling symptoms on trifoliolate orange rootstock in the absence of CEVd (Ito et al., 2002b; Vernière et al., 2006). CBCVd has a broad host range among citrus, citrus relatives, and citrus hybrids as well as herbaceous hosts such as cucumber, tomato,

eggplant, datura, chrysanthemum, gynura, and hop (Jakse et al., 2015; Semancik and Vidalakis, 2005). Specific variants of the *Hop stunt viroid* (HSVd, *Hostuviroid*) are the causal agents of citrus cachexia, a vascular disrupting disease (i.e. phloem gumming and wood pitting) resulting in tree stunting and reduced yields of mandarins (*Citrus reticulata* Blanco), mandarin hybrids and clementines (*C. clementina* Hort. ex Tan.) (Hashemian et al., 2009; Murcia et al., 2015; Palacio-Bielsa et al., 2004; Reanwarakorn and Semancik, 1998, 1999; Vernière et al., 2004, 2006). HSVd is able to infect a large number of non-citrus woody hosts, such as apricot, almond, plum, peach, grapevine, and pomegranate (Gorsane et al., 2010; Polivka et al., 1996; Shikata, 1990). HSVd has also a wide range of herbaceous hosts including hops, cucumber, tomato, eggplant and chrysanthemum (Astruc et al., 1996; Duran-Vila and Semancik, 2003; Ragozzino et al., 2004).

The CEVd, CBCVd and HSVd affecting citrus and non-citrus species are commercially and economically important agricultural crops worldwide (FAO 2015). For example, trifoliate and trifoliate hybrids have become the standard citrus rootstock worldwide due to their tolerance to the *Citrus tristeza virus*, which has destroyed over 100 million citrus trees worldwide (Moreno et al., 2008). CEVd, CBCVd and HSVd have also been reported to induce serious diseases in tomato and hops. More specifically, CEVd was identified as the causal agent of Indian bunchy top disease of tomato while the viroid has been recognized as an important component of costly disease outbreaks of stunted unproductive tomato plants (Fagoaga and Duran-Vila, 1996; Mishra et al., 1991; Verhoeven et al., 2004). In addition, HSVd is the causal agent of the hop stunt disease (Pethybridge et al., 2008) while CBCVd was recently identified as a highly aggressive pathogen of hops resulting in plant death within 3-5 years after infection (Jakse et al., 2015).

CEVd, CBCVd, and HSVd are globally distributed via the movement of infected propagative materials and their transmission occurs through grafting and standard cultural practices. These

viroids are therefore regulated within citrus quarantine, certification, and nursery programs at an international, national, and state level. Successful operation of such programs requires diagnostic tools able to provide rapid and reliable results in high throughput setting with minimal cost, labor, and space inputs (Barbosa et al., 2005; Bostock et al., 2014; Duran-Vila and Semancik, 2003; Gergerich et al., 2015; Navarro, 2015; Osman et al., 2015).

Methods commonly used for the detection of citrus viroids include graft inoculation of sensitive plant indicators (bioassays) (Roistacher, 1991). Since viroids lack a coat protein and any protein-coding ability, serological techniques cannot be employed for their detection (Tabler et al., 2004). To date, molecular-based detection tools are widely used for routine viroid detection and include sequential polyacrylamide gel electrophoresis (sPAGE) (Duran-Vila et al., 1986, Rivera-Bustamante et al., 1986), molecular hybridization (Fonseca et al., 1996, Palacio-Bielsa et al. 1999, Palacio et al. 2000), and reverse transcription polymerase chain reaction (RT-PCR) using species specific primers and/or probes (Gross et al., 1982; Hajizadeh et al., 2012; Ito et al., 2002b; Sano et al., 1988).

The implementation of advanced molecular diagnostic technologies, such as real-time quantitative PCR (qPCR), has significantly accelerated the detection of nucleic acid targets during the past decade (Boonham et al., 2014). The development of various qPCR chemistries such as dsDNA intercalating molecules (e.g. SYBR Green I) and fluorophore-labeled oligonucleotides (e.g. hydrolysis probes), as well as novel instrumentation platforms, has led to improved diagnostic speed, sensitivity, reproducibility, and reduced risk of carry-over contaminations (for a review see; Josefsen et al., 2012; Navarro et al., 2015). SYBR Green I is an asymmetrical cyanine dye that binds non-specifically to the PCR amplicons. A melting curve analysis is required to verify the specificity of the amplified products. A number of different hydrolysis fluorophore probes (e.g.

TaqMan) have been employed in qPCR assays but nearly all share a fluorescent reporter partnered with a quencher (Mackay et al., 2002; Shipley 2007; Navarro et al., 2015). In this system, the reporter is able to fluoresce only after amplification of the targeted DNA (Mackay et al., 2002; Navarro et al., 2015; Shipley 2007; Keer et al., 2008). These hydrolysis qPCR probes are designed to bind to a specific region of the targeted DNA, thus eliminating the need for melting curve analysis. Hydrolysis probes are also conjugated at their 3' end to minor groove binding (MGB) ligands, which are small tripeptides forming non-covalent bonds with dsDNA. As a result, the "target DNA-MGB probe" structure is greatly stabilized (increased melting temperature) providing higher target DNA-binding specificity and sensitivity (Navarro et al., 2015). In addition, the use of differently labeled fluorescent MGB-probes made the simultaneous detection of more than one pathogen possible in multiplex qPCR assays, thus substantially decreasing labor time and cost of diagnostics (Pallas et al., 2009).

RT-qPCR methods targeting one or two citrus viroid species using either SYBR Green I or qPCR probes have been reported (Lin et al., 2015; Loconsole et al., 2013; Monger et al., 2010, Papayiannis, 2014; Rizza et al., 2009, Saponari et al., 2013; Sun et al., 2014; Tessitori et al., 2005). The current study presents the development and evaluation of a multiplex RT-qPCR based on species-specific MGB-hydrolysis probes for the simultaneous detection, identification and quantification of three citrus viroids, CEVd, CBCVd, and HSVd, as they represent important citrus pathogenic viroid species from three different genera. This fast and reliable multiplex RT-qPCR assay was used for the detection of the targeted viroids in citrus and non-citrus hosts and was compared with a high throughput SYBR Green I RT-qPCR assay for the universal detection of citrus viroids (Vidalakis and Wang, 2013). The SYBR Green I assay is currently approved by the California Department Food and Agriculture (CDFA, Permit QC 1354) for use in the mandatory

“Citrus Nursery Stock Pest Cleanliness Program-Senate Bill 140” (Bostock et al., 2014; Osman et al., 2015; Vidalakis 2016). The diagnostic sensitivity and specificity of the developed multiplex RT-qPCR were evaluated to assess the qPCR assay's accuracy. The qPCR assay was then validated using reference viroid isolates, obtained from different geographical areas and different hosts. The developed multiplex RT-qPCR assay can be used in viroid disease management programs to monitor the sanitary status of commercial citrus trees or assist in the prevention of viroid diseases spread in germplasm blocks and nurseries of citrus and other agriculturally significant crops.

2. Materials and methods

2.1. Viroid isolates and plant material

CEVd, CBCVd, and HSVd isolates were maintained *in planta* at the disease bank of the Citrus Clonal Protection Program (CCPP) at the University of California (UC), Riverside (Table 1A). The non-targeted citrus viroids; *Citrus bent leaf viroid* (including variant -LSS), *Citrus dwarfing viroid*, *Citrus viroid-V* (CVd-V) and CVd-VI, and other California endemic citrus graft-transmissible virus and bacteria pathogens, used as non-target controls, were also maintained *in planta* at the CCPP (Table 1B). Non-inoculated citrus seedlings of different varieties representing major citrus types such as sweet orange (*C. sinensis* L. Osbeck), lime (*C. aurantifolia* (Christm.) Swing. (*C. latifolia* Tan.), citron (*C. medica* L.), kumquat (*Fortunella margarita* (Lour.) Swingle and *F. obovata* Hort. ex Tan.), citrange (*Poncirus trifoliata* × *C. sinensis*), lemon (*C. limon* (L.) Burm.f.), grapefruit (*C. paradisi* Macf.), mandarin, and tangelo (*C. reticulata* × *C. paradisi*.), used as negative controls, were provided by CDFA's “Citrus Nursery Stock Pest Cleanliness Program”

(Table 1C). Nucleic acids of citrus pathogens non-endemic to California were provide by the United States Department of Agriculture (USDA), Agriculture Research Service (ARS), Exotic Pathogen Collection of Citrus (EPCC), Beltsville, MD (Table 2A). Citrus samples of unknown viroid status from different geographical areas were provided by the CCPP, CDFA, and USDA-ARS-EPCC (Table 2A).

Citrus samples were collected from phloem-rich tissues such as the bark of the last matured vegetative flush (approximately 12-18 months old) or petioles of fully expanded leaves. Young (current flush) or older (more than 24 months) tissues were not included because they produce low quality nucleic acids as determined by spectrophotometry (i.e. A260/280 and A260/230 less than 1.8) and cytochrome oxidase (COX) based qPCR (Osman et al., 2015; Tan et al. 2015). To account for the possible uneven distribution of the viroids within a plant, 4-6 samples were collected randomly from different branches around the citrus canopy and combined into a single sample per tree. Samples were processed immediately for RNA extraction or stored at -20 °C until processed for RNA extraction.

Non-citrus woody plants, almond, apricot, cherry, peach, plum (*Prunus* sp.) and grapevines (*Vitis* sp.) originated from different geographical areas, were maintained at the USDA-ARS, National Clonal Germplasm Repository (NGCR) for Tree Fruit and Nut Crops and Grapes, Davis, CA (Table 2B and 2C). Samples from trees and grapevines with symptoms suspected to be of viral origin were used in the present study. Considering the possibility of an uneven distribution of viroids within individual plants, samples were collected randomly from different parts of the plant. More specifically, 6-8 leaves with petioles from at least six different branches from each tree, and 6-8 cuttings of lignified canes from at least six different vine sections were collected and combined

into a single sample per tree and grapevine, respectively. All samples were stored at -80 °C until processed for RNA extraction.

Herbaceous ornamental and vegetable plants from various CCPP experiments dating back to 1989 were utilized in this study. More specifically, chrysanthemum (*Dendranthema morifolium* L.), cucumber (*Cucumis sativus* L.), datura (*Datura stramonium* L.), eggplant (*Solanum melongena* L.), gynura (*Gynura aurantiaca* D.C.), luffa (*Luffa aegyptiaca* Mill.), and various tomato (*Solanum lycopersicum* L.) wild tomato species and hybrids (*Solanum* sp.) (Table 2D) were slash inoculated with citrus viroid enriched preparations as previously described (Duran-Vila *et al.* 1986, Hajeri *et al.*, 2011). Following inoculation, plants were maintained under standard greenhouse conditions, and approximately 3-4 months post inoculation, young succulent tissues (stems and leaves) were collected from the inoculated plants and stored at -20 °C until processed for RNA extraction.

2.2. Sample processing and RNA extraction

Citrus and herbaceous plant samples were processed and subjected to total RNA extraction as described by Osman *et al.* (2015) with some modifications. Briefly, 250 mg of coarsely chopped plant tissue were placed in 2 ml Eppendorf Safe-Lock tubes (Hamburg, Germany) and kept at -80 °C for 2 hours or overnight. The frozen tissues were freeze-dried for 26 hours (FreeZone®Triad™ 74000, Labconco®, Kansas City, MO). Two 5/32” stainless steel grinding balls were dropped in each tube when samples were removed from the freeze-dryer. Tubes were then placed in pre-chilled (-80 °C overnight) Geno/Grinder® Cryo-Blocks and placed for 10 min in liquid N₂ in a 2600 CryoStation (SPEX SamplePrep, Metuchen, NJ). Tissues were then ground dry into fine

powder, using a Geno/Grinder® 2010 (SPEX SamplePrep) at 1,680 rpm for 20 s, twice . The pulverized tissues were then homogenized with 750 µl guanidine lysis buffer [4 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5.0, 2 mM EDTA, 2.5% (w/v) PVP-40] using a Geno/Grinder® 2010 at 1,680 rpm for 20 s. Crude extracts were incubated at 4 °C for 10 min and centrifuged for 45 min at 17,200 g at 4 °C. Finally, 150 µl of the supernatant was subjected to total RNA extraction using the 5X MME-96 Viral RNA Isolation Kit (Life Technologies, Grand Island, NY) with the “AM1836_DW_50_v2” program of the MagMAX™ Express-96 (Life Technologies, Grand Island, NY) as described by Osman et al. (2012a) in Method B modified as follows: The lysate plate (slot 1) contained 139 µl of lysis/binding solution concentrate (P/N B8500G5; premixed with isopropanol and carrier RNA as recommended by manufacturer), 20 µl of Bead Mix (P/N 10019G7; 10 µl of RNA Binding Beads + 10 µl of Lysis/Binding Enhancer), 139 µl of isopropanol, and 150 µl of the processed plant tissues supernatant; the wash plates contained 500 µl of wash solutions 1 and 2 (slots 2-3 and 4-5, respectively); the RNA elution plate (slot 6) contained 100 µl of elution buffer.

Tissue processing and RNA extraction from *Prunus* and grapevines was performed using similar equipment as that for citrus and following procedures previously described by Osman et al. (2012b) and Al Rwahnih et al. (2012), respectively. Briefly, 0.2 g of basal (mature) leaves from *Prunus* trees and 0.1 g of cambial scrapings from grapevine canes frozen in liquid nitrogen were ground to fine powder using a Geno/Grinder® 2010 for 1 min at 1,200 rpm. The powdered samples were homogenized in the presence of 1 ml guanidine lysis buffer using a Geno/Grinder® 2010 for 1 min at 1,200 rpm. Homogenates were centrifuged at 4 °C for 8 min at 9,960 g, and 800 µl of the supernatant were subjected to RNA extraction using the MagMax™ Express-96 with the 5X MME-96 Viral RNA Isolation Kit (Life Technologies, Grand Island, NY) as described by Osman

et al. 2017 and Osman et al. 2012a (Method B) for *Prunus* and grapevines, respectively. Total RNA was eluted in 100 µl Diethylpyrocarbonate treated water.

RNA extracts from hops were kindly provided by the Clean Plant Center Northwest, Washington State University Prosser, WA.

2.3. *SYBR Green I RT-qPCR assay*

The forward and reverse degenerate primers for the universal detection of citrus viroids were previously developed based on the genome sequence alignment of CEVd, CBCVd, and HSVd (HSVd citrus variants -IIa, -IIb, -IIc and Ca909) (Vidalakis et al. 2005, Vidalakis and Wang, 2013). The assay was carried out in 20 µl reactions with the iTaq Universal SYBR Green I One-Step Kit and a CFX-96 (Bio-Rad, Hercules, CA), 300 nM of the forward and reverse primers and 1 µL of template RNA were used per reaction. The RT-qPCR protocol was run at: 50 °C for 30 minutes, 95 °C for 5 minutes, 95 °C for 10 seconds, 61 °C for 30 seconds for 35 cycles, 95 °C for 1 minute, 55 °C for 1 minute, and melting curve from 55-95 °C with 0.5 °C increments for 10 seconds (Vidalakis and Wang, 2013).

2.4. *Singleplex and Multiplex RT-qPCR assays*

A total of 385 GenBank accessions (National Center for Biotechnology Information, NCBI) of CEVd (293 accessions), CBCVd (32 accessions) and HSVd (60 accessions) derived from citrus were used for primers and MGB qPCR probe design. The GenBank sequences were aligned separately for each viroid using Sequence Analysis and Molecular Biology Data Management

software Vector NTI Advance™ 11 (Invitrogen; Life Technologies, Grand Island, NY). Viroid sequences were fairly conserved, therefore only one set of primers and qPCR probe was designed for each targeted viroid for singleplex and multiplex RT-qPCR experiments (Table 3).

All primers and qPCR probes were designed using the Primer Express Software v3.0.1 (Life Technologies, Grand Island, NY). The optimum primers' melting temperature (T_m) was 58 °C, and the T_m of the qPCR probes was 10 °C higher to prevent the formation of primer dimers. Amplicon length of the CEVd, CBCVd and HSVd qPCR assays was in the range of 88-105 bp (Table 3). Homology of the primers and qPCR probes was confirmed by BLAST (NCBI) analysis to rule out any cross-reactivity with other organisms.

The fluorophore used for the CEVd MGB-TaqMan qPCR probe was 6-carboxyfluorescein FAM, for CBCVd VIC was used, and for HSVd TET was used (Table 3). In all assays the 3' quencher was the non-fluorescent black hole quencher (BHQ). The qPCR primer/probe mixes were prepared by mixing 20 µl of the forward and reverse primers stock (100 pmol/µl) with 4 µl of the probes stock (80 pmol/µl) for each targeted viroid, and the final volume was made up to 240 µl with water.

Singleplex RT-qPCR reactions (12 µl) were run using the AgPath-ID™ One-Step RT-PCR Kit (Life Technologies, Carlsbad, CA) starting with 2.65 µl water, 6.25 µl 2X RT buffer, 0.6 µl primer/probe mix, 0.5 µl 25X RT mix and 2 µl of RNA template for each reaction. Cycling conditions were 45 °C for 10 min, 95 °C for 10 min for the first cycle, then 40 cycles at 95 °C for 15 seconds and 60 °C for 45 seconds. All samples were analyzed using a 7900HT FAST Real-time PCR system (Life Technologies, Carlsbad, CA). Fluorescent signals were collected during the annealing temperature and the quantitative cycle (C_q) was calculated and exported with a threshold of 0.06 and a baseline of 3-12 for the targets of interest. The C_q was defined as the cycle in which

there is a significant increase in reporter signal, above the threshold, i.e. the cycle in which fluorescence generated within a reaction crosses the fluorescence threshold. It was consequently inversely related to the initial amount of target in the sample (Rasmussen, 2001). The C_q was calculated by the qPCR machine using an algorithm with a set range of cycles at which the first detectable significant increase in fluorescence occurs.

For multiplex RT-qPCR, QuantiFast Multiplex RT-PCR kit (Qiagen, Valencia, CA) was used, starting with total RNA as the template following the manufacturer's instructions. The same primer/probe concentrations used for singleplex were used for multiplex RT-qPCR. Samples were tested using COX (RNA integrity) and viroid qPCR assays (Table 1 and 3) (Osman et al., 2015).

2.5. Singleplex and Multiplex Assays Validation

To assess the efficiency of the singleplex and multiplex RT-qPCR assays for the detection of all three viroids in single and mixed infections across a range of viroid concentrations, a series of CEVd, CBCVd and HSVd infected biological samples were used to create a dilution endpoint analysis of undiluted total RNA and tenfold dilutions of up to $1:10^{5-6}$ of three different samples, each in triplicate. In singleplex RT-qPCR, only one sample corresponding to each viroid was used and amplified. In multiplex RT-qPCR, three samples for each targeted viroid were combined in a single amplification tube for testing. Standard curves were generated for each set of ten-fold serial dilutions of CEVd, CBCVd, and HSVd infected samples by plotting the C_q value against the logarithm of the concentration for the exponential phase of the reaction and fitting a straight line to these data by simple linear regression (Rasmussen, 2001). The amplification efficiency (E) of all assays was calculated from the slope of a standard curve generated on a 10-fold dilution in

triplicate for every RNA sample using the formula $E = 10^{(-1/\text{slope})}$. Optimal qPCR efficiency was achieved at a slope of -3.32 (Rasmussen, 2001).

The inter-assay (reproducibility) and intra-assay (repeatability) variation of the developed singleplex and multiplex RT-qPCR assays was assessed within one plate and in different plates, respectively. More specifically, total RNA from three randomly selected CEVd, CBCVd and HSVd samples was extracted, 10-fold serial dilutions were prepared for each sample and tested by RT-qPCR in duplicates, and the C_q values were calculated. The intra-assay and inter-assay variations were calculated, by determining the percentage of coefficient of variation (CV %), which was calculated for each sample as follows: mean of the standard deviations of the duplicates divided by the grand mean of the duplicates X100.

2.6. Testing the Viroid load in Singleplex and Multiplex RT-qPCR

Quantification of the amount of CEVd, CBCVd and HSVd target in samples was accomplished by generating absolute standard curves, to determine the starting number of copies. The standard curves of CEVd, CBCVd and HSVd qPCR assays were developed for singleplex and multiplex RT-qPCR utilizing their respective fluorophores. More specifically, amplicons for CEVd, CBCVd and HSVd were obtained for each primer set and individually cloned into plasmids (Eurofins MWG Operon, Huntsville, AL) as shown in Table 4. Plasmid DNA was extracted and linearized using HindIII restriction enzyme, to increase the efficiency of dilutions. Serial 10-fold dilutions of plasmids carrying a known copy number of CEVd, CBCVd and HSVd inserts were generated. Singleplex and multiplex RT-qPCR reactions were performed in triplicate to establish

the linear response between the C_q values and the log of known copy numbers. The copy number for each sample was calculated as previously described (Leutenegger, 2001).

Statistical analysis was performed using the PASW Statistics 18 software (SPSS Inc., Chicago, IL). P values < 0.05 were considered significant. Reproducibility of the assay was tested by analysis of variance (ANOVA) comparing the C_q values of the samples.

2.7. *Evaluation of the Singleplex and Multiplex RT-qPCR assays*

A series of citrus plants including, plants graft-inoculated singly or with mixtures of CEVd, CBCVd and HSVd (positive controls, Table 1A), plants not inoculated with any graft-transmissible citrus pathogen (negative controls, Table 1B), and plants inoculated with non-targeted graft-transmissible citrus pathogens such as viroids, viruses, and bacteria (cross reactivity controls, Table 1C) were used for the evaluation of the newly developed singleplex and multiplex RT-qPCR assays. All graft-inoculations and plant maintenance were performed as previously described (Vidalakis et al. 2004). Samples from these citrus plants were used to evaluate the performance of the multiplex RT-qPCR assay and to compare it with the singleplex qPCR assays of each viroid as well as the CDFA approved SYBR Green I RT-qPCR assay for the universal detection of CEVd, CBCVd and HSVd (Bostock et al., 2014). Data were analyzed by comparing the C_q value of each sample using the singleplex, multiplex, and SYBR Green I RT-qPCR assays in combination with an RNA integrity COX-based qPCR assay (Table 1) (Osman et al., 2015). Finally, to evaluate the potential use of the developed multiplex RT-qPCR assay as a general detection tool for CEVd, CBCVd and HSVd for citrus and non-citrus hosts, a series of biological

samples from the field and greenhouse, obtained from various geographical areas, were tested (Table 1 and 2).

3. Results

3.1. Validating and evaluating the RT-qPCR assays

For the validation (i.e. amplification efficiency, reproducibility and repeatability) of the developed RT-qPCR assays the E and CV values were determined. The average E of CEVd, CBCVd and HSVd primer/probe multiplex RT-qPCR were 90.9, 97.2 and 90.3%, respectively. The detection assays for all three viroids showed a linear dynamic range from 10^6 copies down to < 10 copies per μl (Fig. 1). The CV for CEVd in singleplex RT-qPCR was in the range of 0.68-1.11% (intra assay variation) and a value of 1.10% for the inter assay variation. The CV for CBCVd in the singleplex assay was in the range of 0.39-0.64% (intra assay variation) and a value of 0.49% for the inter assay variation. The CV for HSVd in singleplex assay was in the range of 0.28-0.49% (intra assay variation) and a value of 0.47% for the inter assay variation. The CV for CEVd in multiplex RT-qPCR was in the range of 0.30-0.75% (intra assay variation) and a value of 0.77% for the inter assay variation. The CV for CBCVd in multiplex assay was in the range of 0.65-2.85% (intra assay variation) and a value of 1.91% for the inter assay variation. The CV for HSVd in multiplex assay was in the range of 1.32-1.79% (intra assay variation) and a value of 1.43% for the inter assay variation. The CV% of multiplex RT-qPCR thus indicated low variation between different repetitions and different runs.

The developed RT-qPCR assays were evaluated qualitatively by sequence analysis of the amplicons, test of different plant samples and comparison with a previously developed SYBR Green I RT-qPCR assay. The BLAST analysis of the amplicon sequences for CEVd, CBCVd and

HSVd recognized each viroid sequence respectively, confirming the specificity of the designed multiplex RT-qPCR. In addition, the newly developed assays detected and identified all targeted viroids in single and mixed infections in various citrus types (e.g. orange, lemon, and mandarin). The multiplex RT-qPCR was able to identify viroid positive samples and discriminate between different viroid species within each sample (e.g. Table 1A # 39 and # 40). The singleplex and multiplex assays did not cross-react with any non-inoculated negative controls or other graft-transmissible pathogen of citrus, including the four non-targeted citrus viroids (Table 1). Finally, the MGB-probe singleplex and multiplex RT-qPCR results overlapped 100% with the results of the previously developed SYBR Green I RT-qPCR assay approved by the CDFA for the universal detection of citrus viroids (Vidalakis and Wang, 2013, Bostock et al., 2014). The assays detected alike all the viroid inoculated and non-inoculated samples with comparable Cq values. More specifically, the minimum and maximum MGB-probe and SYBR Green I Cq values were 20.05 (HSVd-singleplex #14) and 18.20 (#12) and 31.40 (CEVd-singleplex #1) and 30.91 (#1), respectively (Table 1A). However, the SYBR Green I RT-qPCR assay was not able to identify individual viroid species in the tested samples (Table 1A).

3.2. Testing the viroid load in singleplex and multiplex RT-qPCR

The viroid load of selected samples was calculated to evaluate the quantitative capacity of the developed RT-qPCR assays. Comparison of the viroid load for each viroid species using singleplex and multiplex RT-qPCR assays showed no significant differences for all targeted viroids ($P > 0.05$) (Table 4). Viroid quantification was equally robust in mixed target assays (multiplex) versus single target assay (singleplex).

3.3. *Assessing the multiplex RT-qPCR assay*

The CEVd, CBCVd and HSVd MGB-probe multiplex RT-qPCR assay was assessed by testing 148 samples from various plant types with different geographical origin and viroid load. The tested plants included different types of citrus (44 samples-Table 2A) and non-citrus fruit trees (23 samples-Table 2B) as well as grapevines (46 samples-Table 2C) and herbaceous ornamentals and vegetables (35 samples-Table 2D).

The multiplex RT-qPCR assay successfully detected and identified the three targeted viroids in single and mixed infections in the different tested hosts (Table 2). CEVd, CBCVd and HSVd, were detected in single, double, and triple infections in 48, 39, and 14%, respectively in the citrus samples tested with Cq values ranging from 37.68 (#5, CEVd) and 19.60 (#11, CBCVd) (Table 2A). HSVd was the only viroid detected in the fruit trees and grapevine samples tested (Table 2B & 2C). The Cq values of HSVd detection ranged from 32.12 (#45) to 20.67 (#48) in fruit trees (Table 2B) and from 35.34 (#83) and 29.45 (#86) in grapevines (Table 2C). The newly developed multiplex RT-qPCR assay detected and identified the targeted viroids in the various ornamental, vegetable, and hop hosts with Cq values that ranged from 33.52 (#139, CEVd) to 19.66 (#117, HSVd) (Table 2D).

4. Discussion

Rapid detection and mitigation of graft-transmissible pathogens of citrus, including viroids, promote profitable and sustainable production of citrus (Duran-Vila and Semancik, 2003;

Roistacher 1991). Best citrus disease management practices are based on sensitive, reliable and cost-effective detection tools for large-scale pathogen surveys in orchards, nurseries, and germplasm certification programs (Anonymous, 2010; Belasque et al., 2010; Navarro, 1986; Santos Filho et al., 2000; Von Broembsen and Lee, 1988). Multiplex RT-qPCR has been reported to be a robust and economical detection tool for a broad range of different plant pathogens (Szemes et al., 2005; Balme-Sinibaldi et al. 2006; Dai et al., 2013; Li et al., 2009; Malandraki et al., 2014; Osman et al., 2013, 2015; Price et al. 2010; Qu et al. 2011; Van Doorn et al., 2007). Real-time PCR methods for the quantitative detection of one or two citrus viroids, either by SYBR Green I RT-qPCR or using hydrolysis probes have been reported (Lin et al., 2015; Loconsole et al., 2013; Monger et al., 2010, Papayiannis, 2014; Rizza et al., 2009, Saponari et al., 2013; Sun et al., 2014; Tessitori et al., 2005). This article described the development, validation, and assessment of a one-step multiplex MGB-probe based RT-qPCR assay for the simultaneous detection of three citrus viroids from different genera in a wide range of hosts. The developed multiplex RT-qPCR assay was also compared to the singleplex RT-qPCR assay for the detection of the three targeted viroids as well as a previously developed SYBR Green I RT-qPCR assay for the universal detection of citrus viroids (Vidalakis and Wang 2013).

Genetic variability within a pathogen species is often a drawback in the design of primers and MGB probes for qPCR. Despite their small genome, genetic variability within viroid species has been reported for different hosts and geographical areas (Amari et al., 2001; Fadda et al., 2003; Flores et al., 2005; Ito et al., 2002; Lin et al., 2015; Mohamed et al., 2009; Önelge et al., 2004; Owens, 2008; Semancik et al., 1994; Semancik and Duran-Vila 1999; Szychowski et al., 2005; Wang et al., 2013). Therefore, all available CEVd, CBCVd, and HSVd genome sequences in GenBank were used to design the assays, and a wide range of samples from different hosts and

geographical areas were used to validate and evaluate the assays. As a result, all the designed primers and MGB-qPCR probes were very specific and no cross-reactions occurred among viroid targets, other pathogens, or non-inoculated and non-template controls. In addition, the developed primers/probes detected and specifically identified each targeted viroid in citrus and non-citrus samples of known and unknown viroid infection status. All viroids were detected simultaneously and in an unambiguous way whether present in single, double or triple infections, indicating the robustness and reliability of this multiplex assay. Finally, the specificity and sensitivity of the developed multiplex RT-qPCR were comparable to other multiplex qPCR pathogen detection assays in citrus (Osman et al., 2015), grapes (Osman et al., 2013), pome trees (Malandraki et al., 2014), spinach (Feng et al., 2014), potatoes (Mumford et al., 2000; Li et al., 2009), and 10 more economically important plant pathogens, including oomycetes, fungi and nematodes (Szemes et al., 2005).

Despite the slight increase in C_q values in multiplex RT-qPCR compared to singleplex RT-qPCR, their ratio was always 1 or very close to 1 (Table 1A), indicating that specificity was not affected by combining three singleplex qPCR assays into a multiplex reaction. The multiplex RT-qPCR assay was able to detect and identify each of the three targeted viroids in mixed infected samples as well as in single infected samples, similarly to the singleplex assays. Measuring the intra and inter assay variations confirmed reproducibility and repeatability of the developed singleplex and multiplex RT-qPCR assays.

The SYBR Green I RT-qPCR assay approved by the CDFA for the universal detection of citrus viroids in the “Citrus Nursery Stock Pest Cleanliness Program” has been successfully used in California since 2010 (Bostock et al., 2014). More than 12,000 samples have been tested by the CCPP with this assay and as a result the viroid rate of infection in California’s citrus germplasm

sources has been reduced from 7.8% in 2010 to less than 1% in 2016 (Vidalakis 2016). The results of the newly developed MGB-probes multiplex RT-qPCR assay were in agreement with the SYBR RT-qPCR green assay. This indicated that the multiplex RT-qPCR assay presented here could potentially complement the SYBR Green I RT-qPCR assay. The simpler and more cost-effective SYBR Green I RT-qPCR assay (i.e. no use of fluorophore probes) can be used as a screening tool for a large number of samples (Mackay et al., 2002; Navarro et al., 2015). However, although the SYBR Green I RT-qPCR assay is a universal viroid detection assay, it cannot always reliably differentiate among CEVd, CBCVd or HSVd in a positive sample (data not shown). Therefore, the species-specific MGB-probes of the multiplex RT-qPCR assay could be used as required on a sample by sample basis for the identification of the targeted viroid(s) only in the subset of the SYBR Green I RT-qPCR positive tested samples. The multiplex RT-qPCR assay could also replace or be preferred to the SYBR Green I RT-qPCR assay, depending on the objectives, budget, and structure of the diagnostic project (e.g. field survey, nursery stock testing or germplasm program). It is important, however, to emphasize that further experimentation and testing of a high number of samples is recommended prior to adoption of this newly developed multiplex RT-qPCR assay by different programs and projects.

CEVd, CBCVd and HSVd have a wide host range, thus the developed multiplex RT-qPCR detection assay could be beneficial not only to the citrus industry but also to fruit trees, grapevines, vegetables and ornamental industries, both as diagnostic and research tool (e.g. study competition between viroids in mixed infections).

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Fig. 1. Standard curve analysis of qPCR sensitivity in singleplex qPCR (A) and multiplex qPCR (B) format. The X-axis displays the log copies/well and the Y-axis represents the Cq value. qPCR efficiency is determined by using a 10-fold serial dilutions of samples. CEVd: *Citrus exocortis viroid*, CLBVd: *Citrus bark cracking viroid*, HSVd: *Hop stunt viroid*.

Table 1

Comparison between minor groove binding (MGB) probes based singleplex (S) and multiplex (M) RT-qPCR assays and SYBR Green I based RT-qPCR assay in detecting viroids inoculated citrus plants across a range of different viroid titer concentrations (A) and multiplex RT-qPCR assays test of non-inoculated citrus controls (B) and non-targeted citrus pathogens (C).

A. Viroid inoculated citrus plants						RT-qPCR-Cq values											SYBR Green I
						MGB Probes											
A/A	Sample	Treatment	Variant	Citrus Host	Source	COX	CEVd			CBCVd			HSVd			S-Universal	
1	2765-1a	CEVd	CEVdc	SwO, Pineapple	G	17.28	31.40	30.96	1.01	-	-	-	-	-	-	30.91	
2	2765-1b	CEVd	CEVdc	SwO, Pineapple	G	15.23	20.80	21.02	0.99	-	-	-	-	-	-	21.60	
3	3177a	CBCVd	IV	Ctrn, 'Etrog'	G	19.00	-	-	-	21.12	21.10	1.00	-	-	-	21.77	
4	3177b	CBCVd	IV	SwO, Pineapple	G	15.25	-	-	-	22.19	23.18	0.96	-	-	-	24.68	
5	3220-5	HSVd	909	Ctrn, 'Etrog'	G	16.60	-	-	-	-	-	-	20.34	22.56	0.90	18.63	
6	10A-5-7	HSVd	909	Mand, PSM	F	18.15	-	-	-	-	-	-	21.93	24.41	0.90	22.86	
7	10A-5-8	HSVd	909	Mand, PSM	F	17.72	-	-	-	-	-	-	26.37	26.82	0.98	23.55	
8	2765-9	HSVd	909	SwO, Pineapple	G	17.01	-	-	-	-	-	-	28.12	27.67	1.02	27.23	
9	10C-12-3	HSVd	IIa	NO, Parent	F	17.52	-	-	-	-	-	-	23.29	25.62	0.91	25.70	
10	10C-12-4	HSVd	IIa	NO, Parent	F	16.53	-	-	-	-	-	-	22.92	24.99	0.92	24.84	
11	10A-5-13	HSVd	IIa	Cmac, Alemow	F	17.01	-	-	-	-	-	-	20.94	21.57	0.97	20.46	
12	10A-6-2	HSVd	IIa	Cmac, Alemow	F	17.82	-	-	-	-	-	-	22.51	22.35	1.01	18.20	
13	3044-2	HSVd	IIa (E818)	Ctrn, 'Etrog'	G	15.19	-	-	-	-	-	-	20.64	22.32	0.92	18.85	
14	3172-4	HSVd	IIa (E818)	Ctrn, 'Etrog'	G	16.15	-	-	-	-	-	-	20.05	22.24	0.90	24.33	
15	3172-5	HSVd	IIa (E819)	Ctrn, 'Etrog'	G	16.38	-	-	-	-	-	-	20.40	21.39	0.95	20.54	
16	10C-1-10	HSVd	IIa	Le, Limoneria 8A	F	18.15	-	-	-	-	-	-	24.08	24.31	0.99	22.33	
17	10C-1-11	HSVd	IIa	Le, Limoneria 8A	F	19.06	-	-	-	-	-	-	25.60	24.79	1.03	23.47	
18	10C-2-11	HSVd	IIa	Le, Limoneria 8A	F	18.38	-	-	-	-	-	-	24.57	23.46	1.05	23.97	
19	2765-4	HSVd	IIa (E818)	SwO, Pineapple	G	17.08	-	-	-	-	-	-	25.71	25.00	1.03	26.18	
20	2765-5	HSVd	IIa (E819)	SwO, Pineapple	G	17.49	-	-	-	-	-	-	25.57	23.99	1.07	26.15	
21	10C-12-14	HSVd	IIb	NO, Parent	F	13.12	-	-	-	-	-	-	24.65	26.19	0.94	26.03	
22	10C-13-3	HSVd	IIb	NO, Parent	F	16.98	-	-	-	-	-	-	25.17	27.54	0.91	28.39	
23	2765-6	HSVd	IIb (CA902)	SwO, Pineapple	G	21.65	-	-	-	-	-	-	27.09	24.74	1.09	30.16	
24	2765-8	HSVd	IIb (CA908)	SwO, Pineapple	G	16.98	-	-	-	-	-	-	26.79	28.04	0.96	26.96	
25	2765-7	HSVd	IIb (CCaVd)	SwO, Pineapple	G	17.42	-	-	-	-	-	-	24.36	23.80	1.02	26.08	
26	10C-12-9	HSVd	IIc	NO, Parent	F	17.99	-	-	-	-	-	-	25.45	27.11	0.94	28.20	
27	10C-13-6	HSVd	IIc	NO, Parent	F	15.15	-	-	-	-	-	-	25.91	27.66	0.94	29.33	
28	10C-13-8	HSVd	IIc	NO, Parent	F	17.01	-	-	-	-	-	-	24.74	26.65	0.93	28.72	
29	10A-5-3	HSVd	IIc	Cmac, Alemow	F	17.59	-	-	-	-	-	-	24.33	25.24	0.96	25.27	
30	10A-6-13	HSVd	IIc	Cmac, Alemow	F	18.15	-	-	-	-	-	-	23.29	21.01	1.11	22.85	
31	2765-14	CBCVd+CDVd	IV+IIIa	SwO, Pineapple	G	15.24	-	-	-	22.77	22.93	-	-	-	-	23.79	
32	10C-17-3	CBLVd+HSVd	Ia+IIa	Clem, Algerian	F	18.89	-	-	-	-	-	-	24.36	20.56	1.19	23.16	
33	10C-16-11	CBLVd+HSVd+CDVd	Ia+IIa+IIIb	Clem, Algerian	F	18.46	-	-	-	-	-	-	25.69	25.53	1.01	23.54	
34	10C-17-15	CBLVd+HSVd+CDVd	Ia+IIa+IIIb	Clem, Algerian	F	17.69	-	-	-	-	-	-	23.85	24.49	0.97	23.00	
35	10C-6-5	CBLVd+HSVd+CDVd	Ia+IIa+IIIb	NO, Lane Late	F	18.34	-	-	-	-	-	-	23.03	24.70	0.93	23.23	
36	10C-1-5	CBLVd+HSVd+CDVd	Ia+IIa+IIIb	Le, Limoneira 8A	F	17.76	-	-	-	-	-	-	23.31	23.68	0.98	21.10	
37	10C-1-6	CBLVd+HSVd+CDVd	Ia+IIa+IIIb	Le, Limoneira 8A	F	17.79	-	-	-	-	-	-	23.24	24.10	0.96	20.00	
38	10C-2-4	CBLVd+HSVd+CDVd	Ia+IIa+IIIb	Le, Limoneira 8A	F	17.73	-	-	-	-	-	-	24.77	23.23	1.07	24.67	
39	3207-8	Six viroids	Nine variants	SwO, Pineapple	G	17.13	24.72	25.22	0.98	24.55	24.18	1.0	23.57	25.82	0.91	24.99	
40	3207-4	CEVd+CBCVd	CEVdc+IV	SwO, Pineapple	G	18.61	23.69	25.19	0.94	22.87	23.98	1.0	-	-	-	22.85	
41	10C-15-6	HSVd+CDVd	IIa+IIIb	NO, Parent	F	14.92	-	-	-	-	-	-	24.21	26.26	0.92	24.31	
42	10C-15-7	HSVd+CDVd	IIa+IIIb	NO, Parent	F	14.84	-	-	-	-	-	-	23.21	25.65	0.90	24.11	
43	10C-15-8	HSVd+CDVd	IIa+IIIb	NO, Parent	F	14.77	-	-	-	-	-	-	22.71	25.07	0.91	23.53	
44	2765-13	HSVd+CDVd	IIa+IIIc	SwO, Pineapple	G	16.51	-	-	-	-	-	-	22.05	22.84	0.97	22.25	
45	10C-14-12	HSVd+CDVd	IIb+IIIb	NO, Parent	F	15.37	-	-	-	-	-	-	24.39	26.05	0.94	27.39	
46	10C-15-3	HSVd+CDVd	IIb+IIIb	NO, Parent	F	15.14	-	-	-	-	-	-	24.89	25.98	0.96	25.84	
47	10C-15-5	HSVd+CDVd	IIb+IIIb	NO, Parent	F	14.99	-	-	-	-	-	-	24.34	26.20	0.93	26.73	
48	10C-14-4	HSVd+CDVd	IIc+IIIb	NO, Parent	F	15.97	-	-	-	-	-	-	23.37	25.42	0.92	25.88	
49	10C-14-5	HSVd+CDVd	IIc+IIIb	NO, Parent	F	15.89	-	-	-	-	-	-	24.92	25.42	0.98	27.12	
50	10C-15-14	HSVd+CDVd	IIc+IIIb	NO, Parent	F	14.32	-	-	-	-	-	-	24.56	25.81	0.95	25.07	

B. Non-inoculated citrus controls					C. Non-targeted citrus pathogens						
A/A	Citrus Host	Source	RT-qPCR-Cq values MGB Probes		A/A	Citrus Pathogen	Variant	Source	RT-qPCR-Cq values MGB Probes		
			COX	Viroids (S & M)					COX	Viroids (S & M)	Target
51	NO, Parent	F	15.67	-	74	Citrus bent leaf viroid	Ia	G	14.65	-	18.36
52	NO, Parent	F	15.74	-	75	Citrus bent leaf viroid	LSS	G	15.24	-	22.30
53	BO, Moro	G	16.94	-	76	Citrus dwarfing viroid	IIIb	F	15.48	-	20.51
54	BO, Tarocco	G	15.87	-	77	Citrus viroid V	V	G	15.31	-	18.64
55	NO, Cara Cara	G	17.49	-	78	Citrus viroid VI	VI	G	15.35	-	18.20
56	Citrango, Carrizo	G	13.79	-	79	Citrus tristeza virus	SY558	G	16.21	-	18.25
57	Ctrn, 'Etrog'	G	16.34	-	80	Citrus tristeza virus	SY568	G	16.26	-	21.01
58	Mand, Daisy SL	G	15.29	-	81	Citrus tristeza virus	T517	G	16.61	-	23.75
59	Le, Eureka	G	16.72	-	82	Citrus tristeza virus	T525	G	16.12	-	23.10
60	NO, Fukumoto	G	13.34	-	83	Citrus psorosis virus	P201	G	16.00	-	24.62
61	Gf, Rio Red	G	13.12	-	84	Citrus psorosis virus	P250	G	16.48	-	27.72
62	Gf, Star Ruby	G	17.04	-	85	Citrus tatter leaf virus	TL100	G	16.52	-	(+)
63	Kmqt, Fukushu	G	15.64	-	86	Citrus tatter leaf virus	TL110	G	17.07	-	(+)
64	Kmqt, Nagami	G	12.97	-	87	Citrus tatter leaf virus	TL113	G	16.15	-	(+)
65	Le, Lisbon	G	17.87	-	88	Citrus vein enation virus	VE702	G	16.35	-	(+)
66	Lm, Mexican	G	14.24	-	89	Citrus vein enation virus	VE703	G	15.58	-	(+)
67	Oroblanco	G	17.19	-	90	Citrus vein enation virus	VE704	G	16.62	-	(+)
68	Lm, Persian	G	14.17	-	91	Citrus chlorotic dwarf virus*	Turkey	F	18.13	-	(+)
69	Le, Rough	G	17.79	-	92	Citrus leaf blotch virus	Spain	G	16.36	-	23.93
70	NO, Parent	G	13.27	-	93	Citrus yellow mosaic virus*	Beltsville	G	14.30	-	(+)
71	SwO, Pineapple	G	16.24	-	94	Infectious variegation virus	IVV401	G	16.65	-	22.06
72	Tangelo	G	12.89	-	95	Candidatus Liberibacter sp.*	China	F	15.66	-	22.62
73	Mand, Page	G	14.02	-	96	Spiroplasma citri	C189	G	14.86	-	28.84
					97	Spiroplasma citri	S616	G	14.73	-	28.80
					98	Xylella fastidiosa	Brazil	F	16.41	-	25.52

Cq: quantitative cycle. Source: G-greenhouse, F-field. COX: cytochrome oxidase, internal citrus gene control. CEVd: *Citrus exocortis viroid*, CBCVd: *Citrus bark cracking viroid*, HSVd: *Hop stunt viroid*. Viroids: CEVd, CBCVd, and HSVd. SwO: sweet orange-*Citrus sinensis* L. Osbeck, Ctrn: citron-*C. medica* L., Mand: *C. reticulata* Blanco, PSM: Parson's Special Mandarin, NO: navel orange- *C. sinensis*, Cmac: *C. macrophylla* Wester, Le: lemon Lisbon or Eureka-*C. limon* L. Burm.f., Clem: clementine- *C. clementina* Hort. ex Tan, BO: blood orange- *C. sinensis*, Gf: *C. paradisi* Macf., Kmqt: kumquat-*Fortunella margarita* (Lour.) Swingle and *F. obovata* Hort. ex Tan., Lm: lime- *C. aurantifolia* (Christm.) Swing. and *C. latifolia* Tan. Oroblanco-Gf hybrid: *C. paradisi* Macfadyen, Tangelo: *C. reticulata* x *C. paradisi*. CDVd: citrus dwarfing viroid, CBLVd: citrus bent leaf viroid, CVd-V: citrus viroid V. Six viroids: CEVd+CBCVd+HSVd+CBLVd+CDVd+CVd-V, Nine variants: Ia+IIa+IIb+IIc+IIIb+IV+V+909+CEVd. (+): Positive detection of respective pathogen with conventional RT-PCR. All pathogens isolates were provided by the Citrus Clonal Protection Program (CCPP), University of California, Riverside unless if otherwise noted with (*). *: Nucleic acids of citrus pathogens non-endemic to California were provide by the United States Department of Agriculture, Agriculture Research Service, Exotic Pathogen Collection of Citrus, Beltsville, MD. Citrus samples of unknown viroid status from different. Non-inoculated citrus controls were obtained from CCPP field trials and citrus nursery budwood sources in California's Department of Food and Agriculture, "Citrus Nursery Stock Pest Cleanliness Program". S/M are ratios of Singleplex/multiplex RT-qPCR assay. Source F: field, G: Greenhouse.

Table 2

Multiplex minor groove binding (MGB) probes based RT-qPCR assay test of citrus (A), non-citrus fruit trees (B), grapevines (C), and herbaceous ornamentals and vegetables (D) samples of various geographical origin and viroid load.

and virus load.

A. Citrus				Multiplex RT-qPCR-Cq values		
A/A	Sample	Citrus Host	Origin	CEVd	CBCVd	HSVd
1	B187	SwO	Australia	36.70	34.07	-
2	Az-2	Le	Azerbaijan	23.83	-	28.69
3	Riverside-1873 Parent Navel	NO, Parent Washington	Brazil	-	-	26.29
4	IPPNI22	Satsuma	China	-	-	31.03
5	B63	SwO	China	37.68	-	25.90
6	TL112	SwO	China	-	-	30.55
7	B128	SwO	Colombia	37.22	-	-
8	B310	SwO	Cuba	33.02	-	24.58
9	B305	SwO	Cuba	32.56	-	28.83
10	B296	SwO	Dominican Republic	33.10	-	33.35
11	3210-50	SwO	Greece	-	19.60	22.48
12	3210-44	SwO	Greece	29.25	26.69	25.93
13	3210-46	SwO	Greece	29.65	26.20	26.89
14	3118-29	SwO	Greece	28.80	-	28.24
15	3118-20	SwO	Greece	30.33	27.02	29.24
16	3118-25	SwO, Pineapple	Greece	-	-	27.40
17	3210-56	SwO, Pineapple	Greece	-	24.02	-
18	B194	SwO	India	-	-	34.56
19	B256	SwO	Indonesia	32.08	-	31.27
20	B199	SwO	Israel	36.07	25.28	22.32
21	B198	SwO	Israel	22.71	25.63	24.10
22	B215	SwO	Japan	33.27	-	31.34
23	B185	SwO	Japan	31.97	-	-
24	3170-2	SwO, Pineapple	Japan	-	-	20.73
25	3170-3	SwO, Pineapple	Japan	-	-	21.38
26	3170-1	SwO, Pineapple	Japan	-	-	23.39
27	Pakistan A7	BO, Blood Red	Pakistan	37.40	-	33.94
28	Pakistan A10	BO, Tarocco	Pakistan	36.79	-	34.16
29	Pakistan A9	SwO, Jaffa pure	Pakistan	35.87	-	33.46
30	Pakistan A6	SwO, Kozan	Pakistan	-	-	33.88
31	B134	SwO	Peru	-	-	27.85
32	B46	SwO	South Africa	36.00	-	33.95
33	B7	SwO	South Africa	28.26	32.90	35.10
34	B31	SwO	Spain	33.94	-	31.74
35	B316	SwO	Taiwan	27.16	-	26.77
36	B318	SwO	Taiwan	36.78	-	33.57
37	Riverside-Grapefruit	Gf, Marsh	USA	-	-	26.81
38	CDFA #1	BO, Moro	USA	27.77	-	-
39	CDFA #2	Gf, Star Ruby	USA	30.14	-	-
40	CDFA #3	Le, Frost Lisbon	USA	26.17	-	-
41	CDFA #4	Mand, Fremont	USA	27.83	-	-
42	CDFA #5	Mand, W. Murcott Afourer	USA	-	-	27.77
43	CDFA #6	NO, Cara Cara	USA	-	-	28.13
44	CDFA #7	NO, Cara Cara	USA	29.70	-	-

B. Non-citrus fruit trees

A/A	Sample	Host		Origin	Multiplex RT-qPCR-Cq values		
		Type	Name		CEVd	CBCVd	HSVd
45	A 7 29	Almond	Vesta	USA	-	-	32.12
46	D 3 73	Apricot	Tardifele Bordaneil	France	-	-	21.34
47	D 3 65	Apricot	Cegled De Mamut	Hungary	-	-	20.98
48	D 8 21	Apricot	IC 20089	India	-	-	20.67
49	D 5 17	Apricot	Nonno	Italy	-	-	23.21
50	D 8 1	Apricot	Tsuno Negume	Japan	-	-	24.11
51	D 3 21	Apricot	Dolpa	Pakistan	-	-	26.12
52	D 5 25	Apricot	Saturn	Romania	-	-	22.01
53	D 6 75	Apricot	N/A	Turkey	-	-	28.01
54	D 3 67	Apricot	V/17	Yugoslavia	-	-	21.11
55	C 1 75	Cherry	Mierlose Swarte	Netherlands	-	-	20.87
56	C 5 24	Cherry	Dawson	New Zealand	-	-	21.56
57	C 3 39	Cherry	Ostheim 32/16	Romania	-	-	22.34
58	P 4 25	Peach	Baronesa	Brazil	-	-	23.45
59	P 12 54	Peach	Sdlg Red Have	British Columbia	-	-	29.07
60	P 4 41	Peach	Hakuto	Japan	-	-	29.08
61	P 4 53	Peach	Nishiki	Japan	-	-	21.89
62	P 4 31	Peach	Carlos	Mexico	-	-	27.34
63	P 12 7	Peach	1Neg125	Nepal	-	-	28.76
64	P 4 59	Peach	Kiang Negsi	Spain	-	-	25.66
65	WSU	Plum	Unknown	USA	-	-	27.86
66	E 1 11	Plum	Amphidiploid	Russia	-	-	31.04
67	E 7 65	Plum	Elephant Heart	Unknown	-	-	30.99

C. Grapevines				Multiplex RT-qPCR-Cq values		
A/A	Sample	Grapevine Host	Origin	CEVd	CBCVd	HSVd
68	K5-22	Ghula Dari	Afghanistan	-	-	31.69
69	K7-16	Siar	Afghanistan	-	-	32.76
70	K7-32	Takhani	Afghanistan	-	-	32.21
71	K3-35	Moscatel Ros.	Argentina	-	-	31.65
72	K8-59	Royal	Belgium	-	-	31.54
73	K2-16	Hongjixin	China	-	-	30.10
74	K2-17	Niunai	China	-	-	31.28
75	K2-23	Kastour Rumi	Egypt	-	-	31.84
76	K5-01	Duc De Malakoff	France	-	-	31.82
77	K6-31	Olivette Rose	France	-	-	31.76
78	K1-60	Poet Matabon	France	-	-	33.07
79	K1-23	Servant	France	-	-	30.71
80	K1-02	Adjem Misquet	Germany	-	-	30.68
81	K1-32	Black Hamburg	Germany	-	-	31.50
82	K4-01	Kovalewka	Germany	-	-	30.34
83	K1-06	Murma Isium	Germany	-	-	35.34
84	K4-07	Stambulari	Germany	-	-	33.56
85	K2-13	Hakiki	Greece	-	-	31.69
86	K3-45	Muscat of Hungary	Hungary	-	-	29.45
87	K3-19	Baharat Early	India	-	-	31.79
88	K1-51	Rangspray	India	-	-	30.48
89	K8-61	Salomani	Iraq	-	-	31.20
90	K5-10	Esperia	Italy	-	-	31.75
91	K5-56	Lattuario	Italy	-	-	31.82
92	K1-67	Pirovano 47	Italy	-	-	32.37
93	K7-57	Volta	Italy	-	-	32.29
94	K1-53	Royal Thompson	Mexico	-	-	31.24
95	K7-11	Shami	Palestine	-	-	30.68
96	K1-69	Asma	Russia	-	-	31.76
97	K1-39	Chaush White	Russia	-	-	31.12
98	K1-03	Dschan Im Isium	Russia	-	-	31.11
99	K1-79	Khusaine Red	Russia	-	-	30.37
100	K3-29	Kishmish Early	Russia	-	-	33.89
101	K1-48	Kishmish Hishrau	Russia	-	-	32.22
102	K1-73	Kishmish of Vir	Russia	-	-	30.17
103	K3 - 31	Kishmish-Charni	Russia	-	-	31.13
104	K4 - 03	Noir D'automne	Russia	-	-	31.59
105	K2-73	Shtur Angur	Russia	-	-	30.32
106	K1 - 56	Sochal	Russia	-	-	31.66
107	K1 - 11	Cape Currant	South Africa	-	-	29.96
108	K8 - 54	Red Hanepoot	South Africa	-	-	31.28
109	K1 - 14	Hyaales	Spain	-	-	31.78
110	K1 - 19	Pizzutello	Spain	-	-	31.70
111	K5 - 15	Exotic	USA	-	-	33.40
112	K2 - 11	Aswad	Yemen	-	-	30.93
113	K1 - 15	Kurtelaska	Yugoslavia	-	-	30.31

D. Herbaceous ornamentals and vegetables						Multiplex RT-qPCR-Cq values		
A/A	Sample	Treatment	Variant	Host	Origin	CEVd	CBCVd	HSVd
114	5.28.2003 Blue 8	HSVd	909	Chrysanthemum	USA	-	-	23.08
115	7.14.2003 Blue 12	HSVd	IIC	Chrysanthemum	USA	-	-	24.75
116	7.1.2003 Blue 11	CEVd	CEVdc	Chrysanthemum	USA	24.04	-	-
117	8.5.2002 Red 11	HSVd	909	Cucumber	USA	-	-	19.66
118	8.7.2002 Red 17	HSVd	IIC	Cucumber	USA	-	-	21.76
119	10.19.989 Jar Green 1 SB	CBCVd	IV	Cucumber	USA	-	25.46	-
120	5.25 Jar Green 9 SB	CEVd	M 8-2	Cucumber	USA	31.09	-	-
121	7.14.2003 Blue 21	HSVd	909	Datura	USA	-	-	22.86
122	7.22.2003 Blue 24	HSVd	IIC	Datura	USA	-	-	25.00
123	7.24.2003 Blue 26	CEVd	GV4	Datura	USA	33.17	-	-
124	7.14.2003 Blue 20	HSVd	909	Eggplant	USA	-	-	22.08
125	A33 Jar Green 6 SB	CEVd	M	Gynura	USA	31.05	-	-
126	CPCNW-WSU	HSVd	N/A	Hop	USA	-	-	22.12
127	8.18.2003 Blue 43	HSVd	909	<i>Solanum chilense</i>	USA	-	-	25.86
128	11.12.2003 Grey 2	CEVd	GV4	<i>S. chilense</i>	USA	24.11	-	-
129	7.14.2003 Blue 14	CEVd	GV4	<i>S. chilense</i>	USA	26.90	-	-

130	8.18.2003 Blue 45	HSVd	909	<i>S. habrochaites</i>	USA	-	-	25.57
131	11.12.2003 Grey 4	CEVd	GV4	<i>S. habrochaites</i>	USA	24.90	-	-
132	7.14.2003 Blue 16	CEVd	GV4	<i>S. habrochaites</i>	USA	30.82	-	-
133	8.18.2003 Blue 44	HSVd	909	<i>S. neorickii</i>	USA	-	-	24.79
134	7.14.2003 Blue 15	CEVd	GV4	<i>S. neorickii</i>	USA	30.73	-	-
135	8.18.2003 Blue 46	HSVd	909	<i>S. pennellii</i>	USA	-	-	22.33
136	7.14.2003 Blue 17	CEVd	GV4	<i>S. pennellii</i>	USA	26.28	-	-
137	8.18.2003 Blue 42	HSVd	909	<i>S. peruvianum</i>	USA	-	-	26.39
138	7.14.2003 Blue 13	CEVd	GV4	<i>S. peruvianum</i>	USA	27.68	-	-
139	11.12.2003 Grey 1	CEVd	GV4	<i>S. peruvianum</i>	USA	33.52	-	-
140	10.10.2002 Red 33	HSVd	909	Luffa	USA	-	-	20.08
141	8.7.2002 Red 23	HSVd	Ilc	Luffa	USA	-	-	22.03
142	5.28.2003 Blue 7	HSVd	909	Micro-Tom	USA	-	-	26.71
143	5.28.2003 Blue 6	HSVd	909	Micro-Tom	USA	-	-	26.81
144	7.1.2003 Blue 10	CEVd	CEVdc	Micro-Tom	USA	25.29	-	-
145	8.7.2002 Red 25	HSVd	909	Tomato-Rutgers	USA	-	-	22.09
146	10.10.2002 Orange 4	HSVd	Ilc	Tomato-Rutgers	USA	-	-	28.48
147	5.28.2003 Blue 1	CEVd	GV4	Tomato-Rutgers	USA	24.47	-	-
148	5.28.2003 Blue 4	CEVd	CEVdc	TomP	USA	24.15	-	-

Cq: quantitative cycle. CEVd: *Citrus exocortis viroid*, CBCVd: *Citrus bark cracking viroid*, HSVd: *Hop stunt viroid*. SwO: sweet orange-*Citrus sinensis* L. Osbeck, Le: lemon Lisbon or Eureka-*C. limon* L. Burm.f., NO: navel orange-*C. sinensis*, Satsuma: *C. unshiu* Marcovitch, BO: blood orange- *C. sinensis*, Gf: *C. paradisi* Macf., Mand: *C. reticulata* Blanco. Almond, apricot, cherry, peach, plum (*Prunus* sp.), grapevines (*Vitis* sp.). Chrysanthemum (*Dendranthema morifolium* L.), cucumber (*Cucumis sativus* L.), datura (*Datura stramonium* L.), eggplant (*Solanum melongena* L.), gynura (*Gynura aurantiaca* D.C.), hop (*Humulus lupulus* L.), luffa (*Luffa aegyptiaca* Mill.), Micro-Tom and Tomato-Rutgers (*Solanum lycopersicum* L.), TomP (*S. lycopersicum* L. × *S. peruvianum*). CCPP records list *S. habrochaites* as “*L. hirsutum*” and *S. neorickii* as “*L. parviflorum*”. CPCNW-WSU: Clean Plant Center Northwest, Washington State University. Citrus samples were provided by the Citrus Clonal Protection Program (CCPP), University of California, Riverside, CA, California’s Department of Food and Agriculture (CDFA), “Citrus Nursery Stock Pest Cleanliness Program”, and United States Department of Agriculture (USDA), Agriculture Research Service (ARS), Exotic Pathogen Collection of Citrus, Beltsville, MD. Non-citrus fruit trees and grapevines samples were provide by USDA-ARS, National Clonal Germplasm Repository for Tree Fruit and Nut Crops and Grapes, Davis, CA. Herbaceous ornamental and vegetable samples were provide by the CCPP and CPCNW.

Table 3

Oligonucleotide primers and qPCR probes used for singleplex and multiplex RT-qPCR assay

Primers/probes	Sequence 5'- 3'	Nucleotide Position	Amplicon size (bp)
COX multi-32 F	AATCTGACCTTCTTTCCCATGC	32-53	162
COX multi-194 R	AAGTGATTGTTACGACCACGAAGA	194-171	
COX multi-96 P FAM	ATCCAGATGCTTACGCTGG	96-114	
CEVd -161 F	GTCCAGCGGAGAAACAGGAG	181-200	105
CEVd -258 R	AGAGAAGCTCCGGGCGA	270-286	
CEVd -187 P FAM	TCCTTCCTTTTCGCTGCT	212-228	
CBCVd-44 F	GGGAACATACCTGAAGAGGGATC	113-135	89
CBCVd-133 R	TTCCGGTGCTGGACGC	187-202	
CBCVd-77 P VIC	TCTCTTCAGACTCGTCGAGG	146-165	
HSVd-208 F	GAGACGCGACCGGTGG	216-231	88
HSVd-295 R	GCTCAAGAGAGGATCCGCG	286-304	
HSVd-226 P TET	TCACCTCTCGGTTCGTC	234-250	

COX: Cytochrome oxidase gene of host plants for positive internal control. CEVd: citrus exocortis viroid, CBCVd: citrus bark cracking viroid, HSVd: hop stunt viroid. Nucleotide position is based on GenBank accessions: COX-CX297817, CEVd-HQ284019, CBCVd-KC121568, HSVd-KJ810553. F: forward primer. R: Reverse primer. p: qPCR probe. Primers used for the SYBR Green I based assay for universal detection of citrus viroids reported previously by Vidalakis and Wang, 2013.

Table 4

Comparison between singleplex and multiplex qPCR assays in detecting *Citrus exocortis viroid* (CEVd), *Citrus bark cracking viroid* (CBCVd) and *Hop stunt viroid* (HSVd). Viroid Load/1 mg is shown.

RT-qPCR	CEVd		CBCVd		HSVd	
	Singleplex Cq	Multiplex Cq	Singleplex Cq	Multiplex Cq	Singleplex Cq	Multiplex Cq
Viral load mean (n=3)	6.05E+06	1.79E+06	2.39E+06	7.60E+06	6.16E+06	1.31E+06
Standard deviation	1.42E+07	4.07E+06	5.59E+06	1.98E+07	1.20E+07	3.03E+06
<i>p</i> value	0.23294		0.18193		0.16493	

Statistical analysis was performed using PASW Statistics 18 software (SPSS Inc, Chicago, IL). Analysis of variance (ANOVA) was used to compare the quantitative cycle (Cq) values of the tested samples. n: number of replications= 3