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Increased Efficiency and Sensitivity for Identifying Citrus Greening and *Citrus Tristeza Virus* using Real-time PCR Testing

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All certified citrus nursery trees can trace their origins to fully tested scion and foundation source trees that remain clean and disease-free in screenhouses. Implementation of real-time polymerase chain reaction (PCR) for pathogen testing has lead to an increased requirement for both the testing efficiency and sensitivity in the Florida Bureau of Citrus Budwood Registration testing program. Budwood source citrus trees are tested annually for the vectored diseases *Citrus tristeza virus* (CTV) and citrus greening (HLB) and once every 6 years for other graft-transmissible diseases. To test the current number (more than 6000) of source trees annually, streamlining of collection and extraction of samples was necessary. Advance preparation allowed greater efficiency during the late summer to early fall months that samples are collected, extracted, and tested for HLB. Specialized equipment, including a new type of grinding apparatus, reduced costs and the time needed for processing. The second yearly collection from the same trees had been carried out during Spring 2008 for CTV ELISA testing. Two real-time CTV PCR tests, used in conjunction from a single extraction, have replaced the second collection and CTV enzyme-linked immunosorbent assay (ELISA) test. After extraction, testing for each pathogen can be completed in one month, previously an impossible task by conventional PCR. *Citrus leaf blotch virus* was the first pathogen tested using the same extraction as for HLB. One collection, one sample, and one extraction save money and allow for thorough, sensitive budwood testing for multiple citrus pathogens.

When the Florida Bureau of Citrus Budwood Registration Program (BCBR) changed from a voluntary to a mandatory program in the late 1990s, there were approximately 3200 foundation source trees and 4600 budwood source trees. At that time the Bureau's foundation trees were tested for *Citrus tristeza virus* (CTV) yearly using enzyme-linked immunosorbent assay (ELISA) while scion grove trees were tested by commercial laboratories based on MCA13 reactivity (Sieburth, 2000).

Re-indexing for citrus viroids by biological indexing was done every 6 years with viroid II tested for by reverse transcription polymerase chain reaction (RT-PCR) (Sieburth et al., 2002). More extensive testing was done on incoming parent candidates and shoot tip grafts (STGs). The laboratory staff was increased at that time from one and one-half to three full-time personnel.

With the advent of citrus greening (HLB) in Florida in Fall 2005 (Halbert, 2005), the rule governing citrus propagation was strengthened (Fl Statute 5B-62, 2006). The supply of citrus nursery trees was protected by requiring the screening and frequent testing of citrus budwood source trees. Florida's citrus nursery trees are propagated from either increase blocks or scion budwood source trees. Foundation, scion, increase, and nursery trees all remain clean and disease-free in screenhouses. All nursery budwood source trees originate from foundation source trees, so it is important that foundation trees have the most thorough testing, followed by the scion trees. Other changes instituted with the rule change in 2006 increased required testing. Responsibility for annual CTV testing was assumed for scion grove trees and yearly testing for HLB was also added. The other non-vectored, graft-transmissible diseases are tested for once every 6 years unless disease-incidence indicates otherwise. *Citrus leaf blotch virus* (CLBV), a seed-transmitted virus (Guerri et al., 2004), had not been tested for in all source trees and was a priority for testing. To accommodate this increase in laboratory testing, two Bureau positions were converted to laboratory positions, increasing the laboratory staff to five positions.

To have a sufficiently sensitive test for HLB, a real-time machine was needed. To have the flexibility to use the machine for additional pathogen testing, an Applied Biosystems 7500 Fast real-time machine was chosen to be able to maximize the number of tests that could be run. Seed money for primers and probes from a FCPRAC grant was utilized to test and develop real-time PCR assays for the other pathogens in the testing program. The number of tests that needed to be run also increased, with 1500 foundation trees, parent candidates, and shoot-tip grafts needing more extensive testing, 5000 scion budwood source trees and 3500 seed source trees requiring testing for seed-transmitted diseases.

There was a need to reduce the labor of the collection, extraction, precipitation, and PCR procedures, as well as keep costs to a minimum. It was important while doing this to increase test sensitivity to a maximum to reduce the possibility of false negatives

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that could send thousands of diseased nursery trees throughout the state. The use of random hexamers to create enough RT for all tests being run was compared to using one-step real-time PCR in sensitivity and costs. The objectives of this study were to: 1) utilize a single extraction to start running real-time PCR for HLB and other pathogens; 2) develop a real-time PCR test for CLBV; 3) convert *Citrus tristeza virus* testing from an ELISA format to a real-time PCR assay that could identify CTV strains in samples with low titer, effectively utilize budwood tissue to test for CTV, have enough degeneracy to identify all CTV strains contained in the collection, and reliably differentiate between mild and severe isolates; 4) streamline the collection and extraction process; and 5) reduce testing costs where possible.

Materials and Methods

Tissue collection and preparation of nucleic acid extracts. For all samples (field or greenhouse), young bark tissue was stripped and cut into approximately 2-mm lengths and 500 mg of tissue was placed into Homex universal homogenization bags (STA Laboratories, Longmont, CO) or paper envelopes. Tissue was stored at –20 °C until assayed. Bark tissue was homogenized in 5 mL of extraction buffer (100 mM Tris-HCl; 50 mM NaEDTA; 500 mM NaCl; and10 mM mercaptoethanol) for 40 s using the Kleco (Kinetic Laboratory Equipment Comp., Visalia, CA) or 20 s using the Homex-6 (STA Laboratories). Total nucleic acids were extracted as previously described by Irey et al. (2006). Positive material used for the studies were from the CTV and CLBV isolate collections maintained in citrus plants in a greenhouse at the Bureau of Citrus Budwood Registration (BCBR) in Winter Haven, FL. Citrus greening material was from symptomatic field trees.

HLB detection utilizing real-time PCR. To detect citrus budwood samples infected with *Candidatus* Liberibacter asiaticus (Las), the only HLB strain currently identified in Florida, primers (HLBas and HLBr) and probe (HLBp-FAM) were ordered from Integrated DNA Technologies, Inc. (Coralville, IA) as previously described (Li et al., 2006). Quantitative real-time PCR reactions were set up using Taqman® Fast Universal PCR Master Mix reagents (Applied Biosystems, Foster City, CA), HLBas (200 nM), HLBr (200 nM), HLBp (100 nM), PVP-40 (2.5%), and 1 μL of nucleic acid template (~200 ng). Samples were analyzed with an Applied Biosystems 7500 Fast Real-Time PCR System using previously described conditions (Irey et al., 2006). The C_t value for each sample was determined using the Auto C_t Analysis Setting. Samples with C_t values between 0 and 31.99 were deemed positive, while samples with C_t values from 36.00 to 40.00 were deemed negative for HLB. Samples with C_t values between 32.00 and 35.99 were called "suspicious" and were recollected and retested.

CLBV detection utilizing real-time PCR. A CLBV-specific primer-probe set was developed using a non-redundant set of sequenced CLBV isolates selected from Genbank [National Center for Biotechnology Information (NCBI)] (EF203230, AJ488047, AJ488182, AJ488183, AJ488184, AJ488185, AJ488186, AJ488187, AJ488188, AJ488189, AJ488190, AJ488191, AJ488192, AJ488193) and aligned using ClustalW2. Primer Express 3.0 (Applied Biosystems) was then utilized to identify a primer/probe set specific to a highly conserved region identified from the sequence alignment (AJ488047: bp 295 – bp 357). A 20 μL quantitative real-time PCR reaction containing 1× One-Step TaqMan® RT-PCR Master Mix (Applied Biosystems), 1× RT Enzyme Mix (Applied Biosystems), 0.4 U/μL RNase Inhibitor (Applied Biosystems), CLBf 5´-GCAGCAATTCCAACTGAAGCT-3´ (250 nM), CLBr 5´-TTCTTCATTTGTTGGAGGCCTT-3´ (250 nM), CLBp 5´-/56-FAM/ ATGGGAGYGTTAGAYTGA/ 3IABLFQ/-3´ (150 nM), 1 μL of nucleic acid template, and RNase/DNase free-water was then utilized to screen budwood samples for CLBV infection. For greenhouse CLBV isolates and healthy control trees, two sets of cDNA were made from two trees for each isolate. For field trees, two sets of cDNA were made from a late-Fall 2007 and early-Spring 2008 tissue collection. For each sample, two reactions were made using template from one biological replication, and one reaction was set up using the other replicate (n = 3). Samples were analyzed with an Applied Biosystems 7500 Fast Real-Time PCR System under the following conditions: 48 °C for 30 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 50° C for 1 min. The C_t value for each sample was determined using the Auto C_t Analysis Setting. Samples with C_t values between 0 and 31.99 were deemed positive, while samples with C_t values from 36.00 to 40.00 were deemed negative for CLBV. Samples with C_t values between 32.00 and 35.99 were called "suspicious" and were recollected and retested. The three C_t values for each sample were then averaged, and a standard error was calculated.

CTV detection utilizing real-time RT-PCR. Primer and probe sets initially tested to detect CTV were Ruiz-Ruiz et al. (2007), Saponari et al. (2008), and Yokomi et al (2009). The primer/probe set used for the universal detection of CTV were UTR1, UTR2 and 181T as previously described by Bertolini et al. (2008). Real-time RT-PCR reactions contained 1× TaqMan® One-Step RT-PCR Master Mix (Applied Biosystems), 1× RT Enzyme Mix (Applied Biosystems), 0.4 U/μL RNase Inhibitor (Applied Biosystems), 300 nM UTR1, 50 nM UTR2, 200 nM 181T and 1 μL (~20 ng) of nucleic acid template. RT-PCR consisted of reverse transcription at 48 °C for 30 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 62 °C for 1 min. For each sample one replicate was run from budwood tissue collected in Spring 2008, while two replicates were run from budwood tissue collected in Fall 2009. The resulting C_t values were then averaged and a standard error was calculated. The C_t value for each sample was determined using the Auto C_t Analysis Setting. Samples with C_t values between 0 and 34.99 were determined positive for CTV. Samples with C_t values between 35.00 and 40.00 were determined negative for CTV.

Primers used for mild and severe strain differentiation, CN218 and CN120 as previously described by Cevik et al. (1996), were converted to a Power SYBR® Green real-time RT-PCR assay. Reactions contained 1× Power SYBR® RNA-to-C_t™ One-Step Master Mix (Applied Biosystems), 1× RT Enzyme Mix (Applied Biosystems), 100 nM CN 218, 100 nM CN120 and 2 μL (~40 ng) of nucleic acid template. RT-PCR consisted of reverse transcription at 48 °C for 30 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The C_t value for each sample was determined using the Auto C_t Analysis Setting, and the mean C_t value and standard error were calculated as above. The evaluation of strain differentiation was determined using results from both the universal primer/probe set and primers CN218 and CN120. Samples positive for the universal primer/probe set and with C_t values between 0 and 34.99 for CN218 and CN120 were determined as "severe." Samples positive for the universal primer/probe set and with C_t values between 35.00 and 40.00 for CN218 and CN120 were determined as "mild."

Synthesis of DNA product was confirmed by melting curve

analysis using the Applied Biosystems 7500 Fast software.

Streamline collection and extraction processes and reduce testing costs. To streamline collection and extraction process, time usage was evaluated so that inefficiencies could be eliminated. To reduce costs, all aspects of collection, extraction, and real-time PCR were examined to determine what could be reduced or changed to result in cost savings.

Results

Summary of 2007–08 HLB real-time PCR results. The availability of a nucleic acid extraction for citrus budwood tissue has made it possible to effectively and efficiently screen a large number of samples for infection with HLB. More than 13,000 field and budwood source samples were tested for HLB over the past 2 years (2007–08), and a clear distinction in C_t values between positive and negative samples was observed (Table 1). Thirty-three samples fell in the positive range (C_t values between 0 and 32) with only three samples observed between 30 and 32, indicating a clear boundary between positive and the remaining samples. Samples (12,758) in the 36–40 Ct range or undetermined were deemed negative for HLB, while the remaining samples (101) between 32 and 36 were called suspect and recollected for testing. Overall, 0.2% of the samples tested positive for HLB, with 0.8% of the samples requiring a retest so 99% of the samples tested negative for greening. Negative samples with higher C_t values were observed when testing field samples as compared to budwood source trees where results were predominantly undetermined (Table 2).

CLBV detection utilizing real-time PCR. A primer/probe set was developed and used to test two CLBV isolates in Nagami Kumquat (FS694) and Roble (FS668) from the Bureau of Citrus Budwood Registration (BCBR) Pathogen Collection, along with a healthy Etrog Citron also cultivated in the greenhouse (Fig. 1). The primers/probe effectively amplified a product in the two CLBV isolates [average C_t value: 28.5 (FS694) and 24.3 (FS668)], but did not amplify a measurable product in the healthy control (average C_t value: undetermined). Comparison of these results to those generated using a similar assay (Ruiz-Ruiz et al., 2009) did not show any significant differences (data not shown).

To test if the primer/probe set could also screen field trees for infection with CLBV, two sets of tissue (Spring and Fall) were collected from a subset of field trees from the BCBR Arboretum (Winter Haven, FL) representing a wide variety of citrus including Roble and Nagami kumquat cultivars, both previously shown to be infected with CLBV (Galipienso et al., 2000; Vives et al. 2002). Results from real-time PCR analysis supported these results showing an amplification of CLBV product in the two Roble samples (average C_t value: 30.2 and 27.5), as well as the Nagami kumquat (28.4) (Fig. 1). All other varieties tested did not show a measurable amplification of CLBV product.

Conversion from ELISA to real-time PCR based CTV assays. To transition from ELISA (Permar et al., 1990) to a real-time PCR based assay for the detection and differentiation of mild and severe isolates of CTV, published real-time PCR protocols were tested to determine their effectiveness at screening the BCBR CTV Pathogen Collection (Sieburth and Nolan, 2005). Early CTV tests with real-time PCR on source trees were compared to ELISA results. These results revealed that real-time

Table 1. Quantitative real-time PCR citrus greening results.

Ct range[z]	2007 Samples	2008 Samples
0–24	0	0
25	2	0
26	7	1
27	8	1
28	0	0
29	1	8
30	0	2
31	0	1
32	0	2
33	3	10
34	3	31
35	9	45
36	15	114
37	12	130
38	15	174
39	28	196
Undetermined	4263	7811
Total	**4366**	**8526**

[z]Ct values for reactions run on budwood tissue collected from screenhouse- and field-grown trees sampled from Jan.–Dec. 2007 and Jan.–Dec. 2008. Samples with Ct values from 0–31.9 were considered positive, 32–35.9 suspect (need to be recollected), and 36–40 negative. Samples not assigned a Ct value were labeled undetermined and were also negative.

Table 2. Quantitative real-time PCR citrus greening results.[z]

	Field trees	Greenhouse trees
0–24	0.00	0.00
25–32	0.21	0.00
33–35	0.09	0.00
35–36	0.77	0.08
36–37	0.85	0.49
37–38	2.81	0.67
38–39	2.77	1.49
39–40	92.51	97.26

[z]Values are expressed as a percentage of the total number of samples tested (field trees, N = 2349; greenhouse trees, N = 4892).

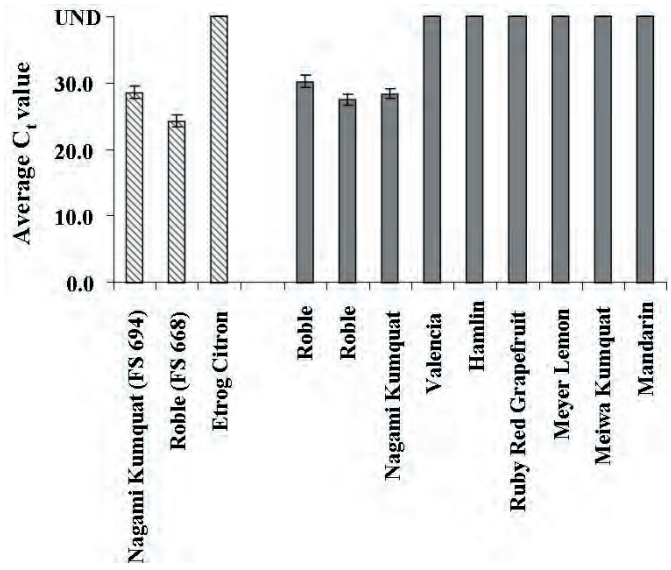

Fig. 1. *Citrus leaf blotch virus* detection utilizing quantitative real-time PCR. Mean Ct values (n = 3, ±SE) were determined from budwood tissue collected from greenhouse (light gray) and field (dark gray) trees. Field tissue was collected in late Fall 2007 and early Spring 2008, while greenhouse tissue was collected in early Spring 2008. UND = undetermined.

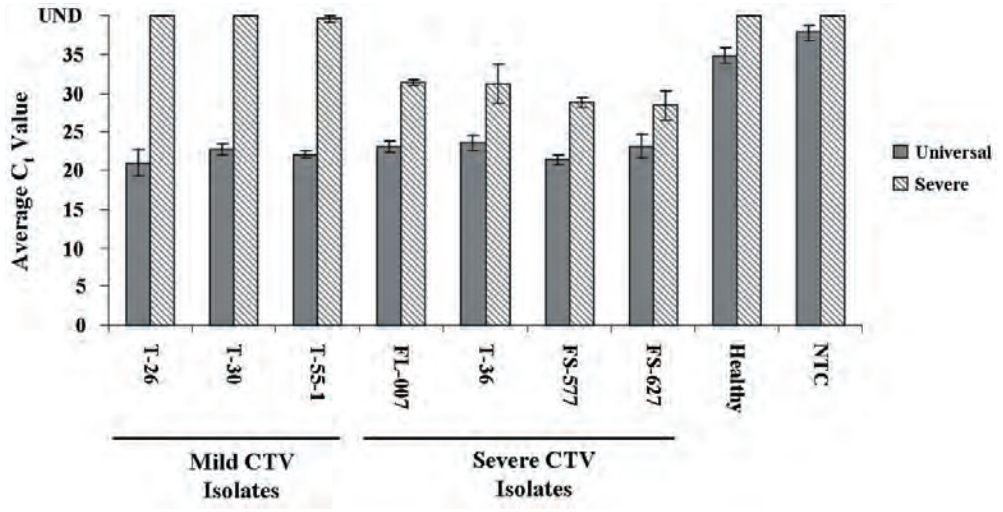

Fig. 2. CTV real-time PCR analysis utilizing a universal and severe-specific primer/probe set. Mean Ct values (n = 3, ±SE) were determined from budwood tissue collected from the BCBR CTV Pathogen Collection during Spring 2008 and Fall 2009. Samples were tested for the presence of any CTV strain (Universal = dark gray) and whether these detected strains were severe stem-pitters (Severe = light gray). UND = undetermined.

PCR was more sensitive for samples collected at sub-optimal times for ELISA testing with a greater percentage of CTV infections detected (data not shown). The initial assays tested did not have the necessary degeneracy to identify all test strains (Ruiz-Ruiz et al., 2007; Saponari et al., 2008) while the subsequent assay tested could not reliably discern decline and stem pitting isolates in the CBRB CTV Pathogen Collection (Yokomi et al., 2009).

To test the effectiveness of a universal primer/probe set (Bertolini et al., 2008), bark tissue was collected from each sample in the CBRB CTV Pathogen Collection both in Spring 2008 and Fall 2009. Samples were tested for infection with any CTV strain. Results from this assay showed that the universal primer/probe set was able to correctly identify the existence of CTV (C_t value less than 35) in 134 out of 134 pathogen collection samples (Fig. 2) previously confirmed to be infected by CTV using ELISA (data not shown).

Pathogen collection samples were then tested for infection with "severe" strains of CTV using a severe-strain specific (CN120/CN218) primer set (Cevik et al., 1996). Results from this assay showed that 89 of the 134 samples tested were positive for a "severe strain" of CTV (C_t less than 35), while the remaining 45 samples were determined to contain only "mild strain" CTV isolates (C_t value greater than 35) (Fig. 2). Out of the 134 samples tested, 133 were in agreement with previous ELISA data with one sample determined to contain a "severe strain" by real-time PCR but a mild strain by ELISA. To confirm the authenticity of the CN120/CN218 Power SYBR® Green assay all resulting PCR products were also analyzed by melting curve analysis. The products of samples containing "severe" strains had a melting temperature of 80.8 ± 0.05 °C, while samples thought to contain products from "mild" strains had a melting temperature of 74.7 ± 0.63 °C (Table 3). Additionally, the melting temperature for samples containing "severe" strains were highly reproducible resulting in a small standard error when compared to samples containing "mild" strains as well as the healthy and non-template controls.

Streamlined collection and extraction processes and reduced testing costs. Advanced preparation for collections enable over 100 samples to be collected per person per day. Prelabeled collection bags (standard address labels) were organized on metal binder rings. To allow quicker organization when samples were returned to the laboratory, bags were not removed from rings during collection.

The extraction process is separated into three steps: chopping, grinding/extraction and precipitation for maximum efficiency. Samples were chopped immediately upon arrival which allowed more than 150 samples to be chopped per person per day. Extraction was divided into the three stations of buffer addition, sample grinding, and sample transfer. The conversion from the Kleco to the Homex-6 eliminated 16 weeks yearly of canister washing and initial sample preparation time. Similar results were obtained from samples ground with the Kleco or Homex-6 and processed identically (data not shown). Equipment used to increase efficiency in the extraction process include a 60-place horizontal drum rotor centrifuge (a large number of samples with pellet at the bottom of tube instead of the side) and bottle top dispensers with attached stylus (dispense buffers without the use of pipette tips). A total of 1000 samples could be extracted in 1 d.

The final step, precipitation, utilizes a vacuum centrifuge (96 samples dried in 12 min) that allows for up to 1000 samples to be dried and resuspended in 1 d. A multichannel adjustable pipette was used for the initial transfer of samples from 1.5-mL tubes to 96 well plates to conveniently store samples. This streamlined subsequent PCR set-up by allowing samples to be transferred directly to 96-well optical plates requiring 12 transfers utilizing an 8-channel pipette (instead of 96 individual transfers).

Table 3. Average melting curve and standard error data for CTV strains tested using the SEV SYBR green real-time assay.

Severe isolates	80.8 ± 0.05
Mild isolates	74.7 ± 0.63
Healthy control	68.2 ± 0.89
NTC	72.7 ± 2.48

[z]NTC, N = 8; Healthy, N = 8; SEV, N = 89; MLD, N = 45.

Discussion

The Bureau of Citrus Budwood Registration (BCBR) requires yearly testing of more than 6000 budwood source trees for multiple pathogens. This process is cost and labor intensive, requiring a large commitment both from the Citrus Budwood Program and well as from the citrus nurseries in lost propagative material during sampling. The development of a real-time PCR based testing program centered on a single yearly collection has been a critical step towards reducing costs and increasing the scope and efficiency of the testing program.

Each year budwood tissue is collected from source trees and a single nucleic acid extraction is used to develop a needed template for real-time PCR testing. Initially, this template was used to test for citrus greening (DNA-based assay); subsequently, it was used for a random-hexamer-based reverse transcriptase (RT) reaction. The resulting RT product formed a universal template that was screened for a wide range of viral (RNA-based) pathogens. However, sensitivity was improved by switching to a one-step RT-PCR reaction that precluded use of the random-hexamer based RT reaction. Following each nucleic acid extraction, samples are aliquoted and placed directly into 96-well stock plates prior to HLB testing.

As one of the world's most destructive citrus diseases (da Graça, 1991), HLB has become a significant problem around the world. Following the discovery of HLB in Florida (Halbert, 2005), the development and implementation of a sensitive real-time PCR assay has become a high priority for the BCBR. In collaboration with other labs around Florida, several variations of HLB assays have been compared to determine the effectiveness of tests for HLB (Irey et al., 2009; Turechek et al., 2009). The use of a primer/probe set specific for the *Candidatus* Liberibacter asiaticus species of HLB (Li et al., 2006, 2007) in conjunction with a Taqman® Fast real-time assay has proved to be a powerful tool for high thorough-put testing of both field- and screenhouse-grown trees. Over the past 2 years, more than 13,000 trees have been tested for HLB as part of the BCBR testing program. Analysis of these results have identified field trees that tested positive for HLB; however, to date HLB has not been detected in any budwood source trees. Field trees that were brought into screened structures have also continuously tested negative. Additionally, a difference in the background has been observed in field trees compared to screenhouse trees. Field trees testing negative for the presence of HLB (C_t value 36–40) averaged a lower C_t value compared to values observed in screen-house grown trees. This indicates that consideration needs to be given as to the type of samples being collected (budwood source trees erring towards false positives, or field trees, erring towards false negatives) when selecting the stringency for the C_t cut off between negative and suspect (C_t value 32–36) samples.

Developing real-time PCR assays that could effectively screen for a wide variety of citrus pathogens using the same template used for HLB testing was critical towards increasing the efficiency of the BCBR testing program. CLBV represented the first step in a comprehensive real-time RT-PCR assay system developed and optimized to screen for viral infection in budwood tissue originally collected for HLB testing. CLBV is a graft-transmissible citrus RNA virus thought to cause bud union crease (Galipienso et al., 2000). Since CLBV has also been shown to be transmitted through seed (Guerri et al., 2004), testing for this virus in seed source trees is a high priority. Previously it has been shown that both the Roble and Nagami kumquat cultivars are infected with CLBV (Galipienso et al., 2000; Vives et al. 2002). To test the effectiveness of a novel CLBV primer/probe set, CLBV isolates (FS694, FS668) from the BCBR pathogen collection, previously shown to be infected with CLBV by conventional PCR (data not shown), were used. Bark tissue was collected and screened for CLBV. The novel CLBV primer/probe set effectively identified CLBV in the FS694 and FS668 isolates, but not in the healthy control variety 'Etrog Citron' or in the field trees, 'Valencia', 'Hamlin', grapefruit, mandarin, and kumquat. Only cultivars previously known to contain CLBV ('Roble' and 'Nagami' kumquat) were infected with CLBV. Comparison of these results with those obtained using a recently published real-time RT-PCR assay (Ruiz-Ruiz et al., 2009) indicates that these two real-time PCR assays are comparable. The increased sensitivity afforded by the real-time PCR assay allows for the effective testing of multiple pathogens from a single nucleic acid extraction from budwood tissue.

Previously, ELISA (Garnsey and Cambra, 1991; Permar et al., 1990) was used for the detection and differentiation of "mild" and "severe" strains of CTV. Running ELISA to test all source trees depends upon shutting down all other testing for three months, in addition to preparation and carrying out an additional sample collection. To have that time back on the schedule, it was essential to convert ELISA to a real-time PCR assay utilizing the single sample extract. Published real-time PCR protocols were tested to identify a method sensitive enough to identify samples with low titer, degenerate enough to account for sequence variability between strains, and specific enough to reliably differentiate between mild and severe isolates of CTV.

The BCBR Florida CTV pathogen collection is a representative sampling of a diverse population of isolates based on geographical and host symptom diversity. These isolates were collected over a 40-year period from 48 counties in Florida and have been characterized by biological, serological, and molecular techniques (Garnsey et al., 1991; Sieburth and Nolan, 2005). It was imperative for the selected real-time RT-PCR test to effectively screen the BCBR Florida CTV pathogen collection as it represents the range of variability among Florida CTV isolates (Hilf and Garnsey, 2002). Results from the universal primer/probe set (Bertolini et al., 2008) on the CTV pathogen collection indicated that this test was capable of accurately detecting a wide range of CTV isolates.

The MCA13 antibody has been the standard in the program for differentiating "severe" from mild strains of CTV. The epitope responsible for MCA13 reactivity is located in a highly conserved region of the coat protein gene (Pappu et al., 1993). Molecular characterization of this epitope revealed that Phenylanaline^{124} was the most conserved amino acid residue among "severe" isolates and is critical for the binding of MCA13 (Pappu et al., 1993). A single nucleotide change (A-T) at nucleotide position 371 regulates MCA13 reactivity. Primers CN120 and CN218 were derived based on this single nucleotide change and were originally developed for a conventional bi-directional RT-PCR assay to identify "severe" and "mild strains" (Cevik et al., 1996). By utilizing this primer set, we were able to convert the conventional RT-PCR assay to a real-time Power SYBR® Green RT-PCR assay. The molecular basis for which primers CN120 and CN218 were derived accounts for the high degree of accuracy in identifying "severe" strains of CTV and correlation with ELISA MCA13 results previously obtained for the CTV pathogen collection. As the BCBR program regulations are based on MCA13 reactivity (Florida Statute 5B-62; Florida Dept. of State, 2006), use of this

accurate procedure fits in well with program requirements. The universal primer/probe set in conjunction with the CN120/CN218 Power SYBR® Green assay effectively identified and differentiated all tested CTV isolates. The conversion from ELISA to real-time PCR increased sensitivity by identifying samples with low CTV titer and increased time efficiency by eliminating one tissue collection and reducing testing time from three months to one month.

Budgetary and personnel constraints produced a need to reduce expenses as well as decrease the time needed for labor intensive tasks. Equipment was an essential part of the solution. Tissue homogenization, the most labor intensive and time consuming task, was switched over to the Homex-6 from the Kleco. This resulted in a substantial reduction of the time necessary for initial sample processing. Transferring template with the adjustable multichannel pipette to 96 well stock plates reduced the time necessary for setting up and running PCR. All test plates were set up with a multichannel pipette from the stock plate, contributing to an increase in the number of plates run per day. Substantial cost savings could be attributed to the use of bottle-top dispensers, reducing the use of expensive filter tips. The incorporation of these changes into our protocols reduced the time and labor necessary to process samples while simultaneously reducing the cost required to test each sample.

Quantitative real-time PCR in conjunction with a high throughput nucleic acid extraction provides for sensitive and time efficient testing of a large number of citrus budwood program samples for a variety of graft transmissible citrus diseases. As part of this comprehensive testing program it was imperative that the extraction and testing method allowed for the screening of a diverse group of pathogens though the use of a single yearly sample collection. By achieving this goal, costs both to the grower and the testing program were substantially reduced.

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