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Real-Time Quantitative PCR Assay Analysis, Evaluation and Optimization for the Cocoa Swollen Shoot Virus

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Cocoa swollen shoot virus (CSSV) sequence was used to design a TaqMan probe-based quantitative polymerase chain reaction (PCR) assay from the nuclear SSR marker (mTcCIR25) in order to determine the cocoa cell number. Artificial fragment from the CSSV genome and microsatellite were used to generate calibration curves from 10^1 to 10^5 fragments per µl which were included in each real-time PCR assay. Generally, there were variations between given concentrations and observed DNA copy concentration of CSSV TaqMan assay decreasing from standard solutions 5 to 1. The accuracy of copy number detection for mTcCIR25 assay was poor at low concentrations (50 to 500 copies) compared with CSSV assay. The closeness of the amplification plots in the mTcCIR25 amplification graphs, the low Ct standard deviation values and the high correlation coefficient of the standard curves indicated that real-time PCR was robust and reproducible for the quantification of the CSSV. This holds great promises for future applications not only in quantitative real-time PCR for CSSV but may be applied to other pathogens.

Key words: Cocoa swollen shoot virus; quantitative polymerase chain reaction; real-time polymerase chain reaction; amplification graphs; standard curves

There have been two major revolutions in the detection of plant pathogens since the 1970s. The first was with the use of antibody-based detection (serological methods) in the mid 1970s and the second with the advent of DNA-based technologies such as PCR. PCR-based tests have a much greater sensitivity than the traditionally serological tests (Van de Vlugt *et al.*, 1997; Cassells and Doyle, 2005). A further break through is the development of multiplexing, where more than one pathogen can be detected per assay (Cassells and Doyle, 2005).

Conventional PCR method involves detection of the products in the plateau phase, using ethidium bromide stained agarose gels. Real-time PCR on the other hand, measures the accumulation of the product in real time at each cycle and the measurement taken in the exponential phase give a more accurate estimation of the starting template.

There are several approaches to real-time PCR, which involves the detection and measurement of amplification products at each cycle of the PCR. Some of the approaches involved the use of the TaqMan

and SYBR green methodologies (Provenzano *et al*., 2001).

The TaqMan methodology uses the 5′-3′ exonuclease activity of Taq DNA polymerase to cleave a dual- labelled probe annealed to a target sequence during PCR amplification. The probe contains both a fluorescent reporter dye at the 5′-end (6-carboxyfluorescein, 6-FAM; emission λ_{max}=518 nm) and a quencher dye at the 3′ (6-carboxytetramethylrhodamine, TAMRA; emission λ_{max} =582 nm) (Provenzano *et al*., 2001). The quencher can only quench the reporter fluorescence when the two dyes are close to each other. Once amplification occurs, the probe is degraded by the 5′-3′ exonuclease activity of the Taq DNA polymerase and the fluorescence will be detected by means of a laser integrated in the sequence detector. The PCR cycle number at which fluorescence reaches a threshold value of 10 times the standard deviation of baseline emission is used for quantitative measurement. This cycle number is called the cycle threshold (Ct) and is inversely proportional to the starting amount of target DNA or cDNA (Provenzano *et al*., 2001).

Real-time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (Mackay *et al*., 2002). The development of real-time PCR has brought true quantification of target nucleic acids out of the pure research laboratory and into diagnostic laboratory.

The tree *Theobroma cacao* also known as cocoa or cacao is a tropical crop with origins in the Amazon basin and dominates the economies of most West African countries. CSSV, a badnavirus, is transmitted by at least 14 species of mealybugs of the family *Pseudococcidae* within the *Coccoidae* (Roivainen, 1976).

Until recently, visual inspection of the cocoa tree for symptoms of the virus has been the mode of diagnosting the disease was found to be inadequate for the detection of latent infections. PCR approach for CSSV detection now enables us to detect and quantify the pathogen well in advance before the appearance of visual symptoms (Quainoo *et al*., 2008a; Quainoo *et al*., 2008b). The aim of this study is to design an easy to quantify the CSSV highlighting the standard curves and the quantification graphs of the essay.

Materials and Methods

DNA extraction

Total genomic DNA was extracted from the leave of symptom CSSV Amelonado cocoa tree (T1). A slightly modified DNeasy TM 96 Plant kit (Qiagen Ltd., UK) protocol was used. 400µl of lysate Buffer AP1 (preheated to 65°C) to dissolve and 2µl of RNase were added to the eppendorf tubes containing the experimental materials and shaken with a tungsten pellet on a Retsch disrupter for 1.4 min x 2 at 25 cycles s^{-1}. DNA extraction quality from the experimental materials was checked on an ethidium bromide stained agarose gel.

Quantitative screening of CSSV

In order to quantify the CSSV a different assay was required. Two PCR assays were designed to quantify a CSSV copy number per cocoa cell. The first one provided a CSSV number while the second one provided a cocoa cell number.

A PCR reaction was performed using DNA from the infected cocoa tree T1 to ensure that the primers selected targeted the CSSV genome correctly. The amplified product was cleaned using Nucleofast 96 plates (Nalgene, Macherey). For direct sequencing, 10 µl volume reactions were performed containing 4 µl Big dye and 1.6 µl of individual primers (1 µM) with 4.4 µl of PCR products and centrifuged at 14,000 rpm (SORVALL® Biofuge pico) for 10 s. The thermo cycling conditions were: 96 °C 30 min, 50 °C 15 min, 60 °C 4.00 min and 10 °C 1 min. The samples were sequenced using ABI

Prism 3100 Genetic Analyzer Capillary Sequencer (16 capillary array model). The run was set up using the ABI Prism 3100 Genetic Analyzer Capillary Sequencer software version 1.0.1. The laser was an argon-ion laser with primary emission lines at 488 nM and 514.5 nM.

The analysis was with the DNA sequencing software Chromas version 2.0. The obtained sequence was compared with the CSSV genome available at GenBank and retrieved by BLAST searches. The alignment of the gene sequences was initially performed using CLUSTAL W subsequently inspected by eye and corrected manually

The CSSV sequence was used to design a TaqMan probe-based quantitative PCR assay with the aid of Primer Express Software version 2. In order to determine the cocoa cell number a quantitative TaqMan assay was designed from the nuclear SSR marker (mTcCIR25). mTcCIR25 occurs at a single locus (Lanaud *et al*., 1999), and the assay was designed within the boundary sequence of the nuclear SSR repeat (EMBL accession number Y16997) (Table 1).

An artificial fragment from the CSSV genome and the microsatellite were used for standard dilution (quantification) using a calibration curve generated ranging from 10^1 to 10^5 fragments per µl. These samples were included in each real-time PCR assay. Each CSSV quantitative PCR reaction contained 12.5 µl of PCR sensimix (QUANTACE), 1.25 µl (400 mM) forward and reverse primers, 1.25 µl (400 mM) TaqMan probe 6 Carboxyl Fluorescein Amidite (FAM), 1.25 µl (6.5 mM) $MgCl_2$, 3.75 Sigma ultra pure water and 5 µl of DNA to bring the final volume to 25µl . To ensure minimal pipetting error the reactions were all set up using a liquid handling robot (Corbett CAS 1200). The quantitative assay was then run on a Rotor-Gene 6000 (Corbett, Australia) and the data was analysed using Rotor-Gene 1.7.28 software. The quantitative PCR reaction conditions with mTcCIR25 were the same as that of the CSSV assay except for the $MgCl_2$ concentration which was decreased to 3.0 mM.

Table 1. Summary of primers and markers used for real-time PCR

Reaction name	Primer/marker name	Primer sequence
Real-time PCR	CSSV forward primer	CCTTAAGAGGCTAACCAAGC
	CSSV reverse primer	GGGCTATCTCTTCTACTAGTC
	TaqMan Probe	TTCCGAGAAAACAAACCACTGTCTGAA
	mTcCIR25 forward primer	CAGATAAGGAAAGGTGGAGTTTGG
	mTcCIR25 reverse primer	CAAGAATGTCTCCTACATTCACTACG
	mTcCIR25 probe	TTCCCGTAAGCTTCGTCCCAGATGC
Standard dilution curve	CSSV artificial fragment	CCTTGAAGAGGCTAACCAAGCAGTTCC GAGAAAACAAACCACTGTCTGAATCT GAAGTAAAGAGACTAGTAGAAGAGAT
	mTcCIR25 artificial fragment	CAGATAAGGAAAGGTGGAGTTTGGTTT CCCGTAAGCTTCGTCCCAGATGCCTTC GTAGTGAATGTAGGAGACATTCTTG

Results and Discussion

The results of the real-time PCR using CSSV and mTcCIR25 TaqMan assays are summarized below.

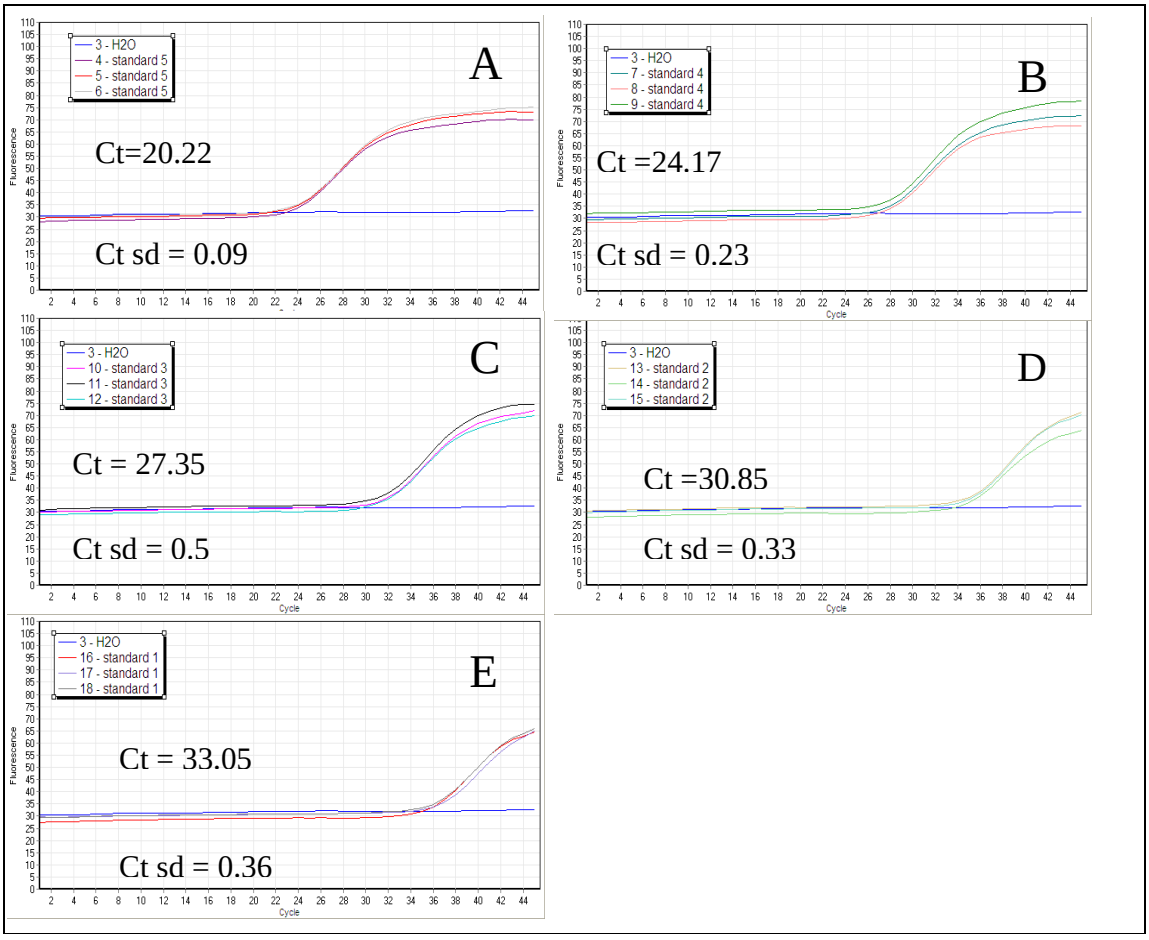


Figure1. mTcCIR25 amplification graphs. Triplicate amplification plots showing cycle number verses normalized fluorescent values of real-time PCR standard dilution curves. A, B, C, D, and E refers to standards 500000, 50000, 5000, 500 and 50 (copy numbers/reaction), respectively. As the standards decreased from A to E, the Ct value increased. Graph was generated from Rotor-gene 6000 series software 1.7. Ct sd is Ct standard deviation.

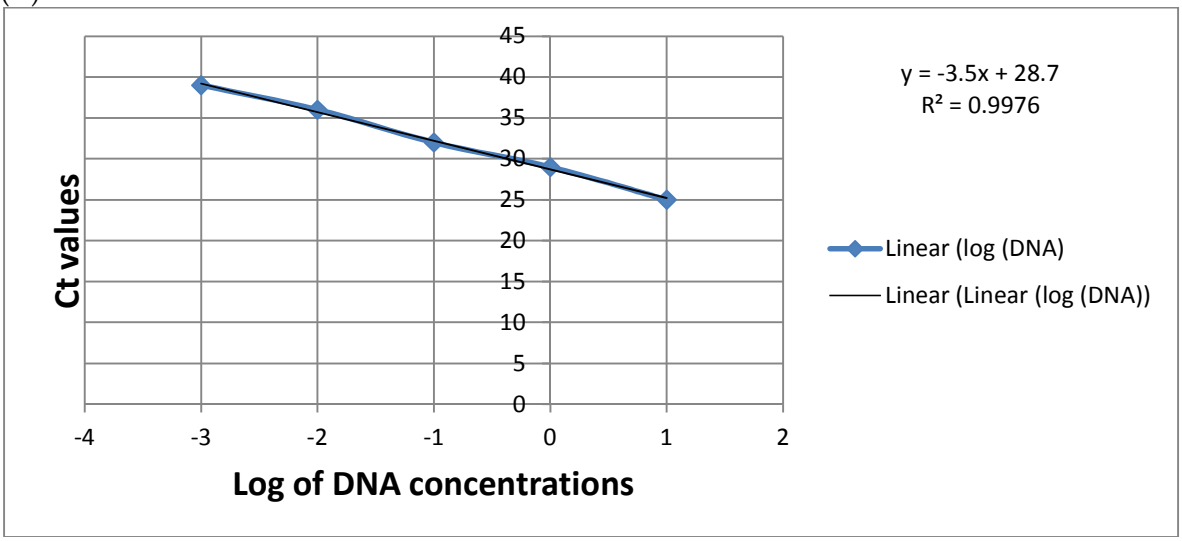
Table 2. Quantitative real-time PCR showing standard concentrations of DNA copies and standard errors, as reveal by CSSV TaqMan assay

Standard	Given concentrations (copy number / reaction)	Observed mean of 15 DNA concentrations	Standard error (SE)	Variation (SE/mean x 100)
1	50	72.21	10.75	14.89
2	500	423.53	25.09	5.92
3	5000	4252.67	316.56	7.44
4	50000	51913.33	2558.90	4.93
5	500000	585066.7	34488.43	5.89

Table 3. Quantitative real-time PCR showing standard concentrations of DNA copies and standard errors, as reveal by mTcCIR25 TaqMan assay

Standard	Given concentrations (copy number/ reaction)	Observed mean of 15 DNA concentrations	Standard error (SE)	Variation (SE/mean x 100)
1	50	69.88	9.56	13.68
2	500	82.0	247.91	30.23
3	5000	3120	232.48	7.45
4	50000	47293.33	2284.80	4.83
5	500000	675200	30055.22	4.45

(A)



(B)

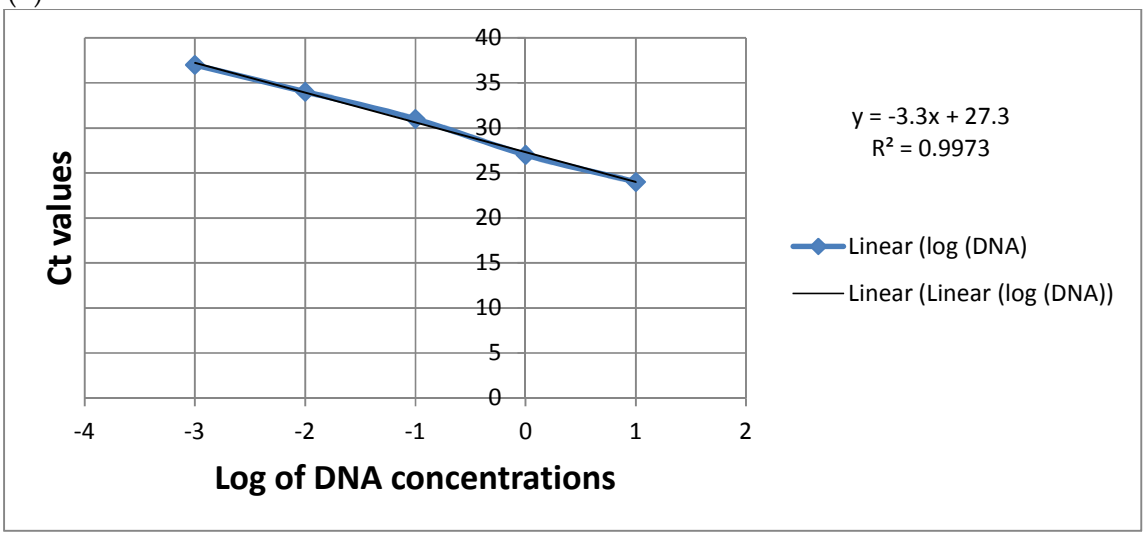


Figure 2. Real-time PCR standard curves: (A) CSSV TaqMan real-time assay and (B) mTcCIR25 TaqMan real-time PCR assay.

As the given concentrations decreases, the observed DNA copy concentrations also decreases (from standard 5 to 1). Within standards there were variations between given concentrations and observed DNA copy concentration decreasing from standards 5 to 1. For mTcCIR25 assay, the accuracy of copy number detection was poor at low concentrations (50 to 500 copies) (Table 3) compared with CSSV assay (Table 2).

The closeness of the triplicate amplification plots of the standards in the mTcCIR25 amplification graphs shows that the real-time PCR was reproducible. The low Ct standard deviation values of the standards indicated the robustness of the real-time assays (Figure 1). When the known concentrations (expressed as logarithmic form, X axis) of target gene are plotted against the corresponding cycle threshold (Ct, Y axis) obtained by the quantitative PCR, the result is a line representing the linear correlation between the two parameters. The equation describing this relationship is used to extrapolate the gene copy number in experimental samples. The assay proved to be highly reproducible, as demonstrated by a high correlation coefficient (R^2 = 0.9976 and R^2 = 0.9973) of the standard curves (Figure 2).

All the amplifications of the standards produced typical fluorescent curves with Ct values decreasing from 50 to 500000. The assay proved to be highly reproducible, as demonstrated by low Ct standard deviation values between triplicates (Figure 1). The quantitative real-time assay is rapid, reproducible, enabled specific detection of CSSV from the explants and adaptable for large scale experiments. Delanoy *et al.* (2003) developed a real-time assay for the detection of episomal *Banana streak virus* (BSV) in banana and plantain which enabled the reproducible and specific detection of episomal BSV from purified DNA and crude extracts from infected plants. The performance of the current real-time PCR assay enabling the detection and estimation of CSSV concentrations in the cells of cocoa conforms to the findings of real-time PCR developed for the rapid detection of episomal BSV (Delanoy *et al.*, 2003).

Conclusion

The quantitative real-time assay proved to be highly reproducible and rapid for the CSSV as demonstrated by low Ct standard deviation values between triplicates.

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