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TaqMan real-time PCR for detection and quantitation of squash leaf curl virus in cucurbits

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A real-time PCR assay based on the TaqMan chemistry was developed for reliable detection and quantitation of the squash leaf curl virus (SLCV) in melon and squash plants. This method was highly specific to SLCV and it was about one thousand times more sensitive than the conventional PCR method. The protocol of the real-time PCR established in this study enabled detection of as little as 10^2 copies of SLCV DNA with CP gene as the target. This TaqMan real-time PCR assay for detection and quantitation of SLCV would be a useful tool for application in quarantine and certification of SLCV in cucurbits as well as in the research of disease resistance and epidemiology.

1. Introduction

The squash leaf curl viruses (SLCV) are circular single-stranded DNA viruses encapsulated in twinned iscoshedral virions. SLCV belongs to subgroup 2 of the genus *Begomovirus* within the family *Geminiviridae* (Padidam et al., 1995). *Geminivirus* is restricted to dicotyledonous plants. SLCV commonly infects squash (*Cucurbita pepo* L.), bottle gourd (*Lagenaria siceraria* (Mol.) Standl), and melon (*Cucumis melo* L.) causing severe leaf curling and plant stunting (Cohen et al., 1983; Flock and Mayhew, 1981; Lazarowitz and Lazins, 1991). The disease can cause severe losses in yield of cucurbitaceous crops such as zucchini (*C. pepo* var. *Pepo* L. Bailey) and squash (*C. pepo* L.) in some tropical and sub-tropical countries (Lazarowitz and Lazins, 1991; Revill et al., 2003; Xie and Zhou, 2003). The disease also caused severe yield losses of watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai), ranging from 30 to 100%, in Texas, USA, particularly in crops planted in the autumn when large populations of whitefly (*Bemisia tabaci* (Gennadius)), vector of SLCV, were present (Isakeit and Robertson, 1994). In Taiwan, SLCV was reported on squash (Tsai et al., 2007) and wax gourd (*Benincasa hispida* Cogn.) (Liao et al., 2007).

Several diagnostic methods have been developed for the detection of SLCV in host plants as well as in the whitefly vector (Rosell et al., 1999). Beside the most widely used PCR based method (Rosell et al., 1999), SLCV can also be detected by other methods such as the ELISA assay (Cohen et al., 1989; Harrison et al., 2002) and the DNA probe method (Harper and Creamer, 1995). The PCR method has several intrinsic disadvantages, such as the requirement for rapid thermal-cycler, and a rather low amplification efficiency. Also, the PCR assay requires skilled technicians and specialized instruments and is time consuming. Recently, we have developed a Loop-mediated isothermal amplification (LAMP) method for detection of SLCV (Kuan et al., 2010). However, both PCR and LAMP methods are capable of detecting the presence of SLCV in diseased tissues but they are incapable of determining SLCV in infected plants quantitatively. The objective of this study was to develop a real-time PCR assay for the rapid and efficient detection of SLCV in infected tissues of squash and melon.

2. Materials and methods

2.1. Virus isolates

Whiteflies were collected in 2008 from a disease field of squash in Tainan, Taiwan, with numerous plants showing symptoms of infection by SLCV. They were released on young seedlings of squash

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(cv. Eastern-Rise) and melon (cv. Silver light) (Known-You Seed Co. Ltd., Kao-Hsiung, Taiwan) in a growth chamber at 28–30 °C under light (12 h light/12 h dark). After incubation for 4–6 weeks, leaves of plants showing symptoms of leaf curling were collected and used in this study.

2.2. DNA extraction

DNA was extracted using the Wizard™ Genomic DNA purification kit (Promega Corporation, Madison, USA) from SLCV-infected leaves of squash and melon. Forty-mg samples of leaf tissues were ground in liquid nitrogen and then using DNA was extracted using the kit, as described by the manufacturer. Control DNA was isolated from healthy leaves.

2.3. PCR amplification

Primers were designed according to coat protein sequence (Table 1), using Primer Express software (Applied Biosystems, Foster, USA). The assays were performed in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster, USA). Amplification reactions were carried out in 25 μl volumes containing 2 μl (10–60 ng) of the template DNA, 1 μl of each primer (20 μM), 2.5 μl of 10x PCR buffers (150 mM Tris–HCl, 500 mM Tris–HCl, pH 8.0 with 25 mM $MgCl_2$), 0.2 μl of dNTPs, 100 mM and 0.15 μl of AmpliTaq DNA polymerase (5 μ/μl) supplied by the manufacturer (Applied Biosystems, Foster, USA). All the genomic DNA were tested for suitability for amplification using the specific primers according to the following protocol: 5 min at 95 °C, 35 cycles of 1 min at 95 °C (denaturation), 20 s at 55 °C (annealing temperature varied by the Tm of specific primers), 1 min at 72 °C (extension) and finally 5 min at 72 °C. PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0), and visualized after staining with ethidium bromide (0.5 mg/l) under UV light.

2.4. TaqMan PCR assay and specificity

TaqMan PCR was performed in a final volume of 25 μl consisted of 1 μl of genomic DNA (10–60 ng/μl), 0.2 μM specific TaqMan probe, 0.3 μM specific primers, and 12.5 μl of universal master mix (Applied Biosystems, Foster, USA). Every dilution was run in duplicate. Real-time PCR was performed in an ABI 7500 system (Applied Biosystems, Foster, USA): 2 min at 50 °C, 10 min at 95 °C, and then at 40 cycles for 15 s at 95 °C and 1 min at 60 °C. The threshold cycle (Ct) is the cycle number at which a significant increase in fluorescence occurs, hence, a Ct value below 40 suggests a positive result. The change in normalized fluorescence (ΔRn) records the amount of DNA product amplified. The specificity of SLCV primers was examined using 10–60 ng of total DNA extracted from SLCV-infected plants and healthy plants as the templates.

2.5. Construction of standard curves for plasmid copy number determination

PCR primers were used to amplify a 693-bp DNA fragment of the partial coat protein gene and nd gene of the SLCV isolate (Table 1). Amplicons of SLCV DNA fragments obtained were purified with Wizard PCR Clean Up kit (Promega Corporation, Madison, USA) and cloned using pGEM-T Eazy vector (Promega Corporation, Madison, USA), then the purified plasmid was quantified with nanoview spectrophotometer (GE Healthcare Life Science, Piscataway, USA). To construct standards, the artificial template was diluted at final concentrations of 10^9 to 1 copies of the target cp gene per microlitre of sterile water. Template DNA solutions for the real-time PCR reactions were prepared by serial dilution of the plasmid until they contained 100 copies/μl.

A standard curve for quantitation was generated, where Ct values were plotted from 10 fold serial dilutions of the plasmid DNA. The Ct values were calculated by the software of the ABI Prism 7500 thermal cycler detects the accumulation of PCR product by the accumulation of fluorescence. Normalized fluorescence relative to established baseline levels is plotted versus cycle number. The estimation of copy number values in samples was performed by computing the estimates of linear regression coefficients. The quantitation of DNA samples were calculated based on the fluorescence (ΔRn) values. All samples were run in duplicates by real-time PCR assay for accuracy of the results obtained.

2.6. Sensitivity comparisons

The relative sensitivity of the TaqMan assay was compared with that of the PCR assay for the detection of SLCV in plant tissues. Plasmid DNA of SLCV insertion was prepared in serial 10-fold dilutions (10^9 to 10^1 copies for each target per microliter) and assayed for the dilution end-point. PCR products were analyzed by electrophoresis as described in Section 2.3. To minimize errors due to pipetting differences, duplicates of each sample were performed on each run and their threshold cycle (Ct) values were averaged during data analysis. In addition, a non-template water control (NTC) as well as a positive control (cloned DNA) and a negative control (total DNA from healthy plant tissue) were used in every PCR or real-time PCR test.

2.7. Evaluation of SLCV concentration in plant samples

Leaves, stems, roots and flowers of SLCV-infected squash plants were collected from the field and tested by real-time and PCR analyses to quantify the number of SLCV present in test samples. Fruits and seeds of squash were also collected from this field and tested for SLCV by real-time and PCR analyses. Tissues of squash were sampled from each infected or uninfected plant and extracted by the procedure described in Section 2.2. Tissues from healthy plants of squash were used as the negative controls. For each real-time PCR, 2 μl of extracted DNA was added. The samples were also tested using PCR of the SLCV-0320-m-693F1/R1 primers. Dilutions of artificial template and target isolates were amplified, by 40 cycles of real-time PCR with SLCV-Taq-130 F1/R1 primers, during the same reaction. Ct-value of each sample was measured in triplicate and the values were plotted against the known copy numbers of the standard samples to determine SLCV concentration.

3. Results

3.1. Specificity of TaqMan assay

Real-time PCR assay was used to test total genomic DNA from healthy plants (control) and SLCV infected plants of squash and melon. No signal was detected using total genomic DNA from healthy control plants and negative control (no template control = NTC). However, the primers and probe set consistently detected SLCV in infected plants of squash (D1-D5) and melon (D6-D10) as well as in the SLCV control DNA (DC) (Fig. 1). Among all the SLCV-infected plants tested, squash and melon showed positive results. In the PCR assay, the 693 bp amplified fragment using primer pair SLCV-0320-m-693F1/R1 was detected in all SLCV-infected plants of squash and melon but was not detected in the healthy plants (data not shown). For the TaqMan real-time PCR assay, two primers encompassing a 139-nt region in coat protein and a 30-nt probe annealing within this region were designed. The primer/probe SLCV set amplified a 139 bp fragment from the coat