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Detection and quantitation of two cucurbit criniviruses in mixed infection by real-time RT-PCR

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Cucurbit chlorotic yellows virus (CCYV) and *Cucurbit yellow stunting disorder virus* (CYSDV) are whitefly-transmitted criniviruses infecting cucurbit crops inducing similar symptoms. Single and multiplex RT-PCR protocols were developed and evaluated on cucurbit samples collected from commercial greenhouses. Primers and probes were designed from the highly conserved heat shock protein 70 homolog (Hsp70h) gene. Conventional RT-PCR and multiplex RT-PCR assays showed high specificity and suitability for routine screening. TaqMan-based quantitative real-time RT-PCR (RT-qPCR) protocols were also developed for the detection and quantitation of both viruses occurring in single or mixed infection. The assays proved to be highly specific with no cross amplification. RT-qPCR assays showed a 100–1000 times improved sensitivity over conventional RT-PCR. Virus titers in mixed infections were compared to singly infected plants by RT-qPCR. CYSDV and CCYV titers decreased in double infected plants. This paper reports highly specific conventional RT-PCR and quantitative real-time PCR assays for detection, quantitation and differentiation between two closely related cucurbit-infecting criniviruses.

1. Introduction

In the Mediterranean region, at least 28 viruses have been reported to infect cucurbit crops (Lecoq and Desbiez, 2012). During the past three decades, the incidence of whitefly transmitted viruses, mainly geminiviruses and criniviruses, has increased tremendously due to the rapid expansion of whitefly populations related to many environmental and man-made factors, such as climatic change and agricultural trade (Navas-Castillo et al., 2011). *Cucurbit yellow stunting disorder virus* (CYSDV) and Cucurbit chlorotic yellows virus (CCYV) are two emergent viruses that belong to the genus *Crinivirus* in the family *Closteroviridae*. CCYV remains a tentative member of the genus *Crinivirus* since it was first fully sequenced in 2010 (Martelli et al., 2012; Okuda et al., 2010). Recently, mixed infections by CYSDV and CCYV were reported in commercially cultivated cucurbits (Abrahamian et al., 2012). Both viruses cause considerable damage mainly to the *Cucurbitaceae* family and can infect field crops or weeds (Okuda et al., 2010; Wintermantel et al., 2009). So far, two other criniviruses infect cucurbits; *Beet pseudo yellows virus* (BPYV) and *Lettuce infectious yellows virus* (LIYV) (Berdiales et al., 1999; Duffus et al., 1986). LIYV incidence has decreased since the 1990s due to displacement of *Bemisia tabaci* New World complex (biotype A) by Middle East-Asia Minor 1 complex (MEAM1) (biotype B) (De Barro et al., 2011; Rubio et al., 1999). BPYV decline is more recent, particularly in the Mediterranean region (Berdiales et al., 1999; Rubio et al., 1999).

CCYV and CYSDV are transmitted by *B. tabaci* Mediterranean (MED) (previously biotype Q) and MEAM1 complexes in a semi-persistent manner (Celix et al., 1996; De Barro et al., 2011; Gyoutoku et al., 2009). CYSDV was first isolated and described in the early 1980s (Hassan and Duffus, 1991). However, CCYV is a newly characterized *Crinivirus*, with a limited geographical distribution and has only been reported in Japan (Gyoutoku et al., 2009), Taiwan (Huang et al., 2010), China (Gu et al., 2011; Zeng et al., 2011), Sudan (Hamed et al., 2011) and Lebanon (Abrahamian et al., 2012). Economic losses due to CYSDV may reach up to 80% on some melon cultivars (Brown et al., 2007); however losses due to CCYV infection have not been determined. Symptoms of criniviruses develop on older leaves and progress toward newer growth inducing nutrition deficiency-like symptoms, interveinal chlorosis, brittleness and thickening of leaves. Symptoms induced by CYSDV, CCYV and other criniviruses are very similar and their differentiation relies on the application of laboratory-based detection techniques (Wintermantel and Wisler, 2006).

Several detection methods have been developed for CYSDV using enzyme-linked immunosorbent assay (ELISA), tissue-blot immuno assay (TIBA), dot-blot immuno assay (DBIA), dot-blot hybridization and reverse transcription (RT)-polymerase chain

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reaction (PCR) (Celix et al., 1996; Hourani and Abou-Jawdah, 2003; Ruiz et al., 2002). On the contrary only a limited number of assays have been developed for CCYV, which include RT-PCR and ELISA (Gyoutoku et al., 2009; Kubota et al., 2011). Serological and hybridization-based detection methods are time consuming, less specific and sensitive for detection and quantitation of viruses (Harper et al., 2011). Quantitative real-time PCR (qPCR) has been used as a platform for rapid, sensitive and efficient amplification of target genes in real time. It has been used extensively in medical, agricultural and veterinary virology research (Mackay et al., 2002). Also, many assays have been developed for the detection and quantitation of several plant RNA viruses (Harper et al., 2011; Price et al., 2010; Quito-Avila and Martin, 2012). Quantitative reverse transcription real-time PCR (RT-qPCR) can be setup in a one-step or two-step format. However, the former is preferred due to its suitability for virus quantitation, reduction in carryover of contaminants and shorter time. In the literature, no assays were reported for the quantitation of CCYV, while three assays have been developed for the quantitation of CYSDV (Abou-Jawdah et al., 2008; Gil-Salas et al., 2007; Papayiannis et al., 2010). However, since CCYV has been reported recently, the latter assays have been designed without including CCYV as a negative control.

The objectives of this study were to: (i) design specific RT-PCR and multiplex RT-PCR (mRT-PCR) assays for routine surveys of CYSDV and CCYV in regions where they may co-exist; (ii) develop and optimize a highly specific TaqMan® based RT-qPCR assay for CCYV and CYSDV detection and quantitation in cucurbits; and (iii) evaluate the assay by relative quantitation of virus titers in mixed infections as compared to single infection in field infected cucumber.

2. Materials and methods

2.1. Virus isolates and plant collection

CCYV and CYSDV maintained on cucumber (*Cucumis sativus* cultivar (cv.) Delta) in insect-proof cages were used as positive controls (Abou-Jawdah et al., 2008; Abrahamian et al., 2012) for development and optimization of RT-PCR and RT-qPCR assay. Samples collected, during September 2012, from 46 symptomatic cucumber (cv. Beit-alpha) plants grown in two different commercial greenhouses in Jiyye, South Lebanon were used for preliminary evaluation of the assay. Later on, assays were performed on an additional pool of 38 samples collected from different regions during surveys conducted in 2011 and 2012. These plants showed symptoms of interveinal chlorosis of middle leaves with complete yellowing on lower leaves. Leaves with mild symptoms positioned directly above more symptomatic leaves were collected 40 days post-transplanting from Jiyye, South Lebanon.

2.2. Total RNA extraction

RNA extraction was performed as described by Accerbi et al. (2010) with minor modifications. About, 60–70 mg leaf tissue (including the midrib area), were mixed with 600 μl of TriReagent (Sigma–Aldrich, MO, USA) instead of 1 g per 10 ml. The slurry was vortexed thoroughly for 1 min and incubated for 10 min at room temperature. Chloroform (200 μl) was added to the mixture, vortexed briefly, and centrifuged at 10,000 × *g* for 15 min at 4 °C. The aqueous phase was transferred to a clean microfuge tube with an equal volume of ice cold isopropanol and mixed gently several times, followed by a 15 min incubation period at room temperature. RNA was precipitated at 10,000 × *g* for 15 min and washed with 70% ethanol. The pellet was eluted in 60 μl of molecular grade water (Sigma–Aldrich, MO, USA). RNA was analyzed in a 1% agarose gel electrophoresis to ascertain the quality of RNA and the absence of DNA contamination. Total RNA was quantified using a NanoDrop 2000c (NanoDrop Technologies, USA) and all samples were diluted to a 30 ng/μl workable concentration and stored at −80 °C.

2.3. TaqMan® probes and primers design

The heat shock protein 70 homolog (Hsp70h) gene, a highly conserved gene in RNA2, was chosen as a target for primer and probe design. Multiple sequence alignment was performed for CYSDV (GenBank accession no. FJ492808; AY242078) and CCYV (GenBank accession no. AB523789; JQ904629; JN126046; JN126045) using ClustalW. The primers and TaqMan® probe for the reference gene were designed from the mRNA sequence of the F-Box domain (GenBank accession no. GW881870) of the cucumber genome. Three sets of TaqMan® probes were designed using Beacon Designer 7 (Premier Biosoft, CA, USA) software and were assessed for secondary structure. Primers and probes specificity were assessed by BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) preset with the highest mismatch score option. No cross detection to any other virus was observed and all CCYV and CYSDV isolates on GenBank were detected. The homology between the Hsp70h genes between the two criniviruses is somewhat high. Therefore, for each target at least two out of the three designed oligonucleotides contained highly polymorphic regions to avoid cross amplification. The 5′ end of TaqMan® probes CcHSP-Taq, FBox-Taq and CyHSP-Taq were labeled with 6-carboxyfluorescein (FAM), Texas Red (TEX) and Yakima Yellow (YAK), respectively. All probes were labeled at the 3′ end with a BlackBerry® Quencher (BBQ) which has a wide fluorescence quenching spectrum. Primers and probes were synthesized by TIB-MOLBIOL (Berlin, Germany).

For development of multiplex RT-PCR, two primer pairs with the same melting temperature were designed to amplify part of the Hsp70h gene of each CCYV and CYSDV. The sequences, amplicon size and corresponding locations of primers and probes are listed in Table 1.

2.4. Conventional and multiplex RT-PCR

Access RT-PCR System (Promega, Madison, WI, USA) kit was used for performing all singleplex and multiplex RT-PCR assays in a one-step reaction. RT-PCR was performed on a Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules CA, USA). Reactions were set in 20 μl volume and contained the following per reaction: 4 μl of AMV/Tfl 5× reaction buffer, 1 mM of $MgSO_4$, 0.2 mM of each dNTP, 0.3 μM for each primer, 2 U of each *Tfl* DNA polymerase and AMV-RT enzyme. The mRT-PCR involved only two targets CCYV-Hsp70h and CYSDV-Hsp70h genes. In order to optimize the mRT-PCR assay, three different factors were taken into consideration, three different RT temperatures (45, 50, and 55 °C), a gradient primer annealing temperature (52–59 °C), and a matrix of primer concentrations (200, 300, 400 nM). The cycling profile was similar for both criniviruses in single and in multiplex RT-PCR. Thermal cycling parameters consisted of a 30 min RT step at 50 °C followed by 3 min inactivation step at 94 °C, then 40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 68 °C, and a final extension step for 7 min.

2.5. RT-qPCR assay

All RT-qPCR assays were run on a CFX96 Touch thermal cycler (Bio-Rad Laboratories, Hercules, USA) in a 96-well hard plate. In order to determine the optimum primer/probe combination for each target, an equimolar primer (100, 200, 300, 400 nM) and probe (100, 200, 300 nM) concentration was used in order to screen for the highest relative fluorescence unit (RFU) and the lowest quantitation cycle (Cq), also known as cycle threshold (Ct).