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Detection and quantitation of *Citrus leaf blotch virus* by TaqMan real-time RT-PCR

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A real-time RT-PCR assay based on the TaqMan chemistry was developed for reliable detection and quantitation of *Citrus leaf blotch virus* (CLBV) in citrus plants. Detection by this method was highly specific and about one thousand times more sensitive than detection by conventional RT-PCR. An external standard curve using *in vitro* synthesized RNA transcripts of the selected target allowed a reproducible quantitative assay, with a wide dynamic range (seven logarithmic units of concentration) and very low variation coefficient values. This protocol enabled detection of as little as 100 copies of CLBV RNA in various tissues and citrus varieties infected with CLBV sources from different geographical origins. The new assay greatly improves current detection methods for CLBV and it has been most helpful for the Spanish citrus sanitation, quarantine and certification programs, and fitness evaluation of infectious cDNA clones of CLBV, useful potentially as viral vectors for citrus.

1. Introduction

Citrus leaf blotch virus (CLBV), a member of the putative genus *Citrivirus*, family *Flexiviridae* (Adams et al., 2005; Martelli et al., 2007), has filamentous virions about 960 nm × 14 nm in size composed of a single-stranded, positive-sense, genomic RNA (gRNA) of 8747 nt and a 41-kDa coat protein (CP) (Galipienso et al., 2001; Vives et al., 2001). The CLBV gRNA has three open reading frames (ORFs) and untranslated regions of 73 and 541 nt at the 5′ and 3′ termini, respectively. ORF1 potentially encodes a ∼227-kDa polyprotein containing methyltransferase, AlkB-like, Out-like peptidase, papain-like protease, helicase and RNA-dependent RNA polymerase motifs; ORF2 encodes a ∼40-kDa polypeptide with a motif characteristic of cell-to-cell movement proteins of the 30K superfamily; and the ∼41-kDa polypeptide encoded by ORF3 was identified as the CP (Martelli et al., 2007; Vives et al., 2001). In addition to the gRNA, CLBV infected tissues contain two 3′-co-terminal and two 5′-co-terminal subgenomic RNAs generated during virus replication and expression (Vives et al., 2002a).

CLBV causes chlorotic leaf blotching in Dweet tangor (*Citrus tangerina* Hort. ex Tan. × *C. sinensis* (L.) Osb.) and stem pitting in Etrog citron (*C. medica* L.) (Galipienso et al., 2000; Navarro et al., 1984). Although CLBV does not induce bud union crease on trifoliate rootstocks (Vives et al., 2008a), it was detected in several citrus species from Australia, USA, Japan, and Spain, usually associated with bud union crease symptoms on citrange (*C. sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.) or citrumelo (*C. paradisi* Macf. × *P. trifoliata*) rootstocks (Galipienso et al., 2004), therefore, an interaction between CLBV and other agent/s to induce bud union crease cannot be discarded. The virus is primarily transmitted by vegetative propagation, but seed transmission at low rate has been observed (Guerri et al., 2004).

Control of graft-transmissible pathogens includes sanitation, quarantine and certification programs (Navarro et al., 2002), but launching these programs requires specific and sensitive procedures for reliable pathogen detection. CLBV diagnosis can be performed by biological indexing on Dweet tangor seedlings, in which it induces chlorotic blotching in young leaves, but due to uneven distribution and low titre of the virus in some hosts, graft-inoculation of at least six indicator plants with several bark pieces is recommended (Galipienso et al., 2000). This procedure is lengthy and costly, requires appropriate greenhouse facilities and well trained personnel, and it is unsuitable for large scale indexing. An antiserum to coat protein expressed in bacteria enabled virus detection in Western blot assays but not in ELISA, thus limiting its potential use for routine diagnosis (Vives et al., 2001). Molecular hybridisation and reverse transcription-polymerase chain reaction (RT-PCR) procedures were reported that readily detected CLBV in infected plants of Eureka lemon (*C. limon* (L.) Burm. f.), Marsh grapefruit (*C. paradisi* Macf.), Nules clementine (*C. clementina* Hort. ex Tan.), Navelina sweet orange (*C. sinensis* (L.) Osb) and Nagami kumquat (*Fortunella margarita* (Lour.) Swing.) grown in a temperature-controlled greenhouse, but not in Pineapple sweet orange, a host cultivar that yielded more than 80% false negatives

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(Galipienso et al., 2004; Vives et al., 2002b). This finding indicates that reliable detection of CLBV in Pineapple sweet orange, and perhaps in other varieties, might require analysing at least ten individual samples per tree to reduce the risk of false negatives. In plants grown in a screen house or in open field detection of CLBV was less reliable than in plants grown in the greenhouse, with only young leaves yielding consistent detection, likely due to lower virus accumulation or uneven distribution within infected trees (Galipienso et al., 2004; Vives et al., 2002b).

Real-time RT-PCR has been reported for detection of several plant viruses in different hosts (López et al., 2006; Osman et al., 2007; Roberts et al., 2000) including citrus plants (Bertolini et al., 2008; Ruiz-Ruiz et al., 2007; Saponari et al., 2008). This technique allows detection of the DNA synthesized during amplification thus avoiding two problems of conventional RT-PCR: electrophoretic analysis of the PCR products, and risk of post-PCR contamination. Furthermore, real-time RT-PCR usually provides increased sensitivity and specificity in comparison with conventional RT-PCR (Bertolini et al., 2008; Saponari et al., 2008), which is important when the viral titre is very low. This paper describes the development and evaluation of a real-time RT-PCR assay based on TaqMan technology, which allows reliable CLBV detection and quantitation in different citrus species even in winter when the virus titre is lowest (Galipienso et al., 2004).

2. Material and methods

2.1. Virus sources and hosts

The following sources of CLBV were used for analysis: (i) Nagami kumquat SRA-153 (K1) (Galipienso et al., 2000), (ii) Nules clementine, Etrog citron and Pineapple sweet orange graft inoculated with bark pieces of kumquat SRA-153 (sources K10, K16 and K12, respectively) to test if detection of CLBV was affected by host variety, (iii) kumquat 497-2 (K7), obtained by shoot-tip grafting *in vitro* from kumquat SRA-153, but differing from this source by symptom expression (Galipienso et al., 2000), (iv) Yamamizaka sweet orange (K25), Hashimoto satsuma (*Citrus unshiu* (Macf.) Marc.) (K27) and another mandarin source (K26) from Japan, (v) a source of Dweet mottle disease from California, USA (Roistacher and Blue, 1968), inoculated in Nules clementine (K3), and (vi) Piña (K2), Lanelate (K14), Newhall (K15) and Navelina (K23) sweet oranges and Seminole tangelo (*C. paradisi* × *C. tangerina*) (K34) sources collected from different commercial citrus orchards in Spain and maintained in a screen house or in the field. Isolates P129 of *Citrus psorosis virus* (CPsV) and T318A of *Citrus tristeza virus* (CTV) from the Instituto Valenciano de Investigaciones Agrarias collection were used as control for specificity of the assay.

Pineapple sweet orange plants were grown as seedlings, Etrog citron was propagated on rough lemon (*C. jambhiri* Lush), and the remaining varieties, including field sources and non-CLBV controls, were propagated on Carrizo or Troyer citrange rootstocks. Except for sources (vi) kept in the field, plants were grown in an insect-proof screen house, using plastic containers with an artificial potting mix (50% sand and 50% peat moss) and a standard fertilizing procedure (Arregui et al., 1982).

2.2. Primers and probe design

The primers and TaqMan probe for specific detection of CLBV targeted the ORF1 of the gRNA and were designed using the Primer Express software (Applied Biosystems) and the nucleotide sequence deposited in GenBank (accession no. NC003877). The forward and the reverse primers were CLBV-F (5′-CAATTGCATGAACACTCACGG-3′, nucleotide positions 2101–2121) and CLBV-R (5′-GGACCCCCCATTAAATTCCA-3′, nucleotide positions 2251–2232), respectively, and the sequence of the TaqMan probe was ATGAAATTCCCACACCGTCAGATGGC (positions 2124–2149). The TaqMan probe was tagged with the reporter dye FAM (6-carboxyfluorescein) at its 5′-end and with the quenching dye TAMRA (6-carboxy-tetramethylrhodamine) at its 3′-end.

2.3. RNA extraction

Total RNA (RNAt) from 1 to 3 g of fresh tissue (young shoots, old leaves, green bark, flowers, rootlets or fruit rind) from healthy or CLBV-infected plants was extracted using (i) TRIzol reagent (Invitrogen), or (ii) a standard protocol with two phenol:chloroform:isoamyl alcohol extractions followed by precipitation with 12 M lithium chloride (Ancillo et al., 2007). RNAt extracts were treated with RNase-free DNase (Turbo DNA-free, Ambion). RNA concentrations were measured in duplicate in a NanoDrop™ spectrophotomer (Thermo Scientific) and then adjusted to approximately 10 ng/μl. Aliquots were stored at −80 °C until use.

2.4. Standard curve

To estimate the number of CLBV RNA copies in RNAt extracts, RNA transcripts of the selected gRNA region were synthesized *in vitro* and serial RNA dilutions were used in real-time RT-PCR assays to generate an external standard curve. The cDNA used as template for *in vitro* transcription was obtained by RT-PCR using RNAt extracts from plants infected with the K-1 CLBV source and the primer set CLBV-T7F and CLBV-R (CLBV-T7F is a modified version of the forward primer CLBV-F that includes the T7-promoter sequence at its 5′ end). Positive sense RNA transcripts were synthesized directly from the PCR amplification products using T7 RNA polymerase (New England BioLabs). Transcripts were purified with the RNaid w/Spin Kit (Q-BIO gene), treated with RNase-free DNase (Turbo DNA-free, Ambion) and RNA concentration estimated spectrophotometrically. Real-time RT-PCR with and without reverse transcriptase (see below) were run in parallel to ensure the absence of DNA template in transcript preparations. To generate an external standard curve 10-fold serial dilutions containing 10^{10} to 10^{1} copies of the target RNA were prepared using RNAt extracts from healthy citrus (10 ng/μl). The transcript RNA concentration (pmol) in each dilution was calculated with the formula: μg of transcript RNA × (10^6 pg/1 μg) × (1 pmol/340 pg) × (1/number of bases of the transcript), and the number of RNA copies using this concentration value and Avogadro's constant. A standard curve was constructed plotting the threshold cycle (C_t) values from two independent assays with four replicates per standard dilution versus the logarithm of the RNA copy number. The amplification efficiency was calculated from the slope of the corresponding curve using the formula $10^{(-1/\text{slope of the standard curve})}$, or the same formula × 100 (when given as a percentage value).

2.5. Conventional RT-PCR

For cDNA synthesis, 2 μl of RNAt preparations and 0.2 μM of each primer (CLBV-F and CLBV-R) were denatured at 85 °C for 5 min and chilled on ice. Then, one-step RT-PCR was performed in a 25 μl reaction volume containing 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 4 units RNase OUT™ ribonuclease inhibitor, 20 units SuperScript™ II Rnase H-reverse transcriptase and 1 unit *Taq* DNA polymerase (Invitrogen)). The reaction mix was incubated at 42 °C for 45 min for RT and then the enzyme inactivated at 94 °C for 2 min. Thermocycling conditions were: 40 cycles of 20 s at 94 °C, 20 s at 60 °C and 30 s at 72 °C, followed by an extension step of 5 min at 72 °C. The PCR reaction