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Real time RT-PCR assay for quantitative detection of *Citrus viroid III* in plant tissues

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A rapid and sensitive real time reverse transcription-PCR (RT-PCR) assay based on SYBR Green I chemistry was developed for the quantitative detection of *Citrus viroid III* (CVd-III) in citrus samples. CVd-III titre was determined at different times in green bark of sour orange, Troyer citrange, trifoliate orange and alemow seedlings inoculated with a CVd-IIIb source. Ten weeks after inoculation the viroid was detected in the four species, without substantial differences in viroid titre among them. Nine weeks later an overall increase of viroid titre was observed. The copy number of CVd-III in sour orange and Troyer citrange was monitored up to 52 weeks after inoculation and a further increase of viroid titre was observed at 35 weeks. For validation purposes, field samples were tested from 58 citrus trees with mixed infections of CVd-III, *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd), as well as from healthy controls. Based on the sensitivity (100%), specificity (96·7%), accuracy (99·2%) and repeatability (Cohen's kappa index 0·98) of the assay, it is suggested that its employment in breeding programmes would be helpful in the evaluation of host resistance and viroid accumulation in plants.

Keywords: citrus, CVd-III, real time RT-PCR, viroid quantification

Introduction

Citrus exocortis viroid (CEVd) and *Hop stunt viroid* (HSVd) (CVd-IIb and CVd-IIc variants) are agents of well-known citrus diseases, exocortis and cachexia, respectively. Both have a wide host range among rutaceous and non-rutaceous plants (Duran-Vila & Semancik, 2003). In contrast, *Citrus viroid III* (CVd-III), a member of the genus *Apscaviroid*, shows a restricted host range confined to the genus citrus and a symptomless replication (Barbosa *et al*., 2002). Dwarfing of citrus plants grafted on trifoliate orange (*Poncirus trifoliata*) and its hybrids as well as on Rangpur lime (*C. limonia*) is associated with CVd-III infection, but the canopy reduction does not impair yield or quality (Polizzi *et al*., 1991; Hutton *et al*., 2000; Vernière *et al*., 2004). For this reason, the potential of this viroid for high density planting has long been investigated (Hutton *et al*., 2000). Its dwarfing effect is currently encouraged in New South Wales (Australia) by inoculating bark patches into host plants one year after the implantation of citrus plants grafted on trifoliate orange or citrange (*P. trifoliata* × *C. sinensis*) (Hardy *et al*., 2004). Therefore, interest in the CVd-III viroid and its relationships with host plants is increasing.

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A CVd-III dwarfing agent (named CMC) was isolated in Italy from a Clementine tree (*C. clementina*) grafted on alemow (*C. macrophylla*) which showed mild stunting (Polizzi *et al*., 1991). CMC is 294 nucleotides in length and belongs to the widely known 'b' variant of CVd-III (Rakowski *et al*., 1994). This variant is known to produce mild vein necrosis on the indicator Etrog citron 'Arizona 861-S1' and mild to slight dwarfing on trifoliate orange and Troyer citrange without any detrimental effects (Albanese *et al*., 1996; Duran-Vila & Semancik, 2003).

Like other viroids, CVd-III has been shown to replicate at different rates in various citrus species and its titre varies considerably depending on the host and environmental conditions (Barbosa *et al*., 2002). Recent studies have shown that inoculation with CVd-III modifies the expression of 18 genes in infected leaves of Etrog citron (*C. medica*) (Tessitori *et al*., 2007) and some of those are diversely modulated in sour orange, alemow and Troyer citrange (Rizza *et al*., 2007). While several reports have investigated the heterogeneous population of its variants by sequential polyacrylamide gel electrophoresis (sPAGE) and sequence analysis (Duran-Vila *et al*., 1988; Rakowski *et al*., 1994) the CVd-III viroid has been shown to be highly conserved (Semancik *et al*., 1997).

For all of the above reasons, a rapid, sensitive and quantitative method for monitoring viroid replication and translocation through plant tissues would be useful to support both basic research and field applications. A

reverse transcription-polymerase chain reaction (RT-PCR) approach for easy detection and identification of citrus viroids, including CVd-III, has been recently reported (Bernad & Duran-Vila, 2006). Nevertheless, the method is qualitative and does not allow ongoing monitoring of viroid replication within the host plant, as a real time PCR protocol could achieve. Real-time PCR methods for the quantitative detection of citrus viroids, such as CEVd and HSVd, as well as potato spindle tuber viroid (PSTVd) have been previously reported (Boonham *et al*., 2004; Tessitori *et al*., 2005). Moreover, a real-time RT-PCR assay enabling the absolute quantification of *Citrus tristeza virus* (CTV) was recently reported (Ruiz-Ruiz *et al*., 2007).

In this study, a novel real time RT-PCR assay has been developed, based on SYBR Green I, for the absolute quantification of CVd-III. The CVd-III copy number was determined in samples from different citrus species growing in the greenhouse or under field conditions, either with multiple or single viroid infections.

Materials and methods

Viroid isolates

The source of CVd-IIIb used for this study (CMC-H6) was a trifoliate orange seedling (H6) inoculated 20 years ago by grafting two pieces of tissue bark taken from the original plant, a Clementine tree grafted on alemow carrying the CMC isolate.

Plant inoculation and sampling

Ten 8-month-old seedlings of sour orange, trifoliate orange, alemow and Troyer citrange were inoculated in August in a greenhouse by T-cut insertion of three pieces of bark taken from CMC-H6, and the same number of uninoculated plants were left as healthy controls. Two months later, both inoculated and uninoculated plants were transferred to a growth chamber with a 16 h light (28°C) and 8 h dark (24°C) cycle, regularly watered with commercial plant nutrients and checked for any suspicious symptoms. Ten weeks after inoculation, at the first sampling (bark tissue from young flush), the seedlings were top cut to promote new sprouts.

For validation purposes, field samples of 43 Clementine trees grafted on trifoliate orange, bark inoculated about ten years ago with a citrus viroid source (named ARA) carrying a mixture of CEVd, HSVd and CVd-IIIb (Polizzi *et al*., 1991) and showing clear symptoms of bark scaling on the rootstock, were collected during the winter. Samples from 35 uninoculated trees were also collected as healthy controls.

In addition, young shoots from 15 Tarocco sweet orange trees grafted on Troyer citrange rootstock, originating from a single mother tree known to be infected with CEVd, HSVd and CVd-III (thanks to previous biological indexing on indicator plants and molecular analysis), were collected in the field at the end of summer together with samples from 15 other Tarocco trees previously untested before for viroids, as healthy controls.

Isolation of total RNA and reverse transcription

Total RNA was extracted from young bark or leaves (100 mg) ground to a fine powder in a mortar with liquid nitrogen, homogenized in TRIZOL Reagent® (Invitrogen) and processed following the manufacturer's instructions. RNA samples were qualitatively analyzed by agarose gel electrophoresis and their concentration was determined by measuring absorbance at 260 nm in a spectrophotometer (Eppendorf BioPhotometer). Samples were used immediately or stored at –80°C prior to use. Total RNA (100 ng) was used for reverse transcription by Thermoscript reverse transcriptase (Invitrogen) and CVd-III specific reverse primer as previously described (Bernad & Duran-Vila, 2006).

SYBR Green I-real time PCR assay

The absolute quantification of CVd-III in tissue samples was based on the preparation of an external standard curve as previously described (Ruiz-Ruiz *et al*., 2007). The entire viroid was amplified using previously reported primers R1 and F1 (Bernard & Duran-Vila, 2006) and the amplification product ligated into the pGEM-T Easy vector (Promega), generating pGEM-T-CVdIII. Integrity of the insert was verified by sequencing in a Ceq 8000 Genetic Analysis System (Beckman Coulter, Inc.). Plasmid concentration was measured by absorbance at 260 nm and the copy number was calculated using the plasmid's molecular weight and Avogadro's constant. The standard curve was generated using previously described R1 and F3 primers (Bernad & Duran-Vila, 2006) and amplifying serial log dilutions of the cloned plasmid in the range of 1×10^8–10^1 copies. Aliquots of these dilutions were stored at –80°C until use. SYBR Green I-based real time PCR reactions were undertaken in two different laboratories equipped with a Stratagene MX 3000 thermal cycler (Stratagene) and a Biorad i-Cycler (Biorad), respectively, using Brilliant SYBR Green Master Mix (Stratagene) and following the manufacturer's instructions. Primer concentration was 150 nM and cycling parameters were: 95°C for 10 min, then 40 cycles of 95°C for 30 s, 60°C for 60 s and 72°C for 30 s with data collection enabled.

For each run, dedicated software of both Real Time instruments plotted the fluorescence intensity against the number of cycles and provided the threshold cycle (Ct) value using the automatic method. Standard curves were obtained by linear regression analysis plotting Ct values vs. the logarithm of the starting plasmid copy number, and the number of target CVd-III copies in each sample was estimated by interpolating the individual Ct values in the standard curve and using the corresponding log values. For detection purposes, a Ct of 35 cycles was established as the cut-off to discriminate positive from negative samples.

Statistical analysis

Stat Graph Pad (version 2·0) was used for statistical analysis. Pearson's correlation coefficient, which measures

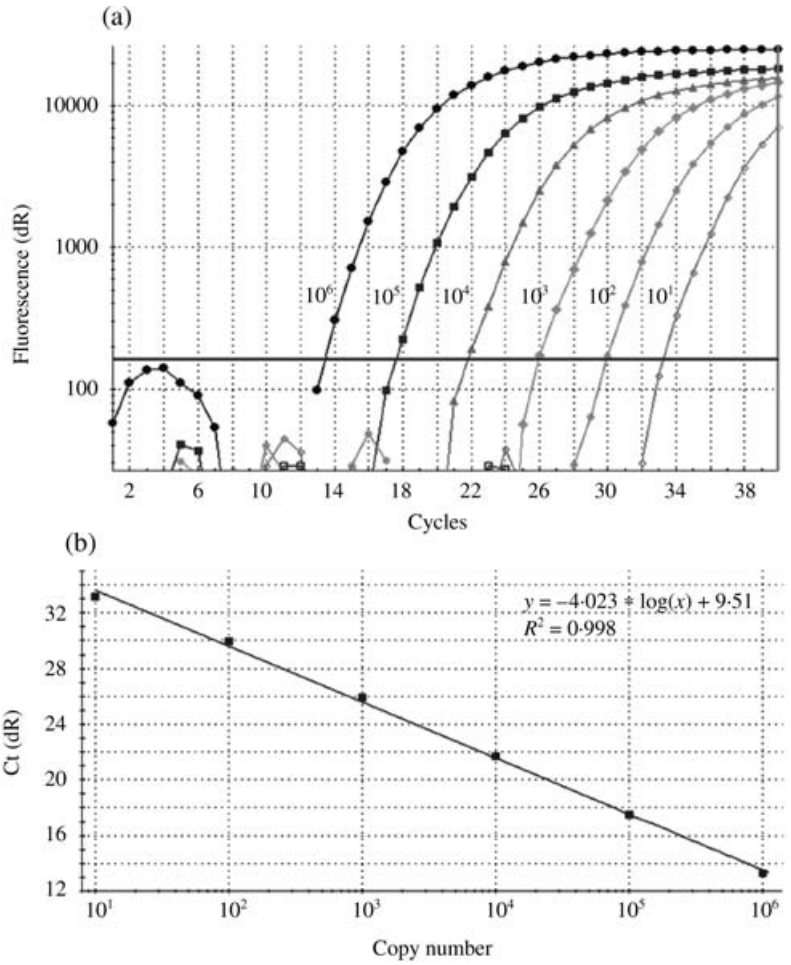

Figure 1 (a) Representative amplification plot of 10-fold serial dilutions of the pGEM-T-CVdIII plasmid obtained with primers R1 and F3 (Bernard & Duran-Vila, 2006). The *x*-axis indicates the PCR cycle number and the *y*-axis the fluorescence intensity (log scale); the threshold line is traced to determine individual threshold cycles (Ct). The initial CVd-III copy number is indicated beside the curves. (b) Standard curve obtained by plotting Ct values vs. actual starting CVd-III copy number. The Ct values for each dilution are the means of three replicates.

the strength of the linear relationship between two variables, revealed a significant linear correlation between viroid titre and weeks after inoculation. Repeatability of the assay was evaluated using Cohen's kappa index (http://homepage.mac.com/aaolmos/KAPPA/kappa.html).

Results and discussion

CVd-III absolute quantification by SYBR Green I-based real time PCR

The need for sensitive, rapid and reliable methods of detection is critical for many plant pathogens including microbes, viruses and viroids. In the case of citrus viroids, best results are achieved after bio amplification of the tested sample obtained by inoculating Etrog citron 861-S1 plants maintained at high temperatures (28–32°C) for at least three to six months. However, in most cases, the citrus species are symptomless and the viroid replication/accumulation rate is unknown. Previous analyses of the replication of citrus viroids, including CVd-III, have used conventional methodologies such as sPAGE and slot-blot hybridization (Barbosa *et al.*, 2002), which are extremely laborious, time consuming and only qualitative/semi-quantitative. To overcome these limitations, a novel real time RT- PCR assay was developed based on SYBR Green I to quantitatively detect CVd-III titre in symptomless citrus host plants.

CVd-III copy number in citrus plant tissues was determined using an external standard curve. As shown in Fig. 1, a linear response was observed for six log dilutions. This large dynamic range allowed a quantitative evaluation of CVd-III in any examined sample. The dissociation curve (Fig. 2) demonstrated the absolute specificity of the expected PCR product without any dimerization of primers.

CVd-III copy number in inoculated citrus plants

CVd-III replication was evaluated, for the first time, in sour orange, Troyer citrange, alemow and trifoliate orange seedlings, all inoculated with the CMC-H6 isolate. Among these citrus rootstocks, selected because of their commercial suitability for citriculture, trifoliate orange

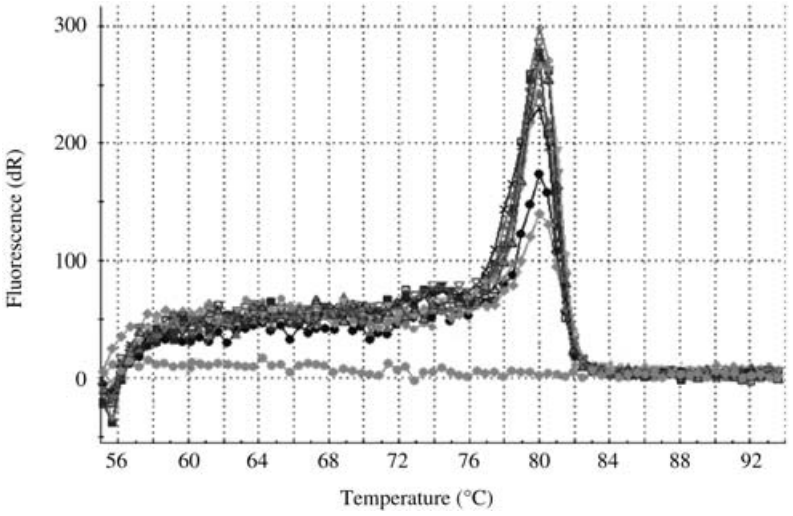

Figure 2 Dissociation curve analysis of amplicons generated in the standard curve (Fig. 1) and no template control (NTC). The *x*-axis indicates the temperature and the *y*-axis the fluorescence intensity; values of the first fluorescence derivative are plotted. No signals of possible aspecific amplicons or primer-dimers are present in standard amplifications and in NTC samples, respectively.

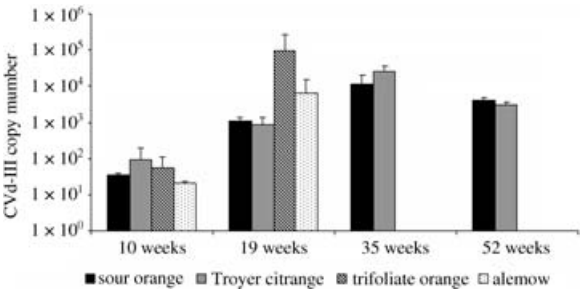

Figure 3 Citrus viroid III (CVd-III) titre determined by real time RT-PCR assay in four citrus species at different sampling times. The *x*-axis indicates the weeks after inoculation and the *y*-axis reports CVd-III copy number/nanogram of total RNA.

and Troyer citrange have been reported to show a certain degree of long-term canopy reduction, whereas sour orange and alemow are practically unaffected (Polizzi *et al*., 1991).

As shown in Fig. 3, a different CVd-III titre was determined in the four citrus species depending on the time of sampling. Ten weeks after inoculation the number of viroid copies/ng of total RNA was in the range of 1×10^1 to 1×10^2 in the four species, without substantial differences between them. Nine weeks later, an increase in the CVd-III titre was observed in the four rootstocks, being higher in alemow (10^4) and trifoliate orange (10^5), compared to sour orange and Troyer citrange (10^2–10^3). The CVd-III titre was subsequently monitored for up to 52 weeks in sour orange and Troyer citrange, proving to be the highest at 35 weeks for both species. A significant correlation between the viroid titre and weeks after inoculation was observed in both sour orange and Troyer citrange up to 35 weeks (data not shown). This result is interesting as these two rootstocks display different phenotypes when infected with CVd-III, and could invite further investigations at the molecular and phenotype level of the relationship, if any, between viroid accumulation and symptom expression.

To ascertain the specificity, sensitivity and accuracy of the assay, field samples were tested from 43 Clementine trees grafted on trifoliate orange bark inoculated with the ARA source, and 15 Tarocco sweet orange trees grafted on Troyer citrange rootstock known to be infected with CEVd, HSVd and CVd-III. Samples from 35 Clementine and 15 Tarocco sweet orange healthy plants were also tested.

CVd-III was detected in all samples from inoculated plants and ranged from $1{\cdot}79 \times 10^2$ to $8{\cdot}77 \times 10^3$ copies/ng of total RNA with median values of $9{\cdot}53 \times 10^2$ for Clementine trees, and from $8{\cdot}61 \times 10^3$ to $5{\cdot}61 \times 10^3$ copies/ng with a median of $6{\cdot}84 \times 10^3$ for Tarocco sweet orange. A difference of almost one log in viroid titre was observed between Clementine and Tarocco sweet orange. This fact may be due to the different sampling season (winter for the former and summer for the latter) and/or host specificity. By contrast, only one healthy plant gave a positive result, due to sample contamination. Therefore, an assay sensitivity of 100%, specificity of 96·7% and accuracy of 99·2% were calculated. Moreover, the repeatability of the assay was assessed by carrying out the protocol in two different laboratories and a Cohen's kappa index of 0·98 was calculated.

Results show that this method is able to detect CVd-III in the range of 1×10^1 to 1×10^6 copies/ng of total RNA. This high sensitivity permits the detection of low titres of CVd-III infection in field samples in months usually unfavourable for viroid analysis (Tessitori *et al*., 1996; Garnsey *et al*., 2002). Based on the sensitivity, specificity, accuracy and repeatability of this protocol, it is suggested that its employment in breeding programmes could improve knowledge on the response of host plants to CVd-III infection and viroid accumulation in plants.

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