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Relative quantitation goes viral: An RT-qPCR assay for a grapevine virus

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A B S T R A C T

Accurate detection and quantitation of viruses can be beneficial to plant–virus interaction studies. In this study, three SYBR green real-time RT-PCR assays were developed to quantitate *grapevine leafroll-associated virus 3* (GLRaV-3) in infected vines. Three genomic regions (ORF1a, coat protein and 3′UTR) were targeted to quantitate GLRaV-3 relative to three stably expressed reference genes (actin, GAPDH and α-tubulin). These assays were able to detect all known variant groups of GLRaV-3, including the divergent group VI, with equal efficiency. No link could be established between the concentration ratios of the different genomic regions and subgenomic RNA (sgRNA) expression. However, a significant lower virus concentration ratio for plants infected with variant group VI compared to variant group II was observed for the ORF1a, coat protein and the 3′UTR. Significant higher accumulation of the virus in the growth tip was also detected for both variant groups. The quantitation of viral genomic regions under different conditions can contribute to elucidating disease aetiology and enhance knowledge about virus ecology.

1. Introduction

A complex network of cellular processes regulates gene expression in plants to ensure normal development and appropriate responses to environmental stresses. Biotic stresses from fungal, bacterial and viral pathogens are a major constraint to the production of agricultural crops. It is therefore imperative to understand plant–pathogen interactions before translating this knowledge into management strategies. Research into plant–pathogen interactions can be approached from the perspective of the host plant or the pathogen (Boyd et al., 2013). Studying the host will lead to the identification of genes involved in partial or permanent pathogen resistance while studying the pathogen leads to the identification of factors that could trigger the plant's defence response. Both approaches benefit from accurate detection and quantitation of the pathogen. Specifically for viruses, the quantitation of not only the viral particles with ELISA, but also different viral genes with RT-qPCR can contribute to our understanding of the disease aetiology.

Reverse transcription quantitative PCR (RT-qPCR) is currently one of the most sensitive techniques for analysing gene expression and has been applied for viral quantitation (Eun et al., 2000; Roberts et al., 2000). RT-qPCR assays can utilise fluorescent dyes or probe-based chemistry (Bustin, 2000) and quantitation will involve either an absolute or relative quantitation strategy (Pfaffl, 2001). For viruses, the detection and quantitation can be complicated by low virus concentration and the presence of diverse variants. Therefore, a sensitive RT-qPCR assay that can detect all virus variants with equal efficiency can aid research focussed at plant–virus interactions.

Grapevine is a highly valuable agricultural commodity that is host to the largest number of viruses of any crop plant (Martelli and Boudon-Padieu, 2006; Prosser et al., 2007). For the purpose of this study we focused on *Grapevine leafroll-associated virus 3* (GLRaV-3), believed to be the main aetiological agent of Grapevine leafroll disease (GLD) (Maree et al., 2013). GLRaV-3 is the type species of the genus *Ampelovirus* in the family *Closteroviridae* (Martelli et al., 2012). Currently, the complete genomes of only ten distinct GLRaV-3 isolates are available that can be divided into four major genetic variant groups (Engel et al., 2008; Maree et al., 2008; Jarugula et al., 2010; Jooste et al., 2010; Gouveia et al., 2011; Sharma et al., 2011; Wang et al., 2011; Bester et al., 2012a; Seah et al., 2012; Fei et al., 2013). The isolates of GLRaV-3 are 91% similar when variant group I is compared to variant group II and 88% when variant group I is compared to variant group III. However, isolates from variant group VI are less than 70% identical to isolates from variant groups I–III

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(Bester et al., 2012a). Three additional variant groups have also been identified, but are only represented by partial sequences (Gouveia et al., 2011; Sharma et al., 2011; Chooi et al., 2013a). Recently, two new GLRaV-3 isolates, GH24 (GenBank: KM058745) and GTG10 (Goszczynski, 2013), were identified. They were found to be more diverse compared to known variant groups and have not yet been assigned to a group. This finding further highlights the great extent of genetic variability between variant groups of GLRaV-3 and warrants the search for a universal detection system.

Currently, the industry standard for GLRaV-3 detection is enzyme-linked immunosorbent assays (ELISA) and conventional end-point RT-PCRs. ELISAs can be time consuming and lack the sensitivity needed for the detection of low virus concentration. Standardised ELISA protocols for GLRaV-3 are also not able to detect all GLRaV-3 variants with equal sensitivity (Cohen et al., 2012). Improvement in the specificity and sensitivity of detection has been achieved by the introduction of qPCR assays based on fluorescent detection systems. Both SYBR green and hydrolysis probe qPCR have been used for the diagnosis and/or quantitation of several grapevine viruses, including GLRaV-3 (Osman and Rowhani, 2006; Osman et al., 2007, 2008; Margaria et al., 2009; Pacifico et al., 2011; Bester et al., 2012b; Tsai et al., 2012; Chooi et al., 2013b; López-Fabuel et al., 2013).

In this study three sensitive SYBR green RT-qPCR assays were developed that are able to detect all variant groups (groups I–III, VI and GH24-like) of GLRaV-3 known to be present in South Africa with equal efficiency. These assays enabled the evaluation of different virus genome regions for their suitability for accurate calculation of GLRaV-3 virus concentration in infected phloem material. The RT-qPCR assays described in this study provide tools for the study of virus ecology.

2. Materials and methods

2.1. Plant material and sample preparation

Six *Vitis vinifera* cultivar Cabernet Sauvignon plants, from a virus isolate collection (Vitis Laboratory, Stellenbosch University, South Africa), were pruned back and left to grow for 60 days in the greenhouse. Only one shoot was allowed to grow and all side shoots were constantly removed. Three plants each, infected with GLRaV-3 variant group II and VI, respectively were selected. Plants were negative for frequently occurring grapevine viruses, except GLRaV-3. GLRaV-3 variant group status of all plants was confirmed using the previously designed real-time RT-PCR high-resolution melting curve analysis assay (Bester et al., 2012b). The shoot from each plant was divided into 5 equal segments to represent different growth stages with segment 1 representing the older (bottom) part of the shoot and segment 5 the actively growing young material at the top of the plant. Due to GLRaV-3 being a phloem-limited virus, phloem material of each segment was collected and stored at −80 °C.

2.2. Total RNA extraction

Total RNA was extracted from 2 g of phloem material using a modified CTAB extraction protocol (Carra et al., 2007). The CTAB buffer contained 2% CTAB, 2.5% PVP-40, 100 mM Tris–HCL (pH8), 2 M NaCl, 25 mM EDTA (pH8) and 2% β-mercaptoethanol. Total RNA was precipitated by adding 2.5 volumes 100% ethanol and 0.1 volumes 3 M sodium acetate (pH5.2) to the upper phase of the 5 M NaCl and chloroform–isoamyl alcohol (24:1) extraction step (Carra et al., 2007). RNA was precipitated for 1 h at −20 °C and centrifuged at 13,500 rpm for 30 min at 4 °C. Pellets were washed with 80% ethanol and resuspended in 100 μl Milli-Q H_2O (Millipore Corporation, Billerica, USA). Integrity and purity was assessed using agarose gel electrophoresis and spectrophotometry (NanoDrop ND-100, NanoDrop Products, Wilmington, USA).

10 μg of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, USA) in 50 μl reactions according to manufacturer's instructions. 450 μl of 10 mM Tris–HCl (pH 8.5) was added to the DNAse treatment mixture and an acidic phenol: chloroform-isoamyl alcohol (5:1) extraction was performed with an ethanol and sodium acetate precipitation (2.5 volumes of 100% ethanol and 0.1 volumes of 3 M Sodium acetate (pH5.2)). After a wash step with 80% ethanol, pellets were dried and resuspended in 30 μl Milli-Q H_2O. Integrity and purity was assessed using agarose gel electrophoresis and spectrophotometry.

2.3. cDNA synthesis

Complementary DNA (cDNA) were synthesised from 1 μg of total RNA using 0.15 μg random hexamers (Promega) and Avian myeloblastosis virus (AMV) reverse transcriptase (Thermo Scientific, Massachusetts, USA) in a final volume of 20 μl according to manufacturer's instructions. 10 μl of each cDNA sample was pooled and a 5-fold dilution series was prepared to construct standard curves for all primer sets. The remaining cDNA was diluted 1:24 and treated as the unknown samples for quantitation. All cDNA dilutions were stored at −20 °C.

2.4. Primer design

Primers were designed targeting three different regions of the GLRaV-3 genome. Open reading frame 1a (ORF1a), ORF6 (coat protein) and the 3′UTR were selected to represent genes/regions with different levels of subgenomic RNAs (sgRNAs) (Jarugula et al., 2010; Maree et al., 2010). By constructing a multiple sequence alignment using CLC main workbench 6.5 (CLC bio, Aarhus, Denmark), conserved regions in the chosen genes/regions of GLRaV-3 were identified. All the GLRaV-3 complete genomes available (GenBank: AF037268.2, GenBank: JQ423939.1, GenBank: JQ655296.1, GenBank: JQ655295.1, GenBank: EU259806.1, GenBank: EU344893.1, GenBank: JX559645.1, GenBank: JQ796828.1, GenBank: GQ352633.1, GenBank: GQ352632.1, GenBank: GQ352631.1, GenBank: GU983863.1, GenBank: KM058745) were included in the multiple sequence alignment in order to design primers that were able to detect all variant groups known to be present in South Africa. All primers were subjected to an NCBI BLAST screen for specificity. Five different primer sets targeting *Vitis vinifera* reference genes were selected from the Reid et al. (2006) study to evaluate their expression stability across all samples used in this study.

2.5. RT-qPCR

2.5.1. PCR and cycle conditions

RT-qPCRs were performed using the Rotor-Gene Q thermal cycler (Qiagen, Venlo, Netherlands) and the SensiMix™ SYBR No-ROX Kit (Bioline, Taunton, USA). Reactions contained 2× SensiMix™ SYBR No-ROX, Milli-Q H_2O and 0.4, 0.48 or 0.56 μM forward and reverse primers (IDT, Coralville, USA), depending on the primer set (Table 1). 2.5 μl cDNA was added to each reaction to a final reaction volume of 12.5 μl. The same cDNA dilution series was used to construct all eight primer-specific standard curves and the same 1:24 dilution of each of the "unknown" samples was screened with the eight primer sets for quantitation. No-template controls, negative plant controls (negative for GLRaV-3) and the third dilution point (1/25) of the five-fold dilution series were included in all runs. To test for the extent of genomic DNA contamination "no-reverse transcription" control qPCRs were performed for all samples using an intron-spanning primer set for the actin gene.