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Rapid detection of *Grapevine leafroll-associated* virus type 3 using a reverse transcription loop-mediated amplification method

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ABSTRACT

Grapevine leafroll disease (GLD) is the most important disease of Grapevines in South Africa. *Grapevine leafroll-associated* virus type 3 (GLRaV-3) has a close association with the disease and is prevalent in South African vineyards. GLD can be controlled using a combination of virus-free planting material, systemic insecticides to control vector populations and removal of infected vines by roguing. Infected vines are identified each autumn using either symptom display (in red cultivars) or ELISA (in white cultivars). While ELISA is a simple, reliable means of testing for GLRaV-3, it is time consuming, laborious and insensitive and a quicker, more sensitive method of detecting GLRaV-3 in the field is needed. A single-tube one-step reverse transcription (RT) loop-mediated isothermal amplification (LAMP) assay combined with a simple RNA extraction protocol was developed for the rapid and easy detection of GLRaV-3. Hydroxy napthol blue was included as an indicator and under isothermal conditions at 60 °C the target viral gene could be amplified in under 2 h and positive results could be easily seen by examining the colour change from violet to sky blue. Using this method, 50 samples could be also pooled together with a single positive sample still being detected. A direct comparison of ELISA, nested PCR and RT-LAMP showed that RT-LAMP is as sensitive as nested PCR and could be performed in a much shorter time with less equipment. This assay is may be a possible alternative to ELISA for the detection of GLRaV-3 in the field.

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1. Introduction

Grapevine leafroll disease (GLD) is the most important disease of grapevines, occurring in every grape-growing country (Martelli, 1993). GLD affects grapevines adversely, where it delays the maturation of the berries, decreases the accumulation of sugars and affects the overall yield and quality of the fruit (Over de Linden and Chamberlain, 1970). This negatively impacts on the wine industry, where it affects the quality and colour of the wines, and the table grapes industry, where yields are affected (Golino et al., 2002).

Globally, five serologically distinct, phloem limited viruses designated *Grapevine leafroll-associated* viruses (GLRaV) 1–4 and 7, are associated with GLD (Fuchs et al., 2009; Martelli et al., 2012, 2002) and of these, GLRaV-3 is the most common (Cabaleiro et al., 2007). GLRaV-3 is part of the Closteroviridae family, where it is type species for the *Ampelovirus* (Martelli et al., 2002). Studies of the genetic diversity of GLRaV-3 has shown that GLRaV-3 can be separated into five phylogenetic groups(Turturo et al., 2005; Jooste et al., 2010; Gouveia et al., 2011). These groups do not seem to be geographically isolated and Group 1 isolates seem to be the most prevalent (Turturo et al., 2005). Initially it was thought that GLRaV-3 was only spread through the use of infected plant propagation material; however it is now known that several species of mealybugs and scale insects act as semi-persistent vectors for the virus with varying efficiencies (Cabaleiro and Segura, 1997; Fuchs et al., 2009; Golino et al., 2002; Walton, 2004).

GLD is the most important disease of grapevines in South Africa and, as it is the most prevalent virus associated with the disease, GLRaV-3 is considered the most important virus associated with GLD (Pietersen, 2004). A study of the variation of GLRaV-3 isolates in South Africa showed that three genetic variants of GLRaV-3; Groups 1, 2 and 3; are present in South African Vineyards (Jooste et al., 2011). Several insects are known to vector the virus in South Africa, however the mealybug *Planococcus ficus* is considered the most important (Douglas and Krüger, 2008).

GLD occurs in all grape varieties, however symptom expression can vary greatly (Over de Linden and Chamberlain, 1970). In red wine cultivars, symptoms are usually expressed as interveinal reddening and down rolling of the leaves and are most distinct in autumn. However white wine cultivars tend to be asymptomatic (with the exception of a few varieties). GLD is usually also

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symptomless in most American wild *Vitis* sp. used as rootstocks and their hybrids (Krake et al., 1999). This variation in symptoms complicates control of the disease as asymptomatic infected individuals can only be detected using either serological (e.g. ELISA) or molecular (e.g. PCR) methods before they can be removed.

In 2002, a study was conducted at a commercial wine farm to prove that GLD could be controlled using an integrated control strategy (Pietersen et al., 2013). The strategy involved the planting of certified material, control of the vector through the use of systemic insecticide and the removal of infected vine material by rogueing (Pietersen and Walsh, 2012; Pietersen et al., 2013). This has been shown to be highly effective for the control of GLD in red cultivars, where symptomatic plants are identified on a vine-for-vine basis at the beginning of autumn each year (Pietersen and Walsh, 2012; Pietersen et al., 2013). However in white cultivars control is more problematic, due to the lack of symptom expression in the majority of varieties.

Currently each season infected white cultivars are identified using Enzyme-linked immunosorbent assay (ELISA) (Ling et al., 2000) before rogueing (Pietersen et al., 2013; Pietersen and Walsh, 2012). ELISA tests are (usually) performed by cellar technicians on the wine farms which have basic facilities such as water baths and fridges but do not have specialised equipment (e.g. thermocyclers) which makes diagnostic test such as RT-PCR unfeasible. ELISA technique is simple and inexpensive (as it requires very little equipment) and can be used for a large number of samples. However, it is less sensitive than molecular techniques (Arora et al., 2006) and is time consuming. Reverse transcriptase polymerase chain reaction (RT-PCR) is sensitive and is less time intensive than ELISA but requires specialised equipment and is more complex than ELISA, usually being performed by trained technicians. The ideal detection technique needs to simple, rapid and specific and would ideally give results in real-time but would not require specialised equipment.

Loop-mediated amplification of nucleic acid (LAMP), a rapid, specific simple means of amplifying nucleic acid, has emerged as a powerful diagnostic technique. (Parida et al., 2008). LAMP relies on the strand displacing DNA polymerase in conjunction with 4 primers (which target 6 specific areas on the target) to amplify target nucleic acid under isothermal conditions within a short period of time (Notomi et al., 2000).

LAMP can be monitored in a number of ways; LAMP amplicons can be viewed using gel electrophoresis; through visual inspection by inspecting turbidity (Mori et al., 2001), colorimetric indicators (Goto et al., 2009; Iwamoto et al., 2003) or intercalating dyes (Maeda et al., 2005).

LAMP has shown to be a highly versatile diagnostic technique and has been used in the detection of a wide variety of pathogens (Parida et al., 2008). There are a number of advantages associated with LAMP; it is isothermal so does not require specialised thermocycling equipment and tests can be heated in a simple heating block or a water bath. The system is highly efficient with no time lost for cycling between temperatures. Secondly because it requires at least 4 primers, it is highly specific. LAMP has also been shown to be more robust than other molecular based techniques and is less affected by biological substances which have been shown to be inhibitory in other techniques (Francois et al., 2011).

This paper reports on the development of a rapid detection technique for GLRaV-3 through the combination of a crude nucleic acid extraction protocol with RT-LAMP and colorimetric assay. This technique has been shown to be rapid, efficient and can reduce the time needed to test a sample from two days by ELISA to 2 h with sensitivity comparable to that of nested RT-PCR. This technique may provide an alternative to ELISA and contribute the control of GLD in white cultivars.

2. Materials and methods

2.1. Plant material and nucleic acid

Petioles were collected from grapevines infected with GLRaV-3 kept in the glasshouses at the University of Pretoria Experimental Farm, Pretoria (01-2839, 01-0257, 01-2639); as well as from the glasshouses at the Agricultural Research Centre (ARC) Plant Protection Research Institute (PPRI), Roodeplaat (623, 621, PL-20, GH 30, 74/2/56, 37/71/84, 74/02/02, 93/0904/74/7/56, 92/1027/74/2/56). Bark scrapings infected with GLRaV-3 were obtained from dormant material from PPRI (623, 621, PL-20). GLRaV-3 strain PL-20 plasmids (pGEM plasmid, Promega) containing the target area (nucleotide positions 5876-8286 on PL-20 genome GQ352633) for the LAMP primers (designated F1, F8 and F9) were obtained from Elize Jooste at the PPRI (Jooste et al., 2010), and were used to assess the LAMP reaction, separately from the reverse transcriptase step.

2.2. LAMP primer design

Primers for LAMP on GLRaV-3 were designed using the 6 available GLRaV-3 whole genomes; GP18 (EU259806) (Maree et al., 2008), 621, 623, PL-20 (GQ352631, GQ352632 and GQ352633) (Jooste et al., 2010), Cl-766 (EU344893) (Engel et al., 2008) and NY-1 (AF037268) (Ling et al., 1998). The genomes were aligned ClustalW in Bioedit (Version 7.0.8, Ibis Bioscience, Carlsbad) and areas with high similarity (>90%) were used as targets. The gene region for RdRp (RNA dependent RNA polymerase) was found to have the highest similarity and was used to design primers. LAMP primers were then designed using Primer Explore V4 (http://primerexplorer.jp/elamp4.0.0/index.html). Possible primers were then compared to available GLRaV-3 genomes (as well as GenBank database) using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and primers with greatest specificity were selected (>98% homology for all primers) (Table 1) and synthesised by IDT (Iowa, USA).

2.3. RT-LAMP

The final LAMP reaction mixture (25 μl total volume in a 0.2 ml tube) was made up as follows; 1.6 μM FIP and BIP, 0.2 μM F3 and B3, 8 U Bst (Lucigen, Middleton, WI) and 1× Bst Buffer B (20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ and 0.1% Triton X-100) (Lucigen), 1 M Betaine (Sigma–Aldrich, St Louis, MO, USA), 1.4 mM dNTPs (Promega, Madison, WI, USA), 7 mM $MgCl_2$ (Fermentas, Vilnuis, Lithuania), 120 μM Hydroxy naphthol blue (HNB) (Acros Organics, Geel, Belgium), 10 U AMV reverse transcriptase (Roche, Palo Alto, CA, USA) and 2 μl of RNA. The mixture was then incubated at 60 °C using a heating block (Eppendorf Thermostat Plus 3130, Hamburg, Germany) for 1 h followed by heating to 80 °C for 10 min to terminate the reaction. Results were analysed by a visual comparison of the colour change of samples to a either a healthy control or a negative (water) control. In order to prevent contamination LAMP mixtures were prepared in a separate laboratory from sample processing and post-LAMP visualisation.

2.4. Optimisation of LAMP

The LAMP reactions were optimised by assessing different incubation temperatures as well as the concentration and ratio of inner: outer primers and Mg^{2+} (4–10 mM). The temperature optimisation (using the optimum primer concentration) was carried out 60, 61, 62, 63, 64 and 65 °C for 1 h and results were analysed using turbidity and confirmed using 2% agarose gel electrophoresis. All the optimisation reactions included negative controls; where no

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