Detection of grapevine leafroll associated virus 3 in South African rootstock clones

by

Shaina Thomasina Facey

13051386

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae Microbiology (MSc)

In the Faculty of Natural & Agricultural Sciences,

Department of Biochemistry, Genetics and Microbiology

University of Pretoria

Pretoria

Submitted November 2019

Declaration of originality

I, Shaina Thomasina Facey, declare that this dissertation, which I hereby submit for the degree Magister Scientiae (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

Date: 13/11/2019

Acknowledgements

I wish to express my sincere gratitude to the following:

My Supervisor, Prof. G. Pietersen for his endless support, encouragement, guidance, knowledge, and technical critique throughout this project.

My Co-Supervisor, Prof. J. Theron for his words of encouragement and academic inputs.

Winetech and the University of Pretoria for the financial support of this project.

My lab mates and friends in the Department of Microbiology and Plant Pathology for their friendship, advice, and positivity.

My brother, Mark, Gareth and Gran for their unconditional love and continued support.

My Father, for granting me the opportunity to pursue my post-graduate studies.

My deepest gratitude to my Mother, for being my biggest inspiration and for always encouraging me to give of my best. This one is for you Mom.

Table of Contents

List of tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of figures

Chapter 5

List of abbreviations

101-14 Mgt	101-14 Millardet et de Grasset
^{o}C	Degrees centigrade
µL	Microlitre
µM	Micromolar
α	Alpha
AA	101-14 Mgt rootstock
ARC	Agricultural Research Council
b	y-intercept
β	Beta
B3	Backward Outer Primer
BIP	Backward Inner Primer
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complementary DNA
C. franc	Cabernet franc
CS	Cabernet Sauvignon
CP	Coat protein
CTAB	Cetyl trimethylammonium bromide
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
E	PCR efficiency
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F3	Forward Outer Primer
FIP	Forward Inner Primer
GFkV	Grapevine fleck virus
GLD	Grapevine leafroll disease
GLRaV	Grapevine leafroll associated virus
GLRaV-3	Grapevine leafroll associated virus 3
GOI	Gene of interest
GVA	Grapevine virus A
H_2O	Water
ha	Hectare
HCl	Hydrochloric acid
HEL	Helicase
Hsp70	Heat shock protein 70
IF	Immunofluorescence
ISEM	Immunosorbent electron microscopy
JA	Jasmonic acid
km^2	Square kilometre
L-Pro	Leader protease
LAMP	Loop-mediated isothermal amplification of nucleic acid
M	Molarity

MET	Methyltransferase
mg	Milligrams
$MgCl_2$	Magnesium chloride
MiRNA	Micro ribonucleic acid
mm	Millimetre
mM	Millimolar
M-MLV	Moloney-Murine Leukemia Virus
mL	Millilitre
N/A	Not applicable
NaCl	Sodium chloride
ng/µL	Nanogram per microlitre
NGS	Next-Generation Sequencing
NH_4	Ammonium
nm	Nanometre
NTC	No template control
nt	Nucleotides
ORFs	Open reading frames
p	Probability value
PCR	Polymerase chain reaction
Pers. Comm.	Personal communication
Pl. ficus	*Planococcus ficus*
Plant SA	South African Plant Improvement Scheme
PPRI	Plant Protection Research Institute
PS	Paulsen rootstock
PS II	Photosystem II
PVP	Polyvinyl-pyrrolidone
qPCR	Real-time PCR
r^2	Coefficient of determination
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RQ	Richter 110 rootstock
RT	Reverse transcriptase
RT-LAMP	Reverse transcription loop-mediated isothermal amplification of nucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
RT-qPCR	Real-time reverse transcription-polymerase chain reaction
RY	Richter 99 rootstock
R99	Richter 99
R110	Richter 110
SA	Salicyclic acid
SAWIS	South African Wine Industry Information & Systems
sgRNA	Subgenomic RNA
SH	Shiraz
SSCP	Single-stranded conformation polymorphism
TAE	Tris-acetate ethylenediaminetetraacetic acid buffer
Tm	Melting Temperature

Tris	Tris(hydroxymethyl)aminomethane
U	µmol/min
UC	US 8-7
USA	United States of America
VIA	South African Vine Improvement Association
V. vinifera	*Vitis vinifera*
w/v	Weight per volume

Abstract

Grapevine leafroll disease (GLD) is recognised as a global, economically significant viral disease of grapevines. The complex of viruses associated with GLD are termed the Grapevine leafroll associated viruses (GLRaVs) and are sequentially numbered in order of discovery. The main etiological agent of GLD is GLRaV-3. In South Africa, a grapevine certification scheme exists to ensure grape growers have access to leafroll-free planting material. Unlike red *Vitis vinifera* cultivars, rootstocks infected with GLD are asymptomatic and cannot be identified through visual diagnosis. To complicate matters even further, GLRaV-3 is thought to be poorly detected in rootstock tissue due to uneven distribution of virus and erratic viral titres. Difficulties in GLRaV-3 detection could result in asymptomatic carriers of GLD being grafted onto healthy vines. To assess the supposed poor detection of GLRaV-3 in rootstocks, a survey was conducted in the Western Cape, South Africa, where various rootstock types and corresponding scions were sampled from 60 vines located in former mother blocks. Samples were tested using a reverse transcription-PCR directed against the conserved GLRaV-3 helicase gene. Detection of GLRaV-3 was significantly higher in scion (83%) than in corresponding rootstock tissue (15%). Several graft transmission experiments were conducted to gain a better understanding of the nature of the poor detection of GLRaV-3 in infected rootstocks. Detection of GLRaV-3 varied depending on the rootstock cultivar used, with Richter 110, Paulsen, and Salt Creek/Ramsey rootstocks appearing to be the least resistant to GLRaV-3 infection and subsequent replication. The variable GLRaV-3 detection in 101-14 Mgt and Richter 99 were suggested to be the result of genetic heterogeneity amongst rootstock clones. The possibility of passive movement of virus in phloem tissue cannot be ruled out and further studies will need to be done to understand this phenomenon. The optimal time of sampling and tissue region of rootstocks most suited for GLRaV-3 detection was investigated using real-time reverse transcription-PCR. Detection of GLRaV-3 was found to be significantly higher in basal tissue than in apical tissue of rootstocks Salt Creek/Ramsey and Richter 110. Rootstock US 8-7 was used as a representative control for *V. vinifera* and, with the exception of R110, was observed to have a significantly higher detection rate over any other rootstock cultivar. The most suitable time of sampling was determined to be late summer/beginning of autumn. A

colourimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay, aided by a crude RNA extraction protocol, was adapted from Walsh and Pietersen (2013) and optimised for possible on-site GLRaV-3 detection in rootstocks. The assay was shown to be rapid (70 minutes), specific, more sensitive than conventional reverse transcription-PCR, and holds promise for detection of the virus in rootstocks. This comprehensive study on GLRaV-3 in rootstocks in South Africa will help bridge the gap in knowledge on the largely unknown GLD dynamics seen in rootstocks.

Chapter 1

Introduction

Grapevine leafroll disease poses a global threat to grapevines and has serious economic impacts on the grape industry (Nel and Engelbrecht, 1972, Namba et al., 1979, Bertamini et al., 2004, Charles et al., 2006, Freeborough and Burger, 2008, Al Rwahnih et al., 2012, Bester, 2012, Jones, 2012, Tsai et al., 2012, Endeshaw et al., 2014), affecting the health and productivity of wine and table grapevines (Cid et al., 2003, Fiore et al., 2008, Chooi et al., 2016). The symptomology of infected vines differs depending on the cultivar type and the environmental conditions at play (Maree et al., 2013). Red cultivars display downward rolling leaves with interveinal reddening and veins that remain green, whereas symptoms in white cultivars are nonexistent or difficult to detect as chlorosis and downward rolling are the only indications of infection (Almeida et al., 2013). Rootstocks are asymptomatic when infected with GLD (Cousins and Striegler, 2005, Chooi et al., 2016).

All commercial *Vitis* farming in South Africa is done through rootstock grafted vines. The reason for this being the presence of *Daktulosphaira vitifoliae,* more commonly known as phylloxera, an aphid limited to feeding on *Vitis* species. Phylloxera primarily targets the roots and leaves of vines with detrimental effects on water and nutrient uptake (Granett et al., 2001). Phylloxera first originated in North America but over the years infested planting material made its way across the globe (Banerjee et al., 2010). *Vitis* species from America that are resistant to phylloxera are bred as rootstocks, onto which *Vitis vinifera* cultivars are grafted, and in this way, the aphid has been combatted since the second half of the 19th century (Campbell, 2004). Rootstocks infected with GLD are asymptomatic carriers of the virus and since GLD is graft transmissible, rootstocks are capable of infecting healthy *V. vinifera* vines (Tsai et al., 2012).

The most effective approach to GLD control is through prevention and limiting spread in and between vineyards (Pietersen et al., 2013). Control strategies include the production of virus-free planting material, monitoring symptomatic vines, performing diagnostic tests on asymptomatic vines, and removing infected individuals.

Diagnostic tests have been develop for GLD detection including: biological indexing, serological, and molecular assays (Bertazzon and Angelini, 2004, Al Rwahnih et al., 2012, Walsh and Pietersen, 2013, Budziszewska et al., 2016). The South African Certification scheme makes use of these diagnostic procedures to ensure that only the most desirable, virus-free grapevines are provided to the wine and table grape industries (Pietersen et al., 2013).

There are several viruses associated with GLD, collectively known as the Grapevine Leafroll associated viruses (GLRaVs) and are proceeded by a number indicating the order of discovery. Grapevine leafroll associated virus (GLRaV-3) is recognised as the main etiological agent of GLD and is also the most prevalent GLRaV is South Africa (Cid et al., 2003, Lee and Martin, 2009, Jooste et al., 2010, Jooste et al., 2011, Almeida et al., 2013). GLRaV-3 has several, highly diverse variants and isolates from each of these variant groups have been detected in South Africa (Maree et al., 2008, Jooste et al., 2010, Bester et al., 2012, Maree et al., 2015).

To successfully control GLRaV-3 in rootstocks within the Wine Grape Certification Scheme the rootstock material supplied to the industry must be free of this virus. It is therefore extremely important to ascertain whether poor detection of GLRaV-3 in rootstocks is due to resistance or another factor. The main objectives of this study were to assist the wine sector manage the virus through further understanding of GLRaV-3 viral tire and seasonal distribution in commercial rootstocks in South Africa, as well as to determine if the difficulties in detection of the virus were due to tolerance or immunity of rootstock clones.

Chapter 2

Literature Review

1. Introduction

1.1 History of the grape industry

It is estimated that around 80 000 km^2 of land worldwide is utilised to cultivate grapes that are then converted to wine, consumed as table grapes, dried to produce raisins, pressed to make juice, or crushed for jams and jellies (Myles et al., 2011, Alabi et al., 2016). This makes the grape one of the most crucial horticultural crops worldwide (Myles et al., 2011).

The *Vitis* genus, belonging to the *Vitaceae* family, is comprised of approximately 68 species that are predominantly located in the Northern Hemisphere (This et al., 2006, Wan et al., 2013). *Vitis vinifera (V. vinifera*), a clonally propagated grapevine, which is predicted to have first arisen around 65 million years ago, is indigenous to Eurasia and is the most commonly used species in the wine industry (This et al., 2006). Today, there are thousands of *V. vinifera* cultivar varieties (Arroyo-García et al., 2006), however, only a select few dominate the global wine market (This et al., 2006).

1.2 Wine grape

The wine grape is a non-climacteric perennial fruit crop which follows two successive developmental stages; berry formation and ripening, which are separated by a lag phase (*véraison*) (CooMbe and McCarthy, 2000). The quality of fruiting berries is influenced by environmental factors and viticultural practices (Dal Santo et al., 2013).

2. Diseases of grapevines

Like most crops, grapevines are susceptible to disease. Viruses, virus-like agents, bacteria, fungi, nematodes, insects, and phytoplasmas are all capable of infecting grapevines and affecting berry production (Jones, 2012, Martelli, 2014). Of all the perennial crops worldwide, grapevine hosts the most viruses (>60) (Martelli, 2006). Most of these are not considered major threats, being of little economic significance,

or confined to a geographic region. However, there are a few that are of great economic importance including Grapevine leafroll disease (GLD) (Martelli, 2006).

3. Grapevine leafroll disease

Grapevine leafroll disease is recognised as one of the most destructive and economically significant disease in grape farming and occurs in all the main grape cultivating areas across the world (Naidu et al., 2014). The disease accounts for approximately 60% of the global grapevine loss (Naidu et al., 2014). The worldwide distribution of GLD is likely a consequence of the local, regional, continental, and intercontinental movement of diseased grapevine material (Almeida et al., 2013). The disease has a rapid rate of spread, impacting both the quality and quantity of grapes in the wine and table grape industry (Chooi et al., 2012, Tsai et al., 2012, Maree et al., 2013). Grapevine leafroll disease is believed to have originated in Afro-Eurasia where the commercial trade of infected plant material led to its current global distribution (Almeida et al., 2013). The earliest accounts of the disease in Europe dates to 1905, where symptoms characterised by reddening of leaves and the downward rolling of laminae were described as “rougeau” in French vineyards (Ravaz and Roos, 1905) and as “rossore” in Italian vineyards (Arcangeli, 1907). The etiology of the disease, however, remained mostly unknown until 1935 when the discovery was made that GLD could be transmitted to vines through grafting practices (Scheu, 1935). In 1979 *Closterovirus*-like particles were found in phloem tissue of GLD infected vines (Namba et al., 1979) and were confirmed through ultrastructure studies in the 1980s (Faoro et al., 1981, Castellano, 1983). Since then, GLD has been found in a total of 36 countries around the globe (Martelli, 2003).

There are several viruses associated with GLD, known as the Grapevine leafroll associated viruses (GLRaVs), which include GLRaV 1-9; all of which belong to the family *Closteroviridae*. The GLRaVs belong to the *Ampelovirus* genus, except for GLRaV-2 which belongs to the *Closterovirus* genus, GLRaV-7 which is a putative member of *Velarivirus* (Ling et al., 2004, Martelli et al., 2012), and GLRaV-8, which is no longer recognised as a virus species as it more closely related to the host plant *V. vinifera* (Martelli et al., 2012). Of all the leafroll associated viruses, GLRaV-3 has the greatest prevalence and the widest distribution around the world, including the Middle East, South and North America, Oceania, Europe, Southern and Northern Africa, and

Asia (Habili et al., 1995, Martin et al., 2005, Charles et al., 2006, Pietersen, 2006, Akbaş et al., 2007, Fiore et al., 2008, Mahfoudhi et al., 2008, Fuchs et al., 2009b, Liu et al., 2013, Moura et al., 2018) It is evident that the cosmopolitan distribution of GLRaV-3 has been as a result of the introduction of virus-infected grapevine material and subsequent propagation and dissemination of GLRaV-3 by insect vectors in most grape-producing areas (Cohen et al., 2003, Tsai et al., 2008, Jooste et al., 2011, Sharma et al., 2011, Almeida et al., 2013).

4. Grapevine leafroll disease in South Africa

4.1 South African grapevine industry

Currently, more than 300 000 people are dependent on the South African wine industry with 3 029 wine farmers cultivating approximately 94 545 hectares (WOSA, 2019). In 2018, the annual harvest reached 1 243 598 tons (960.2 million litres), 86% of which was used for wine production. According to SA Wine Industry Information & Systems (SAWIS), local wine sales contributed R 7,4 million to the regional economy (2018 figures). In terms of international wine production, South Africa is ranked 15th and is responsible for 3.3% of the wine produced worldwide (2018 figures) (SAWIS, 2018).

4.2 Grapevine leafroll associated virus 3

As previously mentioned the consistent reporting of GLRaV-3 in GLD incidence (Martin et al., 2005, Pietersen, 2006) has consequently deemed the virus the main causative agent of GLD (Ling et al., 1998, Martelli et al., 2002). During the 1970s, a high detection rate of virus was found in the Western Cape, South Africa, and thought to be predominantly due to infected rootstocks (Nel and Engelbrecht, 1972). A study by Jooste et al. (2015) in the Western Cape determined the relative abundance of five different GLRaV-3 variants (Jooste et al., 2015). The survey included 315 vines of which 80% tested positive for GLRaV-3 (Jooste et al., 2015). All five GLRaV-3 variants were detected as either single or mixed infections with GLRaV-3 variant group II and group VI observed to be the most dominant as a single infection or in combination with one another or other variant groups (Jooste et al., 2015). Recently, a study by Molenaar et al. (2017) focused on determining the viromes of 17 GLD-infected grapevines (including four rootstock samples) through Next-Generation Sequencing (NGS). An average of four viruses/sample was detected with GLRaV-3 comprising

97.5% of the assembled contigs. The study found rootstock and white-berried cultivars to have more diverse virus populations in comparison to the red-berried cultivars (Molenaar et al., 2017).

4.2.1 Taxonomy and genomic structure of Grapevine leafroll associated virus 3

Grapevine leafroll associated virus 3 belongs to the *Closteroviridae* family, is the type species of the genus *Ampelovirus* (Ling et al., 2004) and a member of subgroup I of this genus (Martelli et al., 2012). GLRaV-3 virions are flexuous filaments of positive-sense single-stranded RNA (Jooste et al., 2015), where the 5' end is likely to be capped and the 3' end is not polyadenylated (Maree et al., 2013). The genome consists of approximately 18,500 nucleotides, is comprised of 13 open reading frames (ORFs) (Figure 1), and is representative of a monopartite C*losterovirus* (Ling et al., 2004, Jooste et al., 2015).

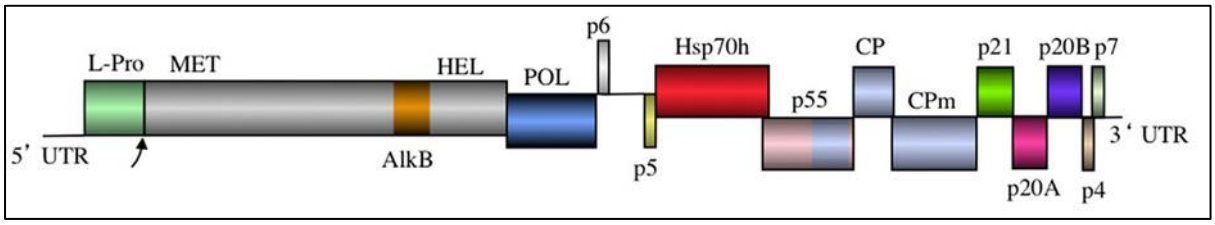


Figure 1: Schematic diagram of the Grapevine leafroll associated virus 3 genome and relative position of genes and ORFs (Maree et al., 2013).

Both ORF1a and ORF1b comprise genes that encode proteins associated with defense and replication. The ORF1a encodes a polyprotein with four domains: leader protease (L-Pro) (Ling et al., 2004), a methyltransferase (MET) (Ling et al., 1998), an AlkB domain (Engel et al., 2008), and a helicase (HEL) (Ling et al., 1998). The ORF1b encodes for an RNA dependent RNA polymerase (RdRp) (Ling et al., 1998), ORF2 encodes a p6 protein involved in cell-to-cell virus transport (Dolja et al., 1994). ORF3 encodes a hydrophobic transmembrane protein and ORF4 encodes the Heat shock 70 (Hsp70) homologue protein, a unique characteristic to closteroviruses (Dolja et al., 1994). ORF5 encodes a 55K protein (Ling et al., 1998) whilst ORF6 encodes the coat protein (CP) gene and ORF7 encodes a copy of the CP (dCP), which is another unique characteristic of closteroviruses (Boyko et al., 1992). The functions of ORF8-12 have not been identified through sequence analysis but are suspected to encode virus silencing suppressors. ORF9 and 10 code for p20 movement proteins (Lu et al., 2004,

Dolja et al., 2006). In a study by Jarugula et al. (2010) the gene expression and *cis*-acting elements of GLRaV-3 were examined and compared to other members of the *Closteroviridae* family. The study found that subgenomic RNA (sgRNA) is required for expression of the 3' ORFs (3-12) in positive-sense single-stranded RNA. The study also suggested that transcription regulation of GLRaV-3 sgRNA is different from that of members of the *Closterovirus* genus (Jarugula et al., 2010).

4.2.2 Genetic diversity of Grapevine leafroll associated virus 3

To further understand the population and genetic variability within GLRaV-3, several techniques have been employed for phylogenetic analysis which include: sequence analysis, single-stranded conformation polymorphism (SSCP), and gene fragment analysis (Turturo et al., 2005, Jooste et al., 2010, Wang et al., 2011). Limited complete GLRaV-3 genome sequences have led to the classification of isolates using the CP, Hsp70, and the RdRp encoding genes (Burger et al., 2017). Ling et al. (2004) were responsible for publishing the first complete nucleotide sequence of GLRaV-3 (isolate NY-1, AF037268) (Ling et al., 2004). Thereafter, numerous studies were conducted investigating the genetic diversity of the GLRaV-3 genome, with Maree et al. (2008) sequencing the GP18 isolate (EU259806) and Engel et al. (2008) having completed the genome sequence of a Chilean isolate (C L-766, EU34489) (Engel et al., 2008, Maree et al., 2008) (Table 1).

Maree et al. (2015) conducted a phylogenetic analysis on complete and incomplete GLRaV-3 genomes and suggested that GLRaV-3 can occur as one of several variants, which are classified into supergroups A-D and represent eight distinct subclades (groups I-VIII of genetic variants) (Maree et al., 2015) (Table 1). Supergroup A is composed of groups I-V, supergroup B (group VI), supergroup C (group VII), and supergroup D (group VIII) (Maree et al., 2015). However, the sequence data for the group VIII isolates from Portugal have been removed from GenBank (Diaz-Lara et al., 2018). A recently published paper which described an isolate from the USA with a novel genetic GLRaV-3 variant proposed the inclusion of a new phylogroup (group IX) (Thompson et al., 2019). In a study by Diaz-Lara et al. (2018), a new RT-qPCR known as 'FPST' was developed to detect all known GLRaV-3 variants. The study found variants that were distantly related to groups I, II, III, V, VI, VI, and IX, and proposed

the addition of a new subclade (group X). To date, there are nine known GLRaV-3 monophyletic groups and four supergroups.

In South Africa, variant groups I, II, III, VI, and VII have all been identified. The complete nucleotide sequences of GLRaV-3 have been recorded and include: 621 (group I), 623 and GP18 (group II), PL-20 (group III), GH30 and GH11 (group VI), and GH24 (group VII) (Table 1). Group II and group VI remain the most dominant variants in South African vineyards (Jooste et al., 2015, Molenaar et al., 2017).

Table 1: Complete Grapevine leafroll associated virus 3 genomes (Diaz-Lara et al., 2018, Harris, 2018, Thompson et al., 2019)

Isolate	GenBank accession	Country	*Vitis vinifera* cultivar	Genome size (nt)	Group	Super Group	Reference
3138-07	JX559645	Canada	*Vitis vinifera*	18498	I	A	(Maree, et al., 2015)
621	GQ352631	South Africa	Cabernet Sauvignon	18498			(Jooste et al., 2010)
CL-766	EU344893	Chile	Merlot	17919*			(Engel, et al., 2008)
NY-1	NC_00466	USA	Pinot Noir	17919*			(Ling, et al., 2004)
WA-MR	GU983863	USA	Merlot	18498			(Jarugula et al., 2010)
623	GQ352632	South Africa	Ruby Cabernet	18498	II		(Jooste et al., 2010)
GP18	EU259806	South Africa	Cabernet Sauvignon	18498			(Maree et al., 2008)
LN	JQ423939	China	Venus Seedless	18563	III		(Fei et al., 2013)
PL-20	GQ352633	South Africa	Cabernet Sauvignon	18433			(Jooste et al., 2010)
185	MH521102	USA	*Vitis vinifera*	18305*	V		(Diaz-Lara et al., 2018)
CA7246	JQ796828	USA	Merlot	18552	VI	B	(Seah et al., 2012)
GH11	JQ655295	South Africa	Cabernet	18671			(Bester et al., 2012)
GH30	JQ655296	South Africa	Cabernet	18576			(Bester et al., 2012)
GH24	KM058745	South Africa	Cabernet Sauvignon	18647	VII	C	(Maree, et al., 2015)
ID45	MH796136	USA	Cabernet Sauvignon	18478	IX	ND	(Thompson et al., 2019)
Trc139	KY764332	USA	Chardonnay	18483*	X	ND	(Diaz-Lara et al., 2018)
139	JX266782	Australia	Sauvignon Blanc	18475	ND	ND	(Rast et al., 2012)

(*)=Genome not fully sequenced

5. Symptomatology

Symptoms associated with GLD can vary significantly with change in season, environmental conditions, the age of the vine, grape cultivar, and scion/rootstock combination (Freeborough and Burger, 2008). Another contributing factor to symptomology is the particular leafroll associated virus responsible for the disease, as different GLRaVs induce varying degrees of severity of symptoms (Martelli et al., 2012). Symptoms are discernible in early to late summer and become more apparent as the season progresses until late autumn (Maree et al., 2013). Red-berried *V. vinifera* cultivars typically have more pronounced symptoms with downward rolling leaf margins and interveinal reddening with veins that remain green (Almeida et al., 2013) (Figure 2A). White-berried *Vitis* cultivars are usually asymptomatic, but may experience some downward rolling with leaf blades that may become chlorotic (Maree et al., 2013). Certain white cultivars infected with the virus, for instance, Chardonnay, have pronounced downward rolling leaves (Figure 2B), while others such as Sauvignon Blanc display subtle and almost unrecognisable leaf rolling (Martelli, 2006, Martelli et al., 2012).

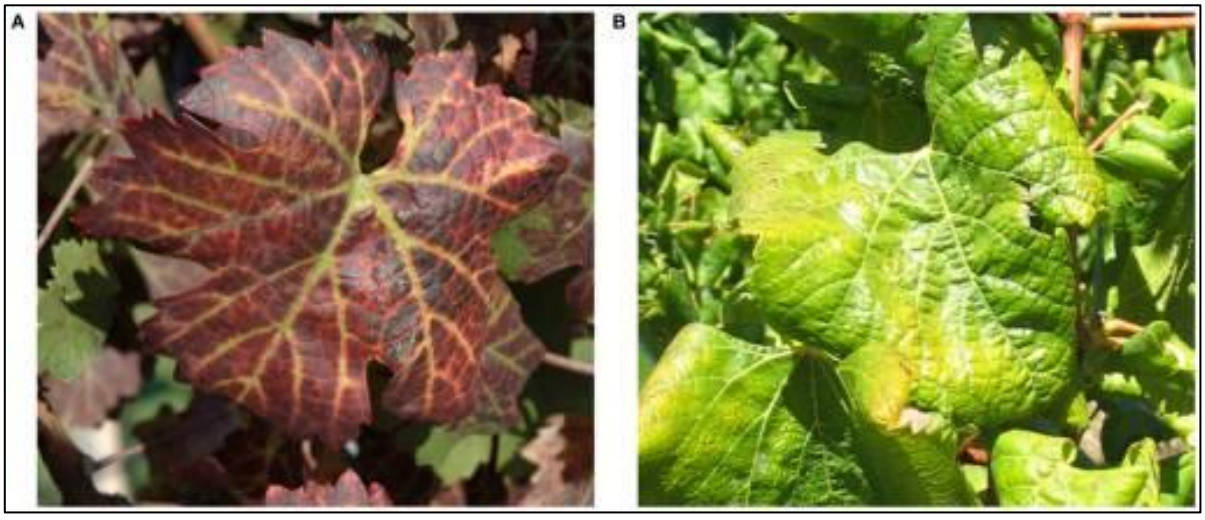


Figure 2: Typical leafroll diseased vines: (A) a red cultivar, *Vitis vinifera* cv Cabernet Franc; (B) a white cultivar, *Vitis vinifera* cv Chardonnay (Maree et al., 2013).

Symptom expression differs between grape varieties and are usually more pronounced in wine grapes than in table grapes. Rootstocks are often *Vitis* species other than V. *vinifera* and usually remain asymptomatic, except for a decline in vigour (Krake et al., 1999, Maree et al., 2013). This is problematic in the grapevine industry

as rootstocks could function as asymptomatic carriers of the virus and pose the risk of disseminating GLD should they be used for grafting and propagation (Martelli, 2006, Martelli et al., 2012). Studies have shown that the use of infected rootstock dramatically increases the occurrence of the GLRaV-3 in a vineyard (Cowham, 2004, Perrone et al., 2017).

A visual assessment of GLD based solely on symptoms is also influenced by other diseases and nutrition deficiencies. These include: any physical injury to grapevine trunks during viticultural practices or wind abrasion between vines, potassium deficiency, insect and herbicide damage, as well as red blotch disease which may resemble GLD symptoms (Jones, 2012). It is, however, possible to reliably use symptoms as a proxy for GLRaV-3 infection in some cultivars under certain circumstances (Bell et al., 2017).

5.1 Economic impact of Grapevine leafroll disease

Grapevine leafroll disease has detrimental physiological effects on infected grapevines, rendering them stressed with low productivity which, in turn, reduces vineyard profitability. More specifically, phloem cells are degraded in leaves, stems, and fruit peduncles, resulting in the accumulation of starch (Namba et al., 1979, Charles et al., 2006, Endeshaw et al., 2014). The interaction between GLRaV-3 and the proteins on the thylakoid membrane in grapevine tissue inhibits the photosystem (PS) II activity of the vine (Bertamini et al., 2004) and depending on the grapevine cultivar, this can result in a 25 to 60% reduction in net photosynthesis activity (Charles et al., 2006). The disease impacts grapes by reducing berry skin phenolic content, lowering Brix (sugar content), lowering pH (increases malic acid and tartaric acids), reducing soluble solids which alters fruit juice composition, altering aromatic profiles, reducing yield and cluster size, and delays fruit ripening (Maree et al., 2013, Naidu et al., 2014). These physiological effects negatively impact the wine industry (Over de Linden and Chamberlain, 1970, Lee and Martin, 2009, Maree et al., 2013).

Studies around the world have suggested that wine produced from grapes harvested on GLRaV-3 infected vines (both red and white cultivars) are of lower quality, with reduced yield, tannins, phenolics, pigmentation, and alcohol in comparison to wines produced from virus-free vines (Borgo et al., 2003, Charles et al., 2006, Legorburu et al., 2009, Alabi et al., 2012, Alabi et al., 2016).

In the absence of control measures, GLRaV-3 results in significant economic losses in both the table and wine industries (Maree et al., 2013). The annual cost of GLD per hectare of *V. vinifera* cv. Cabernet Sauvignon was recorded to be $ 300-2,400 in South Africa in 2008 (Freeborough and Burger, 2008).

6. Spread of Grapevine leafroll disease

There are several modes of transmission linked to GLRaVs including: grafting of infected material, vegetative propagation, and insect vectors (Douglas and Krüger, 2008, Tsai et al., 2008). No evidence has suggested that virus transmission can occur through sap inoculation, i.e. pruning or physical removal of leaves (Martelli et al., 2002). To maintain clonal integrity, grapevine material is vegetatively propagated and planted out either as self-rooted or as grafted vines (Naidu et al., 2014). Cuttings obtained from virus-infected vines and used for propagation allows for the dissemination of GLRaVs with the scion/rootstock material (Martelli, 2001). Virus spread can occur via white and red *V. vinifera* cultivars, native American *Vitis* rootstocks, and hybrid rootstocks (Naidu et al., 2014).

Virus transmission is rapid, with previous studies having recorded an increase in incidence from 11% to 100% over a five-year period (Petersen and Charles, 1997). In South Africa, the incidence of leafroll, measured over 55 vineyards for six years, roughly doubled per year (Pietersen, Pers. Comm.). Whilst global spread of GLD is suggested to be as a result of infected propagative material being used in the industry (Cabaleiro and Segura, 1997, Alkowni et al., 1998, Almeida et al., 2013, Naidu et al., 2014), studies have suggested that insect vectors are expected to be the only contributing factor to inter- and intra-vineyard spread following the establishment of healthy vineyards (Tsai et al., 2008, Daane et al., 2012, Krüger et al., 2015, Herrbach et al., 2017).

6.1 Vectors

Vine-to-vine dissemination of GLRaVs via insect vectors within a vineyard (secondary spread) (Pietersen, 2004) can rapidly alter a vineyard's certification status and is considered the most common means of new infections (Pietersen et al., 2013). Two insect types, both of which belong to the order *Hemiptera* (Johnson and Triplehorn, 2004), are associated with the transmission of GLRaVs namely; soft scale insects

(*Coccidae*) and mealybugs (*Pseudococcidae*) (Douglas and Krüger, 2008, Fuchs et al., 2009a). Virus transmission by both insect vectors occurs in a semi-persistent manner (Charles et al., 2006) with no evidence to suggest vector-virus species specificity (Herrbach et al., 2017).

6.1.1 The family *Coccidae*

Limited research has been done on the transmission of ampeloviruses by soft scale insects. Although 18 soft scale species have previously been observed on grapevine, only two species had been identified as vectors for GLRaVs, *Pulvinaria vitis* (GLRaV-1, and -3) (Belli et al., 1994*)* and *Ceroplastes rusci* (GLRaV-3, -4) (Mahfoudhi et al., 2009). More recently, a survey conducted by Kruger et al. (2013) identified soft scales on grapevines in Vredendal, South Africa, of which three species were selected to determine their ability to transmit GLRaV-3. *Coccus longulus*, *Parasaissetia nigra*, and a *Siaissetia* species were all observed to be vectors of GLRaV-3 (Kruger and Douglas-Smit, 2013). The genera *Neopulvinaria* and *Parthenolecanium* have also been identified as vectors of GLRaV-1 and -3 (Herrbach et al., 2013).

6.1.2 The family *Pseudococcidae*

Several species of mealybug belonging to the genera *Pseudococcus, Planococcus, Phenacoccus,* and *Heliococcus* have been listed as vectors of GLRaVs (Daane et al., 2012, Herrbach et al., 2013). La Maguet et al. (2012) conducted a study in which *Phenacoccus aceris* was shown to transmit six *Ampelovirus* species (Le Maguet et al., 2012) and so, until proven otherwise, all mealybugs on grapevines should be viewed as potential vectors of GLRaVs. The vine mealybug *Planococcus ficus* is a highly efficient vector requiring less than an hour to acquire and inoculate GLRaV-3 (Tsai et al., 2008). Studies have also found that a single viruliferous *Pl. ficus* mealybug nymph is able to alter the health status of a vine (Naidu et al., 2014). For these reasons, the mealybug is deemed the most influential insect vector of GLRaV-3 in South African vineyards (Walton and Pringle, 2004, Douglas and Krüger, 2008, Daane et al., 2012).

Mealybugs are approximately 4 mm in length, elongate-oval shaped, soft-bodied, segmented, grey/pink in colour, covered in a water-repellent waxy secretion, and have hair-like appendages extending from the body (Daane et al., 2012). The ability of mealybugs to transmit ampeloviruses is gender-specific. Male mealybugs have wings

and can fly but lack mouthparts and are therefore unable to feed or transmit virus (Charles et al., 2009). Female mealybugs are phloem feeders and have sap-sucking mouthparts (Millar, 2002) that allow for virus acquisition during feeding and subsequent transmission. The feeding habits of *Pl. ficus* change with the season. In summer the mealybug feeds on berry clusters and during the winter months, when grapes are absent, the mealybugs feed on leaves, cane material, trunks, and the roots of grapevines (Walton and Pringle, 2004). Females are, however, wingless which limits viral transmission to within-vine and between-vine movement (Charles et al., 2009). Generally, female mealybugs have four larval instars whereas males have five (Ben-Dov, 1994). When compared to adult females, the first and second instars, or crawlers, are more efficient at transmitting the virus (Mahfoudhi et al., 2009). This is attributed to their smaller size, increased mobility and lightweight. This ultimately rendering young mealybugs more efficient at dispersal such as: being blown by the wind or carried on workers' clothing, harvesting equipment, and infested plant material (Daane et al., 2012). Although mealybugs can transmit virus during all life stages, the first instar nymphs are the most significant developmental stage in GLD epidemiology (Tsai et al., 2008).

6.2 Epidemiology

In South Africa, the first incidence report of GLD in vineyards was recorded in 1985 (Engelbrecht and Kasdorf, 1985) and in 1990, greenhouse experiments using *Pl. ficus* demonstrated the role of mealybugs in GLD spread (Engelbrecht and Kasdorf, 1990). The spread of GLD has since been studied in multiple countries, including the USA (Golino et al., 2008), New Zealand (Charles et al., 2009), Australia (Habili et al., 1995), South Africa (Pietersen, 2006), France (Le Maguet et al., 2013), and Italy (Gribaudo et al., 2009).

In the Western Cape, South Africa, the spatial distribution and dynamics of GLD were studied between 2001 and 2005. It was found that there are four patterns of spread: 1) primary spread which results in non-uniform spatial patterns, 2) secondary spread (within-field spread) of virus by mealybugs to adjacent vines, 3) the presence of infected vines being found in newly established vineyards in positions where previously infected vines had been improperly removed and 4) the gradient of infection declines from the outskirts to the center of vineyards, which is thought to be due to the

spread of mealybugs via wind, birds, or contaminated farming equipment (Pietersen, 2004, Pietersen, 2006).

7. Control of Grapevine leafroll disease

No natural resistance against GLRaVs has been reported from grapevines, which leaves them vulnerable to infection (Espinoza et al., 2007). Despite the significant worldwide economic impact of GLD, disease management strategies have only quite recently been executed on a large agricultural scale (Pietersen et al., 2013).

Control of GLD in South Africa involves elimination of viruses through the South African Certification scheme, which ensures planting material free of virus is made available to both the wine and table grape industry (Pietersen, 2004). This is followed by an integrated management strategy that includes the virus-free vines, insect vector control using systemic insecticides, and roguing of infected symptomatic grapevines. This is very effective, and can reduce and sustain GLD infection to <1% (Pietersen, 2010).

The transmission of GLRaV-3 in South African vineyards is primarily by mealybugs, *Pl. ficus* and, to a lesser extent, *Ps. longispinus* and several soft scale species (Walton and Pringle, 2004, Douglas and Krüger, 2008, Daane et al., 2012). Control of vector populations and their movements are primarily done using chemical pesticides. However, mealybugs tend to position themselves on the underside of leaves, under bark, or on the roots of vines, making the effective application of contact insecticides difficult (Pietersen et al., 2013).

The removal of virus-infected grapevines from commercial vineyards has been shown to be both an effective and feasible control strategy (Pietersen and Walsh, 2012). A study by Pietersen et al. (2013) at Vergelegen Wine Estate, Somerset West, South Africa, demonstrated roguing of symptomatic vines in combination with continued mealybug control to be highly effective at controlling GLD in red-berried cultivar blocks where viral incidence was reduced from 100% on 41.26 ha (111,431 vines) in 2002 to 0.027% on 77.84 ha (209,626 vines) in 2012 (Pietersen et al., 2013).

Should the majority of vines within a vineyard be infected with virus, and yields and wine quality be reduced, the decision to remove the entire vineyard may be made (Pietersen et al., 2013). The complete removal of residual root and cane material from

a preceding vineyard during a fallow period of up to two seasons aids in reducing virus spread and has been demonstrated in several commercial vineyards (Pietersen and Walsh, 2012). This, however, is only effective when the removal of both above-ground sources of inoculum (canes and trunks) as well as below-ground sources (vine roots) are well implemented as persistent plant material and remnant roots have been noted to be long term reservoirs of GLRaV-3 inoculum (Pietersen, 2004, Pietersen, 2006). Intervening early in the disease cycle may eliminate the possibility of an epidemic (Pietersen et al., 2013).

In summation, for effective GLD management a long-term combination of strategies is required (Almeida et al., 2013), especially when GLRaV-3 can act as a long-term source of inoculum perpetuating the disease cycle. The first step in controlling GLD is to educate growers, followed by access to sanitary planting material and then post-planting management strategies including monitoring and controlling primary and secondary spread.

8. Detection

Regardless of the diagnostic method employed reliable detection is dependent on an appropriate sampling strategy (Tsai et al., 2012). Due to the phloem-limited nature of GLRaVs, low viral titres, and uneven distribution in infected vines the appropriate tissue needs to be sampled at the most ideal time of year to allow for accurate detection (Golino et al., 2008).

Diagnosis of GLD based solely on GLD symptoms in infected vines is mostly unreliable for white-berried cultivars under vineyard conditions and is variable for red-berried cultivars (Naidu et al., 2014). Foliar symptoms are usually only apparent in summer and autumn and several abiotic and biotic factors mimic GLD symptoms as previously described.

Several methodologies have been developed for GLRaV detection and have advanced over the years to establish diagnostic techniques that are sensitive and time efficient for the specific and accurate detection of individual GLRaVs and their variants (Beuve et al., 2007, Constable et al., 2010, Chooi et al., 2012, Budziszewska et al., 2016, Ahn et al., 2019). Diagnostic techniques include biological indexing, serological assays, and molecular methods, as described below.

8.1 Biological indexing

Before the use of serological and molecular assays, hardwood indexing on biological indicators was the main diagnostic technique for GLD (Maree et al., 2013). This technique is also used as a standard for confirming graft transmissibility of GLRaVs and is routinely used to test for GLD in sanitary plant material programs (Naidu et al., 2014).

A small chip bud is removed from the vine under testing and grafted onto an indicator vine by chip-, bench-, or micro-grafting (Rowhani and Golino, 1995, Constable et al., 2010) which is then planted out in the field and observed over two seasons for disease symptoms (Golino et al., 2008). Commonly used indicator plants include *V. vinifera* cultivars Cabernet Franc, Cabernet Sauvignon, and Pinot noir (Pathirana and McKenzie, 2005). Selection of an indicator plant is based on personal preference and on the environmental conditions under which the indicator host is cultivated (Golin et al., 2008).

Biological indexing is, however, laborious and relies on the successful inoculation of the indicator plant with the disease-associated virus (Golino et al., 2008). Low viral titres, as sometime found with GLRaV-3, can affect symptom expression and influence the interpretation of results (Constable et al., 2010). Biological indexing gives an indication of disease, rather than an associated virus and requires a skilled virologist to interpret symptom expression (Maree et al., 2013).

8.2 Serology

Several serological assays exist including: immuno-strip tests, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence (IF) (Pathirana and McKenzie, 2005). Due to its robustness and scalability, ELISA is currently employed for routine testing of vines for GLRaV-3 in basic field laboratories (Ward et al., 2004). The principle of virus detection by ELISA is based on recognition of viral antigen by viral-specific antibodies (immunoglobulins). The locally produced, South African, industry-standard kit can detect GLRaV-3 variant groups I, II, III and VI in grapevine tissue (Bester et al., 2012). Despite the cost-effectiveness, simplicity, and high throughput application of ELISA designed to test numerous samples, it has some limitations. ELISA is less sensitive when compared to molecular assays, i.e. the

polymerase chain reaction (PCR) and may have limited specificity (presence of genetic variant groups may go unrecognised by the available immunoglobulins) (Ward et al., 2004).

8.3 Nucleic acid-based techniques

8.3.1 Polymerase chain reaction

Specific reverse transcription-PCR have shown sensitivities 10-100 fold greater than that of serological methods and biological indexing (Pacifico et al., 2011). Primers target highly conserved regions of the viral genome (RdRp region, Hsp70, helicase, or coat protein) (Ling et al., 2001, Thompson et al., 2019) which increases the specificity of the assay over ELISA (Pacifico et al., 2011). Several RT-PCR assays have been successfully developed for GLRaV-3 detection (Saldarelli et al., 1998, Ling et al., 2001, Nolasco et al., 2006, Bester, 2012).

Quantification of virus target has been taken a step further with real-time RT-PCR (RT-qPCR) where an unknown sample is quantified either relatively or absolutely by comparison to a standard DNA sample or reference gene (Feng et al., 2008). Real-time fluorescent chemistry can be divided into two main groups: double-stranded DNA intercalating molecules (SYBR Green) and fluorophore-labelled oligonucleotides (TaqMan probe) (Navarro et al., 2015). There has been an increase in the use of RT-qPCR over the years with regards to the detection and quantification of GLRaVs in grapevine and insect vectors (Beuve et al., 2007, Osman et al., 2007, Osman et al., 2008, Chooi et al., 2016).

Whilst RT-PCR and RT-qPCR are considered standard diagnostic techniques in science laboratories, most basic field laboratories on wine farms are not equipped to perform these tests which require thermocyclers and skilled personnel. The procedures are also costly to run in comparison to ELISA making them financially unviable testing platforms.

8.3.2 Isothermal amplification of nucleic acid

Recently, an alternative method, RT-loop-mediated isothermal amplification (RT-LAMP) assay, was developed for the successful and rapid detection of GLRaV-3 (Walsh and Pietersen, 2013). The assay isothermally amplifies a target sequence by a strand displacing DNA polymerase using four primers that target six distinct regions.

9. Grapevine leafroll associated viruses within hosts

9.1 Scion

Although ampeloviruses can colonise a wide range of perennial plant taxa including pineapple (Sether and Hu, 2002), plums (Al Rwahnih et al., 2007), and cherries (Bajet et al., 2008), evidence has suggested that GLRaVs are limited to grapevines (*Vitis*) (Almeida et al., 2013, Naidu et al., 2015). Although, in a recent study by Prator et al. (2017), GLRaV-3 was experimentally shown to infect the model plant, *Nicotiana benthamiana* through insect-mediated transmission using the vine mealybug *Pl. ficus*. This model plant has several advantages when studying GLRaV-3 in comparison with its natural host, *V. vinifera*. In the study by Prator et al. (2017), *N. benthamiana* infected with GLRaV-3 was seen to have higher viral protein and virion concentrations compared to *V. vinifera* when subjected to western blot and transmission electron microscopy, respectively. *N. benthamiana* is a herbaceous plant which can be grown year round and relatively quickly (few weeks/months) (Prator et al., 2017) which could make *N. benthamiana* an ideal model plant for future GLRaV-3 research.

9.2 Rootstocks

9.2.1 History

Traditionally grapevines were propagated on their own roots until the late 19th century when the root louse, phylloxera *(Daktulosphira vitifoliae),* was unintentionally imported with American rootstocks from the North Americas to vineyards in Western Europe (Skinkis et al., 2009). The introduction resulted in mass grapevine loss and had devastating effects on the grape industry (Meng et al., 2006). Unlike *V. vinifera*, native American *Vitis* species appeared to have natural resistance to phylloxera, probably due to co-evolution with the insect. French varieties were soon grafted onto these,

establishing an effective and immediate way in which to control the root louse (Smith, 1992). The first species used as cuttings to provide resistance to phylloxera were *V. riparia* and *V. rupestris* (Foëx, 1902). *V. cinerea var. Helleri (V. berlandieri)* soon afterwards became a popular choice due to combined resistance to phylloxera and its adaptation to calcareous soil (Walker and Stirling, 2008, Töpfer et al., 2011). Rootstocks have been noted to display resistance to several other insect pests as well as grapevine diseases, however, there have been no reports of natural resistance to GLRaVs (King et al., 1982, Maree et al., 2013, Naidu et al., 2014).

9.2.2 Rootstock resistance/tolerance

The terms resistance and tolerance each describe a specific virus-host relationship, where resistance is the ability of a plant to limit viral replication by interfering with the disease cycle (Lecoq et al., 2004). The degree to which a host plant is resistant also needs to be considered with the two ends of the spectrum being complete resistance and complete susceptibility. Plants that are incapable of sustaining virus replication and thus do not develop symptoms are said to have complete resistance or immunity, whereas plants that are incapable of hindering a pathogen infection are said to be completely susceptible (Lecoq et al., 2004, Oliver and Fuchs, 2011).

Tolerance is the ability of plants to limit impairment brought on by virus infection and still produce a satisfactory crop despite the presence of virus (Lecoq et al., 2004, Fraile and García-Arenal, 2010). Tolerance can also be further classified with the extremes being complete tolerance and complete intolerance (Oliver and Fuchs, 2011). When a plant is unaffected by the presence of a pathogen, it is said to be completely tolerant to the virus. A completely intolerant or susceptible plant is unable to produce a viable crop in the presence of virus (Oliver and Fuchs, 2011).

Rootstock/scion interactions are influenced by the presence/absence of virus in the scion (Albacete et al., 2015). The response of rootstock to virus can fall somewhere between highly tolerant to highly intolerant, and a rootstock that is sensitive to a scion infected with virus may result in overall vine decline and loss of productivity (Reisch et al., 2012).

9.2.3 Rootstocks in South Africa

The rootstocks that were first used in the industry were Riparia Gloire de Montpellier, Aramon, Rupestris du Lot, and Jacquez (Leipoldt, 1952). Nowadays, the South African wine and table grape industries are dominated by Ramsey, Richter 99 (R99), Richter 110 (R110), and 101-14 Mgt (Fourie and Halleen, 2004, Saayman, 2009). Table 2 provides an overall indication of the rootstock cultivars used in South Africa along with their respective parentage, the percentage distribution in 2012, and their response to certain environmental factors. Jacquez was once a popular choice of rootstock but due to limited resistance to insect pests, phylloxera and nematodes, as well as high drought sensitivity, it is now far less desirable. Ramsey is ranked 4th on the list but is considered a poor choice for wine grapes due to its high potassium uptake. It is, however, widely used in table grape farming as high resistance to nematodes and the ability to grow in sandy soils make it of great benefit in this industry (Table 2) (Saayman, 2009).

Several factors are involved in selecting the best rootstock/scion combinations including: the area of cultivation, environmental conditions (Table 2), soil and drainage, vigour, length of the vegetative cycle of rootstock, scion cultivar, and the presence of nematodes (Poni et al., 2018).

Table 2: Rating of most used rootstocks in South Africa (Malan and Meyer, 1993, Saayman, 2009)

Rootstock	Breeding	% distribution in South Africa 2012	Phylloxera	Nematodes	Phytophthora	Vigour	Lime	Drought
Richter 99	*V. Berlandieri x V. rupestris*	41.2	E-VG	G	VL	VH	G	G
101-14 Mgt	*V. Riparia x V. rupestris*	17.7	G	M=G	M	M	L	M-L
Richter 110	*V. Berlandieri x V. rupestris*	20	VG	G	M-L	H	G	VG
Ramsey	*V. Champinii*	12.6	G	E	VG-G	E	L-G	L
US 8-7	*Jacquez x V. rupestris*	3.9	M-G	M	VG	VH-E	M	M-G
Ruggeri 140	*V. Berlandieri x V. rupestris*	1.4	G	M	M	H-VH	E	E-VG
Paulsen 1103	*V. Berlandieri x V. rupestris*	1.7	VG	M-G	VL	VH	G	VG-E
Jacquez	*V. aestivalis x V. cinereal x V. vinifera*	0.5	VL	L	VG	M-G	M	L-M
143 B Mgt	*V. vinifera x V. riparia*	0.2	M	M	VG	VH-E	M	G
SO4	*V. Berlandieri x V. riparia*	0.3	G	VG	L	G	M-G	L

VL=very low; L=low; M=moderate; G=good; VG very good; H=high; VH=very high and E=exceptional

9.2.4 Detection of Grapevine leafroll associated virus 3 in rootstocks

Several GLRaVs (-1, -2, and -3) have been shown to infect rootstocks (Alkowni et al., 1998, Kominek and Holleinova, 2003, Chooi et al., 2016). However, as previously mentioned, GLRaVs are erratically distributed in infected grapevine tissue which consequently results in the detection of varying viral titres depending on the region of plant tissue sampled (Cohen et al., 2003). A study by Credi et al. (1997) observed GLRaV-3 detection in 125AA *(V. berlandieri x V. riparia)*, Paulsen 1103, R110, Kober 55B *(V. berlandieri x V. riparia),* and *V. rupestris* 'St. George' to be more challenging than detection in *V. vinifera* (Credi, 1997).

Chooi et al. (2016) investigated titre and distribution of Group I and VI GLRaV-3 variants in two rootstock types, 3309C (*V. riparia x V. rupestris)* and Schwarzmann (*V. riparia x V. rupestris)*, and suggested that the poor detection of virus and low viral titres observed in rootstock were linked to previously observed erratic distribution of GLRaV-3 (Chooi et al., 2016). Harris (2017) conducted a survey in the Western Cape, South Africa, to determine the relative composition of GLRaV-3 variants in rootstocks compared to those found in the corresponding scions. Inconsistent detection of GLRaV-3 was observed for R99 and only 43% of rootstocks sampled tested positive for virus, whereas 93% of the scion counterparts tested positive (Harris, 2017). Both rootstock and scions were seen to have mixed infections and variants of group I and group VI were observed to be most dominant. The GLRaV-3 variants detected were not always in agreement between the two tissues types. Harris (2017) also investigated the presence of GLRaV-1,-2, -4-like and, -7 in the rootstocks and scions collected which yielded low infection rates. GLRaV-1 was only detected in 3% of scion and corresponding rootstock and GLRaV-2 had a 6% detection rate in rootstock and 13% detection rate in scion tissue. Harris (2017) was unable to detect GLRaV-4-like and GLRaV-7 in any of the rootstocks and scions tested (Harris, 2017).

In a report published by Cid et al. (2003) the observed low viral titres of GLRaV-3 in infected rootstocks were suggested to be as a result of an unclear natural host resistance mechanism (Cid et al., 2003). It was also noted that viral titres were higher in the basal region in comparison to the apex shoot of the rootstocks tested. Cohen et al. (2003) found similar trends where GLRaV-3 was only detected in the trunks of R110 (Cohen et al., 2003).

Hao et al. (2017) investigated the localization of GLRaV-3 in grapevine micrografts and found that, in general, it took an increased length of time for the virus to infect conjunctions in healthy scion/virus-infected rootstock micrografts versus virus-infected scion/healthy rootstock micrografts (Hao et al., 2017). This finding would suggest the possibility of an accelerated spread of virus from infected scion to healthy rootstock compared to infected rootstock to healthy scion.

Detection in rootstock tissue is challenging due to low and varying viral titres (Malan, 2009, Maree et al., 2013). This is of great concern, as certified virus-free scions could potentially be grafted onto asymptomatic rootstocks infected with virus and in this way increase the spread of GLD in commercial vineyards. Nested RT-PCR and RT-qPCR are more sensitive in detecting GLRaV-3 than conventional methods and serve as alternative diagnostic methods for GLRaV-3 detection (Ling et al., 2001, Malan, 2009). These techniques, however, have some disadvantages, including the need for specialised equipment, trained personnel, and high costs, and are therefore not viable alternatives for basic field laboratories where rootstock testing is routinely performed.

10. The South African Plant Certification Scheme

Since GLRaV-3 can only be acquired and spread from infected *Vitis*, the greatest impact in GLD control is obtained through the use of sanitary grapevine material provided by the South African Plant Certification Scheme for Wine Grapes, known as the South African Vine Improvement Association (VIA) (Pietersen, 2010, Almeida et al., 2013). Grapevines with desirable traits such as vigour, virus-free status, and productivity are selected and propagated under conditions that maintain the virus-free status of the clones (Pietersen et al., 2013). To obtain these vines, plant material is subjected to heat therapy (Almeida et al., 2013) or meristem tip culturing before being used in the establishment of mother blocks (Jooste et al., 2010). Grapevines are planted out in insect-free greenhouses as nuclear material and tested for a range of viruses every three years, eliminating any individuals who test positive for virus (Almeida et al., 2013). ELISA, immunosorbent electron microscopy (ISEM), and PCR are used for the screening of nuclear material (Ling et al., 2000, Narayanasamy, 2008), along with biological indexing for a few virus and virus-like diseases (Rupestris stem pitting disease, Shiraz disease, and Corky bark). With regards to scion material, ELISA, ISEM, and PCR allow for the testing of a large numbers of samples and are all

reliable and time-efficient (Cid et al., 2003). Scions are screened for GVA, GLRaV-1, -2, and -3, and, Grapevine fleck virus (Pietersen, 2004, Almeida et al., 2013). During the first growth season and every three years thereafter, scions undergo testing against GLRaV-1, -2 and -3 using ELISA, whereas only rootstocks US 8-7 and 143 B Mgt are screened (Pietersen, 2017). Grapevines that test negative for all the previously mentioned viruses are awarded a 3-star rating GLRaV-free nuclear material status (Almeida et al., 2013, Pietersen, 2017). Once nuclear material is established, buds from these are propagated and cultivated in foundation blocks. Foundation blocks are no longer necessarily covered areas, but are often an open area of land that is isolated and has no history of use in grapevine propagation (virgin soil) (Almeida et al., 2013). The foundation block also needs to be kept vector-free (Pietersen, 2017). The foundation blocks are treated with systemic insecticides for vector control and inspected annually for GLD symptoms (Pietersen, 2017). Material from foundation vineyards are used to establish mother blocks. These are commonly established at contracted, collaborating commercial wine farmers (Jooste et al., 2011), from whom the planting material is purchased for nurseries. Mother blocks are screened for virus yearly and should the infection rate be above 3% the vineyard can no longer function as a mother block (Walsh and Pietersen, 2013). The scheme is responsible for the continual improvement of planting material and certification of virus-free clones. This ensures that only the finest starting material is made available to the wine industry.

References

AHN, S. J., BAEK, Y. H., LLOREN, K. K. S., CHOI, W.-S., JEONG, J. H., ANTIGUA, K. J. C., KWON, H.-I., PARK, S.-J., KIM, E.-H. & KIM, Y.-I. 2019. Rapid and simple colorimetric detection of multiple influenza viruses infecting humans using a reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic platform. *BMC infectious diseases*, 19**,** 676.

AKBAŞ, B., KUNTER, B. & ILHAN, D. 2007. Occurrence and Distribution of Grapevine Leafroll-associated Viruses 1, 2, 3 and 7 in Turkey. *Journal of Phytopathology,* 155**,** 122-124.

AL RWAHNIH, M., UYEMOTO, J., FALK, B. & ROWHANI, A. 2007. Molecular characterization and detection of plum bark necrosis stem pitting-associated virus. *Archives of virology*, 152**,** 2197-2206.

ALABI, O., GUTHA, L., CASASSA, L., HARBERTSON, J., MIRALES, M., BEAVER, C. & NAIDU, R. 2012. Impacts of grapevine leafroll disease on own-rooted wine grape cultivar in cool climate viticulture. *Proceedings of the 17th Congress of ICVG 7-14*, October, 2012. 170-171.

ALABI, O. J., CASASSA, L. F., GUTHA, L. R., LARSEN, R. C., HENICK-KLING, T., HARBERTSON, J. F. & NAIDU, R. A. 2016. Impacts of grapevine leafroll disease on fruit yield and grape and wine chemistry in a wine grape (Vitis vinifera L.) cultivar. *Public Library of Science one*, 11**,** e0149666.

ALBACETE, A., MARTÍNEZ-ANDÚJAR, C., MARTÍNEZ-PÉREZ, A., THOMPSON, A. J., DODD, I. C. & PÉREZ-ALFOCEA, F. 2015. Unravelling rootstock× scion interactions to improve food security. *Journal of experimental botany*, 66**,** 2211-2226.

ALKOWNI, R., DIGIARO, M. & SAVINO, V. 1998. Viruses and virus diseases of grapevine in Palestine. *European and Mediterranean Plant Protection Organization Bulletin*, 28**,** 189-195.

ALMEIDA, R., DAANE, K., BELL, V., BLAISDELL, G. K., COOPER, M., HERRBACH, E. & PIETERSEN, G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology*, 4**,** 94.

ARCANGELI, G. 1907. Su di un caso di rossore della vite a Careggiano. *Agric. Ital*, 13**,** 18-22.

ARROYO-GARCÍA, R., RUIZ-GARCIA, L., BOLLING, L., OCETE, R., LOPEZ, M., ARNOLD, C., ERGUL, A., SÖYLEMEZO LU, G., UZUN, H. & CABELLO, F. 2006. Multiple origins of cultivated grapevine (Vitis vinifera L. ssp. sativa) based on chloroplast DNA polymorphisms. *Molecular ecology*, 15**,** 3707-3714.

BAJET, N., UNRUH, T., DRUFFEL, K. & EASTWELL, K. 2008. Occurrence of two little cherry viruses in sweet cherry in Washington State. *Plant disease*, 92**,** 234-238.

BELL, V., BLOUIN, A., COHEN, D., HEDDERLEY, D., OOSTHUIZEN, T., SPREETH, N., LESTER, P. & PIETERSEN, G. 2017. Visual symptom identification of grapevine leafrollassociated virus 3 in Red Berry cultivars supports virus management by roguing. *Journal of Plant Pathology*, 99**,** 477-482.

BELLI, G., FORTUSINI, A., CASATI, P., BELLI, L., BIANCO, P. & PRATI, S. 1994. Transmission of a grapevine leafroll associated closterovirus by the scale insect Pulvinaria vitis L. *Rivista di patologia vegetale***,** 43,105-108.

BEN-DOV, Y. 1994. *A systematic catalogue of the mealybugs of the world (Insecta: Homoptera: Coccoidea: Pseudococcidae and Putoidae) with data on geographical distribution, host plants, biology and economic importance*. Intercept Limited, United Kingdom, 169-193.

BERTAMINI, M., MUTHUCHELIAN, K. & NEDUNCHEZHIAN, N. 2004. Effect of grapevine leafroll on the photosynthesis of field grown grapevine plants (Vitis vinifera L. cv. Lagrein). *Journal of Phytopathology*, 152**,** 145-152.

BESTER, R. 2012. Sequencing and detection of a new strain of grapevine leafroll-associated virus 3 in South Africa. Master of Science in Genetics. Stellenbosch University, Stellenbosch.

BESTER, R., MAREE, H. & BURGER, J. 2012. Complete nucleotide sequence of a new strain of grapevine leafroll-associated virus 3 in South Africa. *Archives of virology*, 157**,** 1815-1819.

BEUVE, M., SEMPÉ, L. & LEMAIRE, O. 2007. A sensitive one-step real-time RT-PCR method for detecting Grapevine leafroll-associated virus 2 variants in grapevine. *Journal of virological methods*, 141**,** 117-124.

BORGO, M., ANGELINI, E. & FLAMINI, R. 2003. Effects of grapevine leafroll associated virus 3 on main characteristics of three vineyards. *L'enologo*, 3**,** 99-110.

BOYKO, V. P., KARASEV, A. V., AGRANOVSKY, A. A., KOONIN, E. V. & DOLJA, V. V. 1992. Coat protein gene duplication in a filamentous RNA virus of plants. *Proceedings of the National Academy of Sciences*, 89**,** 9156-9160.

BUDZISZEWSKA, M., WIECZOREK, P. & OBRĘPALSKA-STĘPLOWSKA, A. 2016. One-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) for detection of tomato torrado virus. *Archives of virology*, 161**,** 1359-1364.

BURGER, J., MAREE, H., GOUVEIA, P. & NAIDU, R. 2017. Grapevine leafroll-associated virus 3. *Grapevine Viruses: Molecular Biology, Diagnostics and Management*. Springer, Berlin.

CABALEIRO, C. & SEGURA, A. 1997. Some characteristics of the transmission of grapevine leafroll associated virus 3 by Planococcus citri Risso. *European Journal of Plant Pathology*, 103**,** 373-378.

CASTELLANO, M. 1983. Virus-like particles and ultrastructural modifications in the phloem of leafroll affected grapevines. *Vitis*, 22**,** 23-39.

CHARLES, J., COHEN, D., WALKER, J., FORGIE, S., BELL, V. & BREEN, K. 2006. A review of the ecology of grapevine leafroll associated virus type 3 (GLRaV3). *New Zealand Plant Protection*, 59**,** 330-337.

CHARLES, J., FROUD, K., VAN DEN BRINK, R. & ALLAN, D. 2009. Mealybugs and the spread of grapevine leafroll-associated virus 3 (GLRaV-3) in a New Zealand vineyard. *Australasian Plant Pathology*, 38**,** 576-583.

CHOOI, K., PEARSON, M., COHEN, D. & MACDIARMID, R. 2012. Development of generic and variant-specific molecular assays for the detection of the highly variable Grapevine leafroll-associated virus 3. P*roceedings of the 17th Congress of ICVG7–14,* October, 2012. 142-143.

CHOOI, K. M., COHEN, D. & PEARSON, M. N. 2016. Differential distribution and titre of selected grapevine leafroll-associated virus 3 genetic variants within grapevine rootstocks. *Archives of virology*, 161**,** 1371-1375.

CID, M., CABALEIRO, C. & SEGURA, A. 2003. Detection of Grapevine leafroll-associated virus 3 in rootstocks. *Extended abstracts of the 14th Meeting of ICVG*, Locorotondo, 2003.

COHEN, D., VAN DEN BRINK, R. & HABILI, N. 2003. Leafroll virus movement in newly infected grapevines. *Extended abstracts 14th Meeting ICVG*, Locorotondo, Italy, 2003, 13-17.

CONSTABLE, F., NICHOLAS, P. & RODONI, B. 2010. *Development and validation of diagnostic protocols for the detection of endemic and exotic pathogens of grapevines. en línia]. A: Final report to grape and wine research & development corporation*. Victoria: Australian Government. Department of Primary Industries, 28-29.

COOMBE, B. G. & MCCARTHY, M. 2000. Dynamics of grape berry growth and physiology of ripening. *Australian journal of grape and wine research*, 6**,** 131-135.

COWHAM, S. 2004. *A growers' guide to top-working grapevines*. Phylloxera and Grape Industry Board of South Australia.

CREDI, R. 1997. Characterization of grapevine rugose wood disease sources from Italy. *Plant disease*, 81**,** 1288-1292.

DAANE, K. M., ALMEIDA, R. P., BELL, V. A., WALKER, J. T., BOTTON, M., FALLAHZADEH, M., MANI, M., MIANO, J. L., SFORZA, R. & WALTON, V. M. 2012. Biology and management of mealybugs in vineyards. *Arthropod Management in Vineyards*. Springer, 24-31.

DAL SANTO, S., TORNIELLI, G. B., ZENONI, S., FASOLI, M., FARINA, L., ANESI, A., GUZZO, F., DELLEDONNE, M. & PEZZOTTI, M. 2013. The plasticity of the grapevine berry transcriptome. *Genome biology*, 14**,** 54.

DIAZ-LARA, A., KLAASSEN, V., STEVENS, K., SUDARSHANA, M. R., ROWHANI, A., MAREE, H. J., CHOOI, K. M., BLOUIN, A. G., HABILI, N. & SONG, Y. 2018. Characterization of grapevine leafroll-associated virus 3 genetic variants and application towards RT-qPCR assay design. *Public Library of Science one*, 13**,** e0208862.

DOLJA, V. V., KARASEV, A. V. & KOONIN, E. V. 1994. Molecular biology and evolution of closteroviruses: sophisticated build-up of large RNA genomes. *Annual review of phytopathology*, 32**,** 261-285.

DOLJA, V. V., KREUZE, J. F. & VALKONEN, J. P. 2006. Comparative and functional genomics of closteroviruses. *Virus research*, 117**,** 38-51.

DOUGLAS, N. & KRÜGER, K. 2008. Transmission efficiency of Grapevine leafroll-associated virus 3 (GLRaV-3) by the mealybugs Planococcus ficus and Pseudococcus longispinus (Hemiptera: Pseudococcidae). *European Journal of Plant Patholog*y, 122**,** 207-212.

ENDESHAW, S. T., SABBATINI, P., ROMANAZZI, G., SCHILDER, A. C. & NERI, D. 2014. Effects of grapevine leafroll associated virus 3 infection on growth, leaf gas exchange, yield and basic fruit chemistry of Vitis vinifera L. cv. Cabernet Franc. *Scientia Horticulturae*, 170**,** 228-236.

ENGEL, E. A., GIRARDI, C., ESCOBAR, P. F., ARREDONDO, V., DOMÍNGUEZ, C., PÉREZ-ACLE, T. & VALENZUELA, P. D. 2008. Genome analysis and detection of a Chilean isolate of Grapevine leafroll associated virus-3. *Virus Genes,* 37**,** 110-118.

ENGELBRECHT, D. & KASDORF, G. 1985. Association of a closterovirus with grapevines indexing positive for grapevine leafroll disease and evidence for its natural spread in grapevine. *Phytopathologia mediterranea***,** 101-105.

ENGELBRECHT, D. & KASDORF, G. 1990. Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealybug, Planococcus ficus. *Phytophylactica*, 22**,** 341-346.

ESPINOZA, C., MEDINA, C., SOMERVILLE, S. & ARCE-JOHNSON, P. 2007. Senescence-associated genes induced during compatible viral interactions with grapevine and Arabidopsis. *Journal of Experimental Botany*, 58**,** 3197-3212.

FAORO, F., TORNAGHI, R., FORTUSINI, A. & BELLI, G. 1981. Association of a possible closterovirus with grapevine leafroll in northern Italy. *Rivista di Patologia Vegetale***,** 183-189.

FEI, F., LYU, M.-D., LI, J., FAN, Z.-F. & CHENG, Y.-Q. 2013. Complete nucleotide sequence of a Chinese isolate of Grapevine leafroll-associated virus 3 reveals a 5′ UTR of 802 nucleotides. *Virus genes*, 46**,** 182-185.

FENG, J., ZENG, R. & CHEN, J. 2008. Accurate and efficient data processing for quantitative real-time PCR using a tripartite plant virus as a model. *Biotechniques*, 44**,** 901-912.

FIORE, N., PRODAN, S., MONTEALEGRE, J., ABALLAY, E., PINO, A. & ZAMORANO, A. 2008. Survey of grapevine viruses in Chile. *Journal of Plant Pathology***,** 125-130.

FOËX, G. 1902. *Manual of Modern Viticulture: Reconstitution with American Vines*. R. S. Brain, government printer, Melbourne.

FOURIE, P. & HALLEEN, F. 2004. Occurrence of grapevine trunk disease pathogens in rootstock mother plants in South Africa. *Australasian Plant Pathology*, 33**,** 313-315.

FRAILE, A. & GARCÍA-ARENAL, F. 2010. The coevolution of plants and viruses: resistance and pathogenicity. *Advances in virus research*. Elsevier, 1-32.

FREEBOROUGH, M.-J. & BURGER, J. 2008. *Leafroll: Economic implications*. Wynboer, A technical Guide for Wine Producers, 13-17.

FUCHS, M., MARSELLA-HERRICK, P., LOEB, G., MARTINSON, T. & HOCH, H. 2009a. Diversity of ampeloviruses in mealybug and soft scale vectors and in grapevine hosts from leafroll-affected vineyards. *Phytopathology*, 99**,** 1177-1184.

FUCHS, M., MARTINSON, T., LOEB, G. & HOCH, H. 2009b. Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Disease*, 93**,** 395-401.

GOLINO, D., WEBER, E., SIM, S. & ROWHANI, A. 2008. Leafroll disease is spreading rapidly in a Napa Valley vineyard. *California Agriculture*, 62**,** 156-160.

GRIBAUDO, I., GAMBINO, G., BERTIN, S., BOSCO, D., COTRONEO, A. & MANNINI, F. 2009. Monitoring the spread of viruses after vineyard replanting with heat-treated clones of vitis vinifera 'Nebbiolo'. *Journal of Plant Pathology***,** 741-744.

HABILI, N., FAZELI, C., EWART, A., HAMILTON, R., CIRAMI, R., SALDARELLI, P., MINAFRA, A. & REZAIAN, M. 1995. Natural spread and molecular analysis of grapevine leafroll-associated virus 3 in Australia. *Phytopathology*, 85**,** 1418-1422.

HAO, X. Y., BI, W. L., CUI, Z. H., PAN, C., XU, Y. & WANG, Q. C. 2017. Development, histological observations and Grapevine leafroll-associated virus-3 localisation in in vitro grapevine micrografts. *Annals of Applied Biology*, 170**,** 379-390.

HARRIS, M. 2017. Characterization of viruses associated with Grapevine Leafroll disease in Vitis rootstocks in South Africa. Master of Science in Microbiology. University of Pretoria, Pretoria.

HERRBACH, E., ALLIAUME, A., PRATOR, C., DAANE, K., COOPER, M. & ALMEIDA, R. 2017. Vector transmission of grapevine leafroll-associated viruses. *Grapevine viruses: molecular biology, diagnostics and management*. Springer, Chapter 24.

HERRBACH, E., LE MAGUET, J. & HOMMAY, G. 2013. Virus transmission by mealybugs and soft scales (Hemiptera, Coccoidea*)*. *Vectormediated Transmission of Plant Pathogens.* American Phtyopathological Society Press, USA, Chaper 11.

JARUGULA, S., GOWDA, S., DAWSON, W. O. & NAIDU, R. A. 2010. 3'-coterminal subgenomic RNAs and putative cis-acting elements of Grapevine leafroll-associated virus 3 reveals' unique'features of gene expression strategy in the genus Ampelovirus. *Virology journal*, 7**,** 180.

JOHNSON, N. F. & TRIPLEHORN, C. A. 2004. *Borror and DeLong's Introduction to the Study of Insects*. 7th ed, Brooks/Cole, Belmont, California, USA, 864.

JONES, T. J. 2012. Documentation of grapevine leafroll-associated viruses in wine grape varieties and native grape species in Virginia, and examination of the movement of grapevine leafroll disease to develop management strategies. Master of Science in Life Sciences. Virginia Polytechnic Institute and State University, Virginia.

JOOSTE, A., MAREE, H., BELLSTEDT, D., GOSZCZYNSKI, D., PIETERSEN, G. & BURGER, J. 2010. Three genetic grapevine leafroll-associated virus 3 variants identified from South African vineyards show high variability in their 5′ UTR. *Archives of virology*, 155**,** 1997-2006.

JOOSTE, A. E., MOLENAAR, N., MAREE, H. J., BESTER, R., MOREY, L., DE KOKER, W. C. & BURGER, J. T. 2015. Identification and distribution of multiple virus infections in Grapevine leafroll diseased vineyards. *European journal of plant pathology*, 142**,** 363-375.

JOOSTE, A. E., PIETERSEN, G. & BURGER, J. T. 2011. Distribution of grapevine leafroll associated virus-3 variants in South African vineyards. *European Journal of Plant Pathology*, 131**,** 371-381.

KING, P., MEEKINGS, J. & SMITH, S. 1982. Studies of the resistance of grapes (Vitis spp.) to phylloxera (Daktulosphaira vitifoliae). *New Zealand Journal of Experimental Agriculture*, 10**,** 337-344.

KOMINEK, P. & HOLLEINOVA, V. 2003. Evaluation of sanitary status of grapevines in the Czech Republic. *Plant Soil and Environment,* 49**,** 63-66.

KRAKE, L. R., SCOTT, N. S., REZAIAN, M. & TAYLOR, R. 1999. *Graft-transmitted diseases of grapevines*. CSIRO Publishing, Collington Victoria, Australia.

KRUGER, K. & DOUGLAS-SMIT, N. 2013. Grapevine leafroll-associated virus 3 (GLRaV-3) transmission by three soft scale insect species (Hemiptera: Coccidae) with notes on their biology. *African Entomology*, 21**,** 1-8.

KRÜGER, K., SACCAGGI, D., VAN DER MERWE, M. & KASDORF, G. 2015. Transmission of grapevine leafroll-associated virus 3 (GLRaV-3): Acquisition, inoculation and retention by the mealybugs Planococcus ficus and Pseudococcus longispinus (Hemiptera: Pseudococcidae). *South African Journal of Enology and Viticulture*, 36**,** 223-230.

LE MAGUET, J., BEUVE, M., HERRBACH, E. & LEMAIRE, O. 2012. Transmission of six ampeloviruses and two vitiviruses to grapevine by Phenacoccus aceris. *Phytopathology*, 102**,** 717-723.

LE MAGUET, J., FUCHS, J.-J., CHADŒUF, J., BEUVE, M., HERRBACH, E. & LEMAIRE, O. 2013. The role of the mealybug Phenacoccus aceris in the spread of Grapevine leafroll-associated virus− 1 (GLRaV-1) in two French vineyards. *European journal of plant pathology*, 135**,** 415-427.

LECOQ, H., MOURY, B., DESBIEZ, C., PALLOIX, A. & PITRAT, M. 2004. Durable virus resistance in plants through conventional approaches: a challenge. *Virus research.* Springer,100**,** 31-39.

LEE, J. & MARTIN, R. R. 2009. Influence of grapevine leafroll associated viruses (GLRaV-2 and-3) on the fruit composition of Oregon Vitis vinifera L. cv. Pinot noir: Phenolics. *Food Chemistry*. Elsevier, 112**,** 889-896.

LEGORBURU, F., RECIO, E., LOPEZ, E., BAIGORRI, J., LARREINA, M., REMESAL, A., CIBRIAIN, J., CAMINERO, L., SUBERVIOLA, J. & AGUIRREZÁBAL, F. 2009. Effect of Grapevine leafroll-associated virus 3 (GLRaV-3) on red wine quality. *Proceedings of the 16th congress of ICVG*, 31 August–4 September, 2009, 251.

LEIPOLDT, C. L. 1952. *300 Years of Cape Wine*. Stewart, Cape Town, Chapter 1.

LING, K.-S., ZHU, H.-Y. & GONSALVES, D. 2004. Complete nucleotide sequence and genome organization of Grapevine leafroll-associated virus 3, type member of the genus Ampelovirus. *Journal of General Virology*, 85**,** 2099-2102.

LING, K.-S., ZHU, H.-Y., JIANG, Z.-Y. & GONSALVES, D. 2000. Effective application of DAS-ELISA for detection of grapevine leafroll associated closterovirus-3 using a polyclonal antiserum developed from recombinant coat protein. E*uropean Journal of Plant Pathology*, 106**,** 301-309.

LING, K.-S., ZHU, H.-Y., PETROVIC, N. & GONSALVES, D. 2001. Comparative effectiveness of ELISA and RT-PCR for detecting grapevine leafroll-associated

closterovirus-3 in field samples. *American Journal of Enology and Viticulture*, 52**,** 21-27.

LING, K., ZHU, H., DRONG, R., SLIGHTOM, J., MCFERSON, J. & GONSALVES, D. 1998. Nucleotide sequence of the 3'-terminal two-thirds of the grapevine leafroll-associated virus-3 genome reveals a typical monopartite closterovirus. J*ournal of General Virology*, 79**,** 1299-1307.

LIU, M.-H., LI, M.-J., QI, H.-H., GUO, R., LIU, X.-M., WANG, Q. & CHENG, Y.-Q. 2013. Occurrence of grapevine leafroll-associated viruses in China. *Plant disease*, 97**,** 1339-1345.

LU, R., FOLIMONOV, A., SHINTAKU, M., LI, W.-X., FALK, B. W., DAWSON, W. O. & DING, S.-W. 2004. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. *Proceedings of the National Academy of Sciences*, 101**,** 15742-15747.

MAHFOUDHI, N., DIGIARO, M. & DHOUIBI, M. 2009. Transmission of grapevine leafroll viruses by Planococcus ficus (Hemiptera: Pseudococcidae) and Ceroplastes rusci (Hemiptera: Coccidae). *Plant Disease*, 93**,** 999-1002.

MAHFOUDHI, N., DIGIARO, M. & DHOUIBI, M. H. 2008. Incidence and Distribution of Grapevine Leafroll-associated viruses in Tunisian Vineyards. *Journal of phytopathology*, 156**,** 556-558.

MALAN, A. P. & MEYER, A. 1993. Interaction between a South African population of Xiphinema index and different grapevine rootstocks. *South African Journal of Enology & Viticulture*,14**,** 11-14.

MALAN, S. 2009. Real time PCR as a versatile tool for virus detection and transgenic plant analysis. Master of Science in Genteics. University of Stellenbosch, Stellenbosch.

MAREE, H., FREEBOROUGH, M.-J. & BURGER, J. 2008. Complete nucleotide sequence of a South African isolate of Grapevine leafroll-associated virus 3 reveals a 5′ UTR of 737 nucleotides. *Archives of Virology*, 153**,** 755-757.

MAREE, H. J., ALMEIDA, R. P., BESTER, R., CHOOI, K. M., COHEN, D., DOLJA, V. V., FUCHS, M. F., GOLINO, D. A., JOOSTE, A. E. & MARTELLI, G. P. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology*, 4**,** 82.

MAREE, H. J., PIRIE, M. D., OOSTHUIZEN, K., BESTER, R., REES, D. J. G. & BURGER, J. T. 2015. Phylogenomic analysis reveals deep divergence and recombination in an economically important grapevine virus. *Public Library of Science One*, 10**,** e0126819.

MARTELLI, G. Grapevine virology highlights 2000–2003. *Proceedings of the 14th Meeting of the International Council for the Study of Virus and Virus Diseases of the Grapevine*, Locorotondo, Italy, 2003.

MARTELLI, G. Grapevine virology highlights 2004–2005. *Extended abstracts 15th Meeting of ICVG*, Stellenbosch, South Africa, 200, 13-18.

MARTELLI, G., AGRANOVSKY, A., BAR-JOSEPH, M., BOSCIA, D., CANDRESSE, T., COUTTS, R., DOLJA, V., FALK, B., GONSALVES, D. & JELKMANN, W. 2002. The family Closteroviridae revised. *Archives of virology*, 147**,** 2039-2044.

MARTELLI, G., GHANEM-SABANADZOVIC, N. A., AGRANOVSKY, A., AL RWAHNIH, M., DOLJA, V., DOVAS, C., FUCHS, M., GUGERLI, P., HU, J. & JELKMANN, W. 2012. Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology*, 94**,** 7-19.

MARTELLI, G. P. 2001. Major graft-transmissible diseases of grapevines: nature, diagnosis, and sanitation. *Proceedings of the ASEV 50th Anniversary Annual Meeting*, Seattle, Washington, June 19-23, 2000, 2001. American Society for Enology and Viticulture, ASEV. 231-236.

MARTELLI, G. P. 2014. Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology*, 96**,** 1-136.

MARTIN, R., EASTWELL, K., WAGNER, A., LAMPRECHT, S. & TZANETAKIS, I. 2005. Survey for viruses of grapevine in Oregon and Washington. *Plant Disease*, 89**,** 763-766.

MENG, B., REBELO, A. R. & FISHER, H. 2006. Genetic diversity analyses of grapevine Rupestris stem pitting-associated virus reveal distinct population structures in scion versus rootstock varieties. *Journal of General Virology*, 87**,** 1725-1733.

MILLAR, I. 2002. Mealybug genera (Hemiptera: Pseudococcidae) of South Africa: identification and review. *African Entomology*, 10**,** 185-233.

MOLENAAR, N., ENGELBRECHT, M., MAREE, H. & BURGER, J. 2017. Virus diversity associated with grapevine leafroll disease.WineLand media, viewed 17 June 2019, <https://www.wineland.co.za/virus-diversity-associated-grapevine-leafroll-disease/>

MOURA, C. J. M. D., FAJARDO, T. V. M., EIRAS, M., SILVA, F. N. D. & NICKEL, O. 2018. Molecular characterization of GSyV-1 and GLRaV-3 and prevalence of grapevine viruses in a grape-growing area. *Scientia Agricola*, 75**,** 43-51.

MYLES, S., BOYKO, A. R., OWENS, C. L., BROWN, P. J., GRASSI, F., ARADHYA, M. K., PRINS, B., REYNOLDS, A., CHIA, J.-M. & WARE, D. 2011. Genetic structure and domestication history of the grape. *Proceedings of the National Academy of Sciences*, 108**,** 3530-3535.

NAIDU, R., ROWHANI, A., FUCHS, M., GOLINO, D. & MARTELLI, G. P. 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Disease*, 98**,** 1172-1185.

NAIDU, R. A., MAREE, H. J. & BURGER, J. T. 2015. Grapevine leafroll disease and associated viruses: a unique pathosystem. *Annual Review of Phytopathology*, 53**,** 613-634.

NAMBA, S., YAMASHITA, S., DOI, Y., YORA, K., TERAI, Y. & YANO, R. 1979. Grapevine leafroll virus, a possible member of closteroviruses. *Japanese Journal of Phytopathology*, 45**,** 497-502.

NARAYANASAMY, P. 2008. Molecular techniques for detection of microbial pathogens. *Molecular Biology in Plant Pathogenesis and Disease Management*. Springer, 159-165.

NAVARRO, E., SERRANO-HERAS, G., CASTAÑO, M. & SOLERA, J. 2015. Real-time PCR detection chemistry. *Clinica chimica acta*, 439**,** 231-250.

NEL, A. & ENGELBRECHT, D. 1972. Grapevine virus diseases in South Africa and the influence of latent viruses in the nursery. *Annual Phytopathology n' horsésrie*, 67-74.

NOLASCO, G., SANTOS, C., PETROVIC, N., SANTOS, M. T., CORTEZ, I., FONSECA, F., BOBEN, J., PEREIRA, A. N. & SEQUEIRA, O. 2006. Rupestris stem pitting associated virus isolates are composed by mixtures of genomic variants which share a highly conserved coat protein. *Archives of Virology*, 151**,** 83-96.

OLIVER, J. E. & FUCHS, M. 2011. Tolerance and resistance to viruses and their vectors in Vitis sp.: A virologist's perspective of the literature. *American Journal of Enology and Viticulture*, 62**,** 438-451.

OSMAN, F., LEUTENEGGER, C., GOLINO, D. & ROWHANI, A. 2007. Real-time RT-PCR (TaqMan®) assays for the detection of Grapevine Leafroll associated viruses 1–5 and 9. *Journal of virological methods*, 141**,** 22-29.

OSMAN, F., LEUTENEGGER, C., GOLINO, D. & ROWHANI, A. 2008. Comparison of low-density arrays, RT-PCR and real-time TaqMan® RT-PCR in detection of grapevine viruses. *Journal of Virological Methods*, 149**,** 292-299.

OVER DE LINDEN, A. & CHAMBERLAIN, E. 1970. Effect of grapevine leafroll virus on vine growth and fruit yield and quality. *New Zealand Journal of Agricultural Research*, 13**,** 689-698.

PACIFICO, D., CACIAGLI, P., PALMANO, S., MANNINI, F. & MARZACHÌ, C. 2011. Quantitation of Grapevine leafroll associated virus-1 and-3, Grapevine virus A, Grapevine fanleaf virus and Grapevine fleck virus in field-collected Vitis vinifera L.'Nebbiolo'by real-time reverse transcription-PCR. *Journal of virological methods*, 172**,** 1-7.

PATHIRANA, R. & MCKENZIE, M. J. 2005. A modified green-grafting technique for large-scale virus indexing of grapevine (Vitis vinifera L.). *Scientia horticulturae*, 107**,** 97-102.

PERRONE, I., CHITARRA, W., BOCCACCI, P. & GAMBINO, G. 2017. *Grapevine–virus–environment interactions: an intriguing puzzle to solve*. *New Phytologist*, 213**,** 983-987.

PETERSEN, C. & CHARLES, J. 1997. Transmission of grapevine leafroll-associated closteroviruses by Pseudococcus longispinus and P. calceolariae. *Plant pathology*, 46**,** 509-515.

PIETERSEN, G. 2004. *Spread of grapevine leafroll disease in South Africa-a difficult but not insurmountable problem*. Technical Yearbook 2004/5, WineLand. Viewed: 9 June 2019, <https://www.wineland.co.za/spread-of-grapevine-leafroll-disease-in-south-africa-a-difficult-but-not-insurmountable-problem/ >

PIETERSEN, G. 2006. Spatio-temporal distribution dynamics of grapevine leafroll disease in Western Cape vineyards. *Extended abstracts of the 15th Meeting of the International Council for the Study of Virus and Virus-like diseases of the Grapevine (ICVG)*, 2006. 3-7.

PIETERSEN, G. 2010. *Grapevine leafroll disease control in South Africa*. Wynboer, Viewed: 12 June 2019, < http://www. wineland. co. za/technical/grapevine-leafroll-disease-control-in-southafrica>

PIETERSEN, G. 2017. *Creation of healthy Vitis planting material in the South African wine grape certification scheme*. Istitute for grape and wine sciences, Viewed: 13 June 2019, <http://igws.co.za/article/fact-sheets/leafroll/8-creation-of-healthy-vitis-planting-material-in-the-south-african-wine-grape-certification-scheme>

PIETERSEN, G., SPREETH, N., OOSTHUIZEN, T., VAN RENSBURG, A., VAN RENSBURG, M., LOTTERING, D., ROSSOUW, N. & TOOTH, D. 2013. Control of grapevine leafroll disease spread at a commercial wine estate in South Africa: a case study. *American Journal of Enology and Viticulture*, 64**,** 296-305.

PIETERSEN, G. & WALSH, H. 2012. Development of a LAMP technique for the control of grapevine leafroll associated virus type 3 (GLRaV-3) in infected white cultivar vines by roguing. *Proceedings of the 17th Congress of the International Council for the Study of Virus and Viruslike diseases of the Grapevine (ICVG)*, 2012. 8-11.

PONI, S., GATTI, M., PALLIOTTI, A., DAI, Z., DUCHÊNE, E., TRUONG, T.-T., FERRARA, G., MATARRESE, A. M. S., GALLOTTA, A. & BELLINCONTRO, A. 2018. Grapevine quality: A multiple choice issue. *Scientia horticulturae*, 234**,** 445-462.

PRATOR, C. A., KASHIWAGI, C. M., VONČINA, D. & ALMEIDA, R. P. 2017. Infection and Colonization of Nicotiana benthamiana by Grapevine leafroll-associated virus 3. *Virology*, 510**,** 60-66.

RAST, H., JAMES, D., HABILI, N. & MASRI, S. 2012. Genome organization and characterization of a novel variant of Grapevine leafroll associated virus 3. *Proceedings 17th Congress of ICVG,* 7–14 October, 2012. 61-63.

RAVAZ, L. & ROOS, L. 1905. Le rougeau de la vigne. *Progrés Agric*. Vitic, 22**,** 39-40.

REISCH, B. I., OWENS, C. L. & COUSINS, P. S. 2012. Grape. *Fruit breeding*. Springer, 225-262.

ROWHANI, A. & GOLINO, D. 1995. ELISA test reveals new information about leafroll disease. *California Agriculture*, 49**,** 26-29.

SAAYMAN, D. 2009. Rootstock choice: The South African experience. 6*0th Annual Meeting of the American Society for Enology and Viticulture*, Napa, California, 2009.

SALDARELLI, P., ROWHANI, A., ROUTH, G., MINAFRA, A. & DIGIARO, M. 1998. Use of degenerate primers in a RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero-and vitiviruses of the grapevine. *European Journal of Plant Pathology*, 104**,** 945-950.

SAWIS. 2018. *SA Wine Industry 2018 Statistics* [Online]. Paarl. Viewed: 10 June 2019, <http://www.sawis.co.za/info/download/Book_2018_statistics_year_english_final.pdf>

SCHEU, G. 1935. *Die rollkrankheit des rebstockes*. Der Deutsche Weinbau, 14**,** 222-223.

SEAH, Y. M., SHARMA, A. M., ZHANG, S., ALMEIDA, R. P. & DUFFY, S. 2012. A divergent variant of Grapevine leafroll-associated virus 3 is present in California. *Virology journal*, 9**,** 235.

SETHER, D. & HU, J. 2002. Yield impact and spread of Pineapple mealybug wilt associated virus-2 and mealybug wilt of pineapple in Hawaii. *Plant disease*, 86**,** 867-874.

SHARMA, A. M., WANG, J., DUFFY, S., ZHANG, S., WONG, M. K., RASHED, A., COOPER, M. L., DAANE, K. M. & ALMEIDA, R. P. 2011. Occurrence of grapevine leafroll-associated virus complex in Napa Valley. *Public Library of Science One*, 6**,** e26227.

SKINKIS, P., WALTON, V. M. & KAISER, C. 2009. *Grape phylloxera biology and management in the Pacific Northwest*. Oregon State University. Viewed: 8 June 2019,<https://catalog.extension.oregonstate.edu/sites/catalog/files/project/pdf/ec1463.pdf >

SMITH, E. H. 1992. The grape phylloxera—a celebration of its own. *American Entomologist*, 38**,** 212-221.

THIS, P., LACOMBE, T. & THOMAS, M. R. 2006. Historical origins and genetic diversity of wine grapes. *Trends in Genetics*, 22**,** 511-519.

THOMPSON, B. D., DAHAN, J., LEE, J., MARTIN, R. R. & KARASEV, A. V. 2019. A novel genetic variant of Grapevine leafroll-associated virus-3 (GLRaV-3) from Idaho grapevines. *Plant disease*, 103**,** 509-518.

TÖPFER, R., HAUSMANN, L., HARST, M., MAUL, E., ZYPRIAN, E. & EIBACH, R. 2011. New horizons for grapevine breeding. Methods in temperate fruit breeding. *Fruit, vegetable and cereal science and biotechnology*. Global Science Books, Germany, 5**,** 79-100.

TSAI, C.-W., CHAU, J., FERNANDEZ, L., BOSCO, D., DAANE, K. & ALMEIDA, R. 2008. Transmission of Grapevine leafroll-associated virus 3 by the vine mealybug (Planococcus ficus). *Phytopathology*, 98**,** 1093-1098.

TSAI, C., DAUGHERTY, M. & ALMEIDA, R. 2012. Seasonal dynamics and virus translocation of Grapevine leafroll-associated virus 3 in grapevine cultivars. *Plant Pathology*, 61**,** 977-985.

TURTURO, C., SALDARELLI, P., YAFENG, D., DIGIARO, M., MINAFRA, A., SAVINO, V. & MARTELLI, G. 2005. Genetic variability and population structure of Grapevine leafroll-associated virus 3 isolates. *Journal of General Virology*, 86**,** 217-224.

WALKER, G. & STIRLING, G. 2008. Plant-parasitic nematodes in Australian viticulture: key pests, current management practices and opportunities for future improvements. *Australasian Plant Pathology*, 37**,** 268-278.

WALSH, H. A. & PIETERSEN, G. 2013. Rapid detection of Grapevine leafroll-associated virus type 3 using a reverse transcription loop-mediated amplification method. *Journal of virological methods*, 194**,** 308-316.

WALTON, V. & PRINGLE, K. 2004. A survey of mealybugs and associated natural enemies in vineyards in the Western Cape Province, South Africa. *South African Journal of Enology and Viticulture*, 25**,** 23-25.

WAN, Y., SCHWANINGER, H. R., BALDO, A. M., LABATE, J. A., ZHONG, G.-Y. & SIMON, C. J. 2013. A phylogenetic analysis of the grape genus (Vitis L.) reveals broad reticulation and concurrent diversification during neogene and quaternary climate change. *BMC evolutionary biology*, 13**,** 141.

WANG, J., SHARMA, A. M., DUFFY, S. & ALMEIDA, R. P. 2011. Genetic diversity in the 3′ terminal 4.7-kb region of Grapevine leafroll-associated virus 3. *Phytopathology*, 101**,** 445-450.

WARD, E., FOSTER, S. J., FRAAIJE, B. A. & MCCARTNEY, H. A. 2004. Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology*, 145**,** 1-16.

WOSA. 2019. *Overview*. Cape Town. Viewed: 11 June 2019, <https://www.wosa.co.za/Home/>

Chapter 3

Grapevine leafroll associated virus 3 in rootstocks used in South Africa

1. Abstract

Several viruses, known as the grapevine leafroll associated viruses, are associated with grapevine leafroll disease (GLD). Grapevine leafroll associated virus 3 (GLRaV-3) is the main etiological agent of GLD and in South African vineyards five genetic variants (I, II, III, VI, and VII) have been detected. To control the root louse phylloxera, grapevines (*Vitis vinifera)* are grafted onto rootstocks of different *Vitis* species. Rootstocks infected with GLRaV-3 are asymptomatic and the virus is thought to be poorly detected in infected vines. By conducting a survey comparing the rate of GLRaV-3 detection in infected scion and corresponding rootstock, and through several graft-inoculation trials the study aimed to glean a better understanding of rootstock/GLRaV-3 interactions. Reverse transcription- PCR was utilized for GLRaV-3 status determination in grapevines grown under field and greenhouse conditions. Illumina sequencing was employed to determine GLRaV-3 variants of selected vines. This study found statistically significant differences between the rate of GLRaV-3 detection in scion and corresponding rootstock tissue. No significant difference in the detection rate of GLRaV-3 between top and bottom indicator vines was observed. This suggests that immunity to GLRaV-3 by the intergraft rootstocks could not be demonstrated as virus moved both upward and downward through the intergraft. The possibility of GLRaV-3 moving passively through intergraft phloem tissue to an indicator vine could not be ruled out. Detection of GLRaV-3 between rootstock cultivars varied substantially and a greater overall GLRaV-3 incidence was observed for Paulsen, Richter 110 (R110), and US 8-7 rootstocks. Detection of virus in R110 was suggested to be indicative of a tolerant host defense mechanism, whereas Richter 99 (R99) and 101-14 Mgt were hypothesized to possibly be resistant to single and multiple variant infections, respectively. The dynamics between rootstock and GLRaV-3 require further study to fully understand rootstock host response.

2. Introduction

Grapevines belong to the genus *Vitis* in the family *Vitaceae* and are considered the most widely grown fruit crop across the world (Meng et al., 2006). There are approximately 68 species of *Vitis* (Fortes and Pais, 2016), with the most commercially grown being *Vitis vinifera.* Cultivation of grapevines in South Africa dates back as early as 1688 (Saayman, 2003), but during the late 19th century the destructive aphid, *Dakulosphaira vitifoliae*, family *Phylloxeridae*, commonly known as phylloxera (Smith, 1992), was inadvertently introduced into vineyards worldwide including, South Africa. Phylloxera infests the roots and leaves of grapevines and is cecidogenic (Johnson et al., 2013). Root deformations disrupt water and nutrient uptake, resulting in grape yield reductions and eventual vine death (Granett et al., 2001). Native American *Vitis* species were seen to be naturally resistant to phylloxera and were bred as rootstocks onto which *V. vinifera* cultivars were grafted (Töpfer et al., 2011). This form of biological control was so successful that it has become standard practice in all grape-growing regions where phylloxera is present, including South Africa.

Grapevines are known to host a range of viruses (Martelli, 2017, Perrone et al., 2017) with the most common and persistent being the complex of viruses associated with grapevine leafroll disease (GLD) known as the Grapevine leafroll associated viruses (GLRaVs) which are sequentially numbered in order of discovery. The GLRaVs form part of the *Closteroviridae* family and belong to the genus *Ampelovirus* (Maliogka et al., 2009), with the exception of GLRaV-2 (*Closterovirus*), GLRaV-7 (*Velarivirus*) (Ling et al., 2004) and GLRaV-8, which is no longer recognised as a virus species as it more closely related to the host plant *V. vinifera* (Martelli et al., 2012). Notably, GLRaV-3 has been documented to be the most widespread species in the virus complex (Tsai et al., 2008, Jooste et al., 2011, Almeida et al., 2013, Maree et al., 2013, Walsh and Pietersen, 2013, Thompson et al., 2019).

GLD is a worldwide disease that was first documented in 1936 (Scheu, 1936). The disease results in delayed fruit ripening, decreased Brix and ultimately affects the quality of wine (Over de Linden and Chamberlain, 1970). The virus replicates within the phloem tissue of grapevines, resulting in the disruption of vascular tissue and the reduction of fruit yields with decreased sugar and anthocyanin content (Alabi et al., 2016). Symptoms are usually most obvious in autumn (Naidu et al., 2014) on red

cultivars, which display pronounced leaf reddening with green venation due to an increase in anthocyanin pigments. White cultivars display mild yellowing and infected vines are often not discernible from healthy vines (Naidu et al., 2014). *Vitis* rootstocks infected with the virus are usually asymptomatic except for varying reduction in vigour and are generally poorly detected by ELISA and PCR (Maree *et al*., 2013). This, in turn, complicates the control of GLD in vineyards.

The two main control strategies for GLD are reducing and eliminating leafroll inoculum (GLRaV-3 infected vines) and controlling mealybug incidence and spread (Pietersen, 2010). Thus, the propagation of virus-free material within certification schemes is critical to successful leafroll control. The greatest obstacle in testing rootstock material appears to be low viral titres and erratic distribution of GLRaV-3 (Stewart and Nassuth, 2001, Cid et al., 2003, Maree et al., 2013, Albacete et al., 2015), which could result in the use of asymptomatic carriers of GLD in the establishment of new vineyards.

To successfully control GLRaV-3 in rootstocks within the Wine Grape Certification Scheme, it is imperative to ensure that rootstock material supplied to the industry is free of virus. This study aimed to determine whether the observed poor detection of virus in rootstocks was due to low levels of GLRaV-3 (tolerant host defence mechanism) and, if so, which rootstock cultivars had this property. The study also investigated which, if any, rootstocks were immune (completely lack GLRaV-3 replication) as this would eliminate the need for GLRaV-3 testing before use in the industry.

3. Materials and methods

3.1 Survey to determine GLRaV-3 status of symptomatic leafroll-infected commercial vines and corresponding rootstocks

3.1.1 Plant material and RNA extraction

Vineyard information was obtained from Vititec, Paarl, South Africa, regarding mother blocks no longer utilised due to excessive leafroll infection (an incidence greater than 3%). The growers of these vineyards were contacted, and permission obtained to collect material from these vines. Criteria for sampling of vines were that scions show

leafroll symptoms and that the rootstocks from these have sizeable, lignified sprouts. Basal regions of grapevine stems have been shown to house the highest concentrations of GLRaVs (Monis and Bestwick, 1996). Foliar symptoms are most pronounced during late summer/ early autumn as virus population is believed to increase during this period (Tsai et al., 2012). Sampling was done over two growing seasons namely; 2016/2017 and 2017/2018. These samples represented vines each of Richter 110 (R110), US 8-7, Ruggeri 140, 101-14 Mgt, and Paulsen rootstocks combined with various scions. All vines were tagged with a plant tag, labelled with the appropriate accession number. The differentially corrected latitude/longitude co-ordinate of each was determined to facilitate returning to them (Appendix: Table 1 and Table 2).

For each vine, lignified rootstock suckers were processed by removing outer bark and preparing phloem shavings whereas petioles were cut into small fragments, weighed and homogenized in liquid nitrogen using a mortar and pestle. All plant material was stored at 4°C until utilised for RNA extraction. Total RNA was extracted from 200 mg of phloem material from each sample using a modified cetyltrimethylammonium-bromide-based (CTAB) (2% CTAB, 2.5% PVP-40, 100 mM Tris-HCI pH 8.0, and 3% β-mercaptoethanol) RNA extraction protocol (White et al., 2008).

3.1.2 RNA quality assessment

The quality of total RNA extracted from scion and rootstock samples collected in 2017 was assessed using a NanoDrop 200 Spectrophotometer (Thermo Fisher, Waltham, MA, USA).

3.1.3 Housekeeping gene PCR

To validate the quality of RNA prior to detection of GLRaV-3 by conventional RT-PCR and to prevent false negatives the housekeeping gene, Actin, which is stably expressed in grapevine plants, was used as an internal control. The intron encompassing primer pair used was designed by van der Berg, University of Pretoria (Pers. Comm.) (Table 1). The expression of the Actin gene, as a proxy for RNA, was evaluated in four different rootstock samples as well as in a single healthy *V. vinifera*

control. A 1% (w/v) agarose gel electrophoresis was performed to detect the presence of the 170 bp amplicon.

3.1.4 Complementary DNA synthesis

For both the Actin and the GLRaV-3 specific PCRs, reverse transcription was first accomplished by performing a primer annealing step on the extracted RNA in a total reaction volume of 5 µL containing: 0.5 µM reverse primer (Table 1) (Goszczynski, 2013), 2 µL of RNA, and 2.3 µL nuclease-free water (Thermo Fisher). The reaction mixture was briefly centrifuged and incubated at 70°C for five minutes, followed by cooling at 4°C for five minutes. Reverse transcription was achieved by adding 1x Moloney-murine leukemia virus (M-MLV) reaction buffer, 0.8 mM dNTP mix (Roche, Basel, Switzerland), 8 U RiboLock™ (Thermo Fisher), 120 U M-MLV RT (Promega, Madison, WI, USA), and nuclease-free water (Thermo Fisher) to a final reaction volume of 15 µL. The reaction mixture was incubated at 42°C for 60 minutes. A negative control was included where 5 µL of RNA was replaced by 5 µL of nuclease-free water (Thermo Fisher) and a positive GLRaV-3 control, accession number 15-5080 (82.4 ng/µL).

3.1.5 Polymerase chain reaction amplification

For the Actin and GLRaV-3 helicase gene specific PCRs, the primers listed in Table 1 were used to carry out a conventional PCR assay. Briefly, amplification of first strand cDNA was performed using 2.5 µL cDNA in a final reaction volume of 25 µL consisting of: 1x NH4 BioTaq reaction buffer, 4 mM $MgCl_2$, 0.4 mM dNTPs (Roche), 0.2 µM forward primer, 0.2 µM reverse primer (Table 1) (Goszczynski, 2013), and 1.25 U BioTaq Polymerase (Bioline, London, England). The PCR thermocycling conditions were as follows: denaturation cycle for five minutes at 95°C, 40 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for one minute. One cycle for extension occurred at 72°C for 10 minutes. A negative control was included where 2.5 µL of cDNA was replaced with 2.5 µL of nuclease-free water (Thermo Fisher). The PCR products were held at 4°C. A 2% (w/v) agarose gel electrophoresis was performed to detect the presence of the 549 bp amplicon.

Table 1: Oligonucleotide primers for Grapevine leafroll associated virus 3 detection and the housekeeping host gene, Actin

Target	Primer	Sequence 5'-3'	Product size(bp)	Annealing temp (°C)	Reference
GLRaV-3	Hel2F	GGCGAAGAGTATTCGCTC	549	52	(Gozsczynski., 2013)
	Hel2R	CCAGAAAAGGCCTTCGTC			
Actin	ActinF	ACCGAAGCCCCTCTTAACCC	170	55	(unpublished)
	ActinR	GTATGGCTGACACCATCACC			

3.1.6 Statistical analysis

Significant differences in detection of GLRaV-3 in rootstock and scion material were analysed using the two-proportion hypothesis *z*-test at a 95% confidence interval.

3.2 Determining tolerance/immunity of commercially important rootstock clones

During 2017, in order to obtain single variant GLRaV-3 infected sources, 20 vines (lignified scion canes) were collected from Vergelegen Wine Estate (Somerset West, South Africa) vineyards in which leafroll incidence was less than 0.05%. These vines were selected as they were representative of primary leafroll infections (possibly through long distance dissemination of mealybugs) and had a high likelihood of having had an infection initiated by a single mealybug and hence, being pure sources of a GLRaV-3 variant.

Lignified canes were processed by removing the outer bark and exposing phloem material. This allowed for 200 mg of phloem shavings to be scraped and collected using a sharp blade. RNA was extracted using the modified CTAB (2% CTAB, 2.5% PVP-40, 100 mM Tris-HCI pH 8.0, and 3% β-mercaptoethanol) RNA extraction protocol (White et al., 2008). Samples were tested for GLRaV-3 using PCR directed against the GLRaV-3 helicase gene (refer to sections 3.1.4 and 3.1.5). Samples that tested positive for GLRaV-3, with the highest concentration of amplicon, and with the

most collected cane material (accession numbers: 17-7082, 17-7084, and 17-7093) were analysed for GLRaV-3 homogeneity using Illumina sequencing of the PCR amplicon.

3.2.1 Illumina MiSeq Sequencing

All three samples (from section 3.2) were subjected to column purification (NucleoSpin® Gel and PCR cleanup, Macherey-Nagel) to obtain the required concentrations for Illumina MiSeq submission. For each sample, 450 µL of PCR product was eluted in 30 µL of elution buffer. The concentration of the purified amplicons was determined using the NanoDrop 200 spectrophotometer (Thermo Fisher). The three samples were submitted for Next-Generation Sequencing, using the MiSeq platform at the Agricultural Research Council (ARC), Biotechnology Platform, Pretoria, South Africa.

3.2.2 MiSeq data analysis

CLC Genomics Workbench 6 (Aarhus) was used to carry out trimming and analyses of the three Illumina MiSeq datasets. Data were imported as paired end reads and adapter and quality trimming were performed using default program settings with Nextera V2 transposase adapter sequences
(Transposase 1: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG; Transposase 2: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG). Thereafter, quality control was performed, and each sample reference mapped to the cognate region that Hel2F/Hel2R amplified of GLRaV-3 variant group representatives (Table 2). The parameters used for reference mapping were: 0.9 similarity fraction, 0.9 length fraction, and the use of the ‘ignore’ function where reads capable of multiple mapping were regarded as unmapped.

Table 2: Grapevine leafroll associated virus 3 variant group representatives

Group	Isolate	GenBank accession	Reference
I	NY-1	AF037268.2	(Ling et al., 2004)
	621	GQ352631.1	(Jooste et al., 2010)
	CL-766	EU344893.1	(Engel et al., 2008)
II	623	GQ352632.1	(Jooste et al., 2010)
	GP18	EU259806.1	(Maree et al., 2008)
III	PL-20	GQ352633.1	(Jooste et al., 2010)
VI	GH30	JQ655296.1	(Bester et al., 2012)
VII	GH24	KM058745.1	(Maree et al., 2015)

3.2.3 Graft-inoculations

Vines of healthy Cabernet franc scions grafted onto healthy commonly used rootstock cultivars (R110, R99, 101-14 Mgt, and Ramsey) were obtained from Vititec. These were shipped to the University of Pretoria and planted out in sterilized soil in pots in the insect-free greenhouse at the University of Pretoria Experimental Farm, Proefplaas.

Nine individual canes from the three pure sources of GLRaV-3 variant samples were planted in pots in the adjacent closed-off section of the greenhouse and left to develop roots for four months.

The rooted GLRaV-3 infected scions were then used to graft-inoculate replicates of the rootstock portion of the above-mentioned healthy C. franc/rootstock combinations (for each combination a healthy control was retained). The C. franc indicator scions were monitored for leafroll symptoms over nine months after which total RNA was extracted from all the C. franc indicator vines using the modified CTAB (2% CTAB, 2.5% PVP-40, 100 mM Tris-HCI pH 8.0, and 3% β-mercaptoethanol) RNA extraction protocol (White et al., 2008). The samples were tested for GLRaV-3 infection using conventional RT-PCR directed against the helicase gene (refer to sections 3.1.4 and 3.1.5).

3.3 Determining if transmission of virus was possible through the phloem of putatively immune rootstocks

Three iterations of a graft transmission experiment were conducted at Vititec in 2018. The first involved the use of the nine rooted canes infected with pure sources of GLRaV-3 (from section 3.2). These vines were transported to Vititec where C. franc grafted onto Richter 99 (RY), Richter 110 (RQ),101-14 Mgt (AA), US 8-7(UC), and a single C. franc control were further grafted onto these rooted GLRaV-3 infected sources (scion material on own roots) to form intergrafts (Table 3). Figure 1 under the Appendix is representative of a typical intergraft.

In a second iteration of the graft transmission experiment, C. franc scions were grafted onto a subset of rootstock samples (previously collected May 2017/2018 that had either tested positive or negative for GLRaV-3) where enough material was available (Accessions that had previously tested negative for GLRaV-3: 17-7047, 17-7049, 17-7075, 17-7101, 18-4005, 18-4007, 18-4035, and 18-4037) and (Accessions that had previously tested positive for GLRaV-3: 18-4011, 18-4015, 18-4017, and 18-4019).

Table 3: Graft-inoculations, creating rootstock variety intergrafts using 2017 rooted Grapevine leafroll associated virus 3 infected plant material

Scion	Intergraft	GLRaV-3 infected rootstock	Replicates
CF	RY	17-7093	2
CF	RQ	17-7093	2
CF	AA	17-7082	2
CF	UC	17-7082	2
CF	CF	17-7084	1

CF=Cabernet franc, RY=Richter 99, RQ=Richter 110, AA=101-14 Mgt, and UC=US 8-7

In the third iteration of the above experiments, pure GLRaV-3 from four singly occurring leafroll infected vines within vineyards of more than 3000 vines each were identified at Vergelegen Wine Estate in 2018. All the cane material of these vines were collected to be used later as GLRaV-3 inoculum sources. Total RNA was extracted

from all four samples and submitted to Rachel Bester, University of Stellenbosch, to determine the GLRaV-3 status of the vines using real-time PCR. Samples contained GLRaV-3 variants II, III, and VI (Vine 18-0061), II (Vine 18-0062), II and III (Vine 18-0063) or II (Vine 18-0064). Samples were also tested for the presence of Viti- and Foveaviruses using a nested reverse transcription-PCR (Dovas and Katis, 2003) (Natalie Nel, Stellenbosch University Honours student). Samples 18-0061, 18-0062, and 18-0063 all contained a Viti- or Foveavirus in addition to GLRaV-3, these were identified by Sanger sequencing as being isolates of GVA in the case of 18-0061 and 18-0063, with 18-0062 probably having a mixture of Viti- or Foveaviruses. Only sample 18-0064 appeared to contain only GLRaV-3. Samples 18-0061 and 18-0064 as GLRaV-3 sources were used by Mr. Dirk Visser (Vititec) for the creation of further intergrafts at Vititec (Table 9). These grafts were monitored for callusing and rooting and planted out in mistbeds with high humidity. The vines were treated with imidachlorpid and maintained under insect-free conditions at Stellenbosch University. The C. franc components were monitored for symptoms and sent to the University of Pretoria where RNA was extracted (White et al., 2008) and GLRaV-3 testing took place (refer to section 3.1.4 and 3.1.5).

4. Results

4.1 Survey to determine GLRaV-3 status of symptomatic leafroll-infected commercial vines and corresponding rootstocks

A total of 42 and 18 vines samples (rootstock and corresponding scion from the same vine) were collected in May 2017 and May 2018, respectively (Appendix: Table 1 and Table 2), and the number of each combination is provided in Table 4. The rootstocks collected represent those most often used in the South African wine industry and included R110, US 8-7, Ruggeri 140, 101-14 Mgt, and Paulsen 1103. R99 was excluded as this rootstock had been assessed in a previous study (Harris, 2017). Sampling criteria required scions to have clear leafroll symptoms and sizeable rootstock suckers growing from the stems (Figure 1). However, diligent wine farmers usually prune rootstocks, thus locating vines of any description (even small unlignified suckers) was challenging.

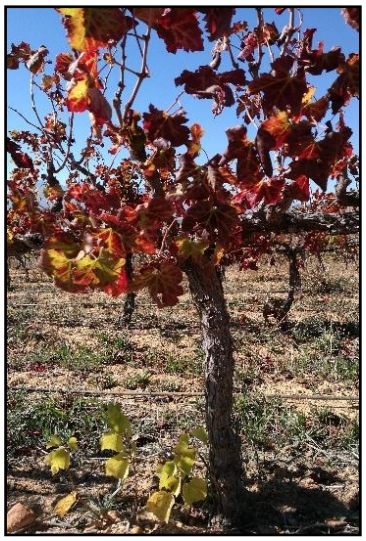


Figure 1: A grapevine that meets the sampling criteria, where the scion displays clear leafroll symptoms and has sizeable, lignified rootstock suckers growing.

When assessing RNA quality, rootstock tissue was generally seen to have higher concentrations of total RNA (average of 45.18±74.92 ng/uL) compared to scion tissue (average of 24.46±65.40 ng/uL) of any given combination. However, RNA concentrations for both tissue types were relatively low. The A260/280 ratios for both scion and rootstock tissue were found to be acceptable, averaging around 2.09 for rootstock tissue and 2.08 for scion. The A260/230 ratios for both scion and rootstock were slightly lower than the recommended range of 2.0-2.2, indicating the presence of contaminants which absorbed at 230 nm. The average A260/230 ratio for rootstock material was 1.28 and for scion 1.27. Detailed information on the NanoDrop readings can be found in Table 3 under the Appendix.

Four representative grapevine rootstocks were selected, namely: R110 (Accession: 17-7003), US 8-7 (Accession: 17-7007), 101-14 Mgt (Accession: 17-7043), and Ruggeri 140 (Accession: 17-7023) and screened against the housekeeping Actin gene using conventional RT-PCR (Figure 2). These accessions were representative of both the higher and lower ends of the total RNA concentrations obtained from rootstocks (64.2 ng/uL, 50.5 ng/uL, 15.3 ng/uL, and 7.1 ng/uL, respectively).

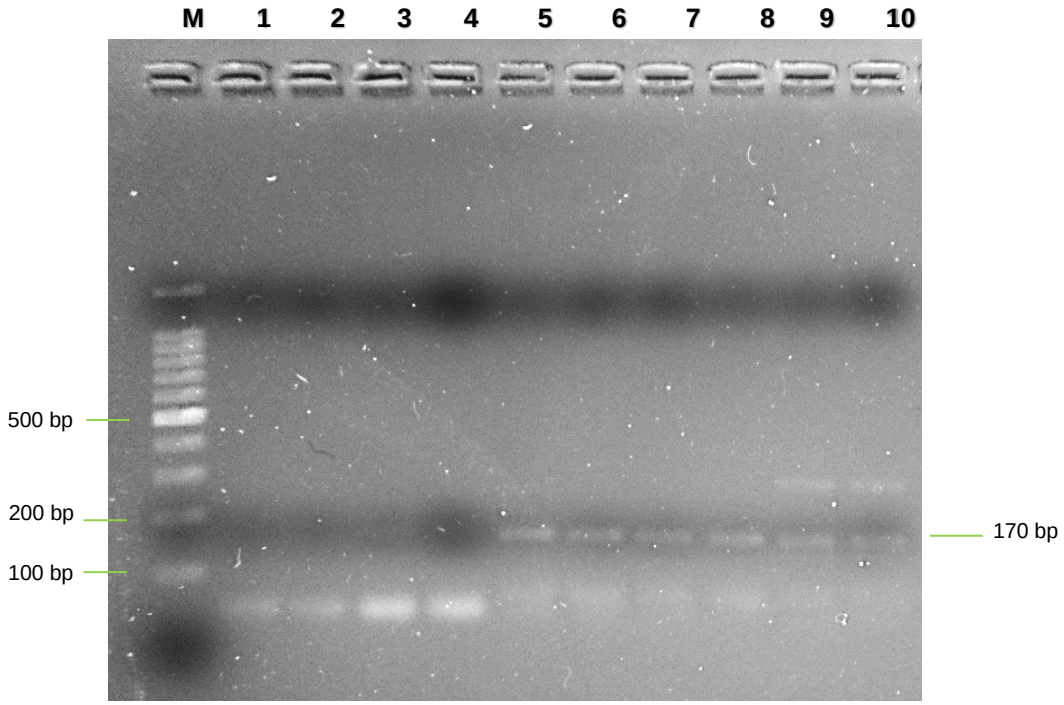


Figure 2: A 1% (w/v) agarose gel electrophoresis following Actin housekeeping plant host gene RT-PCR of scion and rootstock tissue. Lanes: (M) 100 bp DNA Ladder (0.13 µg/µL) (Promega), (1) cDNA synthesis negative control 1, (2) cDNA synthesis negative control 2, (3) PCR negative control 1, (4) PCR negative control 2, (5) Richter 110 (17-7003), (6) US 8-7 (17-7007), (7) Ruggeri 140 (17-7023), (8) 101-14 Mgt (17-7043), (9) healthy *V. vinifera* control 1, (10) GLRaV-3 positive *V. vinifera* control 2.

All four rootstock types yielded clear bands indicative of intact Actin RNA, as shown by the 170 bp amplicon product observed on the agarose gel electrophoresis image depicted in Figure 2, indicating successful total RNA extractions.

A total of 60 vines were tested for GLRaV-3 with majority of the rootstocks from these vines having had a negative GLRaV-3 status compared to corresponding scion. GLRaV-3 was detected in nine out of 60 (15%) rootstock samples and in 50 out of 60 (83%) scions. When positive, rootstock samples yielded lower amplicon concentrations than corresponding scions, despite the lower total RNA concentrations of scion used as templates in the PCRs. This would suggest that the concentration of initial RNA template used was of little importance.

Figure 2 in the Appendix is representative of a typical GLRaV-3 assay agarose gel electrophoresis image indicating the differences in rootstock and scion amplicon band strengths. Eight out of 60 (13%) samples tested positive for GLRaV-3 in both

rootstocks and scions and included one R110, one US 8-7, and six Paulsen rootstocks (Figure 3). The detection rate of GLRaV-3 differed significantly (z=7.307, p<0.05) between scion and respective rootstocks (Figure 2).

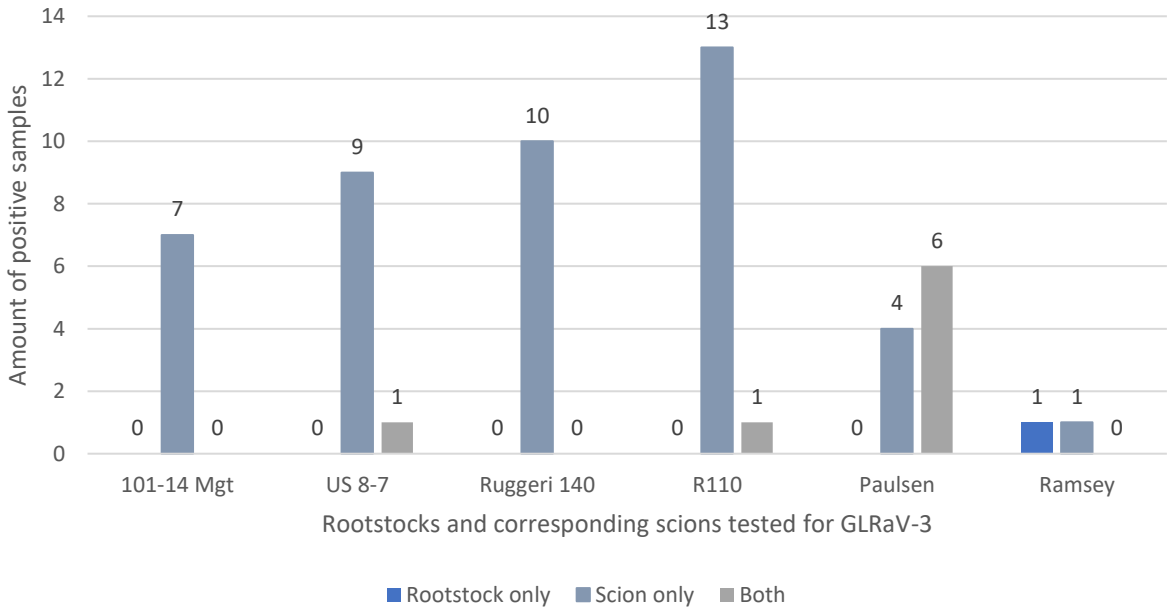


Figure 3: Graph indicating Grapevine leafroll associated virus 3 positives found in scion and rootstock tissues. The different colour bars represent the tissues in which the positives were found per rootstock type.

Table 4: Grapevine leafroll associated virus 3 positive samples per rootstock/scion combination

Rootstock/scion combination		Roostocks GLRaV-3 positive	Scions GLRaV-3 positive	N
Rootstock	Scion			
101-14 Mgt	Merlot	0	7	10
US 8-7	Touriga Nacional (TN) 1A	1	10	14
Ruggeri 140	Merlot	0	10	10
R110	Merlot	1	4	5
R110	Cabernet Sauvignon	0	3	3
R110	Shiraz	0	2	2
R110	Touriga Nacional (TN) 1A	0	2	4
Paulsen	Merlot	3	7	7
Paulsen	Ruby Cabernet	3	3	3
Ramsey	Crimson	1	1	2
Total		9	49	60

N=Total amount of vines tested

The GLRaV-3 status for both US 8-7 and R110 was one out of 14 (7%), Paulsen had six out of ten (60%) positives, Ramsey had one out of two (50%), and both Ruggeri 140 and 101-14 Mgt had zero out of ten (0%) (Table 4). In one instance, a rootstock (Ramsey) tested positive for GLRaV-3 and the corresponding scion negative (Figure 3). Detailed information on GLRaV-3 presence, absence, and amplicon band strength can be found in Table 1 under the Appendix.

4.2 Determining tolerance/immunity of commercially important rootstock clones

All 20 vines collected from Vergelegen in 2017 tested positive for GLRaV-3. Three samples with clear amplicon bands and from which the most cane material was available were selected and prepared for Illumina MiSeq sequencing. These were: 17-7082 (232 ng/µL), 17-7084 (342 ng/µL), and 17-7093 (263 ng/µL). The samples submitted for Illumina MiSeq analysis yielded a maximum and minimum of 2 061 000 and 949 000 reads, respectively, and after trimming had average lengths of 206 bases.

As seen in Table 5 the vast majority of reads of all three sources mapped only to GLRaV-3 variant VI.

Table 5: Percentage reads mapped of various Grapevine leafroll associated virus 3 variant groups of three scion samples

Accession	Scion	GLRaV-3 variant %				
		Group I	Group II	Group III	Group VI	Group VII
17-7082	Cabernet Sauvignon	0	0	0	100	0
17-7084	Cabernet Sauvignon	0	1	0	99	0
17-7093	Cabernet Sauvignon	0	9	0	91	0

Majority of the grafted vines supplied by Vititec died due to an irrigation system failure. The rootstock portion of the remaining scion/rootstock combinations were graft-inoculated with the GLaV-3 variant samples and included six of 101-14 Mgt combinations, seven of R99, four of Ramsey, and 11 of R110. Vines were monitored for symptoms over nine months with initial symptoms appearing at three months (Figure 4). Vines were tested for GLRaV-3 after 9 months (Table 6). A detailed layout of the inoculated vines can be seen in the Appendix under Table 4, together with an agarose electrophoresis gel image of the tested samples (Appendix: Figure 3).

Figure 4: Image depicting the interveinal reddening and downward rolling of leaf margins observed on scions three months post graft-inoculation of the rootstock component of scion/rootstock combinations.

Table 6: Grapevine leafroll associated virus 3 positive scion material post graft-inoculation of the rootstock component of any given scion/rootstock combination infected with Grapevine leafroll associated virus 3 variant sources

Scion/rootstock combination		Number or rootstocks infected with each GLRaV-3 source						Scion GLRaV-3 positive	N
Scion	Rootstock	17-7082		17-7084		17-7093			
		N	(+)	N	(+)	N	(+)		
Cabernet franc	R110	3	3	4	4	4	2	9	11
Cabernet franc	101-14 Mgt	2	1	2	1	2	1	3	6
Cabernet franc	R99	3	0	2	2	2	2	4	7
Cabernet franc	Ramsey	2	2	1	1	1	1	4	4
Total		10	6	9	8	9	6	20	28

Thirty-two grapevine scions (including one healthy control per scion/rootstock combination) were tested for GLRaV-3, with majority of the scions testing positive, i.e. 20 out of the 28 (71%) graft-inoculated samples (Table 6). The GLRaV-3 status for C. franc grafted onto R110 was nine out of 11 (82%), C. franc/101-14 Mgt had three out of six (50%) positives, C. franc/R99 had four out of seven (57%) and, C. franc/Ramsey had four out of four (100%) positives. The four healthy controls were confirmed to be virus-free.

Six out of ten (60%) vines graft-inoculated with 17-7082 (variant VI) virus source were positive for GLRaV-3, eight out of nine (89%) vines graft-inoculated with 17-7084 (variants VI and II) virus source were positive, and six out of nine (67%) graft-inoculated with 17-7093 (variants VI and II) virus source were positive (Table 6).

4.3 Determining if transmission of virus was possible through the phloem of putatively immune rootstocks

During the 2018 graft experiments, C. franc grafted onto R99, R110, 101-14 Mgt, US 8-7, and a single C. franc control were further grafted onto the rooted GLRaV-3 infected pure sources (scion material on own roots) to form intergrafts (Table 6 and Table 7). Unfortunately, five out of the original nine grafts did not take and in the end only four vines could be tested for GLRaV-3. Overall, one out of the four indicators scions (25%) tested positive for the virus, this was a C. franc scion grafted onto R99 with one out of two (50%) positives. The C. franc indicators grafted onto US 8-7 and R110 both tested negative for GLRaV-3. The small sample size did not provide enough evidence to suggest that detection of GLRaV-3 differed amongst these three rootstock types.

Table 7: Grapevine leafroll associated virus 3 positive scion samples per graft-inoculation intergraft using 2017 rooted Grapevine leafroll associated virus 3 infected plant material

Rootstock-intergraft-scion combination				
Rootstock	Intergraft	Scion	Scion GLRaV-3 positive	N
17-7093	R99	Cabernet franc	1	2
17-7093	R110	Cabernet franc	0	1
17-7082	US 8-7	Cabernet franc	0	1
Total			1	4

N=Total amount of vines tested

In the second iteration of the experiment, C. franc scions were grafted onto rootstock material collected in 2017/2018 where sufficient material was left after RNA extraction. The rootstocks collected in 2017 had all previously tested negative for GLRaV-3 (when subjected to PCR, as previously mentioned) and in order to confirm these results plant material was transported to Vititec for grafting. Unfortunately, these grafts were unsuccessful and only one vine, C. franc grafted onto Ramsey (Accession: 17-7101), took. The GLRaV-3 specific PCR revealed that the C. franc indicator vine was negative for the virus following grafting. The C. franc/rootstocks collected in 2018 grafts were successful (Table 8). The R110 rootstocks previously tested negative for GLRaV-3 (when subjected to PCR, as previously mentioned), however, 3 out of 4 (75%) of the C. franc scions grafted onto these rootstocks tested positive for GLRaV-3 (Table 8). C. franc scions grafted onto GLRaV-3 negative Paulsen 1103 rootstocks had 1 out of 3 (33%) GLRaV-3 positives. This would suggest that either the RT-PCR employed was not sensitive enough to detect the virus in these rootstock samples or that the rootstocks themselves have a defence mechanism against the virus, which resulted in decreased virus populations that were low enough to go undetected by conventional PCR but high enough to be transmitted to grafted scion material.

Table 8: Grapevine leafroll associated virus 3 positive scion indicator samples per graft using Paulsen and Richter 110 rootstocks collected in 2018. Scions were tested 11 months post graft-inoculation.

Rootstock	Scion	C. franc GLRaV-3 positive	N
Paulsen (18-4005, 18-4007) (-)	Cabernet franc	1	3
Paulsen (18-4011, 18-4015, 18-4017, and 18-4019) (+)		5	8
R110 (18-4035) (-)		0	1
R110 (18-4037) (-)		3	3
Cabernet franc GLRaV-3 positive control		2	2

(-)=GLRaV-3 negative; (+)=GLRaV-3 positive, N=Total amount of vines tested

The table below gives an overview of the results pertaining to the third iteration of the graft transmission experiment where various intergrafts were done.

Table 9: Grapevine leafroll associated virus 3 positive scion samples per graft-inoculation intergraft using 2018 collected Grapevine leafroll associated virus 3 infected cane material

Rootstock-intergraft-scion combination				
Rootstock	Intergraft	Scion	C. franc GLRaV-3 positive	N
GLRaV-3 var II, III, VI, and Viti/Foveavirus (18-0061)	R110	Cabernet franc	2	3
	R99		0	1
	Salt Creek		3	3
	Us 8-7		3	4
	Paulsen		3	3
	Cabernet franc		3	4
GLRaV-3 var II virus (18-0064)	101-14 Mgt	Cabernet franc	5	5
	R110		4	5
	R99		3	4
	Salt Creek		3	5
	US 8-7		4	5
	Paulsen		1	4
	Cabernet franc		1	5
101-14 Mgt	None (healthy controls)	Cabernet franc	0	2
R110			0	1
R99			0	2
Salt Creek			0	2
US 8-7			0	1
Paulsen			0	2
Cabernet franc			0	2
Cabernet franc	101-14 Mgt	GLRaV-3 var II, III, VI, and Viti/Foveavirus (18-0061)	1	5
	R110		2	3
	R99		3	3
	Salt Creek		5	5
	US 8-7		2	3
	Paulsen		3	3
	Cabernet franc		1	2
Cabernet franc	101-14 Mgt	GLRaV-3 var II virus (18-0064)	1	1
	R110		1	3
	R99		0	2
	US 8-7		2	2
	Cabernet franc		1	3

N=Total amount of vines tested

Eleven months post graft-inoculation grapevine leafroll associated virus 3 detection was compared between C. franc indicator vines grafted onto different rootstock types further grafted onto either a single or multiple GLRaV-3 variant group source. Irrespective of whether C. franc indicator vine was the top or bottom (rooted portion) of the graft combination with an intergrafted 101-14 Mgt rootstock further grafted with a single variant source, there was a 100% detection rate. Whereas only 20% of the rooted indicator vines intergrafted with 101-14 Mgt further grafted with a multiple variant source were seen to be positive. Rootstock 101-14 Mgt appeared to have no resistance to a single variant infection, namely GLRaV-3 group II, but exhibited some resistance in the presence of a multiple variant infection. For R110, irrespective of whether C. franc indicator vine was the top or bottom (rooted portion) of the graft combination, there was a 67% detection rate when the virus source was GLRaV-3 (multiple variant group). Rootstock 99 produced interesting results in that the detection rate of GLRaV-3 was dependent on the position of the C. franc indicator vine (top versus rooted) and was in contradiction between the single and multiple variant groups. For Salt Creek/Ramsey and Paulsen rootstock intergrafts the position of C.franc was irrelevant as a 100% detection rate was seen for GLRaV-3 multiple variant source. Salt Creek/Ramsey and Paulsen intergrafts with a single infection behaved differently. This indicated that these rootstocks were either susceptible to the virus infection, replication and subsequent transmission, or GLRaV-3 passively moved through phloem tissue from the intergraft rootstock into the indicator portion of the vine. As expected, C. franc with a US 8-7 rootstock intergraft grafted with a single and a multiple GLRaV-3 source resulted in a high (80% and 75%) detection rate, respectively. The 12 C. franc scions grafted onto the different rootstock cultivars that were not grafted with virus sources (healthy controls) retained their GLRaV-3 free status.

A total of 51 C. franc indicator scions and 35 rooted C. franc indicator vines were tested for GLRaV-3 with majority of the samples having had a positive GLRaV-3 status. GLRaV-3 was detected in 35 out of 51 (69%) C. franc scions and in 22 out of 35 (63%) rooted C. franc vines (Table 9). There was, however, not enough evidence to suggest that the detection rate of GLRaV-3 differed significantly (z=0.5561, p>0.05) between C. franc scions and rooted C. franc vines and hence rootstock immunity could not be

established. The virus was able to move both upward and downward through the intergraft. An expanded view of the intergrafts, GLRaV-3 presence, absence, and amplicon concentrations can be seen under the Appendix: Table 5, together with the GLRaV-3 assay agarose gel electrophoresis images (Appendix: Figure 4-6).

5. Discussion

The GLRaV-3 survey conducted in this study provided significant evidence to conclude that GLRaV-3 is more readily detected in scion than in corresponding rootstock tissue of the same vine (Table 4). The poor detection of GLRaV-3 in rootstocks tested in this study support the study of Harris (2017) who found 88 out of 95 (93%) scion samples to be positive for GLRaV-3 compared to 41 out of 95 (43%) corresponding rootstocks (Harris, 2017). Chooi et al. (2016) did a study on the titre and distribution of GLRaV-3 variants in selected rootstocks and found that 15 out of the 32 (47%) rootstocks tested, namely, 3309C (*V. riparia* x *V. rupestris*) and Schwarzmann (*V. riparia* x *V. rupestris*) showed inconsistent GLRaV-3 detection. Studies over the years have observed low viral titres and erratic distribution of GLRaV-3 in rootstocks (Rowhani and Golino, 1995, Monis and Bestwick, 1996, Cid et al., 2003, Cohen et al., 2003, Tsai et al., 2012, Chooi et al., 2016). Monis and Bestwick (1996), using serological methods, and Tsai et al. (2012), using RT-qPCR, performed studies on *V. vinifera* and found that erratic distribution of GLRaV-3 was not limited to rootstocks but also occurred in *V. vinifera* (Monis and Bestwick, 1996, Tsai et al., 2012). The erratic distribution and low virus titres could account for the discrepancies seen in GLRaV-3 detection between scion and rootstock.

It was also evident that the detection of GLRaV-3 in the sampled rootstock types differed from cultivar to cultivar. It should also be noted that the results are related to a specific tissue analysed in a certain period of the year. Paulsen rootstock had a higher detection rate than any other rootstock, with 60% positives, followed by Ramsey (50%), and then R110 and US 8-7 each with a 7% detection rate. The sample size for Ramsey, however, was too small to draw conclusions and would require further samples to be tested. The rate of detection of GLRaV-3 in Paulsen was similar to that found by Harris (2017), who observed Paulsen rootstock to have the highest detection rate with two out of two (100%) positives. The detection rate observed in R110 and 101-14 Mgt rootstocks were dissimilar to those found by Walsh and Pietersen (2013)

who observed a 67% and 71% detection rate for R110 and 101-14 Mgt, respectively. That being said, these two rootstock cultivars had the lowest detection rate out of the five rootstocks tested in the study. It should also be noted that rootstocks were subjected to testing using RT-LAMP, which has a higher sensitivity compared to RT-PCR (Walsh and Pietersen, 2013). Rootstock R99 was not selected for during this study as Harris (2017) did extensive sampling and testing of R99 in 2017 and found 28 out of 76 (39%) positives. Harris (2017) also confirmed that the differences in detection between scion and corresponding rootstock were not due to the presence of PCR inhibitory substances in the selected rootstocks, as Vitiviruses were easily detected in the same rootstocks using PCR.

The virus status of all plant samples was determined using an end-point GLRaV-3 RT-PCR assay utilising primers that targeted the helicase gene, which is highly conserved amongst GLRaV-3 variants and amplifies a sequence that varies amongst the GLRaV-3 variants. This allowed for the specific GLRaV-3 variants present in the three scion samples selected to be determined following Illumina sequencing. The most abundant GLRaV-3 variants found in all three scion samples were group VI and to a much lesser extent group II (Table 5). This was in agreement with Jooste et al. (2015) and Diaz-Lara et al. (2018) where group II and group VI were predominantly found in scions in South Africa (Jooste et al., 2010, Diaz-Lara et al., 2018). In the scion samples tested by Harris (2017), group I followed by group VI were found to be the most prevalent.

Screening of the C. franc indicator component of the rootstock graft-inoculated greenhouse plants revealed that the majority (71%) of the vines tested positive for GLRaV-3, suggesting efficient infection and replication of the virus in the rootstock cultivars. The vines were only screened at a single time-point (nine months post-inoculation), whereas future studies should include multiple screening time-points to monitor and compare rootstock responses to different GLRaV-3 variant inoculum sources.

The detection rate of GLRaV-3 in the scions of various scion/rootstock combinations differed depending on the virus source used to graft-inoculate the rootstock. Scions grafted onto rootstocks infected with virus source 17-7082 (variant VI only) had a 60% detection rate, whereas 17-7084 (variants VI and to a lesser extent II) produced an 89% detection rate, and 17-7093 (variants VI and to a lesser extent II) a 67% detection

rate. There was however no statistical significance between them percentages. Further studies incorporating a larger sample size would need to be carried out to determine if rootstocks infected with multiple variants, i.e. group VI and II were less likely to overcome virus replication and subsequent transmission to the scion component of the vines. The virus concentrations of the three inoculum grafting sources were not measured but may have been a contributing factor to the differences in GLRaV-3 detection rates. Grafting material with lower virus titres would likely have reduced GLRaV-3 variant groups and, in turn, reduce virus transmission and replication in rootstock tissue.

C. franc indicator vines grafted onto Ramsey rootstocks that were graft-inoculated exhibited a 100% GLRaV-3 detection rate (Table 6). Ramsey rootstocks are rarely used in the wine industry, but are common place in the table grape industry where they are more suited (Saayman, 2009). Ramsey also has a distinctively different parentage (*V. Champinii* and *Vitis* species) (Malan and Meyer, 1993) compared to other rootstock types. The 100% infection rate could imply that Ramsey rootstock does not have any form of resistance to GLRaV-3. When Salt Creek/Ramsey acted as an intergraft rootstock, C. franc indicator vines were observed to have an 85% GLRaV-3 detection rate. This supports the results of both Harris (2017) who found Salt Creek/Ramsey to have an 89% infection rate as well as Walsh and Pietersen (2013) who observed a 91% infection rate in the rootstock using an RT-LAMP assay.

C. franc indicator vines grafted onto R110 graft-inoculated with GLRaV-3 had an 82% GLRaV-3 incidence rate. This is interesting to note as the survey conducted in 2017 found R110 grafted on a variety of symptomatic scions to have only a 7% GLRaV-3 rate (Table 6). When C. franc scions were grafted onto R110 rootstocks (that had previously tested negative for GLRaV-3 using RT-PCR), 75% of the C. franc scions tested positive for GLRaV-3 (Table 8). This could be indicative of a tolerant host response in which R110 harbours the virus at a low concentration but is able to transmit virus to scion tissue. Another possible explanation could be that the results obtained in this study are reflective of replication in relation to sap flow and hormonal levels, which differ depending on whether the rootstock under question is alone or grafted, forming part of scion/rootstock combination (Kriton Kalantidis, Pers. Comm.). Future research should focus on investigating rootstocks on their own versus

scion/rootstock combinations for comparison studies. R110 intergrafts produced a 64% incidence rate in C. franc indicator vines which, once again, supports the possibility that R110 may be tolerant to GLRaV-3 (virus at sub-detectable levels but nevertheless present and replicating).

C. franc grafted onto R99 graft-inoculated with 17-7093 and 17-7084 virus sources (variants VI and II) had 100% infection, whereas C.franc/R99 graft-inoculated with 17-7082 (variant VI only) had a 0% infection rate (Table 6). The graft-inoculations on all three R99 rootstocks took and thus it can be ruled out that the observed poor detection of GLRaV-3 in scion was due to unsuccessful grafts. It could therefore be hypothesized that R99 is resistant to only certain GLRaV-3 variants, however, further studies would have to be done to confirm this. The detection rate of virus in C. franc when R99 functioned as an intergraft are interesting, in that when C. franc was a rooted indicator the findings support the notion that the rootstock was likely to overcome a single variant infection (0% infection rate) and that the rootstock appeared to be completely susceptible to a multiple variant infection (100% infection rate). However, when C. franc was the top indicator component and R99 the intergraft there was a 75% infection rate (single variant virus source). This could be as a result of a higher virus titre in the graft source or as a result of genetic variability amongst rootstock types.

C. franc grafted onto 101-14 Mgt had a 50% detection rate, irrespective of the virus source the rootstock was graft-inoculated with (Table 6). The study by Chooi et al. (2016) made use of Schwarzmann and 3309C rootstocks (as previously mentioned) which have the most similar parentage to that of 101-14 Mgt. The detection rate of GLRaV-3 in C. franc grafted onto 101-14 Mgt was similar to that found in the rootstocks used by Chooi et al. (2016). The inconsistent detection could be attributed to tolerance by the rootstock, however, the sample size was too small to infer any conclusions regarding this and therefore experimental trials on a much larger sample size would need to be conducted. Rootstock 101-14 Mgt intergraft was seen to have had no resistance to a single GLRaV-3 infection but was seen to display possible resistance to a multiple variant infection (20% incidence rate). This could suggest that 101-14 Mgt favours single variant infection and is partially resistant to a multiple variant infection. The differences in detection between rootstocks of the same cultivars

suggests that there may be some genetic heterogeneity amongst individual vines. Future studies will need to focus on investigating the degree to which rootstocks are genetically uniform.

C. franc grafted onto Paulsen rootstocks collected in May 2018 (eight of which previously tested positive for GLRaV-3 and two negative) had a 60% detection rate. The C. franc indicator vines with a Paulsen intergraft rootstock grafted with single and multiple variant GLRaV-3 sources were seen to have an overall detection rate of 70%. These results could possibly indicate that Paulsen rootstock is susceptible to GLRaV-3 infection, however, the possibility of virus transmission through an intergraft rootstock could also be as a result of the passive movement of virus through phloem, and not necessarily due to replication in the intergraft. The same can be said for US 8-7 rootstock which resulted in an overall detection rate of 79% in C. franc indicator vines.

Aldrich et al. (2019) found a statistically significant difference in virus concentration ratios between grapevines with single variant infections relative to vines with multiple GLRaV-3 variants (Aldrich et al., 2019). This result implied that GLRaV-3 variants did not have an antagonistic relationship but rather a type of synergy, i.e. variants were able to co-express suppressors of silencing. This would explain why a multiple variant infection could possibly be more pathogenic in rootstock/scion tissue compared to a single variant GLRaV-3 infection. Cabernet franc grafted onto C. franc intergrafts further grafted with single and multiple variant GLRaV-3 sources support the findings by Aldrich *et al*. (2019).

Common host defense mechanisms are regulated by hormone-mediated signaling pathways, predominantly, jasmonic acid (JA) and salicylic acid (SA) (Pieterse et al., 2009). A host plant responds to a viral infection through the SA pathway, resulting in an increase in reactive oxygen species which, in turn, initiates cell wall fortification and localized programmed cell death in infected tissue. This hypersensitive response defense mechanism limits viral proliferation (Glazebrook, 2005, Stange et al., 2008). This response, however, has not been reported in the *Vitis* genus (Martelli, 2014). There are a limited number of studies focused on plant-pathogen interactions in GLRaV-3 infections, mainly due to the complex etiology of the virus and varying symptomatic expression in red and white cultivars (Naidu et al., 2014). RNA

interference (RNAi) is a common strategy employed by host plants to combat viral infections (Guleria et al., 2011). One of two mechanisms of RNAi include; synthesis of microRNAs (miRNAs), which silence the expression of target genes by cleaving sequence-specific target mRNA or alternatively repress transcription/translation of invading viral genomes (Khraiwesh et al., 2012). Investigating the response of miRNA expression in GLRaV-3 infected rootstocks may provide a greater insight into the potential tolerant immune response of infected vines. The use of miRNA as an internal control for tolerance and immunity could facilitate further studies on GLRaV-3-rootstock interactions, with specific reference to the genetic variability of rootstock cultivars.

6. Conclusion

This study confirmed deductions made by previous studies that GLRaV-3 is inconsistently detected in rootstocks (Walsh and Pietersen, 2013, Chooi et al., 2016, Harris, 2017) and is less frequently detected in rootstocks than in corresponding scion tissue. The detection rate of GLRaV-3 was found to be highest in Paulsen, Salt creek/Ramsey and US 8-7, indicating that these rootstocks may have no inherent resistance to GLRaV-3 infection. When C. franc was grafted onto rootstock 101-14 Mgt the detection rate was seen to be higher when infected by a single variant GLRaV-3 source rather than multiple variants. The opposite was true for R99. Cabernet franc grafted onto R110 was seen to have a similar detection pattern, irrespective of single or multiple variants and irrespective of C. franc being the top component of the vine or rooted. However, GLRaV-3 detection in R110 itself was poor. This suggests that R110 may have a tolerant host response to GLRaV-3 infection. Evaluating rootstock genetic variability and miRNA expression pre- and post-GLRaV-3 infection would aid in further understanding the mechanisms underlying symptom development and a lack thereof in rootstock cultivars.

Acknowledgements

This research was financially supported by Winetech. The authors would like to thank all the grape growers who granted permission to sample from their vineyards. We would especially like to thank Vititec and specifically Mr. Dirk Visser for his help in performing the grafts.

References

ALABI, O. J., CASASSA, L. F., GUTHA, L. R., LARSEN, R. C., HENICK-KLING, T., HARBERTSON, J. F. & NAIDU, R. A. 2016. Impacts of grapevine leafroll disease on fruit yield and grape and wine chemistry in a wine grape (Vitis vinifera L.) cultivar. *Public Library of Science one,* 11**,** e0149666.

ALBACETE, A., MARTÍNEZ-ANDÚJAR, C., MARTÍNEZ-PÉREZ, A., THOMPSON, A. J., DODD, I. C. & PÉREZ-ALFOCEA, F. 2015. Unravelling rootstock× scion interactions to improve food security. *Journal of experimental botany,* 66**,** 2211-2226.

ALDRICH, D., BESTER, R., BURGER, J. & MAREE, H. 2019. Characterisation of different GLRaV-3 variant infections by determining virus concentration ratios and miRNA expression profiles. *Vitis: Journal of Grapevine Research,* 58**,** 79-86.

ALMEIDA, R., DAANE, K., BELL, V., BLAISDELL, G. K., COOPER, M., HERRBACH, E. & PIETERSEN, G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology,* 4**,** 94.

BESTER, R., MAREE, H. & BURGER, J. 2012. Complete nucleotide sequence of a new strain of grapevine leafroll-associated virus 3 in South Africa. *Archives of virology,* 157**,** 1815-1819.

CHOOI, K. M., COHEN, D. & PEARSON, M. N. 2016. Differential distribution and titre of selected grapevine leafroll-associated virus 3 genetic variants within grapevine rootstocks. *Archives of virology,* 161**,** 1371-1375.

CID, M., CABALEIRO, C. & SEGURA, A. Detection of Grapevine leafroll-associated virus 3 in rootstocks. *Extended abstracts of the 14th Meeting of ICVG*, Locorotondo, 2003.

COHEN, D., VAN DEN BRINK, R. & HABILI, N. Leafroll virus movement in newly infected grapevines. *Extended abstracts 14th Meeting ICVG*, Locorotondo, Italy, 2003. 13-17.

DIAZ-LARA, A., KLAASSEN, V., STEVENS, K., SUDARSHANA, M. R., ROWHANI, A., MAREE, H. J., CHOOI, K. M., BLOUIN, A. G., HABILI, N. & SONG, Y. 2018. Characterization of grapevine leafroll-associated virus 3 genetic variants and application towards RT-qPCR assay design. *Public Library of Science one,* 13**,** e0208862.

DOVAS, C. & KATIS, N. 2003. A spot nested RT-PCR method for the simultaneous detection of members of the Vitivirus and Foveavirus genera in grapevine. *Journal of Virological Methods,* 107**,** 99-106.

ENGEL, E. A., GIRARDI, C., ESCOBAR, P. F., ARREDONDO, V., DOMÍNGUEZ, C., PÉREZ-ACLE, T. & VALENZUELA, P. D. 2008. Genome analysis and detection

of a Chilean isolate of Grapevine leafroll associated virus-3. *Virus Genes,* 37**,** 110-118.

FORTES, A. M. & PAIS, M. S. 2016. Grape (Vitis species). *Nutritional Composition of Fruit Cultivars.* Elsevier.

GLAZEBROOK, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology,* 43**,** 205-227.

GOSZCZYNSKI, D. E. 2013. Brief Report of a New Highly Divergent Variant of Grapevine leafroll-associated virus 3 (GLR aV-3). *Journal of Phytopathology,* 161**,** 874-879.

GRANETT, J., WALKER, M. A., KOCSIS, L. & OMER, A. D. 2001. Biology and management of grape phylloxera. *Annual review of entomology,* 46**,** 387-412.

GULERIA, P., MAHAJAN, M., BHARDWAJ, J. & YADAV, S. K. 2011. Plant small RNAs: biogenesis, mode of action and their roles in abiotic stresses. *Genomics, proteomics & bioinformatics,* 9**,** 183-199.

HARRIS, M. 2017. Characterization of viruses associated with Grapevine Leafroll disease in Vitis rootstocks in South Africa. Master of Science in Microbiology. University of Pretoria, Pretoria.

JOHNSON, S. N., HILTPOLD, I. & TURLINGS, T. C. 2013. *Behaviour and physiology of root herbivores*, Elsevier.

JOOSTE, A., MAREE, H., BELLSTEDT, D., GOSZCZYNSKI, D., PIETERSEN, G. & BURGER, J. 2010. Three genetic grapevine leafroll-associated virus 3 variants identified from South African vineyards show high variability in their 5′ UTR. *Archives of virology,* 155**,** 1997-2006.

JOOSTE, A. E., PIETERSEN, G. & BURGER, J. T. 2011. Distribution of grapevine leafroll associated virus-3 variants in South African vineyards. *European Journal of Plant Pathology,* 131**,** 371-381.

KHRAIWESH, B., ZHU, J.-K. & ZHU, J. 2012. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms,* 1819**,** 137-148.

LING, K.-S., ZHU, H.-Y. & GONSALVES, D. 2004. Complete nucleotide sequence and genome organization of Grapevine leafroll-associated virus 3, type member of the genus Ampelovirus. *Journal of General Virology,* 85**,** 2099-2102.

MALAN, A. P. & MEYER, A. 1993. Interaction between a South African population of Xiphinema index and different grapevine rootstocks. *South African Journal of Enology & Viticulture*,14**,** 11-14.

MALIOGKA, V. I., DOVAS, C., LOTOS, L., EFTHIMIOU, K. & KATIS, N. 2009. Complete genome analysis and immunodetection of a member of a novel virus species belonging to the genus Ampelovirus. *Archives of virology,* 154**,** 209.

MAREE, H., FREEBOROUGH, M.-J. & BURGER, J. 2008. Complete nucleotide sequence of a South African isolate of Grapevine leafroll-associated virus 3 reveals a 5′ UTR of 737 nucleotides. *Archives of Virology,* 153**,** 755-757.

MAREE, H. J., ALMEIDA, R. P., BESTER, R., CHOOI, K. M., COHEN, D., DOLJA, V. V., FUCHS, M. F., GOLINO, D. A., JOOSTE, A. E. & MARTELLI, G. P. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology,* 4**,** 82.

MAREE, H. J., PIRIE, M. D., OOSTHUIZEN, K., BESTER, R., REES, D. J. G. & BURGER, J. T. 2015. Phylogenomic analysis reveals deep divergence and recombination in an economically important grapevine virus. *Public Library of Science One,* 10**,** e0126819.

MARTELLI, G., GHANEM-SABANADZOVIC, N. A., AGRANOVSKY, A., AL RWAHNIH, M., DOLJA, V., DOVAS, C., FUCHS, M., GUGERLI, P., HU, J. & JELKMANN, W. 2012. Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology*, 94**,** 7-19.

MARTELLI, G. P. 2014. Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology,* 96**,** 1-136.

MARTELLI, G. 2017. An overview on grapevine viruses, viroids, and the diseases they cause. *Grapevine viruses: molecular biology, diagnostics and management.* Springer, Switzerland.

MENG, B., REBELO, A. R. & FISHER, H. 2006. Genetic diversity analyses of grapevine Rupestris stem pitting-associated virus reveal distinct population structures in scion versus rootstock varieties. *Journal of General Virology,* 87**,** 1725-1733.

MONIS, J. & BESTWICK, R. K. 1996. Detection and localization of grapevine leafroll associated closteroviruses in greenhouse and tissue culture grown plants. *American Journal of Enology and Viticulture,* 47**,** 199-205.

NAIDU, R., ROWHANI, A., FUCHS, M., GOLINO, D. & MARTELLI, G. P. 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Disease,* 98**,** 1172-1185.

OVER DE LINDEN, A. & CHAMBERLAIN, E. 1970. Effect of grapevine leafroll virus on vine growth and fruit yield and quality. *New Zealand Journal of Agricultural Research,* 13**,** 689-698.

PERRONE, I., CHITARRA, W., BOCCACCI, P. & GAMBINO, G. 2017. Grapevine–virus–environment interactions: an intriguing puzzle to solve. *New Phytologist,* 213**,** 983-987.

PIETERSE, C. M., LEON-REYES, A., VAN DER ENT, S. & VAN WEES, S. C. 2009. Networking by small-molecule hormones in plant immunity. *Nature chemical biology,* 5**,** 308.

PIETERSEN, G. 2010. *Grapevine leafroll disease control in South Africa*. Wynboer, Viewed: 12 June 2019, < http://www. wineland. co. za/technical/grapevine-leafroll-disease-control-in-southafrica>

ROWHANI, A. & GOLINO, D. 1995. ELISA test reveals new information about leafroll disease. *California Agriculture,* 49**,** 26-29.

SAAYMAN, D. 2003. The South African vineyard and wine landscapes: heritage and development. *Technical Yearbook 2004/5*. Viewed: 13 August 2019, < https://www.wineland.co.za/the-south-african-vineyard-and-wine-landscapes-heritage-and-development/>

SAAYMAN, D. 2009. Rootstock choice: The South African experience. 6*0th Annual Meeting of the American Society for Enology and Viticulture*, Napa, California, 2009.

SCHEU, G. 1936. *Mein Winzerbuch*. Reichsnährstand Verlags-Ges, Berlin, 1936, 274.

SMITH, E. H. 1992. The grape phylloxera—a celebration of its own. *American Entomologist,* 38**,** 212-221.

STANGE, C., MATUS, J. T., DOMÍNGUEZ, C., PEREZ-ACLE, T. & ARCE-JOHNSON, P. 2008. The N-homologue LRR domain adopts a folding which explains the TMV-Cg-induced HR-like response in sensitive tobacco plants. *Journal of Molecular Graphics and Modelling,* 26**,** 850-860.

STEWART, S. & NASSUTH, A. 2001. RT-PCR based detection of Rupestris stem pitting associated virus within field-grown grapevines throughout the year. *Plant Disease,* 85**,** 617-620.

THOMPSON, B. D., DAHAN, J., LEE, J., MARTIN, R. R. & KARASEV, A. V. 2019. A novel genetic variant of Grapevine leafroll-associated virus-3 (GLRaV-3) from Idaho grapevines. *Plant disease,* 103**,** 509-518.

TÖPFER, R., HAUSMANN, L., HARST, M., MAUL, E., ZYPRIAN, E. & EIBACH, R. 2011. New horizons for grapevine breeding. Methods in temperate fruit breeding*. Fruit, vegetable and cereal science and biotechnology*. Global Science Books, Germany, 5**,** 79-100.

TSAI, C.-W., CHAU, J., FERNANDEZ, L., BOSCO, D., DAANE, K. & ALMEIDA, R. 2008. Transmission of Grapevine leafroll-associated virus 3 by the vine mealybug (Planococcus ficus). *Phytopathology,* 98**,** 1093-1098.

TSAI, C., DAUGHERTY, M. & ALMEIDA, R. 2012. Seasonal dynamics and virus translocation of Grapevine leafroll-associated virus 3 in grapevine cultivars. *Plant Pathology,* 61**,** 977-985.

WALSH, H. A. & PIETERSEN, G. 2013. Rapid detection of Grapevine leafroll-associated virus type 3 using a reverse transcription loop-mediated amplification method. *Journal of virological methods,* 194**,** 308-316.

WHITE, E. J., VENTER, M., HITEN, N. F. & BURGER, J. T. 2008. Modified Cetyltrimethylammonium bromide method improves robustness and versatility: The benchmark for plant RNA extraction. *Biotechnology Journal: Healthcare Nutrition Technology,* 3**,** 1424-1428.

Chapter 4

Tissue and time of collection for optimal detection of GLRaV-3 in rootstock petioles

1. Abstract

Several viruses have been associated with grapevine leafroll disease (GLD) and are named grapevine leafroll associated viruses (GLRaVs), followed by various numbers from 1 to 9. The most prevalent of these is Grapevine leafroll associated virus 3 (GLRaV-3). No resistance to GLRaV-3 is known in the *Vitis vinifera* cultivars commonly utilised in the commercial wine and table grape industries. *V. vinifera* scions are generally grafted onto rootstocks which are different species of *Vitis* to control phylloxera which typically attacks *V. vinifera* roots. The relationship between GLRaV-3 and rootstock is largely unknown, but infected rootstock is generally observed to have low viral titres and an erratic distribution of virus. In this study, we investigated the optimal time and tissue region most suited for GLRaV-3 detection in commonly used rootstocks using real-time reverse transcription-PCR. GLRaV-3 detection was shown to be affected by virus distribution and viral titre amongst the rootstock cultivars, with US 8-7 having significantly higher GLRaV-3 viral titres in basal rootstock tissue when compared to all other rootstock cultivars, except Richter 110. Viral titres of GLRaV-3 were found to be significantly higher in the basal region of rootstocks R110 and Salt Creek/Ramsey compared to their respective apical regions. Rootstocks R110, Ruggeri 140, and Salt Creek/Ramsey displayed a general decrease in viral titre t[over the years.

2. Introduction

Grapevine leafroll associated virus 3 is a single-stranded positive-sense RNA virus belonging to the *Ampelovirus* genus within the *Closteroviridae* family (Martelli, 2006, Maree et al., 2013,). GLRaV-3 is the main causative agent of GLD and has a detrimental impact on grapevine health, resulting in yield losses ranging from 36% to 68% worldwide (Maree et al., 2013, Bester et al., 2014, Chooi et al., 2016).

As part of an active disease management approach to grapevines, growers in the process of establishing a new vineyard are encouraged to plant certified material derived from virus-tested stocks (Maree et al., 2013). Vineyards then require monitoring for infected grapevines that can be physically removed (rogued). Red cultivars display distinctive interveinal reddening and leafroll which are easily identifiable. However, it has been shown that most infected white cultivars and rootstocks are symptomless and can serve as reservoirs for GLD transmission (Maree et al., 2013, Bester et al., 2014, Montero et al., 2016). Failure to detect GLRaV-3 infections in rootstock material greatly contributes to virus spread in vineyards (Beuve et al., 2007).

Reliable diagnostic techniques are essential in eliminating the introduction and spread of virus in vineyards (Chooi et al., 2016). In the grapevine industry, serological, enzyme-linked immunosorbent assays (ELISA) and end-point nucleic acid-based methods are employed for GLRaV-3 detection (Bester et al., 2014, Bruisson et al., 2017). The development of RT-qPCR assays has allowed for increased sensitivity and specificity of diagnostic testing, as well as quantification of virus targets (Bester et al., 2014, López-Fabuel et al., 2013).

GLRaV-3 is known to occur in low concentrations in rootstock material (Stewart and Nassuth., 2001). This in conjunction with non-uniform virus distribution in the host, renders reliable on-site diagnostic testing difficult (Walsh and Pietersen, 2013). Polyphenolic and polysaccharide compounds known to interfere with PCR detection are present in high concentrations in grapevines and these compounds vary among tissues and across seasons (Minafra and Hadidi, 1994). However, petioles and the basal region of actively growing grapevine stems have been shown to house the

highest concentrations of GLRaVs (Monis and Bestwick, 1996) and for this reason petioles were chosen as the sampled tissue type.

To investigate the effect of *in planta* distribution and seasonal fluctuations of virus titre on the success of molecular detection of GLRaV-3 in grapevine rootstocks, a SYBR green RT-qPCR assay was employed to detect and quantify GLRaV-3 in infected rootstock phloem material.

3. Materials and methods

3.1 Plant material and sample preparation

Rootstock material successfully inoculated with GLRaV-3 sources was obtained from a previous trial where the sensitivity of immuno electron microscopy and ELISA in the Wine Grape Certification Scheme was conducted at the PPRI, Roodeplaat (Kasdorf, 2006). These plants were maintained in an insect-free screenhouse with no temperature, humidity, or light control. The trial consisted of virus-free rootstocks that had been cleft grafted with various virus sources from *V. vinifera*. The inoculated rootstocks were maintained at the PPRI in a screen-cage since the conclusion of the 2006 trial. Only those rootstock individuals inoculated with GLRaV-3 containing sources and with sufficient surviving replicates of vines with large and voluminous canes were useful for analysis. These were the Black Spanish 2 (90/0246) and Ohanez (92/1023) inoculated rootstock clones namely: Richter 99, Ruggeri 140, Richter 110, Salt Creek/Ramsey, and 101-14 Mgt. Black Spanish (90/0246) canes contained GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-4 strain 5, grapevine virus A (GVA) and grapevine fleck virus (GFkV). Ohanez (92/1023) canes contained GLRaV-3 and GLRaV-4 strain 5 (Table 1). Apart from the Kasdorf trial canes, canes of GLRaV-3 infected US 8-7 (which contain a *V. vinifera* parental line) vines were obtained from Tobie Oosthuizen, Vititec, to serve as a *V. vinifera* containing parental line control. These plants were only added to the trial shortly before sampling commenced.

Samples were collected every six weeks during the growing season between 2014/15 and 2015/16 (over 10-time points) by a former MSc student (M. Harris). Samples consisted of petioles collected from either the bottom one third (older part of the shoot)

or the top one third (actively growing young material) of three biological replicates of each rootstock cultivar inoculated with GLRaV-3 containing sources. A total of 576 samples were stored at -80^{o}C until use (Table 1).

Table 1: Date and number of replicates of each treatment collected for RT-qPCR analysis

	Black Spanish 2										Ohanez										GLRaV-3 (Vititec)	
	R99		R110		101-14 Mgt		Ruggeri 140		SC		R99		R110		101-14 Mgt		Ruggeri 140		SC		US 8-7	
Date collected	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
2014/11/03	3	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1
2014/12/17	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1
2015/01/26	3	3	3	3	3	3	3	1	-	-	3	3	3	3	3	3	-	-	-	-	1	1
2015/03/10	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1
2015/04/28	3	3	3	3	3	3	3	3	2	3	3	3	3	1	3	3	2	3	3	3	1	1
2015/11/04	3	3	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	-	-
2015/12/22	3	3	3	3	3	1	3	3	3	3	3	2	3	3	3	3	3	3	3	3	1	1
2016/01/27	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1
2016/03/15	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	-	-
2016/04/06	3	3	3	3	2	1	2	2	3	3	3	3	1	2	3	3	3	3	3	3	-	-

SC= Salt Creek/Ramsey; (-) =Not collected

3.2 Total RNA extraction

Total RNA was isolated from 200 mg of phloem material using a modified cetyltrimethylammonium-bromide-based (CTAB) RNA extraction protocol (Bester et al., 2014). Plant material was weighed out and freeze-dried at -60^{o}C using the AdVantage Plus Bench Top Freeze Dryer (Mason Technology, NY, USA). A metal bead was added to freeze-dried plant material, followed by processing with the Genogrinder (SPEX SamplePrep, NJ, USA). The CTAB buffer contained: 2% CTAB, 2% soluble polyvinylpyrrolidone K-40, 25 mM EDTA, 2 M NaCI, 100 mM Tris-HCI pH

8.0, and 3% β-mercaptoethanol. To each sample, 1.2 mL CTAB buffer was added and incubated at 65ºC for 30 minutes. Samples were centrifuged for 10 minutes at 12303 x g. A chloroform-isoamyl (24:1) extraction step was performed twice by adding an equal volume to the supernatant, vortexing for 30 seconds and centrifugation for 15 minutes at 14269 x g at 4ºC. Following an overnight Lithium Chloride (2M) treatment at 4ºC, RNA was precipitated by centrifugation at 12 303 x g for 60 minutes at 4ºC. After a wash step with 70% ethanol, pellets were dried and resuspended in 50 µL molecular grade H_2O (Life Technologies, MA, USA). Integrity and purity of RNA were assessed by agarose gel electrophoresis (2% Tris-acetate-EDTA (TAE) agarose gel) and spectrophotometry (NanoDrop 200 Spectrophotometer) (Thermo Fisher, Waltham, MA, USA).

3.3 Complementary DNA synthesis

In initial tests to produce standard curves, healthy and cloned GLRaV-3 material were used as templates. Healthy *V. vinifera* RNA (accessions: 16/0008, 16/0009, and 16/0010) was used to produce cDNA by a M-MLV Reverse Transcriptase-mediated reaction, followed by determining the concentration using a fluorometer. Separate 10-fold serial dilutions were performed on the healthy cDNA and a gel-purified 144 base pair (bp) ORF1a amplicon with nuclease-free molecular grade H_2O (Life Technologies) producing cDNA to H_2O ratios of 1:1; 1:10; 1:100; 1:1000; and 1:10 000 and amplicon to H_2O ratios of 1:1 000 000; 1:10 000 000; 1:100 000 000; 1:1000 000 000; and 1:10 000 000 000 000. The diluted cDNA/amplicon was subjected to qPCR conditions, as described below, utilizing the α-tubulin reference gene primer pair (Reid et al., 2006) and the LR3 virus-specific primer pair (Bester et al., 2014). Ultimately, only relative quantification was performed.

Primer-specific cDNA were synthesized from 5 µL of total RNA using 0.5 µM of GLRaV-3 (ORF1a) (Bester et al., 2014) or *V. vinifera* α-tubulin reverse primers (Reid et al., 2006), and M-MLV RT polymerase (120 U/µL) (Thermo Fisher) in a final reaction volume of 15 µL according to the manufacturer's instructions. All cDNA was diluted (100 ng/µL) and treated as the unknown samples for quantification. All cDNA was stored at -20 ºC.

3.4 Quantitative reverse transcription-polymerase chain reaction

The RT-qPCRs were performed using the QuantStudio K12 Flex thermal cycler (Thermo Fisher) and PowerUp SYBR Green Master Mix (Thermo Fisher). Reactions contained 2x PowerUp SYBR Green Master Mix, molecular grade H_2O (Life technologies), and 0.4 µM forward and reverse primers (IDT, Coralville, USA). One µL cDNA was added to each reaction to a final reaction volume of 10 µL. The 100 ng/µL dilution of each 'unknown' sample was screened with the two primer sets for relative quantification. No-template controls, negative plant controls (negative for GLRaV-3), and positive controls (positive for GLRaV-3) were included in all runs. All reactions were performed in triplicate in FG-384-well clear reaction plates and sealed with optical adhesive strips. Thermocycling conditions included: an initial denaturation at 95ºC for 10 minutes followed, by 45 cycles of 95ºC for 15 seconds, primer-specific annealing temperature for 15 seconds (Table 1) and 72ºC for 15 seconds. Acquisition of the green channel was recorded at the end of the extension step. Melting curve analysis was performed with temperatures ranging from 65ºC to 95ºC with a 0.2ºC increase in temperature every second to identify primer-dimers and non-specific amplification.

Table 2: Oligonucleotide primers targeting a GLRaV-3 genomic region and *Vitis vinifera* reference gene

Primer	Sequence 5'-3'	Amplicon size	Target	Annealing temperature (ºC)	Primer concentration	Reference
LR3_6995F	GGGRACGGARAAGTGTTACC	144	GLRaV-3 ORF1a	53	0.4	Bester et al., 2014
LR3_6138R	TCCAAYTGGGTCATRCACAA					Bester et al., 2014
Vv_α-tubulin_F	CAGCCAGATCTTCACGAGCTT	119	*Vitis vinifera* alpha-tubulin	55	0.4	Reid et al., 2006
Vv_α-tubulin_R	GTTCTCGCGCATTGACCATA					Reid et al., 2006

3.5 Data analysis

The PCR efficiency (E) for each of the targets was calculated using the slope of the standard curve constructed with the 10-fold dilution series over the linear dynamic range (Equation 1). Relative quantification values were calculated based on the method described by Pfaffl (Equation 2) (Pfaffl, 2001). The original use of Equation 2

is for gene expression, however, in this study it was used to determine relative viral titres. The QuantStudio 12K Flex software version 1.1 (Thermo Fisher) was used to calculate primer efficiencies and all Ct values.

$$E = 10^{(-1/slope)} \quad (1)$$

$$Gene\ expression\ ratio = \frac{(Etarget)^{\Delta Ct\ target\ (control-sample)}}{(Eref)^{\Delta Ct\ ref\ (control-sample)}} \quad (2)$$

4. Results

4.1 RNA extractions

Total RNA extraction resulted in acceptable yields (average of 249.70± 23.62 ng/µL) with average A260/A280 ratios of 1.82±0.038 and an average A260/A230 ratio of 1.91±0.046.

4.2 Primer specificity

Specificity of primers were analysed based on gel electrophoresis and melting curve analysis. Gel electrophoresis showed a single amplicon of the desired size for both primer sets (Table 1). The QuantStudio 12K Flex melting curve analyses resulted in a single amplicon-specific melting temperature curve for the α-tubulin reference gene and, for the most part, a single amplicon-specific melting temperature curve for GLRaV-3 (Figure 1).

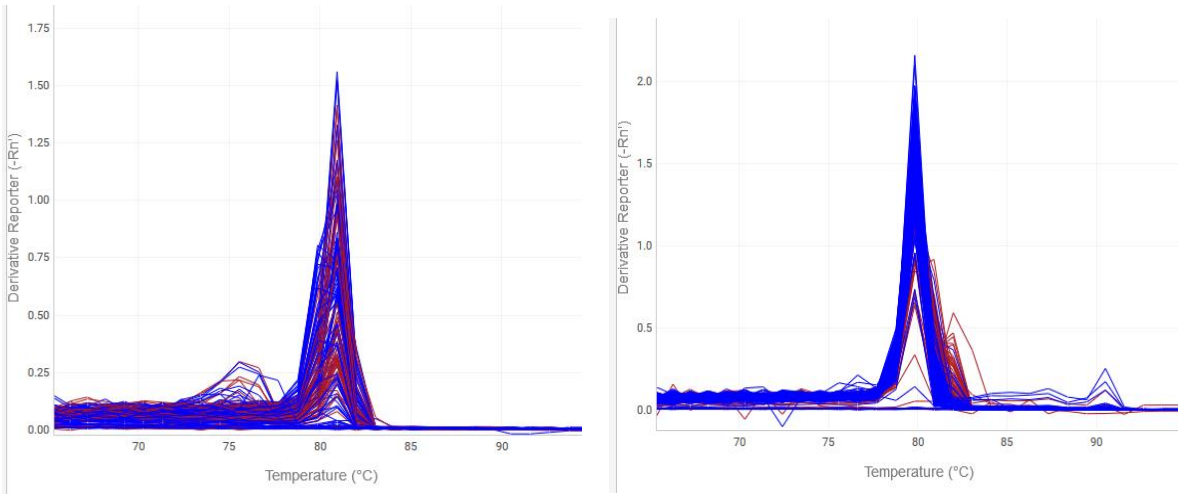


Figure 1: Single amplicon-specific melt curve plots for GLRaV-3 ORF1a (left) and the α-tubulin host reference gene (right), respectively.

4.3 Primer efficiency

The PCR efficiencies for the LR3 and α-tubulin primer pairs were calculated from the standard curve slopes using the QuantStudio 12K Flex software version 1.1 (Thermo Fisher). The PCRs performed showed relatively poor efficiencies of 0.74 and 0.84 for the α-tubulin host reference gene and GLRaV-3, respectively, and high linearity with $r^2 > 0.98$ (Table 3). These differences in efficiency of amplification required the use of a corrected transformation of data (Equation 2).

Table 3: Efficiency, coefficients of determination (r^2), slope, and y-intercept (b) of the two standard curves constructed using the QuantStudio 12K Flex software

Assay	Efficiency	r^2	Slope	y- intercept (b)
α-tubulin	0.81	0.98	-3.87	23.9
ORF1a	0.74	0.99	-4.15	27.8

4.4 Grapevine leafroll associated virus 3 relative quantification

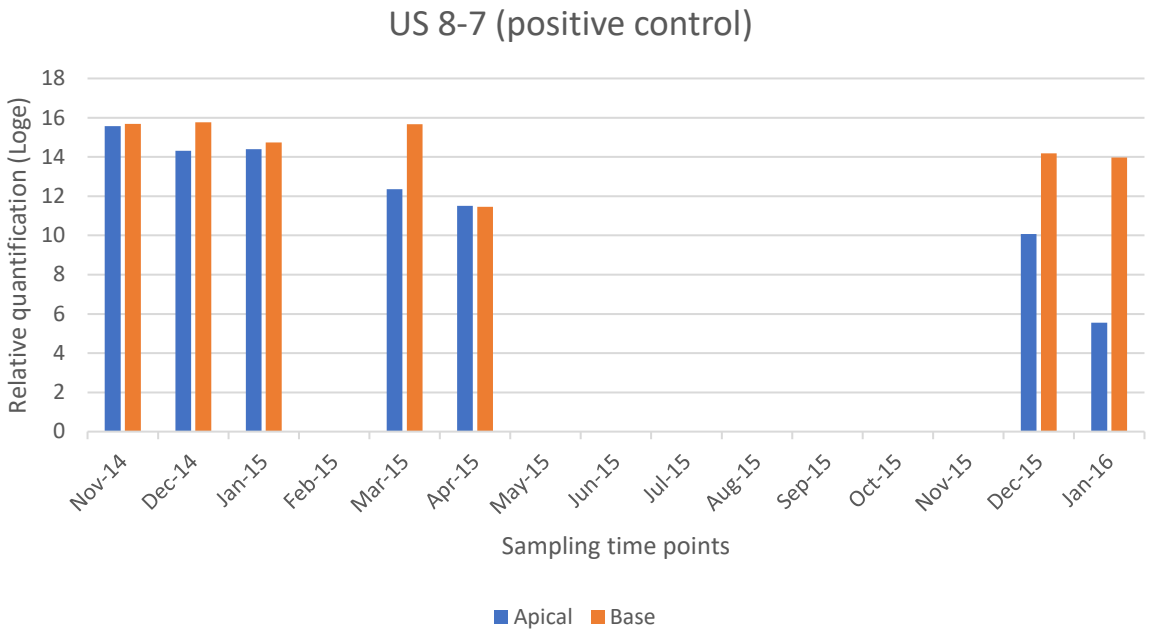


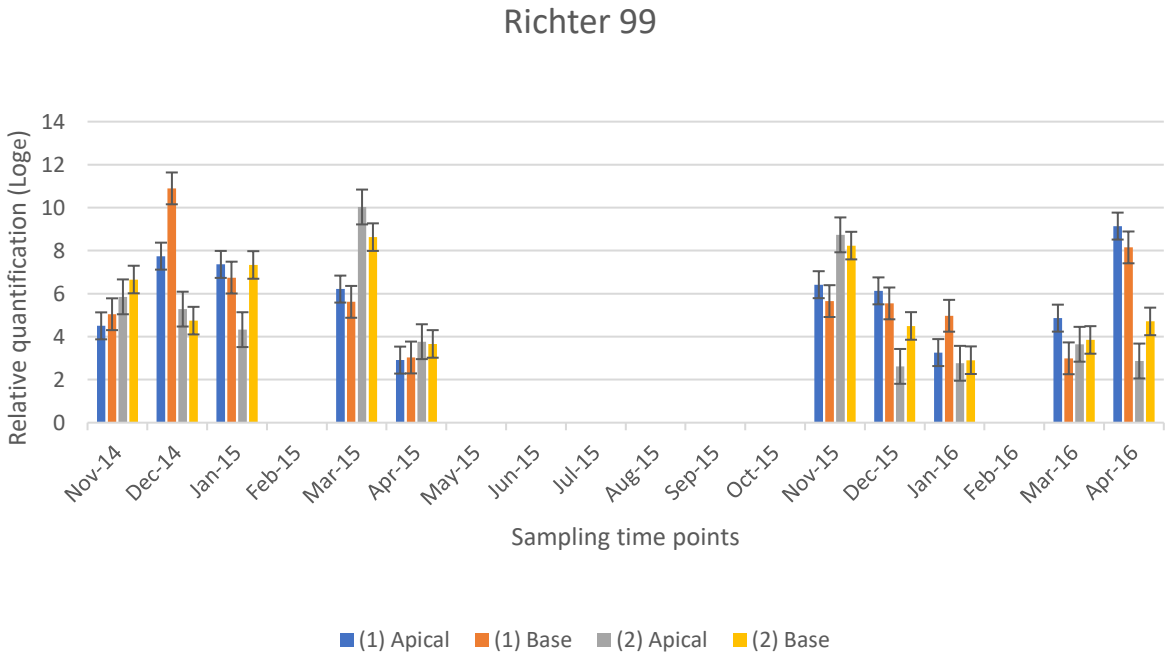
Figure 2: Relative quantification of GLRaV-3 infected US 8-7 (which contains a *V. vinifera* parental line) basal and apical tissue. Only one apical and one basal sample were collected every six weeks during the 2014/15 and 2015/16 growing seasons, therefore, no standard errors could be included on the graph. Zero values on the graph indicate a lack of samples collected over that timepoint.

During March 2015, with the exception of R110, virus replication of the gene of interest (GOI), namely, GLRaV-3 ORF1a, was significantly higher in US 8-7 (for both virus sources) compared to Richter 99 (R99) (*t*-test, p=0.0359, p=0.0046, respectively), 101-14 Mgt (*t*-test, p=0.0001, p=0.0022, respectively), Ruggeri 140 (*t*-test, p=0.0004, p=0.0001, respectively) and Salt Creek/Ramsey (*t*-test, p=0.0042, p=0.0006, respectively) (Table 4).

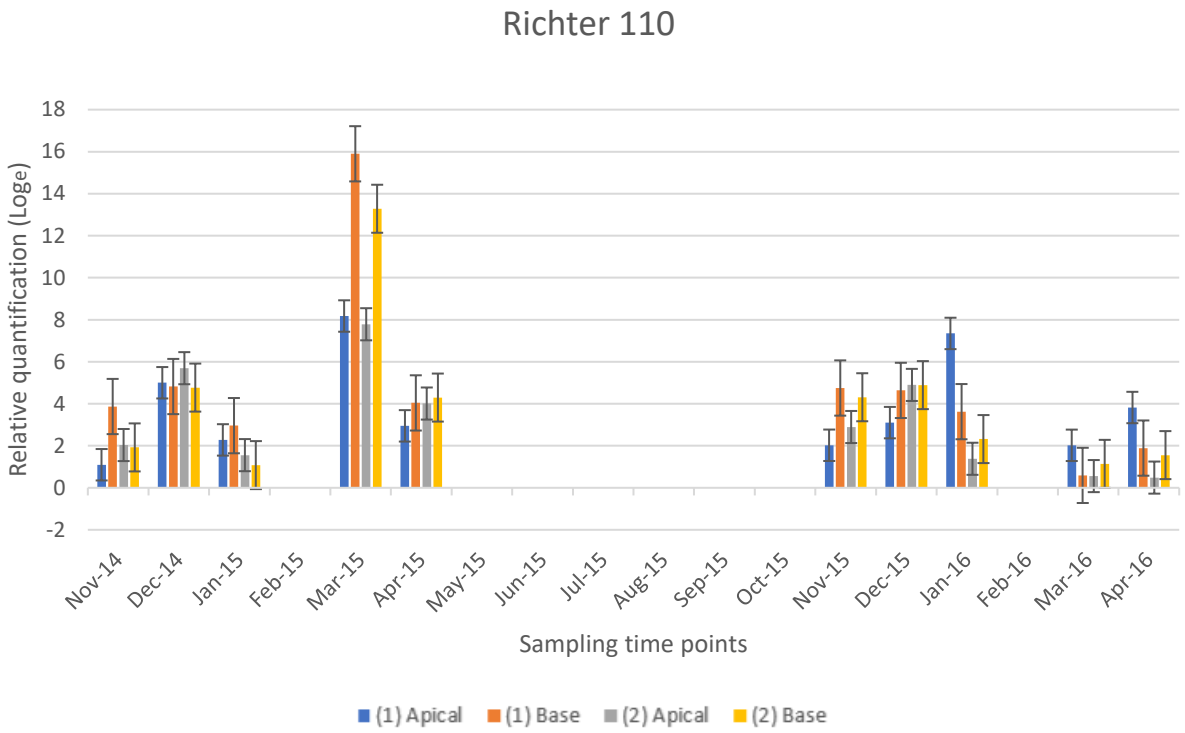
Table 4: Two-sided *t*-tests to determine statistically significant differences in relative viral titres between US 8-7 and different rootstock types in March 2015 for each of the two virus sources. Significant *p*-values are indicated in bold. A *p*-value significance threshold of 0.05 was selected

Rootstock	March 2015 sampling time point	Black Spanish *p*-value	Ohanez *p*-value
R99	US 8-7 *vs* R99	**0.0359**	**0.0046**
R110	US 8-7 *vs* R110	0.6368	0.2651
101-14 Mgt	US 8-7 *vs* 101-14 Mgt	**0.0001**	**0.0022**
Ruggeri 140	US 8-7 *vs* Ruggeri 140	**0.0004**	**0.0001**
Salt Creek/ Ramsey	US 8-7 *vs* Salt Creek/Ramsey	**0.0042**	**0.0006**

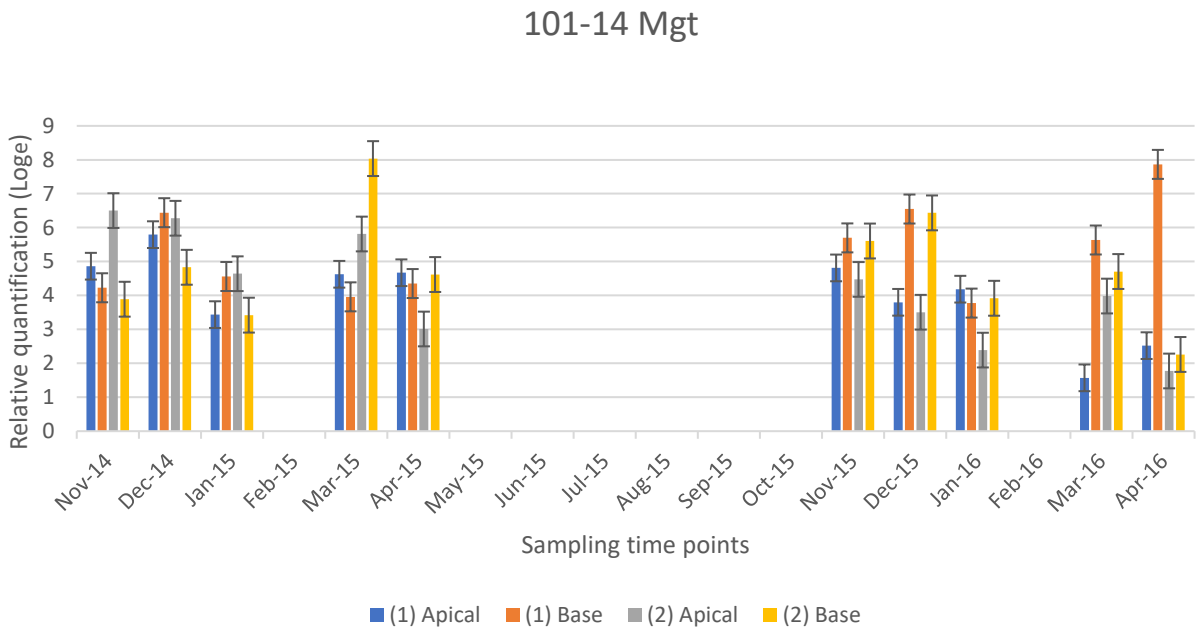
To distinguish virus replication of the GOI in basal and apical rootstock tissue during different sampling time points, namely, over the 2014/15 and 2015/16 growing seasons, from those that merely have the same expression level with a higher cDNA concentration, the following logarithmic graphs were constructed (Figure 3).



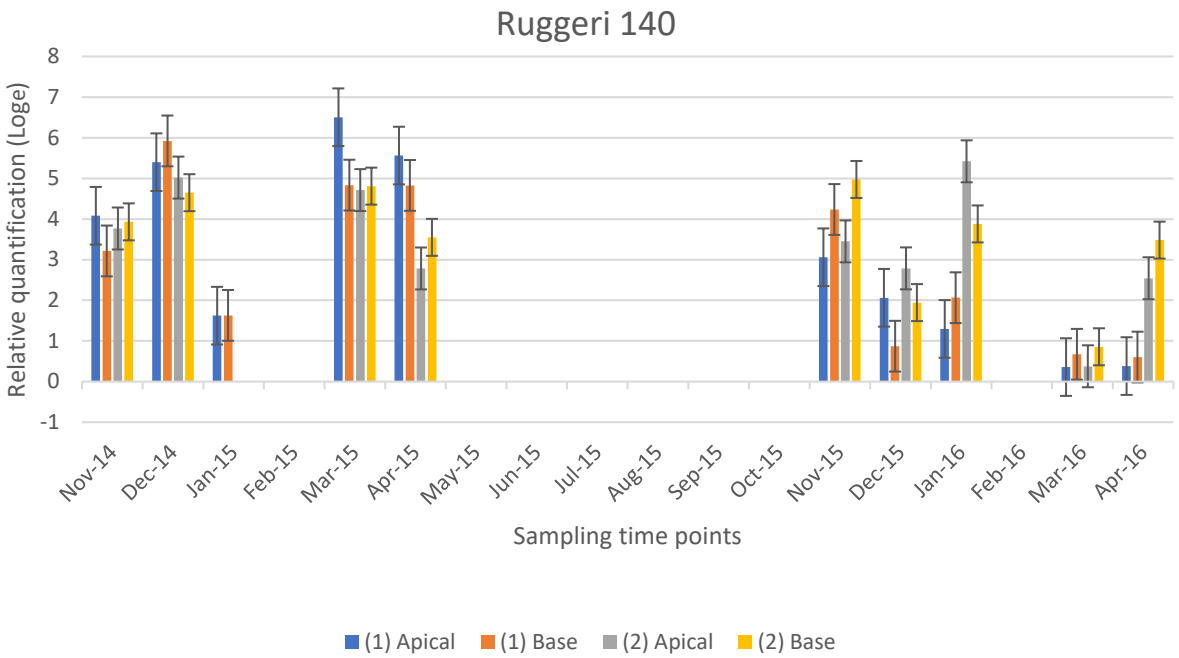
(a)


Richter 110
Relative quantification (Loge)
18
16
14
12
10
8
6
4
2
0
-2
Nov-14
Dec-14
Jan-15
Feb-15
Mar-15
Apr-15
May-15
Jun-15
Jul-15
Aug-15
Sep-15
Oct-15
Nov-15
Dec-15
Jan-16
Feb-16
Mar-16
Apr-16
Sampling time points
(1) Apical
(1) Base
(2) Apical
(2) Base

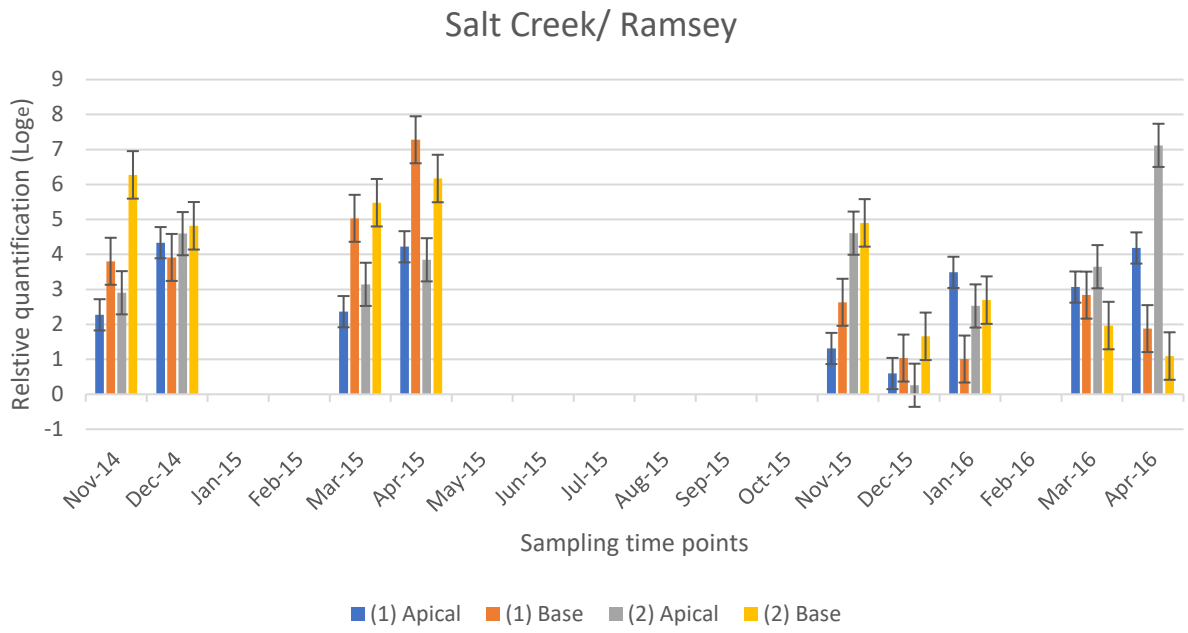
(b)


101-14 Mgt
Relative quantification (Loge)
9
8
7
6
5
4
3
2
1
0
Nov-14
Dec-14
Jan-15
Feb-15
Mar-15
Apr-15
May-15
Jun-15
Jul-15
Aug-15
Sep-15
Oct-15
Nov-15
Dec-15
Jan-16
Feb-16
Mar-16
Apr-16
Sampling time points
(1) Apical
(1) Base
(2) Apical
(2) Base

(c)



(d)



(e)

Figure 3 (a-e): Relative quantification of GLRaV-3 in rootstocks Richter 99, R110, 101-14 Mgt, Ruggeri 140, and Salt Creek/Ramsey during the growing season between November 2014 and April 2016. Petioles were collected from the base and the apical region of all canes of three biological replicates of (1) Black Spanish (90/0246) and (2) Ohanez (90/1023) inoculated virus-sources. Bars indicate standard errors.

Table 5: Two-sided *t*-tests to determine statistically significant differences in viral titre between plant segments in March 2015 for each of the two virus sources. Significant *p*- values are indicated in bold. A *p*-value significance threshold of 0.05 was selected

Rootstock	March 2015 sampling time point	Black Spanish *p*-value	Ohanez *p*-value
R99	Apical *vs* basal	0.7627	0.5766
R110	Apical *vs* basal	**0.0075**	**0.0067**
101-14 Mgt	Apical *vs* basal	0.9228	0.1066
Ruggeri 140	Apical *vs* basal	0.4367	0.6281
Salt Creek/Ramsey	Apical *vs* basal	**0.0284**	**0.0360**

There were significantly higher GLRaV-3 viral titres in basal rootstock tissue for R110 for both virus sources (*t*-test, *p*=0.0075, *p*=0.0067, respectively) compared to apical tissue (Table 5). The same could be said for Salt Creek/Ramsey (*t*-test, *p*=0.0284, *p*=0.0360, respectively). No significant differences were observed between apical and basal tissue for R99, 101-14 Mgt, or Ruggeri 140 in March 2015.

Table 6: Two-sided *t*-tests to determine statistically significant differences in viral titres between basal tissue over three specific sampling timepoints. Significant *p*-values are indicated in bold. A *p*-value significance threshold of 0.05 was selected

Rootstock	Month of sampling	*p*-value
R99	March 2015 *vs* November 2014	0.5333
	March 2015 *vs* April 2016	0.7898
	November 2014 *vs* April 2016	0.7893
R110	March 2015 *vs* November 2014	**0.0189**
	March 2015 *vs* April 2016	**0.0103**
	November 2014 *vs* April 2016	0.3642
101-14 Mgt	March 2015 *vs* November 2014	0.4448
	March 2015 *vs* April 2016	0.8136
	November 2014 *vs* April 2016	0.7545
Ruggeri 140	March 2015 *vs* November 2014	0.0809
	March 2015 *vs* April 2016	0.2042
	November 2014 *vs* April 2016	0.4108
Salt Creek/Ramsey	November 2014 *vs* November 2015	0.7292
	March 2015 *vs* April 2016	**0.0134**
	November 2014 *vs* April 2016	0.2145

GLRaV-3 viral titre analysis revealed that in general the virus titre decreased towards the apex of the rootstock shoot, ranging from 0.5-to 6-fold reductions across rootstock cultivars. A significant difference in ORF1a viral titre between basal and apical sampling positions was observed in March 2015 for R110 and Salt Creek/ Ramsey (*t*-test, p=0.0377 and p=0.0034, respectively) (Figure 3). Notably higher basal viral titre levels were observed for R110 in March 2015 compared to November 2014, and April 2016 (*t*-test, p=0.0189 and p=0.0103, respectively). Salt Creek/Ramsey displayed higher basal viral titres in March 2015 compared to April 2016 (*t*-test, p=0.0134) (Table 6).

Table 7: Two-sided *t*-tests to determine statistically significant differences in viral titres in basal tissue between early and late sampling timepoints in the growing season. Significant *p*-values are indicated in bold. A *p*-value significance threshold of 0.05 was selected

Rootstock	Month of sampling	*p*-value
R99	November 2014 *vs* November 2015	0.0878
	December 2014 *vs* December 2015	0.4694
	January 2015 *vs* January 2016	0.0966
	March 2015 *vs* March 2016	0.1520
	April 2015 *vs* April 2016	0.2200
R110	November 2014 *vs* November 2015	0.2406
	December 2014 *vs* December 2015	0.1225
	January 2015 *vs* January 2016	0.3698
	March 2015 *vs* March 2016	**0.0105**
	April 2015 *vs* April 2016	**0.0043**
101-14 Mgt	November 2014 *vs* November 2015	0.2370
	December 2014 *vs* December 2015	0.4025
	January 2015 *vs* January 2016	0.8285
	March 2015 *vs* March 2016	0.7313
	April 2015 *vs* April 2016	0.8521
Ruggeri 140	November 2014 *vs* November 2015	0.1753
	December 2014 *vs* December 2015	**0.0424**
	March 2015 *vs* March 2016	**0.0008**
	April 2015 *vs* April 2016	0.3099
Salt Creek/ Ramsey	November 2014 *vs* November 2015	0.5279
	December 2014 *vs* December 2015	**0.0324**
	March 2015 *vs* March 2016	**0.0255**
	April 2015 *vs* April 2016	**0.0163**

There was no significant change in GLRaV-3 viral titre in basal tissue between November 2014 and April 2016 for R99 and 101-14 Mgt. For rootstock Salt Creek/Ramsey, there was a significantly higher viral titre in December 2014 compared to December 2015, resulting in a 3-fold reduction of relative viral titre (*t*-test, p=0.0324), a 4-fold reduction between March 2015 and March 2016 (*t*-test, p=0.0255) and a 5-fold reduction between April 2015 and April 2016 (*t*-test, p=0.0163) (Table 7).

Rootstock R110 had a 13-fold reduction in viral titre in March 2016 compared to March 2015 (*t*-test, *p*=0.0105) and a 2-fold reduction in April 2016 compared to April 2015 (*t*-test, *p*=0.0043). Ruggeri 140 with a 4-fold reduction had a significantly higher viral titre in December 2014 compared to December 2015 (*t*-test, *p*=0.0424) and a 4-fold reduction in March 2016 compared to March 2015 (*t*-test, *p*=0.0008).

5. Discussion

The aim of this study was to determine the optimal tissue and time of collection best suited for GLRaV-3 detection in rootstocks. Two different regions were collected from each grapevine and a single primer set targeting the GLRaV-3 ORF1a genomic region was used to measure relative virus concentration. Relative quantification compensates for the drawbacks of absolute quantification by measuring only the relative change in GLRaV-3 viral titre without attempting to determine exact copy number. To ascertain reliable relative quantification results, accurate normalization of the gene of interest is required (Pfaffl, 2007). The gene of interest was normalized to a previously validated internal reference gene (Reid et al., 2006) undergoing simultaneous and similar amplification. Factors such as RNA integrity, loading errors, and primer efficiencies affect quantification (Fleige and Pfaffl, 2006).

The specificity of the LR3 primer set was confirmed by gel-electrophoresis and melt curve analysis. The LR3 primer pair targets and amplifies a 144 bp region within the conserved ORF1a gene contained by all variants of GLRaV-3. In this study, the relative viral titre of one reference gene in GLRaV-3 infected phloem rootstock material was analysed, namely; α-tubulin. It should, however, be noted that the use of only one reference gene for normalization can result in under- or overestimations of relative transcript abundance (Kozera and Rapacz, 2013). The reference gene primers, which were designed based on *V. vinifera* sequences, were used in this study to detect α-tubulin in rootstock cultivars (*Vitis* species other than *vinifera)* and this may have contributed to a lower efficiency in detection.

When the individual rootstock types were examined, no universal trends could be discerned, and the data was difficult to interpret. The results of this study did, however, indicate that relative viral titre of GLRaV-3 was significantly higher in the basal tissue compared to apical tissue of R110 and Salt Creek/Ramsey (Table 5). This contradicts

results found by Bester et al. (2014) who observed higher titres of GLRaV-3 in new growth material (Bester et al., 2014). The study did correspond with the results found by Chooi et al. (2016), where variants of GLRaV-3 had high detectability at the base sampling region (Chooi et al., 2016). Figure 3 (a-e) indicated that over the course of the years there was a general decrease in viral titre between basal and apical tissues, ranging from 0.5- to 6-fold reductions across all rootstock cultivars excluding 101-14 Mgt. Significant differences in viral titre between 2014-2016 were observed in basal tissue for R110, Salt Creek/Ramsey and Ruggeri 140 (Table 6 and Table 7). These reductions in titre suggest that the best time of the year to sample is late in the growing season during the early stages of grapevine development and would be best in March/April for R110, in December/March for Ruggeri 140 and December, March, or April for Salt Creek/Ramsey. Rootstock R99 and 101-14 Mgt had no significant change in viral titre in basal tissue over the growing season. The detection of higher viral concentrations within the basal region of rootstock suggests that the virus accumulates within older more established tissue, resulting in a higher viral load when compared to younger rootstock tissue.

A *V. vinifera* lineage representative rootstock (Figure 2) was included to compare differences between *Vitis* species. Previous studies have indicated that erratic viral titres also occur in *V. vinifera* and have been observed to be seasonal (Monis and Bestwick, 1996, Tsai et al., 2012). Detection of GLRaV-3 in US 8-7 was found to be significantly higher than all other rootstock cultivars, except for R110. The difference in detection between US 8-7, which contains a *V. vinifera* parental line, and other rootstocks tested, which do not, confirm that detection in rootstock material is indeed poorer than in *V. vinifera* (scion material).

Salt Creek/Ramsey (parentage and *Vitis* species) differs significantly from other rootstock cultivars (Malan and Meyer, 1993) and is not considered to be an ideal rootstock for wine grapes due to its high potassium uptake. The rootstock is, however, predominantly utilised in the table grape industry where it performs well in sandy soil (Saayman, 2009). The distinct differences in parentage could perhaps explain the different GLRaV-3 detection pattern exhibited by Salt Creek/Ramsey when compared to the other rootstock cultivars. The rootstocks used by Chooi et al. (2016) were 3309C

and Schwarzmann, which have the most similar parentage to 101-14 Mgt used in this study and showed similar results.

Out of the 576 rootstock samples tested, 42% were unsuccessful for GLRaV-3 detection. A negative result from grapevines infected with GLRaV-3 may be due to the absence of the virus or a low viral titre in the tissue samples tested. Factors such as plant age, the season of sampling, and the genomic region selected for detection used in this study may have also contributed to the negative results.

The growing season for grapes in South Africa lasts from October through to March yearly. Following this pattern, all rootstock petioles were collected during the growing season between November 2014 and April 2016 to determine a suitable timeframe for rootstock sample collection and testing. The data showed that, in general, phloem material collected in March during the first year of growth provided the highest viral titre (Table 6 and Table 7). Viral load was found to be lowest in January-April, just over a year post-initial sampling, possibly due to higher levels of polyphenolic compounds having inhibited the RT-qPCR reactions. Future studies should include amplification with an endogenous reference at different dilutions of template in order to calculate the PCR efficiency and determine if there is PCR inhibition. To increase the reliability and accuracy of diagnostic tests, it is recommended that GLRaV-3 infected grapevines be tested in December-March when plant material is still juvenile.

6. Conclusion

This study provides useful guidelines for increased reliability and accuracy of GLD detection. It is recommended that samples consisting of petioles collected from mature basal leaves are tested during late summer/beginning of autumn(December-March). The proposed sampling and collection strategy can form part of a throughput diagnostic system to compliment the sensitive detection methods available for grapevine screening and the certification programs. Further research is, however, required to substantiate these possibilities which should include virus testing throughout the year to determine whether GLRaV-3 is more prevalent in the growing or dormant seasons. Due to the high diversity of the GLRaV-3 genome and the influence of GLRaV-3 on the host plant, a relative quantification model with multiple reference genes would provide more reliable quantification data.

Acknowledgements

This research was supported by Winetech and the University of Pretoria. The authors would like to thank Dr. J.L Staphorst and Mr. G.G.F Kasdorf at the Agricultural Research Council-Plant Protection Research Institute, Roodeplaat (ARC-PPRI) for allowing the continued use of their inoculated rootstocks.

References

BESTER, R., PEPLER, P., BURGER, J. & MAREE, H. 2014. Relative quantitation goes viral: An RT-qPCR assay for a grapevine virus. *Journal of virological methods,* 210**,** 67-75.

BEUVE, M., SEMPÉ, L. & LEMAIRE, O. 2007. A sensitive one-step real-time RT-PCR method for detecting Grapevine leafroll-associated virus 2 variants in grapevine. *Journal of virological methods,* 141**,** 117-124.

BRUISSON, S., LEBEL, S., WALTER, B., PREVOTAT, L., SEDDAS, S. & SCHELLENBAUM, P. 2017. Comparative detection of a large population of grapevine viruses by TaqMan® RT-qPCR and ELISA. *Journal of virological methods,* 240**,** 73-77.

CHOOI, K. M., COHEN, D. & PEARSON, M. N. 2016. Differential distribution and titre of selected grapevine leafroll-associated virus 3 genetic variants within grapevine rootstocks. *Archives of virology,* 161**,** 1371-1375.

FLEIGE, S. & PFAFFL, M. W. 2006. RNA integrity and the effect on the real-time RT-qPCR performance. *Molecular aspects of medicine,* 27**,** 126-139.

KASDORF, G. G. F. 2006. *Final report: Detection of grapevine viruses in grapevine rootstocks.*Sawis library. Viewed: 12 March 2018, <http://www.sawislibrary.co.za/dbtextimages/FinalReport118.pdf

KOZERA, B. & RAPACZ, M. 2013. Reference genes in real-time PCR. *Journal of applied genetics,* 54**,** 391-406.

LÓPEZ-FABUEL, I., WETZEL, T., BERTOLINI, E., BASSLER, A., VIDAL, E., TORRES, L. B., YUSTE, A. & OLMOS, A. 2013. Real-time multiplex RT-PCR for the simultaneous detection of the five main grapevine viruses. *Journal of Virological Methods,* 188**,** 21-24.

MALAN, A. P. & MEYER, A. 1993. Interaction between a South African population of Xiphinema index and different grapevine rootstocks. *South African Journal of Enology and Viticulture*, 14**,**

MAREE, H. J., ALMEIDA, R. P., BESTER, R., CHOOI, K. M., COHEN, D., DOLJA, V. V., FUCHS, M. F., GOLINO, D. A., JOOSTE, A. E. & MARTELLI, G. P. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology,* 4**,** 82.

MARTELLI, G. P. 2006. *Directory of infectious diseases of grapevines and viroses and virus-like diseases of the grapevine: Bibliographic report 1998-2004*. Centre international de hautes études agronomiques méditerranéennes, Paris, France.

MINAFRA, A. & HADIDI, A. 1994. Sensitive detection of grapevine virus A, B, or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. *Journal of virological methods,* 47**,** 175-187.

MONIS, J. & BESTWICK, R. K. 1996. Detection and localization of grapevine leafroll associated closteroviruses in greenhouse and tissue culture grown plants. *American Journal of Enology and Viticulture,* 47**,** 199-205.

MONTERO, R., MUNDY, D., ALBRIGHT, A., GROSE, C., TROUGHT, M., COHEN, D., CHOOI, K., MACDIARMID, R., FLEXAS, J. & BOTA, J. 2016. Effects of Grapevine Leafroll associated Virus 3 (GLRaV-3) and duration of infection on fruit composition and wine chemical profile of Vitis vinifera L. cv. Sauvignon blanc. Food chemistry*. South African Journal of Enology and Viticulture,* 197**,** 1177-1183.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research,* 29**,** e45-e45.

PFAFFL, M. W. 2007. Relative quantification. *Real-time PCR.* International University Line, California, USA, Chapter 3, 63-82.

REID, K. E., OLSSON, N., SCHLOSSER, J., PENG, F. & LUND, S. T. 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC plant biology,* 6**,** 27.

SAAYMAN, D. 2009. Rootstock choice: The South African experience. *In: Oral presentation at Rootstock Symposium, SEV 60th Annual Meeting,* Napa, California, 23.

STEWART, S. & NASSUTH, A. 2001. RT-PCR based detection of Rupestris stem pitting associated virus within field-grown grapevines throughout the year. *Plant Disease,* 85**,** 617-620.

TSAI, C., DAUGHERTY, M. & ALMEIDA, R. 2012. Seasonal dynamics and virus translocation of Grapevine leafroll-associated virus 3 in grapevine cultivars. *Plant Pathology,* 61**,** 977-985.

WALSH, H. A. & PIETERSEN, G. 2013. Rapid detection of Grapevine leafroll-associated virus type 3 using a reverse transcription loop-mediated amplification method. *Journal of virological methods,* 194**,** 308-316.

Chapter 5

Grapevine leafroll associated virus 3 specific reverse transcription loop-mediated isothermal amplification for the detection of GLRaV-3 in grapevine rootstocks

1. Abstract

Grapevine leafroll disease (GLD) is the most economically significant viral disease of grapevines in South Africa with grapevine leafroll associated virus 3 (GLRaV-3) being the primary pathogen of GLD. Disease control focuses on ensuring that vineyards receive virus-free planting material, followed by post-planting management including insecticide treatments as well as the identification and removal of infected vines. In established vineyards, infected red cultivar vines are easily identifiable in autumn through symptomatic display, whereas white cultivars require viral testing (ELISA). Although ELISA is simple and reliable, it has a relatively low sensitivity which has prompted the need for field-ready diagnostic methods that are rapid, easy-to-use, provide sensitivity, and specificity. A single-tube, reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay, together with a crude RNA extraction protocol, were adapted and optimised for rapid, on-site GLRaV-3 detection. The WarmStart Colorimetric LAMP 2x Master mix (New England Biolabs, Ipswich, MA, USA) was used in this assay under isothermal conditions at 65°C. Visible colour changes from successful amplification were seen in 70 minutes. The assay was found to be highly specific and more sensitive than reverse transcription-PCR. The use of microcrystalline wax capsules reduced the risk of contamination resulting from the high quantity of amplicons produced. The developed platform could be a possible alternative diagnostic technique to ELISA for on-site GLRaV-3 detection.

2. Introduction

Grapevine leafroll disease (GLD) is one of the most destructive and economically damaging diseases occurring in all major grape-growing regions around the world (Alabi et al., 2016). There are several viruses within the *Closteroviridae* family associated with GLD including Grapevine leafroll associated virus type 3 (GLRaV-3), the most prevalent virus in South African grapevines (Pietersen, 2010).

The disease is currently managed through an integrated control strategy of which the use of certified plant material is a crucial component (Pietersen, 2010, Almeida et al., 2013). The first line of defence is the careful selection of virus-free rootstock/scion material, thereafter post-planting management includes the removal of infected vines known as roguing, and controlling insect vectors through insecticides (Pietersen and Walsh, 2012). This strategy can be implemented in red-berried cultivars by visually monitoring vineyards due to discernible symptomology (Bell et al., 2017), but can only be implemented in asymptomatic white-berried cultivars following virus-specific tests. This is especially difficult in rootstocks where no symptoms are observed and virus tests are unreliable (Cid et al., 2003, Cousins and Striegler, 2005, Maree et al., 2013).

Propagating rooted grapevines directly from cuttings was common practice until the late 19th century when the discovery was made that unlike susceptible *Vitis vinifera* vines grown on their own roots, native American *Vitis* species had natural resistance to the soil-borne aphid, phylloxera (*Daktulosphaira vitifoliae*) (Foëx, 1902, Ollat et al., 2014). From then on, European vines were grafted onto American rootstocks. Now, more than 80% of vineyards worldwide, including South Africa, use grafted plants consisting of a *V. vinifera* scion onto a rootstock of single American *Vitis* species or *Vitis* species hybrids that combine desirable traits of their parentage (Töpfer et al., 2011, Ollat et al., 2014). Rootstocks have been noted to display resistance to several other pests and grapevine diseases (Cousins and Goolsby, 2011, Ferris et al., 2012), however, no complete resistance to GLRaVs has been observed in rootstocks (Golino, 1993, Golino et al., 2000, Maree et al., 2013). Nevertheless, it has been suggested that some rootstocks are less susceptible to GLRaV-3 than others (Ioannou et al., 1999). This, in conjunction with GLRaV being found in very low concentrations in rootstock material, renders detection of the virus problematic (Beuve et al., 2007,

Malan, 2009), especially when virus-free scion is grafted onto symptomless, infected rootstock.

The South African Plant Improvement Scheme (PlantSA) is the authority tasked to ensure the monitoring and control of high-quality plant material in the grapevine industry. Various plant improvement organisations, under the auspices of PlantSA, establish foundation blocks and mother blocks from virus-free nuclear material (through heat therapy or meristem tip culturing) (Almeida et al., 2013). The blocks, in turn, provide plant material free of the viruses specified in the scheme for the establishment of healthy vineyards (Pietersen et al., 2013). Mother blocks are screened yearly for the presence of viruses and where infection exceeds 3% the vineyard is no longer utilised as a mother block for further planting material (Walsh and Pietersen, 2013).

Nuclear stocks used for propagation undergo serological testing using ELISA or nucleic acid-based methods such as reverse transcription-PCR. Although ELISA is less sensitive compared to RT-PCR, its scalability makes it a popular choice for routine diagnostic work in the grapevine industry (Maree et al., 2013). The serological assay is ideally suited for testing scion material but in the case of rootstocks, ELISA is not sensitive enough to detect GLRaVs, especially when rootstocks lacking *V. vinifera* parental lines are used (Cid et al., 2003). The lag time between infection and the time when viral titre is high enough for detection by conventional methods potentially allows for infected vines to be used in the establishment of vineyards (Pietersen, 2004).

Previous studies have observed GLRaV-3 to be present in low concentrations and erratically distributed in rootstock material, rendering reliable detection challenging (Stewart and Nassuth, 2001, Walsh and Pietersen, 2013, Chooi et al., 2016, Harris, 2017). Although molecular tests such as real-time reverse transcription PCR (RT-qPCR) have increased sensitivity, they are usually only available at centralized laboratories with specialised equipment where their operation requires skilled personnel. This is not ideal for basic field laboratories where rootstock material is usually tested.

Developments of *in vitro* nucleic acid synthesis have led to the possibility of amplifying nucleic acids in isothermal (single) temperatures without the requirement of thermocycling equipment. RT-LAMP is an alternative DNA amplification technique to

PCR which can be used to test GLRaV-3 suspected samples (Walsh and Pietersen, 2013).

The GLRaV-3 specific RT-LAMP assay developed by Walsh and Pietersen (2013) is not ideal for on-site GLRaV-3 detection due to difficulties in accurate interpretation of positive results and the high risk of contamination associated with the large quantity of amplicon produced. In this study, RT-LAMP was adapted for on-site detection of GLRaV-3 in rootstock tissue.

3. Materials and Methods

3.1 Plant material and sample preparation

Grapevine leafroll associated virus 3 positive samples used for validation of the RT-LAMP assay included scion petioles (Accessions: 17-7000, and 17-7008) and cane material from scion samples (Accessions: 17-7080, 17-7092, and 17-7096) previously collected during an official survey for GLRaV-3 in 2017 from vineyards in the Western Cape, South Africa. Detailed information on GLRaV-3 presence, absence, and amplicon band strength can be found in Table 1 and Table 2 under the Appendix. In addition, for the comparative study of RT-LAMP and RT-PCR, 50 rootstock samples were collected in 2017 from vines where the scions displayed clear leafroll symptoms. The rootstock samples were tested in parallel. Table 1 lists the five different rootstock types tested and the respective parentage. Ten samples per rootstock type were tested.

Table 1: Five different rootstock clones (and respective parentage) sampled and used in reverse transcription loop-mediated isothermal amplification of Grapevine leafroll associated virus 3

Rootstock	Cross source
US 8-7	*V. berlandieri x V. rupestris x Jacquez*
101-14 Mgt	*V. riparia x V. rupestris*
Paulsen 1103	*V. berlandieri x V. rupestris*
Ruggeri 140	*V. berlandieri x V. rupestris*
Richter 110	*V. berlandieri x V. rupestris*

3.2 Total RNA extraction

Total RNA was isolated from 200 mg of phloem material using a modified cetyltrimethylammonium-bromide-based (CTAB) RNA extraction protocol (White et al., 2008). Plant material was weighed out and homogenized in liquid nitrogen using a mortar and pestle. To each sample, 1.2 mL CTAB buffer (2% CTAB, 2% soluble PVP-40, 25 mM EDTA, 2 M NaCI, 100 mM Tris-HCI pH 8.0, and 3% β-mercaptoethanol) was added and incubated at 65^{o}C for 30 minutes. Samples were centrifuged for 10 minutes at 12303 x g. A chloroform-isoamyl (24:1) extraction step was performed twice by adding an equal volume to the supernatant, vortexing for 30 seconds and centrifugation for 15 minutes at 14269 x g at 4 ^{o}C. Following an overnight Lithium Chloride (2 M) precipitation at 4^{o}C, RNA was precipitated by centrifugation at 12303 x g for 60 minutes at 4^{o}C. After a wash step with 70% ethanol, pellets were dried and resuspended in 50 µL molecular grade H_2O (Life Technologies; Waltham, MA, USA). The quality of RNA was assessed by spectrophotometry, using the NanoDrop 200 Spectrophotometer (Thermo Fisher, Waltham, MA, USA) and gel electrophoresis (2% Tris-acetate-EDTA (TAE) agarose gel).

3.3 Reverse transcription loop-mediated isothermal amplification primer design

Ten available GLRaV-3 whole genome sequences (Table 2) were retrieved from GenBank (www.ncbi.nih.gov) and imported into BioEdit Sequence Alignment Editor software version 7.2.5 (Hall, 1999). A multiple alignment was generated using ClustalW. Conserved regions were identified and used in the primer design to flank partial sequences of the RNA-dependent RNA polymerase gene (RdRp).

Table 2: Grapevine leafroll associated virus 3 complete genome sequences obtained from GenBank (www.ncbi.nih.gov) used in the reverse transcription loop-mediated isothermal amplification assay primer design

Isolate	Group	GenBank accession number	Reference
GP18	II	EU259806	(Maree et al., 2008)
621	I	GQ352631	(Jooste et al., 2010)
623	II	GQ352632	(Jooste et al., 2010)
PL-20	III	GQ352633	(Jooste et al., 2010)
CI-766	I	EU344893	(Engel et al., 2008)
NY-1	I	AF037268	(Ling et al., 1998)
LN	III	JQ423939	(Fei et al., 2013)
GH11	VI	JQ655295	(Bester et al., 2012)
GH30	VI	JQ655296	(Bester et a., 2012)
GH24	VII	KM058745	(Maree et al., 2015)

In addition to the LAMP primers designed by Walsh and Pietersen (2013) (Table 3), two additional primers sets were designed using Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html).

Table 3: Published primer sequences used in this study for reverse transcription loop-mediated isothermal amplification of Grapevine leafroll associated virus 3 (Walsh and Pietersen, 2013)

Primer	Type	Position	Sequence (5'-3')	Reference
F3	Forward Outer Primer	7728-7747	GAAGTGTAACCTCGTCACGT	(Walsh and Pietersen, 2013)
B3	Backward Outer Primer	7941-7958	GCCCGCTTGAGAGACTTG	(Walsh and Pietersen, 2013)
FIP	Forward Inner Primer (F1c-TTTT-F2)	F1c: 7793-7813 F2: 7752-7771	CATGCGCCACAGAGTCGTCA CTTTT-AAATGGGAATTTCAACGCCG	(Walsh and Pietersen, 2013)
BIP	Backward Inner Primer (B1c-TTTT-B2)	B1c: 7851-7871 B2: 7909-7928	GCTCGTTTAGCAGAGGTGAC GGTTTT-GCCCTTTTGTCCAACCAATC	(Walsh and Pietersen, 2013)

Primers were compared to GLRaV-3 genomes using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and primers with the greatest specificity were selected (>98% homology) (Table 4) and synthesised by Integrated DNA Technologies (Iowa, USA).

Table 4: Additional primer sequences for reverse transcription loop-mediated isothermal amplification of Grapevine leafroll associated virus 3

Primer	Position	Sequence (5'-3')
2890F3	75- 94	TCGGACTTTGTCGACAGGAT
2890B3	281- 300	AGCGAATACTCTTCGCCCTA
2890FIP	F1c: 141-161	CGCCCCAACGGCATACTCAAA-
	F2: 95- 114	CTATTCCTACGCGCTCAAC
2890BIP	B1c: 196- 215	GTCGCGTCGTCCACAGCAAG-
	B2: 248- 267	GAGCCATAACAGCGACAACT
6506F3	75- 92	TATGGTGGGCGAATCGTT
6506B3	239- 259	CCAAATTCGGATCTCTCTTCT
6506FIP	F1c: 138- 160	CGTCCACTAACATCGTCGTCTTA-
	F2: 98-122	CTTTTGAATACAAGTGCTATAATGC
6506BIP	B1c: 165- 185	CGTCAAGTCACCCAATAGCAC-
	B2: 218- 237	CACCGCCATATTTATGTCCT

3.4 Reverse transcription loop-mediated isothermal amplification assay

All RT-LAMP reactions were performed in a 25 µL reaction mixture that contained: 12.5 µL WarmStart Colorimetric LAMP 2x Master mix (New England Biolabs, Ipswich, MA, USA), 2.5 µL LAMP quadruple primer mixture (1.6 µM FIP, 1.6 µM BIP, 0.2 µM F3, and 0.2 µM B3), 3.5 µL DEPC (Thermo Fisher), 5.5 µL nuclease-free H_2O (Life Technologies) and 1 µL of RNA. The reaction mixture was incubated at 65^{o}C using a heating block (Eppendorf Thermostat Plus 3130, Hamburg, Germany) for 80 minutes. A sample was recognised as positive when the reaction colour changed from pink to yellow.

3.5 Sensitivity of the reverse transcription loop-mediated isothermal amplification assay

The analytical sensitivity of the assay was evaluated by testing GLRaV-3 positive RNA of accession 17-7096 (109.6 ng/µL) that was diluted in nuclease-free water. Ten-fold dilutions (1:10; 1:100; 1:1000; and 1:10 000) were prepared and analysed with the RT-

LAMP assay for the detection GLRaV-3 against all three primer sets. The samples were analysed in triplicate per primer set.

3.6 Optimisation

The RT-LAMP reactions were optimized by assessing different incubation temperatures and varying lengths of incubation. Temperature optimisation was carried out at: 60, 62, 64, and 65 ^{o}C. Incubation time ranged from 40-80 minutes where colour changes were monitored every ten minutes. All optimisation reactions included two negative controls: negative RT-LAMP control (where no RNA template was present) and a healthy host control (uninfected grapevine RNA sample).

3.7 Specificity and selectivity

The analytical specificity was assessed throughout the study by including a host control (uninfected RNA) in each set of reactions. The developed assay was designed to discriminate *Vitis* species RNA from GLRaV-3 RNA. The selectivity of the assay was evaluated by testing different grapevine cultivars and plant material, i.e. scion petioles, cane material, and rootstock tissue.

3.8 Comparative study of reverse transcription loop-mediated isothermal amplification assay and reverse transcription-polymerase chain reaction

The sensitivity of the RT-LAMP assay was compared to that of an established GLRaV-3 RT-PCR assay (Goszczynski, 2013) by testing five different rootstock cultivars consisting of 10 samples each. Reverse transcription-PCR was performed in a final reaction volume of 25 µL, as previously described (Goszczynski, 2013), using the primers listed in Table 5 to produce an amplicon representing the helicase gene. A 2% agarose gel electrophoresis was performed to detect the presence of the 549 bp amplicon. The RT-LAMP reactions were carried out at 65°C for 70 minutes according to the method described in section 3.4. Differences in detection of GLRaV-3 in rootstock material between the two assays were statistically analysed using the two-proportion hypothesis *z*-test at a 95% confidence interval.

Table 5: Oligonucleotide primers for Grapevine leafroll associated virus 3 detection using RT-PCR

Target	Primer	Sequence 5'-3'	Product size(bp)	Annealing temp (°C)	Reference
GLRaV-3	Hel2F Hel2R	GGCGAAGAGTATTCGCTC CCAGAAAAGGCCTTCGTC	549	52	(Gozsczynski, 2013)

3.9 Crude RNA extraction

For nucleic acid purification from petioles, 200 mg of sample was homogenized using a mortar and pestle in the presence of 1 mL extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2% (w/v) polyvinylpyrrolidone (PVP), and 1% Tween-20) for approximately 30 seconds (Zou et al., 2017). A 4 mm^2 disc was punched from Whatman No.1 filter paper and transferred into the mortar for three seconds. The disc was then placed in 200 µL of wash buffer (10 mM Tris pH 8.0 and 0.1% Tween-20) for one minute and directly immersed into the RT-LAMP reaction tube where the reaction was performed to completion without removing the disc.

3.10 Microcrystalline wax capsule

The opening of post-amplification RT-LAMP reaction tubes, albeit accidently, holds the risk of contamination due to the high copy number of amplified LAMP products (Tomita et al., 2008). Even a small amount of aerosolised amplicon released in the laboratory could result in contamination that is extremely difficult to rid. This potential risk of contamination would drastically reduce the suitability of RT-LAMP as an alternative on-site tool for GLRaV-3 detection.

Microcrystalline wax has a higher molecular weight and melting temperature (95°C) compared to paraffin waxes and is easier to cast into a mould (Tao et al., 2011). The use of a microcrystalline wax capsule would ensure that contamination is prevented, should reaction vessels unintentionally be opened in the field laboratory setting.

According to the method outlined by Tao et al. (2011) the distal end of a 1 mL disposable syringe was removed, and the remaining stem and plug used as a mould. Microcrystalline wax was placed in a stainless-steel laboratory spoon and melted over

an open flame. Approximately 0.3 mL of liquid wax was poured into the syringe mould, left to cool and solidify, and then pushed out the syringe using the plug. Capsules were prepared in batches and stored at 4ºC.

4. Results

4.1 RNA extraction and Grapevine leafroll associated virus 3 reverse transcription-PCR

RNA was successfully extracted and positive controls were confirmed through GLRaV-3 specific RT-PCR directed against the conserved helicase gene producing a product size of 549 bp (Goszczynski, 2013). Amplicon products were viewed by agarose gel electrophoresis (Figure 1).

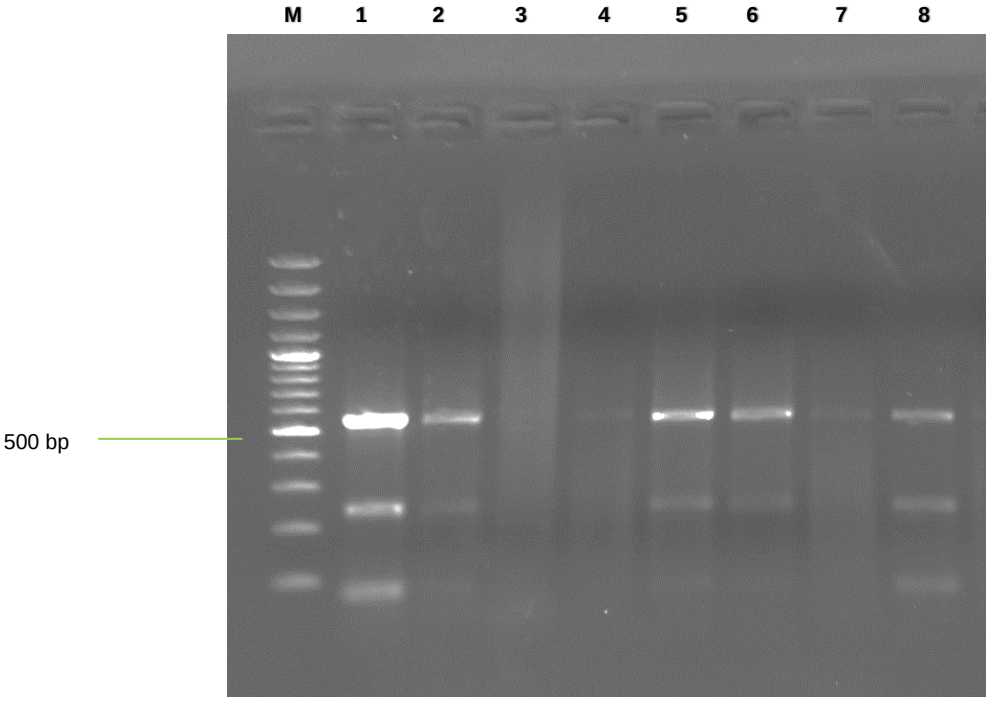


Figure 1: 1% (w/v) Agarose gel electrophoresis following Grapevine leafroll associated virus 3 specific PCR of six grapevine scion samples. Lanes: (M) O'GeneRuler 100 bp Plus DNA ladder (0.1 µg/µL) (Thermo Fisher), (1) Grapevine leafroll associated virus 3 amplicon PCR control, (2) cDNA synthesis positive control (3) healthy control, grapevine infected accessions:(4) 17-7000, (5) 17-7008, (6) 17-7080, (7) 17-7092, (8) 17-7096.

4.2 Reverse transcription loop-mediated isothermal amplification evaluation

The RT-LAMP assay was preliminarily evaluated using the WarmStart Colorimetric LAMP 2x Master mix (New England Biolabs) and the three sets of GLRaV-3 specific primers. The performance of the RT-LAMP assays (using all three primer sets) was initially tested on five GLRaV-3 positive samples, one GLRaV-3 negative sample, and on a negative control where RNA was substituted for nuclease-free H_2O (Life Technologies). The LAMP products were not analysed using agarose gel electrophoresis due to high
contamination implications as seen in the study by Walsh and Pietersen (2013). The RT-LAMP assays were run at 65°C. Amplification was seen to occur for all positive samples against all three primer sets, whilst no amplification was observed in either the GLRaV-3 negative control nor in the no template control (Figure 2). Having established that all three sets of primer pairs were able to successfully detect GLRaV-3 at the selected temperature of amplification the assay was optimised to determine sensitivity and the shortest time of positivity for each given primer set.

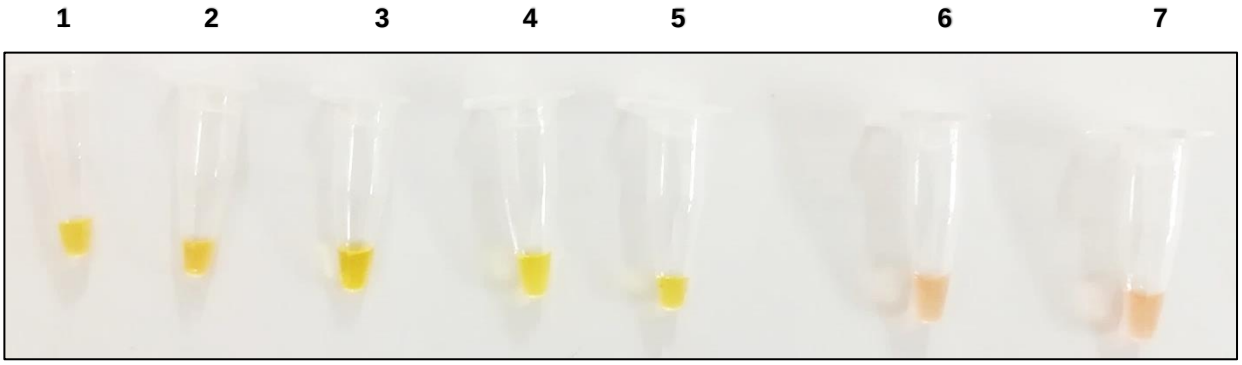


Figure 2: Visualisation of colour changes of the reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay. Tubes: grapevine infected accessions:(1) 17-7000, (2) 17-7008, (3) 17-7080, (4) 17-7092, (5) 17-7096, (6) healthy host control, and (7) no template control. Example provided of primer set 6506.

4.3 Sensitivity of the reverse transcription loop-mediated isothermal amplification assays

The relative detection limit for the RT-LAMP assay was determined using RNA of accession 17-7096 (109.6 ng/µL) against all three primer sets. Negative controls were included. The testing of a dilution series showed that the RT-LAMP assay using the

primer pair designed by Walsh and Pietersen (2013), primer pair 6506, and primer pair 6810 detected as low as a 1:100 dilution. All three replicates were indicated as positive for each of the three primer sets (Figure 3). Although all three primer sets had equal sensitivity the primer set designed by Walsh and Pietersen (2013) was selected for all further RT-LAMP assay optimisation as the established primer pair allowed for direct comparison between the two LAMP assays.

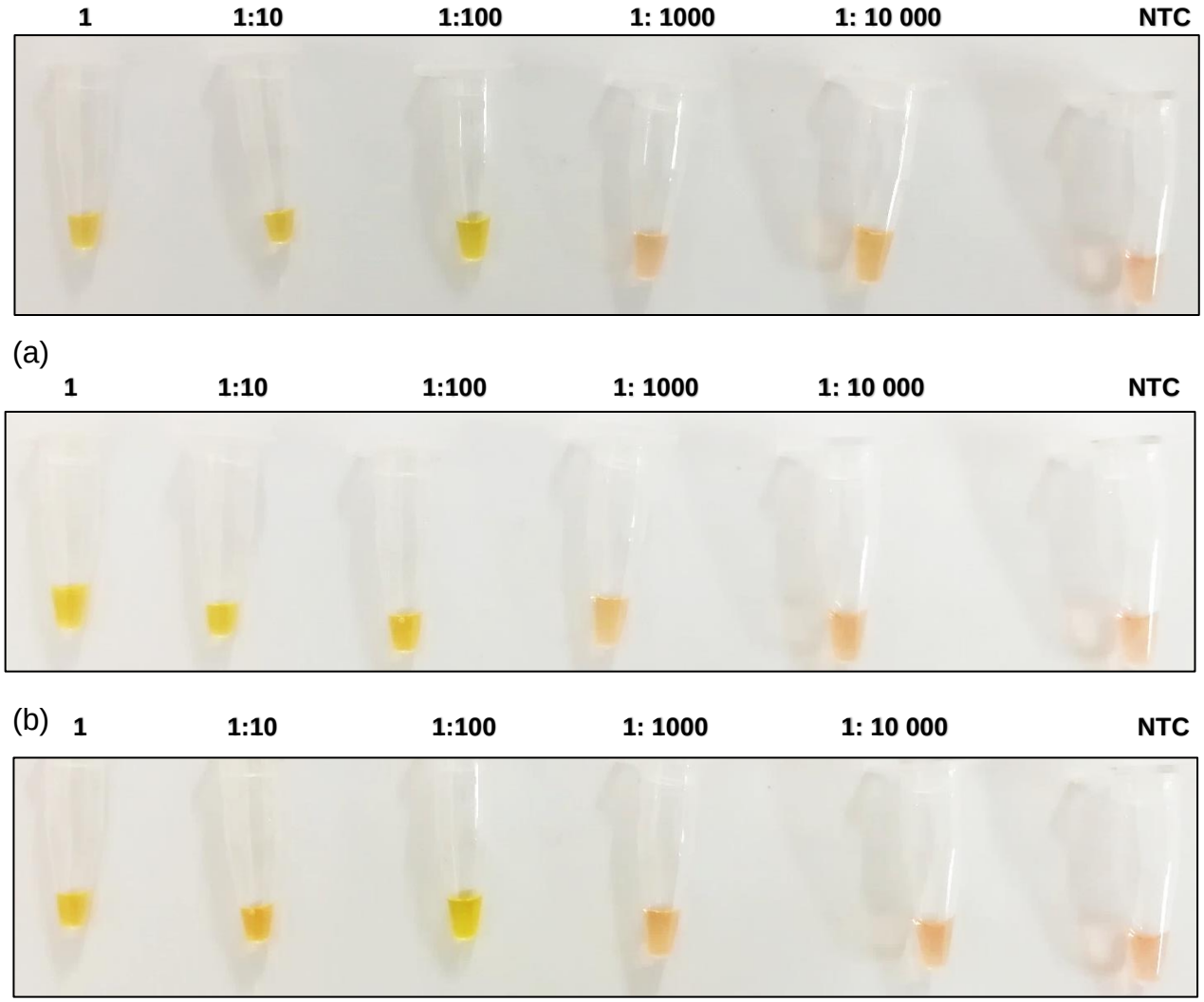


Figure 3: Detection limits of the Grapevine leafroll associated virus 3 reverse transcription loop-mediated isothermal amplification assay of RNA (Accession: 17-7096), (a) primer set designed by Walsh and Pietersen (2013), (b) primer set 6506, and (c) primer set 6810. NTC=No template control.

4.4 Optimisation

To determine the optimal temperature, RT-LAMP reactions were incubated at: 60, 62, 64, and 65^{o}C where reactions were monitored at 10-minute intervals between 40-80 minutes of incubation. Reaction efficiency was measured by the time of positivity indicated by a colour change from pink to yellow. Temperatures of 60-63°C resulted in no colour changes in any of the reactions during the incubation time period, whereas reactions incubated at 64°C and 65°C had colour changes after 80 minutes and 70 minutes, respectively (Figure 4).

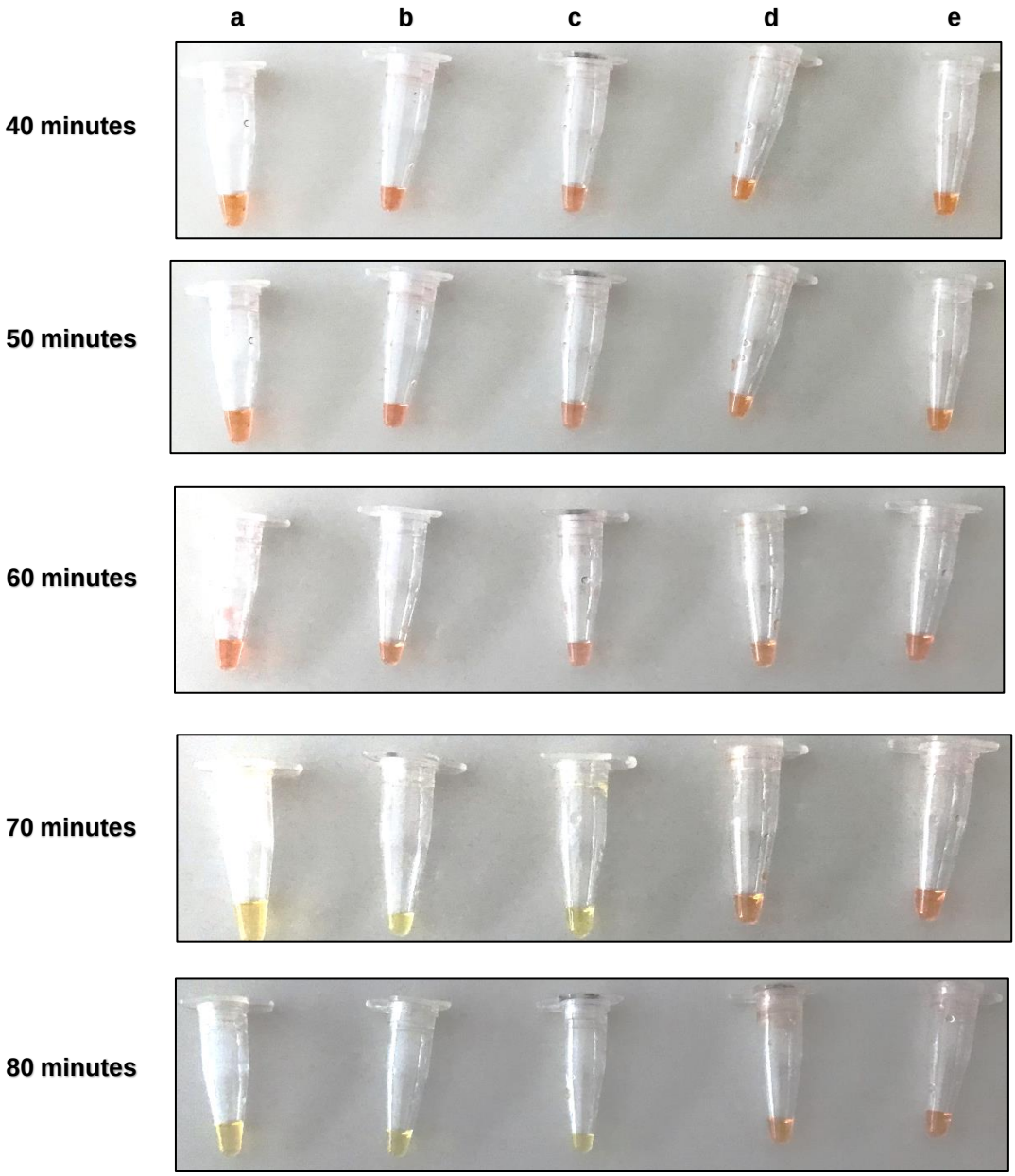


Figure 4: Image depicting results of reverse transcription loop-mediated isothermal amplification of known GLRaV-3 positive samples, a healthy plant control and a no template control at 65°C using the primer set designed by Walsh and Pietersen (2013). Reactions were monitored at 10-minute intervals from 40-80 minutes. Tubes: grapevine infected accessions (a-c): (a) *17*-7000, (b) 17-7080, (c) 17-7092, (d) healthy host control, and (e) no template control.

4.5 Specificity and selectivity

No cross-reactivity of the RT-LAMP reaction was observed with host RNA, as no positive result was obtained with healthy controls, confirming the validity of the assay. In terms of selectivity, results were found to be consistent amongst GLRaV-3 positive scions, irrespective of cultivar and tissue type tested.

4.6 Comparative study of the detection rate of GLRaV-3 in rootstocks through reverse transcription loop-mediated isothermal amplification and reverse transcription-PCR

When using the primer set designed by Walsh and Pietersen (2013) the RT-LAMP results indicated that of the 50 rootstock samples tested, 26 samples (52%) were positive for GLRaV-3 whereas, 48% of rootstock samples tested negative (Table 6). Conventional RT-PCR (Gozsczynski, 2013) of the same samples resulted in six out of 50 (24%) positives with the remaining 36 rootstocks (76%) testing negative for GLRaV-3. The detection rate of GLRaV-3 in rootstocks was significantly higher ($z=3.9279$, $p<0.05$) in RT-LAMP than in RT-PCR.

Table 6: Results comparing detection rate of Grapevine leafroll associated virus 3 in different rootstocks using reverse transcription loop-mediated isothermal amplification with primers designed by Walsh and Pietersen (2013) and reverse transcription-polymerase chain reaction (Gozscynski, 2013)

Rootstock clone	RT-PCR		RT-LAMP		N	Tissue	
	+	-	+	-	10	Petioles	Bark
R110	0	10	3	7	10	4	6
US 8-7	0	10	7	3	10	4	6
101-14 Mgt	0	10	2	8	10	2	8
Ruggeri 140	0	10	5	5	10	7	3
Paulsen 1103	6	4	9	1	10	7	3
Total	6	44	26	24	50		

N=Total amount of vines tested

With the RT-LAMP assay, rootstock Paulsen was seen to have the highest detection rate, with nine out of ten (90%) positives compared to 101-14 Mgt with the least number of infected individuals, two out of ten (20%) positives.

It should also be highlighted that two out of the seven US 8-7 samples, one out of three R110 samples, and one out of five Ruggeri 140 samples that tested positive for GLRaV-3 were RNA extracts from bark scrapings and not petioles.

4.7 Crude extraction protocol

Rootstock Paulsen clones, one with a high GLRaV-3 titre, Accession: 18-4015 (72.4 ng/µL), and one with a low GLRaV-3 titre, Accession:18-4019 (10.8 ng/µL), were tested in conjunction with a healthy control (uninfected RNA) to ensure the specificity of the method. Detailed information on the samples tested is given in Table 2 under the Appendix.

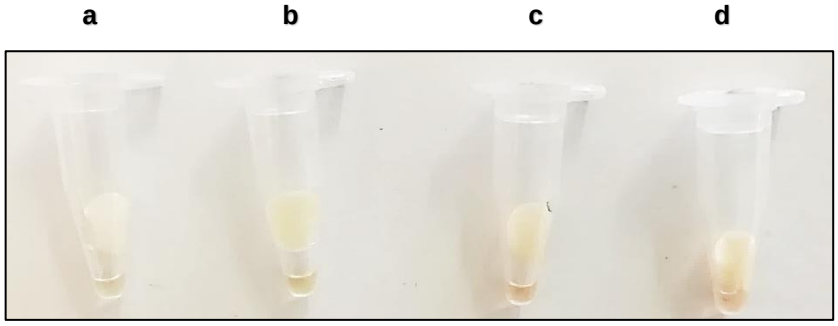


Figure 5: Image depicting results of reverse transcription loop-mediated isothermal amplification on crude homogenates. Tubes: (a) reverse transcription loop-mediated amplification of low viral titre indicated by a pale-yellow colour, (b) reverse transcription loop-mediated amplification of high viral titre indicated by a slightly darker yellow colour, (c) healthy plant control indicated by pink colour, and (d) negative RT-LAMP control indicated by pink colour.

The crude RNA extractions were successful in producing the desired colour changes for the appropriate samples (Figure 5), however, colour changes were delayed by up to 10 minutes compared to the conventional RNA extraction method on the same accession numbers which were tested in parallel.

4.8 Microcrystalline wax capsule

The microcrystalline wax blocks were inserted into 0.2 mL Eppendorf tubes after the RT-LAMP mixture and RNA had been added. The wax blocks remained situated above the LAMP reactions during amplification and no contact between the wax and the reagents/ RNA was observed. After 70 minutes (the predetermined time of positivity) the reaction temperature was increased to 95^{o}C for five minutes to melt the wax capsules. The reaction tubes were removed from the heating block at which point the wax immediately cooled and solidified, forming seals over the reaction mixtures (Figure 6).

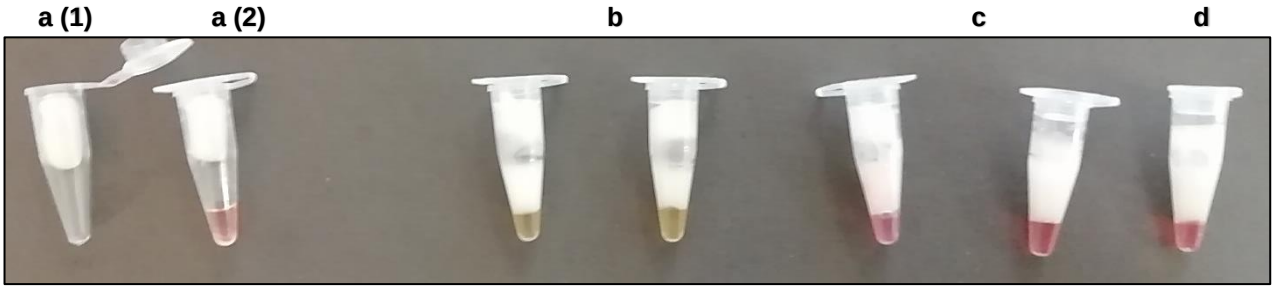


Figure 6: Use of microcrystalline wax capsules in reverse transcription loop-mediated isothermal amplification. Tubes: (a) (1) an example of how the wax capsule fit into the Eppendorf tube (a) (2) an example of the wax capsule inserted in the Eppendorf tube once the reaction mixture and RNA had already been added, indicating the absence of contact between the wax and reagents prior to amplification, (b) infected samples after isothermal amplification and 5 minutes at 95°C the wax capsule remained intact and formed a solid barrier over the reaction mixture, indicated by yellow colour change, (c) two healthy controls, and (d) a reverse transcription loop-mediated amplification negative control.

5. Discussion

Previous studies have observed the extraction of RNA from grapevine plant material to be hindered by the presence of inhibitory enzymes and other substances, which decrease both sensitivity as well as inhibit RT-PCR (Al Rwahnih et al., 2012). Extraction methods are usually cumbersome, expensive, or require specialised equipment, rendering them ineffective for large-scale on-site testing. The results obtained suggest that the crude RNA extraction method was capable of purifying nucleic acids from inhibitors, whilst keeping RNA intact. It should be noted, however, that whilst the crude RNA extraction method has been proven successful, the time of positivity was delayed by 10 minutes when compared to conventional RNA extraction methods (Figure 5). This could have been as a result of: (1) a large quantity of RNA loss during the extraction or (2) the result of some inhibitory substances still present.

The aim of the study was to optimise a GLRaV-3 specific RT-LAMP assay which could potentially be used as an additional in situ diagnostic tool for GLRaV-3 detection in grapevine rootstocks. The RT-LAMP assay was shown to be sensitive (Figure 3) and amplified with a high level of specificity and efficiency at an isothermal temperature of 65°C. The assay used four individual primers to detect a conserved region of the GLRaV-3 genome with the WarmStart Colorimetric LAMP 2x Master mix kit (New England Biolabs). This, in conjunction with the simple extraction method and microcrystalline wax capsules (Figure 6), reduced the complexity and potential contamination of the assay. The assay together with the cost-effective extraction method, could be easily performed in a basic laboratory.

The robustness of the LAMP assay can be affected by the choice of indicator (Kaarj et al., 2018). The WarmStart Colorimetric LAMP 2x Master mix (New England Biolabs) contains a visible pH indicator that allowed for clear visual detection of isothermal amplification (Figure 2) and has already been used in a number of studies for virus detection (Kaarj et al., 2018, Ahn et al., 2019, Yu et al., 2019). Extensive DNA polymerase activity results in protons being produced and subsequently, a drop in the pH of the solution produces a colour change from pink to yellow providing a discernible positive result. This contrasted the RT-LAMP assay developed by Walsh and Pietersen (2013), which made use of a Hydroxy Naphthol Blue (HNB) indicator which resulted in a positive colour change from violet to sky blue. The poor colour change of

this indicator would potentially increase the possibility of inconclusive results when performing on-site testing.

Grapevine rootstocks are notoriously known for low viral titres and erratic viral distribution (Cid et al., 2003, Saayman, 2009, Almeida et al., 2013, Maree et al., 2013). It has been suggested that the poor detection of virus in rootstock tissue is a combination of the low viral titres (Fiore et al., 2008) and diagnostic techniques that have low sensitivity (Maree et al., 2013). LAMP, however, is independent of starting material amount (Tomlinson et al., 2010, Thai et al., 2004), thus allowing for the detection of even small amounts of virus present in rootstocks. This was seen by the significantly higher GLRaV-3 detection rate in rootstock cultivars using RT-LAMP over RT-PCR (Table 6). This was once again made apparent when the crude RNA extraction method was used (Figure 5), even though it was carried out only in a low number of samples.

Studies of translocation of GLRaV-3 in *V. vinifera* grapevines found that virus particles group together in plant tissue, however, this was not the case in any studied rootstock variety (Cid et al., 2003). This suggests that rootstocks may possess a biological or chemical defence against GLRaV-3. Overall 52% of rootstock samples tested positive for GLRaV-3 against GLRaV-3 specific RT-LAMP with varying percentage positives between the different cultivars. Paulsen had the highest (90%) detection rate, whilst 101-14 Mgt had the lowest (20%) detection rate. These results were similar to those found a previous study (Alkowni et al., 2004). The differences in detection rates amongst rootstocks could potentially be attributed to varying degrees of susceptibility of rootstock cultivars to GLRaV-3 infection. Research has shown there to be a close association between rootstock cultivar, virus, and rootstock response to viral infection (Maree et al., 2013).

The RT-LAMP assay had sensitivity comparable to that of qPCR but was unable to detect GLRaV-3 in all the rootstock samples tested this could be due to (1) a lack of infection in the rootstock altogether or (2) the erratic distribution of virus in rootstock tissue resulting in false negatives. In this study, only petiole and bark scrapings collected over May 2017/2018 were tested and although four out of seven bark scrapings tested positive for GLRaV-3, further investigation into the most prominent

material sampled needs to be conducted. A study by Bell et al. (2009) found grapevine root material to contain persistent viral titres of GLRaV-3.

The findings of this study may contribute to the successful management of GLRaV-3 in rootstocks within the Wine Grape Certification Scheme by identifying rootstock cultivars that are more likely to have lower viral titres and thus require additional modes of GLRaV-3 testing for confirmation of results prior to use in the industry. The use of LAMP technology is a promising alternative isothermal technique due to its simplistic workflow, compactness and mobility of equipment, and the time efficiency. However, further studies need to be done to assess the best rootstock material for testing (petiole, bark scrapings, or roots) and the most suitable time of year for sampling.

6. Conclusion

The RT-LAMP assay is a relatively rapid (70 minutes) and simple method that serves as an ideal alternative diagnostic tool for on-site qualitative results regarding the detection of GLRaV-3 in vineyards. The GLRaV-3 specific RT-LAMP assay can be implemented in the detection of virus in rootstocks where it has been shown to be more efficient than RT-PCR. The RT-LAMP assay has already developed into a method of choice for the rapid and cost-effective detection of several diseases (Budziszewska et al., 2016, Carrillo et al., 2018, Xia et al., 2019). The on-site application of the assay could potentially increase response times to outbreaks, reduce spread, and ultimately contribute to the control of GLD. Upon further understanding of the erratic distribution of GLRaV-3 in rootstock material the RT-LAMP assay would be a worthwhile addition to the molecular diagnostic tests currently employed for viral detection.

References

AHN, S. J., BAEK, Y. H., LLOREN, K. K. S., CHOI, W.-S., JEONG, J. H., ANTIGUA, K. J. C., KWON, H.-I., PARK, S.-J., KIM, E.-H. & KIM, Y.-I. 2019. Rapid and simple colorimetric detection of multiple influenza viruses infecting humans using a reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic platform. *BMC infectious diseases,* 19**,** 676.

AL RWAHNIH, M., OSMAN, F., SUDARSHANA, M., UYEMOTO, J., MINAFRA, A., SALDARELLI, P., MARTELLI, G. & ROWHANI, A. 2012. Detection of Grapevine leafroll-associated virus 7 using real time qRT-PCR and conventional RT-PCR. *Journal of virological methods,* 179**,** 383-389.

ALABI, O. J., CASASSA, L. F., GUTHA, L. R., LARSEN, R. C., HENICK-KLING, T., HARBERTSON, J. F. & NAIDU, R. A. 2016. Impacts of grapevine leafroll disease on fruit yield and grape and wine chemistry in a wine grape (Vitis vinifera L.) cultivar. *Public Library of Science One,* 11**,** 2-15.

ALBACETE, A., MARTÍNEZ-ANDÚJAR, C., MARTÍNEZ-PÉREZ, A., THOMPSON, A. J., DODD, I. C. & PÉREZ-ALFOCEA, F. 2015. Unravelling rootstock× scion interactions to improve food security. *Journal of experimental botany,* 66**,** 2211-2226.

ALKOWNI, R., ROWHANI, A., DAUBERT, S. & GOLINO, D. 2004. Partial characterization of a new ampelovirus associated with grapevine leafroll disease. *Journal of Plant Pathology***,** 123-133.

ALMEIDA, R., DAANE, K., BELL, V., BLAISDELL, G. K., COOPER, M., HERRBACH, E. & PIETERSEN, G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology,* 4**,** 94.

BELL, V. A., BONFIGLIOLI, R. G. E., WALKER, J. T. S., Lo, P. L., MACKAY, J. F., & McGREGOR, S. E. 2009. Grapevine leafroll-associated virus 3 persistence in *Vitis vinifera* remnant roots. *Journal of Plant Pathology.* 91, 527–533.

BELL, V., BLOUIN, A., COHEN, D., HEDDERLEY, D., OOSTHUIZEN, T., SPREETH, N., LESTER, P. & PIETERSEN, G. 2017. Visual symptom identification of grapevine leafrollassociated virus 3 in Red Berry cultivars supports virus management by roguing. *Journal of Plant Pathology,* 99**,** 477-482.

BESTER, R. 2012. Sequencing and detection of a new strain of grapevine leafroll-associated virus 3 in South Africa. Master of Science in Genetics. Stellenbosch University, Stellenbosch.

BESTER, R., PEPLER, P., BURGER, J. & MAREE, H. 2014. Relative quantitation goes viral: An RT-qPCR assay for a grapevine virus. *Journal of virological methods,* 210**,** 67-75.

BEUVE, M., SEMPÉ, L. & LEMAIRE, O. 2007. A sensitive one-step real-time RT-PCR method for detecting Grapevine leafroll-associated virus 2 variants in grapevine. *Journal of virological methods,* 141**,** 117-124.

BUDZISZEWSKA, M., WIECZOREK, P. & OBRĘPALSKA-STĘPLOWSKA, A. 2016. One-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) for detection of tomato torrado virus. *Archives of virology,* 161**,** 1359-1364.

CARRILLO, C., WERBAJH, S., MALNERO, C., STOLOWICZ, F., LAROCCA, L., MALIRAT, V. & VOJNOV, A. 2018. Development of a colorimetric RT-LAMP amplification assay adapted to an early and easy detection of Dengue virus. *International Journal of Infectious Diseases,* 73**,** 171.

CHOOI, K. M., COHEN, D. & PEARSON, M. N. 2016. Differential distribution and titre of selected grapevine leafroll-associated virus 3 genetic variants within grapevine rootstocks. *Archives of virology,* 161**,** 1371-1375.

CID, M., CABALEIRO, C. & SEGURA, A. 2003. Detection of Grapevine leafroll-associated virus 3 in rootstocks. *Extended abstracts of the 14th Meeting of ICVG*, Locorotondo, 2003.

COUSINS, P. & GOOLSBY, J. Grape rootstock variety influence on Pierce's disease symptoms in Chardonnay. P*roceedings of the 2011 Pierce's Disease Research Symposium*, 2011. 99-100.

COUSINS, P. & STRIEGLER, R. K. 2005. Grapevine Rootstocks: Current Use, Research, and Application. *2005 Rootstock Symposium*. Osage Beach, Missouri: Mid-America Viticulture and Enology Center, Southwest Missouri State University, 2005.

FEI, F., LYU, M.-D., LI, J., FAN, Z.-F. & CHENG, Y.-Q. 2013. Complete nucleotide sequence of a Chinese isolate of Grapevine leafroll-associated virus 3 reveals a 5′ UTR of 802 nucleotides. *Virus genes*, 46**,** 182-185.

FERRIS, H., ZHENG, L. & WALKER, M. 2012. Resistance of grape rootstocks to plant-parasitic nematodes. *Journal of nematology,* 44**,** 377.

FIORE, N., PRODAN, S., MONTEALEGRE, J., ABALLAY, E., PINO, A. & ZAMORANO, A. 2008. Survey of grapevine viruses in Chile. *Journal of Plant Pathology***,** 125-130.

FOËX, G. 1902. *Manual of Modern Viticulture: Reconstitution with American Vines*. R. S. Brain, government printer, Melbourne.

GOLINO, D., SIM, S. & ROWHANI, A. 2000. The role of GLRV-2 and GVB in virus-induced rootstock decline in California grapevines. *Phytopathology,* 90**,** 1-5.

GOLINO, D. A. 1993. Potential interactions between rootstocks and grapevine latent viruses. *American Journal of Enology and Viticulture,* 44**,** 148-152.

GOSZCZYNSKI, D. E. 2013. Brief Report of a New Highly Divergent Variant of Grapevine leafroll-associated virus 3 (GLR aV-3). *Journal of Phytopathology,* 161**,** 874-879.

HALL,T.A.1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*. Information Retrieval Ltd., London. 95-98.

HARRIS, M. 2017. Characterization of viruses associated with Grapevine Leafroll disease in Vitis rootstocks in South Africa*.* Master of Science in Microbiology. University of Pretoria, Pretoria.

IOANNOU, N., HADJINICOLIS, A. & HADJINICOLI, A. 1999. Mealybug transmission and natural spread of grapevine closterovirus in Cyprus. *Options Mediterraneennes*, 47-64.

KAARJ, K., AKARAPIPAD, P. & YOON, J.-Y. 2018. Simpler, faster, and sensitive Zika virus assay using smartphone detection of loop-mediated isothermal amplification on paper microfluidic chips. *Scientific reports,* 8**,** 12438.

MALAN, S. 2009. Real time PCR as a versatile tool for virus detection and transgenic plant analysis. Master of Science in Genetics. University of Stellenbosch, Stellenbosch.

MAREE, H. J., ALMEIDA, R. P., BESTER, R., CHOOI, K. M., COHEN, D., DOLJA, V. V., FUCHS, M. F., GOLINO, D. A., JOOSTE, A. E. & MARTELLI, G. P. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology,* 4**,** 82.

OLLAT, N., BORDENAVE, L., TANDONNET, J.-P., BOURSIQUOT, J.-M. & MARGUERIT, E. 2014. Grapevine rootstocks: origins and perspectives. *I International Symposium on Grapevine Roots 1136*. Rauscedo, Italy, October 2014. 11-22.

PIETERSEN, G. 2004. *Spread of grapevine leafroll disease in South Africa-a difficult but not insurmountable problem*. Technical Yearbook 2004/5, WineLand. Viewed: 4 May 2019, <https://www.wineland.co.za/spread-of-grapevine-leafroll-disease-in-south-africa-a-difficult-but-not-insurmountable-problem/ >

PIETERSEN, G. 2010. *Grapevine leafroll disease control in South Africa*. Wynboer, Viewed: 4 May 2019, < http://www. wineland. co. za/technical/grapevine-leafroll-disease-control-in-southafrica>

PIETERSEN, G., SPREETH, N., OOSTHUIZEN, T., VAN RENSBURG, A., VAN RENSBURG, M., LOTTERING, D., ROSSOUW, N. & TOOTH, D. 2013. Control of grapevine leafroll disease spread at a commercial wine estate in South Africa: a case study. *American Journal of Enology and Viticulture,* 64**,** 296-305.

PIETERSEN, G. & WALSH, H. 2012. Development of a LAMP technique for the control of grapevine leafroll associated virus type 3 (GLRaV-3) in infected white

cultivar vines by roguing. *Proceedings of the 17th Congress of the International Council for the Study of Virus and Viruslike diseases of the Grapevine (ICVG)*, 2012. 8-11.

SAAYMAN, D. 2009. Rootstock choice: The South African experience. *60th Annual Meeting of the American Society for Enology and Viticulture*, 2009.

STEWART, S. & NASSUTH, A. 2001. RT-PCR based detection of Rupestris stem pitting associated virus within field-grown grapevines throughout the year. *Plant Disease,* 85**,** 617-620.

TAO, Z.-Y., ZHOU, H.-Y., XIA, H., XU, S., ZHU, H.-W., CULLETON, R. L., HAN, E.-T., LU, F., FANG, Q. & GU, Y.-P. 2011. Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of Plasmodium vivax infection. *Parasites & vectors,* 4**,** 115.

THAI, H. T. C., LE, M. Q., VUONG, C. D., PARIDA, M., MINEKAWA, H., NOTOMI, T., HASEBE, F. & MORITA, K. 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. *Journal of Clinical microbiology,* 42**,** 1956-1961.

TOMITA, N., MORI, Y., KANDA, H. & NOTOMI, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature protocols,* 3**,** 877.

TOMLINSON, J., BOONHAM, N. & DICKINSON, M. 2010. Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. *Plant Pathology,* 59**,** 465-471.

TÖPFER, R., HAUSMANN, L., HARST, M., MAUL, E., ZYPRIAN, E. & EIBACH, R. 2011. *N*ew horizons for grapevine breeding. Methods in temperate fruit breeding. *Fruit, vegetable and cereal science and biotechnology*. Global Science Books, 5**,** 79-100

WALSH, H. A. & PIETERSEN, G. 2013. Rapid detection of Grapevine leafroll-associated virus type 3 using a reverse transcription loop-mediated isothermal amplification method. *Journal of virological methods,* 194**,** 308-316.

XIA, H., ZHAO, N., ZHAO, L., WANG, Y., ZHAO, W. & YUAN, Z. 2019. Rapid detection of Banna virus by reverse transcription-loop-mediated isothermal amplification (RT-LAMP). *International Journal of Infectious Diseases,* 78**,** 93-98.

YU, L.-S., RODRIGUEZ-MANZANO, J., MALPARTIDA-CARDENAS, K., SEWELL, T., BADER, O., ARMSTRONG-JAMES, D., FISHER, M. C. & GEORGIOU, P. 2019. Rapid and Sensitive Detection of Azole-Resistant Aspergillus fumigatus by Tandem Repeat Loop-Mediated Isothermal Amplification. *The Journal of Molecular Diagnostics,* 21**,** 286-295.

ZOU, Y., MASON, M. G., WANG, Y., WEE, E., TURNI, C., BLACKALL, P. J., TRAU, M. & BOTELLA, J. R. 2017. Nucleic acid purification from plants, animals and microbes in under 30 seconds. *Public Library of Science One,* 15**,** 2-22.

Chapter 6

Concluding remarks

An aim of this study was to determine the GLRaV-3 status of rootstocks commonly used in South Africa. Scion and rootstock tissue were collected from individual vines from former mother blocks in the Western Cape, South Africa, allowing for comparison of GLRaV-3 detection between two tissues of a single vine. Although GLRaV-3 was detected in rootstocks, the detection was significantly lower than that of corresponding scion tissue. Only one out of 14 (7%) R110 and US 8-7 tested positive for GLRaV-3. Six out of ten (60%) of Paulsen and one out of two (50%) Salt Creek/Ramsey rootstocks tested positive for the virus. Both Ruggeri 140 and 101-14 Mgt had zero out of ten (0%) positives. This poor detection was not due to PCR inhibitory substances as the grapevine housekeeping gene, Actin, was easily detected in a subset of rootstock samples. The total RNA concentration extracted from scion and rootstock were generally low, however, this could not be attributed to poor detection as, in general, RNA extracted from scion tissue was lower than that of rootstock.

Various graft experiments were performed to determine the graft transmissibility of GLRaV-3 in rootstocks. C. franc grafted onto commonly used rootstock clones graft-inoculated with pure sources of GLRaV-3 revealed differences in virus transmission and replication within various rootstock cultivars. This was further emphasised when rootstocks were used for the creation of intergrafts, where Salt Creek/Ramsey was observed to have high susceptibility to GLRaV-3 replication and subsequent transmission. This was corroborated by previous studies (Walsh and Pietersen, 2013, Harris, 2017). Rootstock Paulsen and US 8-7 were seen to be more susceptible to GLRaV-3 infection and transmission. Rootstock R110 was suggested to have a tolerant host defence mechanism in which GLRaV-3 was harboured in low concentrations but was graft-transmissible. The discrepancies seen in GLRaV-3 detection in R99 and 101-14 Mgt suggests that there may be genetic heterogeneity amongst individual vines, which could explain the erratic behaviour of GLRaV-3 in rootstocks (Cid et al., 2003, Cohen et al., 2003, Chooi et al., 2016, Harris, 2017). This study shed some light on the plant-pathogen relationship, however, further studies

would need to be undertaken to gain further insights into the dynamics between GLRaV-3 and rootstocks.

The successful detection of GLRaV-3 in rootstocks is largely dependent on the sampling strategy employed. The second aim of this study was to understand the distribution and virus titre of GLRaV-3 in rootstocks throughout the grapevine growing season. An RT-qPCR assay using primers designed by Bester et al. (2014) was employed to determine the relative quantification of virus in apical and basal tissue of petioles from different rootstock cultivars over 10 sampling points. No definitive pattern amongst rootstocks could be discerned from the data collected, though, the study showed significantly higher virus titres in rootstocks R110 and Salt Creek/Ramsey basal tissue compared to that of apical tissue. This result corresponded with that found by Chooi et al. (2016). A *V. vinifera* lineage representative was included in order to compare differences between *Vitis* species. The detection of GLRaV-3 was observed to be significantly higher in US 8-7 than in other rootstock cultivars, apart from R110. The differences in detection between rootstocks containing a *V. vinifera* parental line from those that do not corroborates the finding that GLRaV-3 detection in rootstock tissue is poorer than in *V. vinifera* (Cid et al., 2003, Tsai et al., 2012, Harris, 2017). Based on the results of this study, it is recommended that rootstock material be collected in March during the first grapevine growing season to increase the accuracy and reliability of GLRaV-3 diagnostic tests.

The third objective of this study was to adapt an RT-LAMP assay developed by Walsh and Pietersen (2013) for on-site GLRaV-3 detection in rootstocks. A single-tube GLRaV-3 specific RT-LAMP assay combined with a crude RNA extraction protocol (Tao et al., 2011) and the possible incorporation of a microcrystalline wax capsule aiding in contamination prevention was established. The assay was shown to have a high level of specificity and sensitivity higher than that of RT-PCR. The WarmStart Colorimetric LAMP 2x Master mix kit (New England Biolabs) was used as it contains a visible pH indicator that allowed for discernible positive results. The RT-LAMP assay was shown to have a significantly higher detection rate of GLRaV-3 in rootstocks over conventional RT-PCR.

The study yielded an in-depth look at GLRaV-3 detection in commercial rootstocks commonly used in the South African grapevine industry. Comparison of the detection of GLRaV-3 in scion versus that of rootstock tissue of the same vine, combined with the relative quantification study and the RT-LAMP assay provided further insights into the largely unknown dynamics between GLRaV-3 and rootstock cultivars. The observations made in this study emphasise the need for further research on this subject.

References

AL RWAHNIH, M., OSMAN, F., SUDARSHANA, M., UYEMOTO, J., MINAFRA, A., SALDARELLI, P., MARTELLI, G. & ROWHANI, A. 2012. Detection of Grapevine leafroll-associated virus 7 using real time qRT-PCR and conventional RT-PCR. *Journal of virological methods,* 179**,** 383-389.

ALMEIDA, R., DAANE, K., BELL, V., BLAISDELL, G. K., COOPER, M., HERRBACH, E. & PIETERSEN, G. 2013. Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology,* 4**,** 94.

BANERJEE, A., DUFLO, E., POSTEL-VINAY, G. & WATTS, T. 2010. Long-run health impacts of income shocks: Wine and phylloxera in nineteenth-century France. *The Review of Economics and Statistics,* 92**,** 714-728.

BERTAMINI, M., MUTHUCHELIAN, K. & NEDUNCHEZHIAN, N. 2004. Effect of grapevine leafroll on the photosynthesis of field grown grapevine plants (Vitis vinifera L. cv. Lagrein). *Journal of Phytopathology,* 152**,** 145-152.

BERTAZZON, N. & ANGELINI, E. 2004. Advances in the detection of Grapevine leafroll-associated virus 2 variants. *Journal of Plant Pathology***,** 283-290.

BESTER, R. 2012. Sequencing and detection of a new strain of grapevine leafroll-associated virus 3 in South Africa. Master of Science in Genetics. Stellenbosch University, Stellenbosch.

BESTER, R., MAREE, H. & BURGER, J. 2012. Complete nucleotide sequence of a new strain of grapevine leafroll-associated virus 3 in South Africa. *Archives of virology,* 157**,** 1815-1819.

BESTER, R., PEPLER, P., BURGER, J. & MAREE, H. 2014. Relative quantitation goes viral: An RT-qPCR assay for a grapevine virus. *Journal of virological methods,* 210**,** 67-75.

BUDZISZEWSKA, M., WIECZOREK, P. & OBRĘPALSKA-STĘPLOWSKA, A. 2016. One-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) for detection of tomato torrado virus. *Archives of virology,* 161**,** 1359-1364.

CAMPBELL, C. 2004. *Phylloxera: how wine was saved for the world*, 4th edition, HarperCollins Publishers, London.

CHARLES, J., COHEN, D., WALKER, J., FORGIE, S., BELL, V. & BREEN, K. 2006. A review of the ecology of grapevine leafroll associated virus type 3 (GLRaV3). *New Zealand Plant Protection,* 59**,** 330-337.

CHOOI, K. M., COHEN, D. & PEARSON, M. N. 2016. Differential distribution and titre of selected grapevine leafroll-associated virus 3 genetic variants within grapevine rootstocks. *Archives of virology,* 161**,** 1371-1375.

CID, M., CABALEIRO, C. & SEGURA, A. 2003. Detection of Grapevine leafroll-associated virus 3 in rootstocks. *Extended abstracts of the 14th Meeting of ICVG*. Locorotondo, 2003.

COHEN, D., VAN DEN BRINK, R. & HABILI, N. 2003. Leafroll virus movement in newly infected grapevines. *Extended abstracts 14th Meeting ICVG*, Locorotondo, Italy, 2003, 13-17.

COUSINS, P. & STRIEGLER, R. K. 2005. Grapevine Rootstocks: Current Use, Research, and Application. *2005 Rootstock Symposium*. Osage Beach, Missouri: Mid-America Viticulture and Enology Center, Southwest Missouri State University, 2005.

ENDESHAW, S. T., SABBATINI, P., ROMANAZZI, G., SCHILDER, A. C. & NERI, D. 2014. Effects of grapevine leafroll associated virus 3 infection on growth, leaf gas exchange, yield and basic fruit chemistry of Vitis vinifera L. cv. Cabernet Franc. *Scientia Horticulturae,* 170**,** 228-236.

FIORE, N., PRODAN, S., MONTEALEGRE, J., ABALLAY, E., PINO, A. & ZAMORANO, A. 2008. Survey of grapevine viruses in Chile. *Journal of Plant Pathology***,** 125-130.

FREEBOROUGH, M.-J. & BURGER, J. 2008. *Leafroll: Economic implications*. Wynboer, A technical Guide for Wine Producers, 13-17.

GRANETT, J., WALKER, M. A., KOCSIS, L. & OMER, A. D. 2001. Biology and management of grape phylloxera. *Annual review of entomology,* 46**,** 387-412.

HARRIS, M. 2017. Characterization of viruses associated with Grapevine Leafroll disease in Vitis rootstocks in South Africa. Master of Science in Microbiology. University of Pretoria, Pretoria.

JONES, T. J. 2012. Documentation of grapevine leafroll-associated viruses in wine grape varieties and native grape species in Virginia, and examination of the movement of grapevine leafroll disease to develop management strategies. Master of Science in Life Sciences. Virginia Polytechnic Institute and State University, Virginia.

JOOSTE, A., MAREE, H., BELLSTEDT, D., GOSZCZYNSKI, D., PIETERSEN, G. & BURGER, J. 2010. Three genetic grapevine leafroll-associated virus 3 variants identified from South African vineyards show high variability in their 5′ UTR. *Archives of virology,* 155**,** 1997-2006.

JOOSTE, A. E., PIETERSEN, G. & BURGER, J. T. 2011. Distribution of grapevine leafroll associated virus-3 variants in South African vineyards. *European Journal of Plant Pathology,* 131**,** 371-381.

LEE, J. & MARTIN, R. R. 2009. Influence of grapevine leafroll associated viruses (GLRaV-2 and-3) on the fruit composition of Oregon Vitis vinifera L. cv. Pinot noir: Phenolics. *Food Chemistry*. Elsevier, 112**,** 889-896.

MAREE, H., FREEBOROUGH, M.-J. & BURGER, J. 2008. Complete nucleotide sequence of a South African isolate of Grapevine leafroll-associated virus 3 reveals a 5′ UTR of 737 nucleotides. *Archives of Virology,* 153**,** 755-757.

MAREE, H. J., ALMEIDA, R. P., BESTER, R., CHOOI, K. M., COHEN, D., DOLJA, V. V., FUCHS, M. F., GOLINO, D. A., JOOSTE, A. E. & MARTELLI, G. P. 2013. Grapevine leafroll-associated virus 3. *Frontiers in Microbiology,* 4**,** 82.

MAREE, H. J., PIRIE, M. D., OOSTHUIZEN, K., BESTER, R., REES, D. J. G. & BURGER, J. T. 2015. Phylogenomic analysis reveals deep divergence and recombination in an economically important grapevine virus. *Public Library of Science One,* 10**,** e0126819.

NAMBA, S., YAMASHITA, S., DOI, Y., YORA, K., TERAI, Y. & YANO, R. 1979. Grapevine leafroll virus, a possible member of closteroviruses. *Japanese Journal of Phytopathology,* 45**,** 497-502.

NEL, A. & ENGELBRECHT, D. 1972. Grapevine virus diseases in South Africa and the
influence of latent viruses in the nursery. *Annual Phytopathology n' horsésrie,* 67-74.

PIETERSEN, G., SPREETH, N., OOSTHUIZEN, T., VAN RENSBURG, A., VAN RENSBURG, M., LOTTERING, D., ROSSOUW, N. & TOOTH, D. 2013. Control of grapevine leafroll disease spread at a commercial wine estate in South Africa: a case study. *American Journal of Enology and Viticulture,* 64**,** 296-305.

TAO, Z.-Y., ZHOU, H.-Y., XIA, H., XU, S., ZHU, H.-W., CULLETON, R. L., HAN, E.-T., LU, F., FANG, Q. & GU, Y.-P. 2011. Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of Plasmodium vivax infection. *Parasites & vectors,* 4**,** 115.

TSAI, C., DAUGHERTY, M. & ALMEIDA, R. 2012. Seasonal dynamics and virus translocation of Grapevine leafroll-associated virus 3 in grapevine cultivars. *Plant Pathology,* 61**,** 977-985.

WALSH, H. A. & PIETERSEN, G. 2013. Rapid detection of Grapevine leafroll-associated virus type 3 using a reverse transcription loop-mediated amplification method. *Journal of virological methods,* 194**,** 308-316.

Appendix

Table 1: Grapevine leafroll associated virus 3 status and PCR band strength of scion and rootstock tissue combinations of individual grapevines collected May 2017

Accession #	Vineyard	GPS Co-ordinates			Scion/rootstock combination		Scion GLRaV-3 status	Rootstock GLRaV-3 status
		Latitude	Longitude	Altitude	Scion	Rootstock		
17-7000/7001	Kuikenvlei	34 01 46.35470 S	18 47 25.0685161 E	55.827	Touriga Nacional	R110	+	-
17-7002/7003	Kuikenvlei	34 01 46.19135 S	18 47 25.2233392 E	55.768	Touriga Nacional	R110	-	-
17-7004/7005	Kuikenvlei	34 01 46.25698 S	18 47 25.1658034 E	55.695	Touriga Nacional	R110	-	-
17-7006/7007	Kuikenvlei	34 01 45.88184 S	18 47 24.3795184 E	56.361	Touriga Nacional	US 8-7	+	-
17-7008/7009	Kuikenvlei	34 01 45.82589 S	18 47 24.3749138 E	56.313	Touriga Nacional	US 8-7	++	-
17-7010/7011	Kuikenvlei	34 01 45.79450 S	18 47 24.6432245 E	55.453	Touriga Nacional	US 8-7	++	-
17-7012/7013	Kuikenvlei	34 01 45.97505 S	18 47 24.6112773 E	55.721	Touriga Nacional	US 8-7	-	-
17-7014/7015	Kuikenvlei	34 01 45.95037 S	18 47 25.0654513 E	55.778	Touriga Nacional	US 8-7	+ ++	-
17-7016/7017	Kuikenvlei	34 01 45.61200 S	18 47 25.3338263 E	57.71	Touriga Nacional	US 8-7	+ ++	-
17-7018/7019	Kuikenvlei	34 01 46.00673 S	18 47 24.8417951 E	56.266	Touriga Nacional	US 8-7	-	-
17-7020/7021	Kuikenvlei	34 01 46.06769 S	18 47 24.8252234 E	55.612	Touriga Nacional	US 8-7	-	-
17-7022/7023	Stellenzicht	33 59 27.02090 S	18 51 35.2308652 E	160.808	Merlot	Ruggeri140	+ ++	-
17-7024/7025	Stellenzicht	34 04 32.13569 S	18 47 45.1748038 E	160.193	Merlot	Ruggeri140	++	-
17-7026/7027	Stellenzicht	34 04 40.70560 S	18 47 14.1978215 E	153.423	Merlot	Ruggeri140	+	-
17-7028/7029	Stellenzicht	34 04 39.50523 S	18 47 22.6115323 E	143.172	Merlot	Ruggeri140	+	-
17-7030/7031	Stellenzicht	34 04 32.90171 S	18 47 10.0451233 E	163.127	Merlot	Ruggeri140	++	-
17-7032/7033	Stellenzicht	34 04 41.03781 S	18 47 12.7699021 E	161.220	Merlot	Ruggeri140	+	-
17-7034/7035	Stellenzicht	34 04 37.06780 S	18 47 23.3907563 E	154.652	Merlot	Ruggeri140	+	-
17-7036/7037	Stellenzicht	34 04 44.81907 S	18 47 24.6184953 E	156.313	Merlot	Ruggeri140	+	-
17-7038/7039	Stellenzicht	34 04 41.71200 S	18 47 14.5482756 E	155.243	Merlot	Ruggeri140	+	-
17-7040/7041	Stellenzicht	34 04 41.97505 S	18 47 24.6105733 E	155.172	Merlot	Ruggeri140	+	-
17-7042/7043	Backsberg	33 49 55.13551 S	18 54 43.4158470 E	247.346	Merlot	101-14 Mgt	-	-
17-7044/7045	Backsberg	33 49 54.54427 S	18 54 43.8689101 E	246.381	Merlot	101-14 Mgt	+	-
17-7046/7047	Backsberg	33 49 54.52828 S	18 54 43.5322660 E	246.483	Merlot	101-14 Mgt	+	-
17-7048/7049	Backsberg	33 49 53.92389 S	18 54 44.1255426 E	244.11	Merlot	101-14 Mgt	++	-
17-7050/7051	Backsberg	33 49 53.84604 S	18 54 44.1739576 E	243.9	Merlot	101-14 Mgt	-	-
17-7052/7053	Backsberg	33 49 51.94965 S	18 54 45.3573799 E	238.157	Merlot	101-14 Mgt	++	-
17-7054/7055	Backsberg	33 49 51.83585 S	18 54 45.4317927 E	238.231	Merlot	101-14 Mgt	+ ++	-
17-7056/7057	Backsberg	33 49 49.66926 S	18 54 45.8202094 E	233.242	Merlot	101-14 Mgt	++	-

Accession #	Vineyard	GPS Co-ordinates			Scion/rootstock combination		Scion GLRaV-3 status	Rootstock GLRaV-3 status
		Latitude	Longitude	Altitude	Scion	Rootstock		
17-7058/7059	Backsberg	33 49 49.40294 S	18 54 46.2601648 E	232.623	Merlot	101-14 Mgt	+	-
17-7060/7061	Backsberg	33 49 50.28184 S	18 54 45.3020000 E	235.664	Merlot	101-14 Mgt	+ ++	-
17-7062/7063	Backsberg	33 50 20.30250 S	18 54 50.3735149 E	336.252	Merlot	R110	-	-
17-7064/7065	Quoin Rock	33 50 20.21729 S	18 54 50.6332006 E	336.014	Merlot	R110	+	-
17-7066/7067	Quoin Rock	33 52 44.16669 S	18 53 34.0821712 E	422.88	CabernetSauvignon	R110	++	-
17-7068/7069	Quoin Rock	33 52 43.19160 S	18 53 31.7926213 E	413.571	CabernetSauvignon	R110	++	-
17-7070/7071	Quoin Rock	33 52 43.38680 S	18 53 31.9276778 E	415.232	CabernetSauvignon	R110	+ ++	-
17-7072/7073	Fransmanskraal	33 54 28.48533 S	18 47 37.0996748 E	246.928	Shiraz	R110	++	-
17-7074/7075	Fransmanskraal	33 54 25.34468 S	18 47 36.6070786 E	250.085	Shiraz	R110	++	-
17-7076/7077	Kuikenvlei	34 01 40.26176 S	18 47 29.3960734 E	66.433	Touriga Nacional	US 8-7	++	-
17-7078/7079	Kuikenvlei	34 01 41.18802 S	18 47 28.5305365 E	65.004	Touriga Nacional	US 8-7	-	-
17-7080	Vergelegen	34 04 48.47211 S	18 54 02.1485571 E	123.809	CS46A	N/A	+ ++	N/A
17-7081	Vergelegen	34 04 44.29180 S	18 54 10.1347244 E	131.176	CS46A	N/A	++	N/A
17-7082	Vergelegen	34 04 42.37291 S	18 54 10.0714329 E	153.443	CS15M	N/A	++	N/A
17-7083	Vergelegen	34 04 47.04690 S	18 54 06.7269499 E	143.906	SH 9C	N/A	++	N/A
17-7084	Vergelegen	34 04 48.47747 S	18 54 10.1116170 E	156.314	CS 46 C	N/A	+ ++	N/A
17-7085	Vergelegen	34 04 45.28863 S	18 54 10.0814801 E	153.377	CS 46 C	N/A	++	N/A
17-7086	Vergelegen	34 04 42.55289 S	18 54 08.6007767 E	143.072	CS 46 C	N/A	+	N/A
17-7087	Vergelegen	34 04 48.58455 S	18 54 12.0822925 E	163.147	CS 46 C	N/A	++	N/A
17-7088	Vergelegen	34 04 48.53216 S	18 54 12.0804151 E	161.226	CS 46 C	N/A	++	N/A
17-7089	Vergelegen	34 04 42.87018 S	18 54 13.3581175 E	154.602	CS 15 M	N/A	++	N/A
17-7090	Vergelegen	34 04 42.37555 S	18 54 13.1556782 E	152.21	CS 15 M	N/A	++	N/A
17-7091	Vergelegen	34 04 49.11475 S	18 54 19.5175592 E	177.591	CS 169 B	N/A	+	N/A
17-7092	Vergelegen	34 04 50.61747 S	18 54 20.2157202 E	181.058	CS 169 B	N/A	+	N/A
17-7093	Vergelegen	34 04 48.09630 S	18 54 18.0790794 E	173.401	CS 169 B	N/A	+ ++	N/A
17-7094	Vergelegen	34 04 49.00313 S	18 54 16.9875670 E	174.652	CS 169 B	N/A	+ ++	N/A
17-7095	Vergelegen	34 04 50.81924 S	18 54 17.7661489 E	177.316	CS 169 B	N/A	+ ++	N/A
17-7096	Vergelegen	34 04 50.92384 S	18 54 17.7700313 E	177.664	CS 169 B	N/A	+ ++	N/A
17-7097	Vergelegen	34 04 52.45801 S	18 54 21.2448925 E	182.558	CS 169 B	N/A	++	N/A
17-7098	Vergelegen	34 04 52.57970 S	18 54 21.2399347 E	182.311	CS 169 B	N/A	+	N/A
17-7099	Vergelegen	34 04 38.11428 S	18 54 21.4104792 E	157.074	SH 22 F	N/A	++	N/A
17-7100/7101	Trawal	N/A	N/A	N/A	Crimson	Ramsey	+	-
17-7102/7103	Wellington	N/A	N/A	N/A	Crimson	Ramsey	-	++

CS=Cabernet Sauvignon, SH=Shiraz, N/A=Not applicable, only scion collected, (-)=Negative for GLRaV-3, (+)=Weak PCR band in GLRaV-3 PCR, (++)=Medium PCR band in GLRaV-3 PCR, and (+++)=Strong band in GLRaV-3 PCR

Table 2: Grapevine leafroll associated virus 3 status and PCR band strength of scion and rootstock tissue combinations of individual grapevines collected May 2018

Accession #	Vineyard	GPS Co-ordinates			Scion/rootstock combination		Scion GLRaV-3 status	Rootstock GLRaV-3 status
		Latitude	Longitude	Altitude	Scion	Rootstock		
18-4000/4001	Hugoskraal	-33.583848834	19.216995122	269.140	Merlot	Paulsen	++	-
18-4002/4003	Hugoskraal	-33.583202555	19.217286282	268.740	Merlot	Paulsen	++	+
18-4004/4005	Hugoskraal	-33.583495208	19.217196771	267.529	Merlot	Paulsen	++	-
18-4006/4007	Hugoskraal	-33.584368891	19.216916189	267.579	Merlot	Paulsen	++	-
18-4008/4009	Hugoskraal	-33.583187809	19.217295045	267.637	Merlot	Paulsen	++	-
18-4010/4011	Witelsrivier	-33.600873386	19.212886166	279.693	Ruby Cabernet	Paulsen	+++	++
18-4012/4013	Witelsrivier	-33.601584226	19.213876906	279.814	Ruby Cabernet	Paulsen	+	+++
18-4014/4015	Witelsrivier	-33.600465497	19.212502675	280.923	Ruby Cabernet	Paulsen	+	+++
18-4016/4017	Hugoskraal	-33.585926228	19.218763866	264.473	Merlot	Paulsen	+	++
18-4018/4019	Hugoskraal	-33.585800434	19.218726124	263.192	Merlot	Paulsen	++	++
18-4022/4023	Kuikenvlei	-34.029465242	18.790186943	86.613	Touriga Nacional	US 8-7	+	++
18-4024/4025	Kuikenvlei	-34.028583702	18.791044175	94.487	Touriga Nacional	US 8-7	++	-
18-4026/4027	Kuikenvlei	-34.029506825	18.790247882	87.269	Touriga Nacional	US 8-7	+	-
18-4028/4029	Kuikenvlei	-34.029527045	18.790271588	87.412	Touriga Nacional	US 8-7	++	-
18-4032/4033	Kuikenvlei	-34.029108514	18.791130845	90.526	Touriga Nacional	R110	+++	-
18-4034/4035	Backsberg	-33.834617227	18.912683509	305.130	Merlot	R110	+	-
18-4036/4037	Backsberg	-33.834605239	18.912674455	303.461	Merlot	R110	+	-
18-4038/4039	Backsberg	-33.834470851	18.912511780	302.653	Merlot	R110	++	++

(-)=Negative for GLRaV-3; (+)=Weak PCR band in GLRaV-3 PCR; (++)=Medium PCR band in GLRaV-3 PCR; and (+++)=Strong PCR band in GLRaV-3 PCR

Table 3: RNA concentration and NanoDrop 200 readings (A260, A280, 260/280, and 260/230) of scion and rootstock tissue combinations of individual grapevines collected May 2017

Accession #	Scion/rootstock	Concentration (ng/µL)	A260	A280	260/280	260/230
17-7000	Touriga Nacional	1.5	0.037	0.017	2.21	0.30
17-7001	R110	5.3	0.132	0.068	1.93	0.64
17-7002	Touriga Nacional	56.2	1.405	0.536	2.02	1.82
17-7003	R110	64.2	1.605	0.599	2.30	1.44
17-7004	Touriga Nacional	1.2	0.029	0.018	1.84	0.75
17-7005	R110	2.2	0.054	0.029	1.90	0.63
17-7006	Touriga Nacional	1.4	0.034	0.012	2.83	0.51
17-7007	US 8-7	50.5	1.283	0.432	2.92	1.60
17-7008	Touriga Nacional	20.4	0.511	0.104	2.64	1.78
17-7009	US 8-7	12.2	0.305	0.135	2.25	1.15
17-7010	Touriga Nacional	9.5	0.237	0.102	2.34	1.70
17-7011	US 8-7	94.3	2.358	1.056	2.23	1.55
17-7012	Touriga Nacional	55.3	1.382	0.596	2.32	1.68
17-7013	US 8-7	53.0	1.326	0.525	2.52	1.83
17-7014	Touriga Nacional	26.8	0.669	0.310	2.16	2.00
17-7015	US 8-7	57.4	1.435	0.627	2.29	1.50
17-7016	Touriga Nacional	15.2	0.379	0.155	2.45	1.69
17-7017	US 8-7	27.1	0.678	0.282	2.41	1.54
17-7018	Touriga Nacional	6.9	0.172	0.072	2.39	1.44
17-7019	US 8-7	17.8	0.445	0.217	2.05	1.14
17-7020	Touriga Nacional	22.2	0.544	0.237	2.34	1.72
17-7021	US 8-7	58.1	1.453	0.649	2.24	1.33
17-7022	Merlot	2.3	0.057	0.038	1.49	1.38
17-7023	Ruggeri140	7.1	0.177	0.100	1.76	1.82
17-7024	Merlot	5.9	0.148	0.074	1.99	1.46
17-7025	Ruggeri140	20.6	0.515	0.235	2.19	1.60
17-7026	Merlot	4.7	0.117	0.065	1.80	1.20
17-7027	Ruggeri140	43.6	1.089	0.519	2.10	1.49
17-7028	Merlot	11.3	0.282	0.141	2.01	1.56
17-7029	Ruggeri140	13.9	0.347	0.179	1.94	1.59
17-7030	Merlot	8.7	0.216	0.104	2.03	1.78
17-7031	Ruggeri140	19.9	0.497	0.252	1.90	1.11

Accession #	Scion/rootstock	Concentration (ng/µL)	A260	A280	260/280	260/230
17-7032	Merlot	2.6	0.064	0.031	2.07	0.55
17-7033	Ruggeri140	3.8	0.095	0.058	1.64	0.95
17-7034	Merlot	9.0	0.225	0.107	2.11	1.52
17-7035	Ruggeri140	2.0	0.050	0.041	1.21	0.50
17-7036	Merlot	16.8	0.421	0.187	2.25	1.64
17-7037	Ruggeri140	5.0	0.124	0.070	1.78	0.88
17-7038	Merlot	15.8	0.395	0.195	2.02	1.51
17-7039	Ruggeri140	9.3	0.233	0.138	1.70	0.72
17-7040	Merlot	13.0	0.326	0.164	1.98	1.52
17-7041	Ruggeri140	2.8	0.071	0.047	1.51	0.70
17-7042	Merlot	16.3	0.408	0.198	1.91	2.13
17-7043	101-14 Mgt	15.3	0.382	0.207	1.84	2.12
17-7044	Merlot	17.1	0.426	0.193	2.2	1.70
17-7045	101-14 Mgt	2.1	0.053	0.024	2.26	0.69
17-7046	Merlot	1.7	0.041	0.032	1.29	0.73
17-7047	101-14 Mgt	31.0	0.774	0.371	2.09	1.43
17-7048	Merlot	8.6	0.216	0.119	1.81	1.16
17-7049	101-14 Mgt	51.4	1.285	0.64	2.01	1.62
17-7050	Merlot	21.3	0.533	0.254	2.1	1.58
17-7051	101-14 Mgt	23.7	0.593	0.308	1.93	1.13
17-7052	Merlot	35.6	0.889	0.521	1.71	0.55
17-7053	101-14 Mgt	29.0	0.725	0.364	1.99	1.59
17-7054	Merlot	11.1	0.278	0.665	1.93	0.06
17-7055	101-14 Mgt	107.8	2.698	0.243	1.86	0.04
17-7056	Merlot	10.5	0.261	0.107	1.68	0.01
17-7057	101-14 Mgt	15.4	0.384	3.644	1.92	2.32
17-7058	Merlot	24.5	0.614	3.541	1.91	2.09
17-7059	101-14 Mgt	82.7	1.589	0.617	1.87	1.65
17-7060	Merlot	10.2	0.254	0.125	2.00	1.13
17-7061	101-14 Mgt	11.4	0.286	1.218	2.14	1.33
17-7062	Merlot	1.8	0.033	0.13	2.10	1.06
17-7063	R110	122.5	3.062	0.155	2.23	1.05
17-7064	Merlot	4.4	0.092	0.251	2.44	1.63
17-7065	R110	17.1	0.410	0.722	2.17	2.06
17-7066	Cabernet Sauvignon	1.4	0.036	0.106	2.38	1.15
17-7067	R110	32.9	0.824	0.125	2.14	1.04
17-7068	Cabernet Sauvignon	16.8	0.421	0.028	1.54	0.59

Accession #	Scion/rootstock	Concentration (ng/µL)	A260	A280	260/280	260/230
17-7069	R110	7.8	0.196	1.075	2.85	1.47
17-7070	Cabernet Sauvignon	8.1	0.203	0.044	2.46	1.22
17-7071	R110	8.3	0.207	0.187	2.29	1.47
17-7072	Shiraz	4.5	0.103	0.0015	2.25	0.66
17-7073	R110	113.9	2.847	0.326	2.51	1.49
17-7074	Shiraz	5.6	0.141	0.102	1.92	0.49
17-7075	R110	5.6	0.139	0.103	1.72	0.47
17-7076	Touriga Nacional	15.3	0.381	0.083	2.44	1.57
17-7077	US 8-7	25.9	0.672	0.079	2.65	0.98
17-7078	Touriga Nacional	78.4	1.960	0.902	2.17	1.77
17-7079	US 8-7	172.2	4.306	1.536	2.60	1.35
17-7100	Crimson	1.9	0.046	0.028	1.65	0.27
17-7101	Ramsey	1.3	0.032	0.015	2.05	0.39
17-7102	Crimson	425.6	10.63	4.830	2.20	2.24
17-7103	Ramsey	450.1	11.25	5.147	2.19	2.25

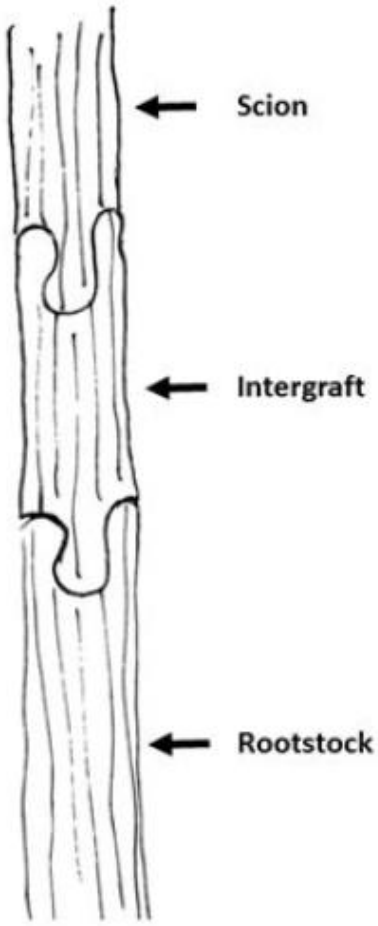


Figure 1: Image depicting a typical scion-intergraft-rootstock combination.

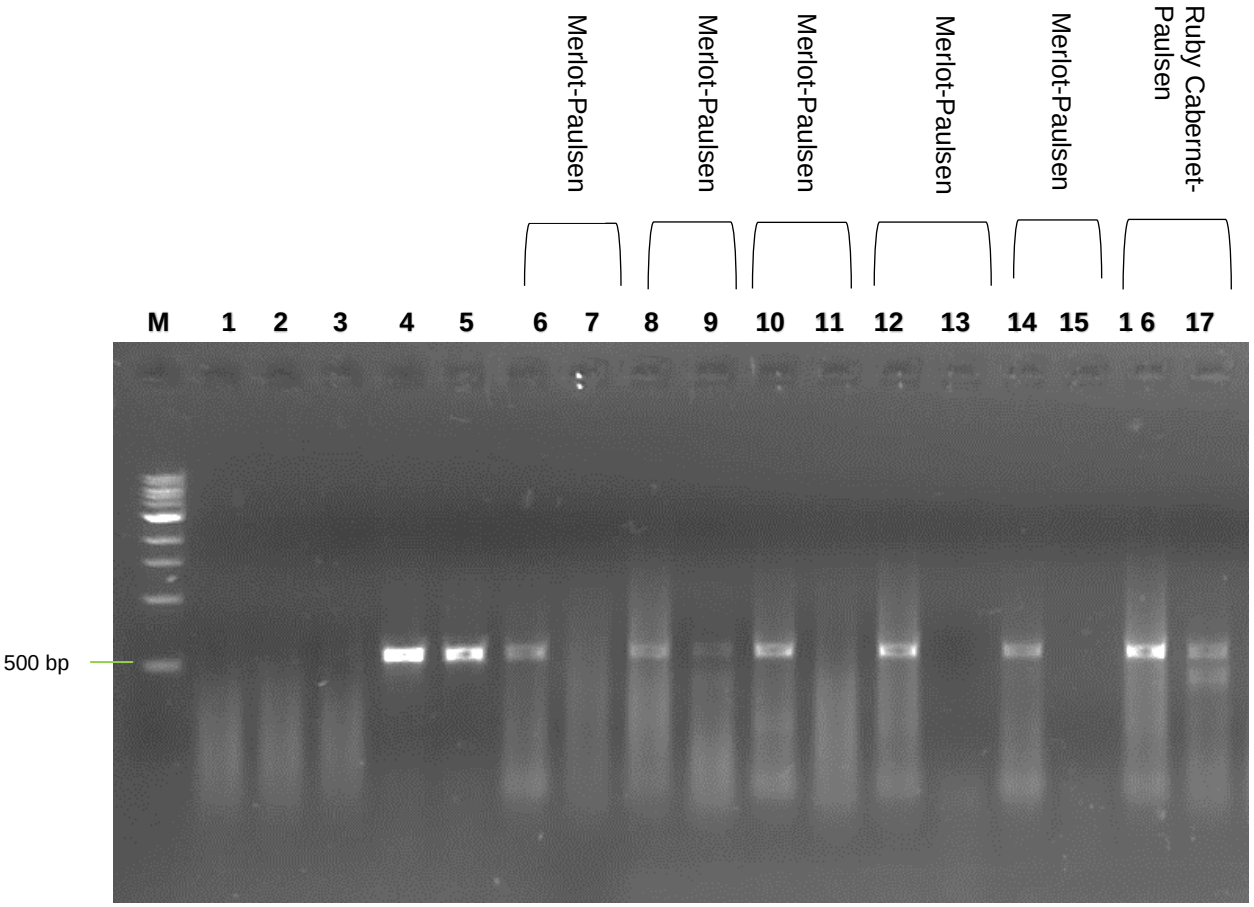


Figure 2: A 2% (w/v) agarose gel electrophoresis following Grapevine leafroll associated virus 3 specific RT-PCR of scion and rootstock tissues of the same vine. Lanes: (M) Quick-Load 1 kb Extended DNA Ladder (0.5 µg/µL) (New England Biolabs), (1) cDNA synthesis negative control, (2) PCR negative control, (3) Healthy control, (4) RNA cDNA synthesis positive control, (5) Grapevine leafroll associated virus 3 amplicon positive control (6) Merlot (17-7058), (7) 101-14 Mgt (17-7059), (8)Merlot (18-4004), (9) Paulsen 18-8005, (10) Shiraz (17-7074), (11) Richter 110 (17-7075), (12) Touriga Nacional (17-7076), (13) US 8-7 (17-7077), (14) Cabernet Sauvignon (17-7066), (15) Richter 110 (17-7067), (16) Merlot (18-4010), (17) Paulsen (18-8011).

Table 4: Grapevine leafroll associated virus 3 status and PCR band strength of scion tissue of individual grapevines grafted onto commonly used rootstock clones graft-inoculated with Grapevine leafroll associated virus 3 variant sources

Accession # (Scion)	Rootstock-scion combination		GLRaV-3 VI source	Symptomatic for leafroll	Scion GLRaV-3 status
	Rootstock	Scion			
17-7104	RQ 28 C	Cabernet franc 1A	17-7082	-	++
17-7105	RQ 28 C	Cabernet franc 1A	17-7082	✓	++
17-7107	RQ 28 C	Cabernet franc 1A	17-7082	-	+
17-7111	RQ 28 C	Cabernet franc 1A	17-7084	-	++
17-7112	RQ 28 C	Cabernet franc 1A	17-7084	-	+
17-7113	RQ 28 C	Cabernet franc 1A	17-7084	-	++
17-7114	RQ 28 C	Cabernet franc 1A	17-7084	-	+
17-7115	RQ 28 C	Cabernet franc 1A	17-7093	✓	++
17-7116	RQ 28 C	Cabernet franc 1A	17-7093	-	-
17-7117	RQ 28 C	Cabernet franc 1A	N/A	-	HC
17-7118	RQ 28 C	Cabernet franc 1A	17-7093	-	-
17-7121	RQ 28 C	Cabernet franc 1A	17-7093	-	+
17-7133	AA 219 F	Cabernet franc 1A	N/A	-	HC
17-7135	AA 219 F	Cabernet franc 1A	17-7082	-	-
17-7136	AA 219 F	Cabernet franc 1A	17-7082	-	+++
17-7137	AA 219 F	Cabernet franc 1A	17-7084	-	-
17-7138	AA 219 F	Cabernet franc 1A	17-7084	-	++
17-7139	AA 219 F	Cabernet franc 1A	17-7093	✓	+++
17-7152	AA 219 F	Cabernet franc 1A	17-7093	-	-
17-7156	RY 13 C	Cabernet franc 1A	17-7082	-	-
17-7161	RY 13 C	Cabernet franc 1A	17-7082	-	-
17-7163	RY 13 C	Cabernet franc 1A	17-7082	-	-
17-7168	RY 13 C	Cabernet franc 1A	17-7084	-	+
17-7169	RY 13 C	Cabernet franc 1A	17-7084	-	++
17-7175	RY 13 C	Cabernet franc 1A	17-7093	-	++
17-7177	RY 13 C	Cabernet franc 1A	17-7093	-	+
17-7178	RY 13 C	Cabernet franc 1A	N/A	-	HC
17-7185	SC 18 AB	Cabernet franc 1K	N/A	-	HC
17-7192	SC 18 AB	Cabernet franc 1K	17-7082	-	+
17-7196	SC 18 AB	Cabernet franc 1K	17-7082	✓	+
17-7202	SC 18 AB	Cabernet franc 1K	17-7082	✓	++
17-7203	SC 18 AB	Cabernet franc 1K	17-7082	✓	++

RQ=Richter 110; AA=101-14 Mgt; RY=Richter 99; SC=Salt Creek/Ramsey; N/A=Not applicable; (-)=Negative for GLRaV-3; (+)=Weak PCR band; (++)=Medium PCR band; and (+++)=Strong PCR band; HC=Healthy control (✓)=Leafroll symptoms present

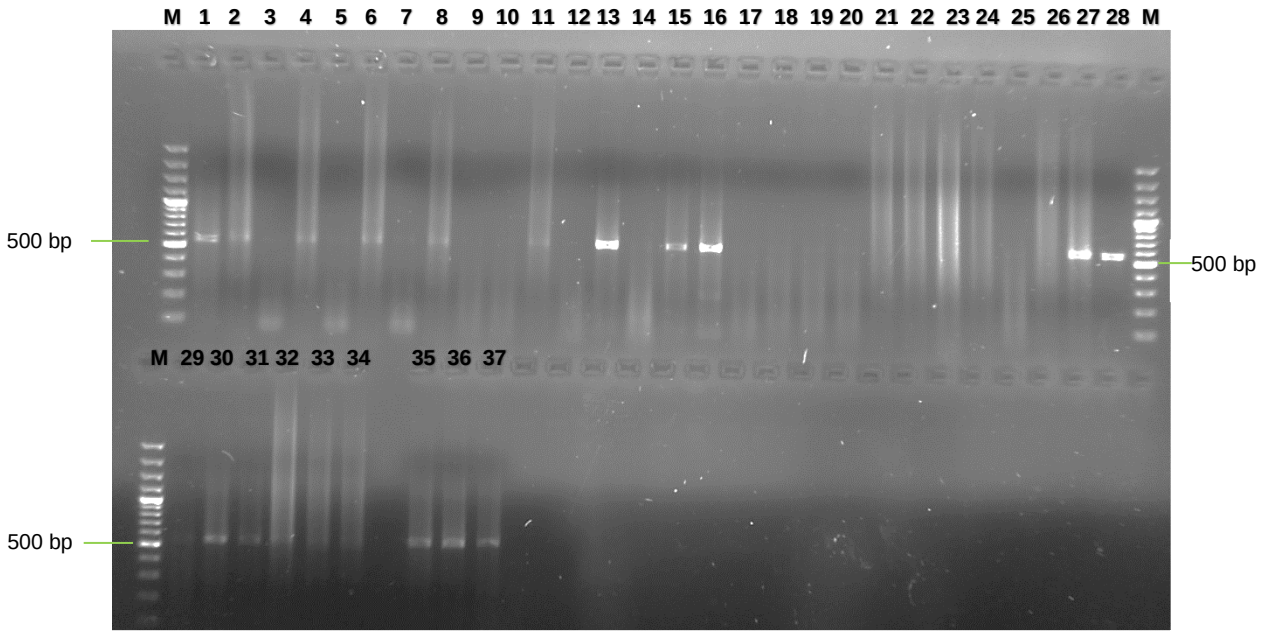


Figure 3: 1% (w/v) Agarose gel electrophoresis following Grapevine leafroll associated virus 3 specific RT-PCR of scion tissue obtained from scion/rootstock combinations that had been graft-inoculated with Grapevine leafroll associated virus 3 variant VI sources. Lanes: (M) O'GeneRuler 100 bp Plus DNA ladder (0.1 µg/µL) (Thermo Fisher), Lanes 1-11 (C. franc/Richter 110 combinations): (1) 17-7104, (2) 17-7105, (3) 17-7107, (4) 17-7111, (5) 17-7112, (6) 17-7113, (7) 17-7114, (8) 17-7115, (9) 17-7116, (10) 17-7118, (11) 17-7121, Lanes 12-18 (C.franc/101-14 Mgt combinations): (12) 17-7135, (13) 17-7136, (14) 17-7137, (15) 17-7138, (16) 17-7139, (17) 17-7140, (18) 17-7152, Lanes 19-20 (C. franc/Richter 99 combinations): (19) 17-156, (20) 17-7161, (21) cDNA synthesis negative control, (22) PCR negative control, (23) R110 Healthy control (17-7117), (24) 101-14 Mgt healthy control (17-7133), (25) R99 healthy control (17-7178), (26) Salt Creek/Ramsey healthy control (17-7185), (27) RNA cDNA synthesis positive control, (28) Grapevine leafroll associated virus 3 amplicon PCR control, Lanes 29-33 (C. franc/Richter 99 combinations): (29) 17-7163, (30) 17-7168, (31) 17-7169, (32) 17-7175, (33) 17-7177, Lanes 34-37 (C. franc/Salt Creek/Ramsey combinations): (34) 17-7191, (35) 17-7196, (36) 17-71202, (37) 17-71203.

Table 5: Grapevine leafroll associated virus 3 status of scion tissue of grapevines of rootstock-intergraft-scion combinations

Sample #	Rootstock-intergraft-scion combination			Scions GLRaV-3 positive	N
	Rootstock	Intergraft	Scion		
1	PS1103 18-4005	N/A	Cabernet franc	0	1
2-3	PS1103 18-4007	N/A	Cabernet franc	1	2
4	PS1103 18-4011	N/A	Cabernet franc	0	1
5-6	PS1103 18-4015	N/A	Cabernet franc	2	2
7	PS1103 18-4017	N/A	Cabernet franc	1	1
8-10	PS1103 18-4019	N/A	Cabernet franc	2	3
11	+ control 18-4021	N/A	Cabernet franc	1	1
12	+ control 18-4031	N/A	Cabernet franc	1	1
13	R110 18-4035	N/A	Cabernet franc	0	1
14-16	R110 18-4037	N/A	Cabernet franc	3	3
17-21	18-0064	AA	Cabernet franc	5	5
22-24	18-0061	RQ	Cabernet franc	2	3
25-29	18-0064	RQ	Cabernet franc	4	5
30-34	18-0061	RY	Cabernet franc	3	5
35-37	18-0061	SC	Cabernet franc	3	3
38-42	18-0064	SC	Cabernet franc	3	5
43-46	18-0061	UC	Cabernet franc	3	4
47-51	18-0064	UC	Cabernet franc	4	5
52-54	18-0061	PS	Cabernet franc	3	3
55-58	18-0064	PS	Cabernet franc	1	4
59-62	18-0061	CF	Cabernet franc	3	4
63-64, 79-81	18-0064	CF	Cabernet franc	1	5
65-78	N/A	AA 219F RQ 28C RY 13C SC 18B UC 274A PS 28I CF 1K	Cabernet franc	0	12
82-86	Cabernet franc	AA	18-0061	1	5
89	Cabernet franc	AA	18-0064	1	1
92-96	Cabernet franc	RQ	18-0061	2	3
97-99	Cabernet franc	RQ	18-0064	1	3
102-106	Cabernet franc	RY	18-0061	3	3
107-108	Cabernet franc	RY	18-0064	0	2
110-114	Cabernet franc	SC	18-0061	5	5
121	Cabernet franc	UC	18-0061	2	3
125, 129	Cabernet franc	UC	18-0064	2	2
130, 131, 134	Cabernet franc	PS	18-0061	3	3
140-141	Cabernet franc	CF	18-0061	1	2
146-149	Cabernet franc	CF	18-0064	1	3
150-151	17-7093	RY	Cabernet franc	1	2
152	17-7093	RQ	Cabernet franc	0	1

Sample #	Rootstock-intergraft-scion combination			Scions GLRaV-3 positive	N
	Rootstock	Intergraft	Scion		
154	17-7082	UC	Cabernet franc	0	1
159	N/A	17-7101 (Ramsey)	Cabernet franc	0	1

RQ=Richter 110; RY=Richter 99; SC=Salt Creek/Ramsey; UC=US 8-7; PS=Paulsen; CF=Cabernet franc; N/A=Not applicable; 18-0061=GLRaV-3 variant II, III, VI, and Viti/Foveavirus; 18-0064=GLRaV-3 variant II virus

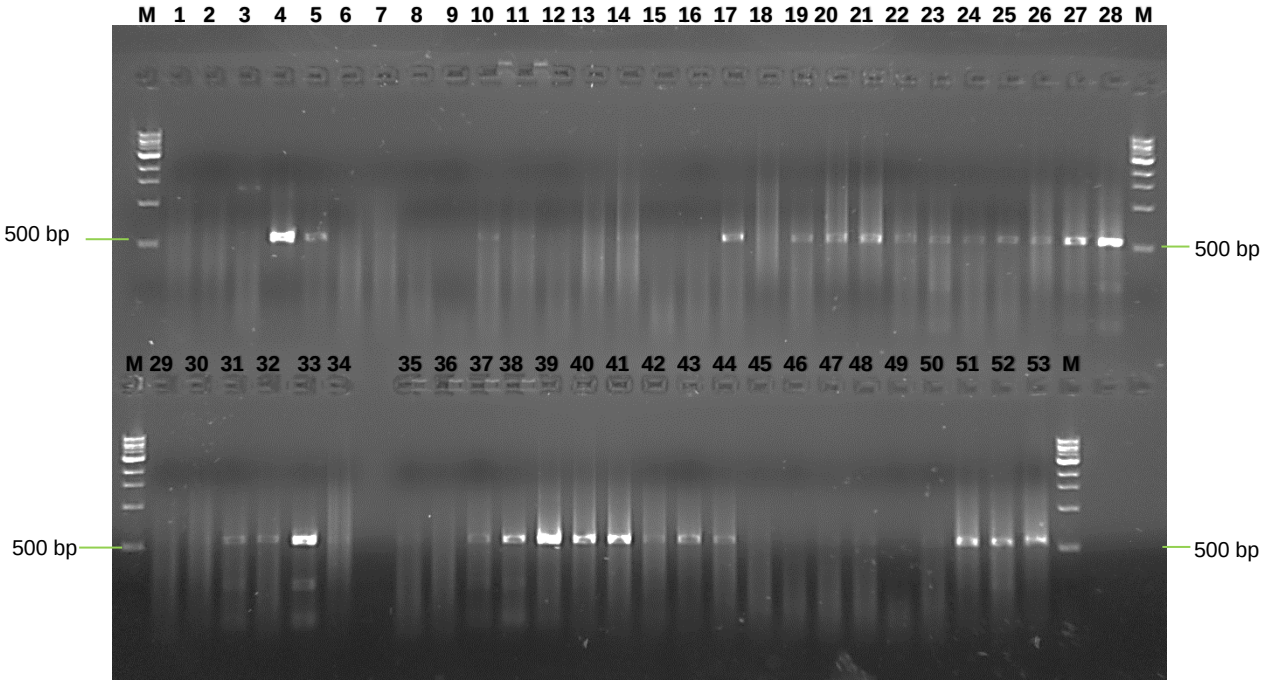


Figure 4: 1% (w/v) Agarose gel electrophoresis following Grapevine leafroll associated virus 3 specific RT-PCR of scion tissue obtained from rootstock-intergraft-scion combinations. Lanes: (M) Quick-Load Purple 1 kb DNA Ladder (0.5 µg/µL) (New England Biolabs), (1) cDNA synthesis negative control, (2) PCR negative control, (3) Healthy control, (4) RNA cDNA synthesis positive control, (5) Grapevine leafroll associated virus 3 amplicon PCR control, C. franc grafted onto Paulsen 1103 samples collected May 2018 (6) -(15), Positive controls, (16)-(17), C. franc grafted onto Richter 110 samples collected May 2018 (18)-(21), For the following combinations scion was the top component, rootstock as intergraft, and the virus infected source was rooted: Rootstock 101-14 Mgt as intergraft (22)-(26), Rootstock Richter 110 as intergraft (27)-(34), Rootstock Richter 99 as intergraft (35)-(39), Rootstock Salt Creek/Ramsey as intergraft (40)–(47), Rootstock US 8-7 as intergraft (48)-(53).

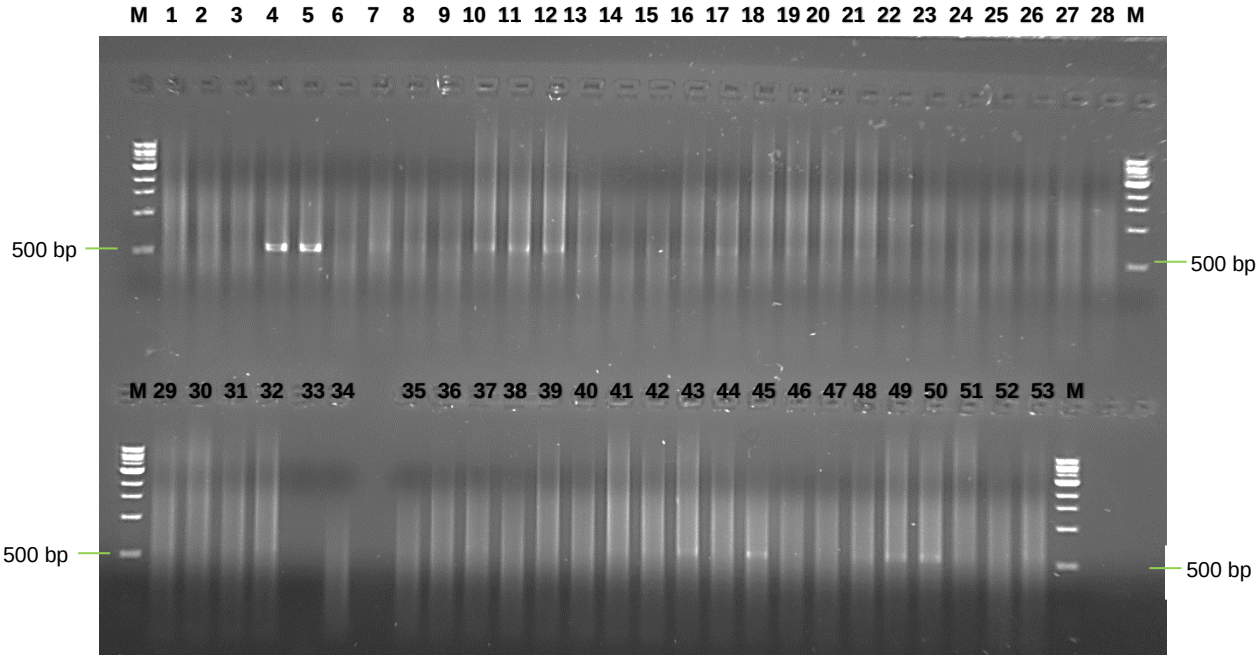


Figure 5: 1% (w/v) Agarose gel electrophoresis following Grapevine leafroll associated virus 3 specific RT-PCR of scion tissue obtained from rootstock-intergraft-scion combinations. Lanes: (M) Quick-Load Purple 1 kb DNA Ladder (0.5 µg/µL) (New England Biolabs), (1) cDNA synthesis negative control, (2) PCR negative control, (3) healthy control, (4) RNA cDNA synthesis positive control, (5) Grapevine leafroll associated virus 3 amplicon PCR control, For the following combinations scion was the top component, rootstock as intergraft, and the virus infected source was rooted: Rootstock US 8-7 as intergraft (6)-(8), Rootstock Paulsen 1103 as intergraft (9)-(15), C. franc as intergraft (16)-(21), C. franc grafted onto health rootstocks (22)-(33), C. franc as intergraft (34)-(36), For the following combinations virus infected source was the top component, rootstock as intergraft, and C. franc was rooted: Rootstock 101-14 Mgt as intergraft (37)-(42), Rootstock Richter 110 as intergraft (43)-(48), Rootstock Richter 99 as intergraft (49)-(53).

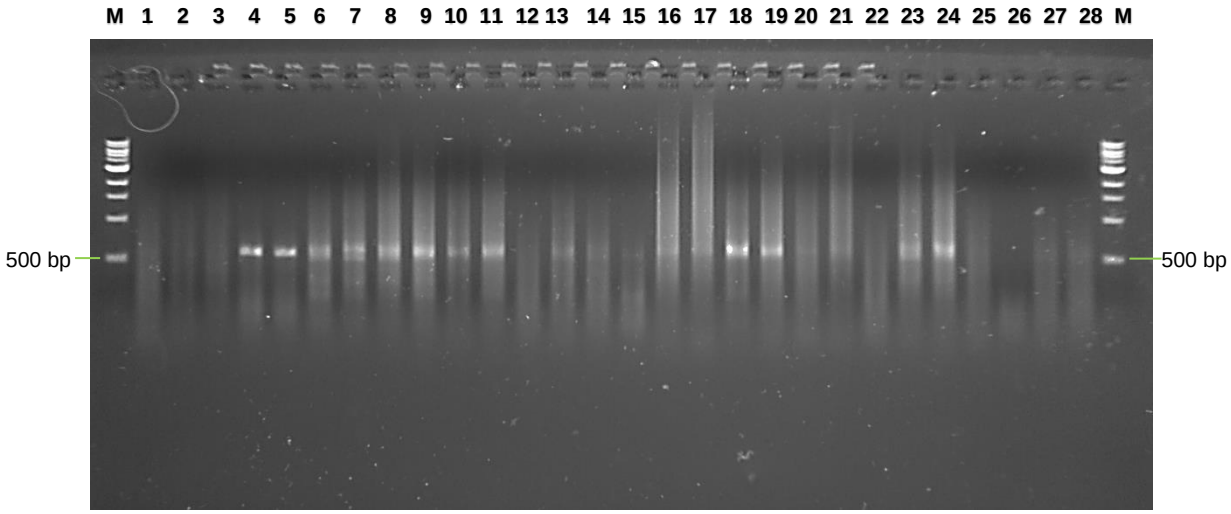


Figure 6: 1% (w/v) Agarose gel electrophoresis following Grapevine leafroll associated virus 3 specific RT-PCR of scion tissue obtained from rootstock-intergraft-scion combinations. Lanes: (M) Quick-Load Purple 1 kb DNA Ladder (0.5 µg/µL) (New England Biolabs), (1) cDNA synthesis negative control, (2) PCR negative control, (3) Healthy control, (4) RNA cDNA synthesis positive control, (5) Grapevine leafroll associated virus 3 amplicon PCR control, For the following combinations virus infected source was the top component, rootstock as intergraft, and C. franc was rooted: Salt Creek/Ramsey as intergraft (6)-(10), US 8-7 as intergraft (11)-(15), Paulsen 1103 as intergraft (16)-(18), C. franc as intergraft (19)-(23), rootstock 17-7093 as rooted component with Richter 110 as intergraft (24)-(26), rootstock 17-7082 as rooted component with US 8-7 as intergraft (27), C. franc grafted onto 17-7107 (Ramsey rootstock) (28).