Molecular characterization of grapevine virus E in South Africa

by

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Thesis presented in fulfilment of the requirements for the degree
Master of Science in Genetics at Stellenbosch University

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December 2012

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Abstract

Grapevine virus E (GVE) is a newly identified virus that has been detected in an established vineyard in South Africa. This virus is a member of the genus *Vitivirus*, family *Flexiviridae*. Members of this genus are known to infecte grapevine and are associated with various disease complexes, such as the Rugose wood complex (RWC) and Shiraz disease (SD). However, the role and impact of GVE in South African vineyards are still unknown. It is important to study these viruses to determine how they infect and the possible impact they may have on vine health.

The accurate and early detection of grapevine viruses is the first important step in disease management. In this study, reverse transcription-polymerase chain reaction (RT-PCR), double antibody sandwich enzyme linked immunesorbent assay (DAS-ELISA) and quantitative (q)RT-PCR were used for the detection of GVE in the vineyard (*Vitis vinifera* cv Merlot) where GVE was first identified in South Africa. Reverse transcription-PCR was used for detection and determining the incidence of GVE. The incidence was as low as 3% in the vineyard surveyed. All the GVE positive plants were co-infected with GLRaV-3 and no disease association could therefore be made. Evaluation of the Bioreba Grapevine virus A (GVA) DAS-ELISA kit showed that it did not detect GVE. No cross-reactivity occurred with epitopes of GVE, confirming this kit to be a valid and specific assay for GVA infection. The relative virus titer of GVE was calculated over the growing season of 2010/2011, using qRT-PCR. No fluctuation in virus titer was observed during that growing season.

Transmission experiments were performed in an attempt to transfer GVE from grapevine to an alternative host. Three different transmission buffers as well as nine different herbaceous plant species, that have shown to be susceptible to several plant viruses in previous studies, were evaluated. In these experiments, GVE could not be transmitted to any of the herbaceous species. To further characterize GVE, chimeric clones were constructed with GVA. The ORF2 and ORF5 of GVE were cloned into previously constructed GVA ORF2 and ORF5 deletion mutants. Construction of the chimeric clones, 35S-GVA-GR5-ΔORF2-GVE-ORF2 and 35S-GVA-118-ΔORF5-GVE-ORF5 were successful and they were evaluated for their infectivity in *N. benthamiana*. The 35S-GVA-GR5-ΔORF2-GVE-ORF2 chimera was able to infect and replicate in these plants and disease symptoms such as yellowing of veins and leaf curling were observed. Virus, derived from this vector, was detected by TPIA, RT-PCR and DAS-ELISA. The 35S-GVA-118-ΔORF5-GVE-ORF5 chimeric vector was not able to infect *N. benthamiana* as no disease symptoms were observed in any of the infiltrated plants and virus was not detected with serological analysis and RT-PCR.

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This study was aimed at further characterizing the recently identified virus GVE. Here, insight is given into the prevalence of this virus in the vineyard where it was first identified and attempts to biologically characterize GVE were made.

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Opsomming

Grapevine virus E (GVE) is ‘n nuut geïndetifiseerde virus wat onlangs in ‘n gevestigde wingerd in Suid Afrika opgespoor is. Hierdie virus vorm deel van die genus *Vitivirus*, familie *Betaflexiviridae*. Spesies in hierdie genus is bekend vir wingerdinfeksies en word met ‘n verskeidenheid wingerd siektes geassosieer, soos bv. Rugose wood complex (RWC) en Shiraz siekte (SD). Die rol en impak van GVE is nog onbekend. Dit is dus belangrik om die virus te bestudeer om te bepaal hoe dit infekteer en of dit enige impak het op wingerd gesondheid.

Akkurate en vroeë opsporing van virusse is die eerste belangrike stap vir virussiekte beheer. In hierdie studie word tru-transkripsie (TT) – polimerase ketting reaksie (PKR), dubbel teenliggaam (DAS) -ensiem gekoppelde immuno-absorberende analise (ELISA) en qTT-PKR gebruik vir die opsporing van GVE in die wingerd (*Vitis vinifera* cv Merlot) waar dit vroeër in Suid Afrika geïdentifiseer was. Vir opsporing en bepaling van verspreiding is TT-PKR gebruik. Daar is bepaal dat 3% van die wingerd met GVE geïnfekteer is. Al die GVE-positiewe stokke het ook positief getoets vir GLRaV-3 en geen assosiasie met siekte simptome kon gemaak word nie. Evaluering van die Bioreba GVA DAS-ELISA met GVE positiewe stokke het nie GVE opgespoor nie. Geen kruisreaktiwiteit het plaasgevind met epitope van GVE nie en dus is die DAS-ELISA ’n betroubare toets vir GVA infeksie. Die relatiewe virus titer van GVE was ook bepaal oor die groeiseisoen van 2010/2011 deur qTT-PKR te gebruik. Geen fluktuasie in virus titer gedurende die groeiseisoen is waargeneem nie.

Transmissie eksperimente is gedoen om GVE vanaf wingerd na ’n alternatiewe gasheer oor te dra. Drie verskillende transmissie buffers en tien verskillende sagteplant spesies, wat voorheen vatbaarheid vir plantvirusse getoon het, is gebruik. In die transmissie eksperimente kon GVE nie na enige van die sagteplante oorgedra word nie.

Om GVE verder te karakteriseer is hibried-virusse met GVA gemaak. Die leesraam (ORF) 2 en ORF5 van GVE gekloneer in GVA ORF2 en -ORF5 delesie konstrukte, 35S-GVA-GR5-ΔORF2 en 35S-GVA-118-ΔORF5, onderskeidelik (Blignaut, 2009; Du Preez, 2010). Klonering van die hibried konstrukte, 35S-GVA-GR5-ΔORF2-GVE-ORF2 en 35S-GVA-118-ΔORF5-GVE-ORF5, was suksesvol en is in *N. benthamiana* geëvalueer. Virus afkomstig van die 35S-GVA-GR5-ΔORF2-GVE-ORF2 hibried konstruk, kon plante suksesvol infekteer en kon repliseer binne hierdie plante. Siektesimptome soos vergeling van die are en rolblaar is ook waargeneem in plante geïnfekteer met hierdie hibried konstruk. Plante is getoets met weefsel afdruk immuno analise (TPIA), TT-PKR en DAS-ELISA en is positief gevind vir virus afkomstig van hierdie konstruk. Die 35S-GVA-118-ΔORF5-GVE-ORF5 hibried kon nie *N. benthamiana* infekteer nie en geen siektesimptome is

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waargeneem in enige van die plante geïnfiltreer met hierdie konstruk. Serologiese analise en TT-PKR het ook nie virus in die *N. benthamiana* plante opgespoor nie.

Die doel van hierdie studie was om GVE te karakteriseer. In hierdie studie word insig gegee oor die verspreiding van hierdie virus in Suid Afrika en pogings is gemaak om GVE biologies te karakteriseer.

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Abbreviations

+ss	Positive-sense single-stranded
aa	Amino acid
AMV	Avian Myeloblastosis Virus
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
bp	Base pair
CaMV	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
cfu	Colony forming units
CP	Coat protein
Ct	Threshold cycle
CTAB	N-Cetyl-N,N,N-trimethyl Ammonium Bromide
DAS-ELISA	Double antibody sandwich ELISA
DNA	deoxyribonucleic acid
dpi	Days post-infiltration (-inoculation)
DTT	1,4-Dithiothreitol
e	qPCR reaction efficiency
EDTA	Ethylene Diamine Tetra-Acetic Acid di-sodium salt
ELISA	Enzyme-linked immunosorbent assay
GFP	Green Fluorescent Protein
GOI	Gene of interest
GVA	Grapevine virus A
GFLV	Grapevine fanleaf virus
GLRaV-1	Grapevine leafroll associated virus-1
GLRaV-2	Grapevine leafroll associated virus-2
GLRaV-3	Grapevine leafroll associated virus-3
GLRaV-5	Grapevine leafroll associated virus-5
GLRaV-9	Grapevine leafroll associated virus-9
GRSPaV	Grapevine stem-pitting associated virus
GUS	β-Glucuronidase
MCS	Multiple cloning site
ORF	Open reading frame
qPCR	Quantitative real-time PCR
qRT-PCR	Quantitative real time reverse transcription-PCR

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R^2	Correlation coefficient
rDNA	Ribosomal DNA
REST	Relative Expression Software Tool
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription - polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
S.E.	Standard error
sgRNA	Subgenomic RNA
T-DNA	Transfer DNA
Ti-plasmid	Tumour-inducing plasmid
TMV	Tobacco mosaic virus
TPIA(s)	Tissue-print immuno-assay(s)
VIGS	Virus-induced gene silencing

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Acknowledgements

I would like to express my sincerest gratitude and appreciation to the following people and institutions:

- My supervisor Prof JT Burger and study leaders Dr HJ Maree and Dr D Stephan for the opportunity to do this research and their guidance, encouragement and support throughout this study;
- Dr MJ Freeborough and Dr J du Preez for their intellectual input;
- Mr A le Grange for statistical analysis;
- The National Research Foundation (NFR) and Stellenbosch University for financial support;
- Kanonkop wine estate for providing grapevine material;
- All the members of the Vitis lab for the great working environment and their support;
- All who help with sampling;
- My family and friends for their support throughout my studies;
- My Creator.

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Dedicated to my mother, Wilhelmina

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Chapter 1

Introduction

1.1 Background

As the 7th largest wine producing country in the world, South Africa contributes 3.5 % to the global production. In 2010, the harvest amounted to 1 261 309 tons of which 79% was used for wine production. The wine industry also contributes to the employment opportunities in South Africa (SA) with approximately 275 600 people being employed by this industry (www.wosa.co.za). Grapevine is an economically important commodity crop, which is susceptible to numerous pathogens and pests, which include fungi, insects, bacteria, nematodes, phytoplasmas viruses and viroids.

Viruses are important pathogens infecting grapevine, causing numerous disease complexes. These diseases lower the quality of grapes by reducing the sugar content and berry weight. The most common viral disease complexes affecting grapevine in SA are: Leafroll disease (LRD), Rugose wood complex (RWC) and Shiraz disease (SD). Viruses that are thought to be involved in these disease complexes are spread through the use of infected propagation material or insect vectors. Due to the lack of direct treatments or natural resistance, viral diseases have a considerable impact on grapevine health (Espinoza *et al*., 2007). It has been found that more than 60 different virus species can infect grapevine (Martelli and Boudon-Padieu, 2006).

Grapevine virus E (GVE) is a recently detected virus, first identified in Japan by Nakaune *et al*. (2008) in *Vitis labrusca*. Two sequence variants of GVE were identified: TvAQ7 and TvP15. More recently, GVE was detected in an established South African vineyard displaying both typical and atypical leafroll disease symptoms (Coetzee *et al*., 2010a). Another GVE isolate was discovered in a plant displaying typical Shiraz disease symptoms (Coetzee *et al*., 2010b). However, the role and impact in South African vineyards are still unknown. It is therefore important to study this virus on a molecular and biological level, to understand the host-virus interaction and determine the effect this virus may have on vine health, as well as to determine whether it is associated with any disease complexes.

1.2 Aims and objectives

This study was performed to characterize the recently identified GVE. Firstly, it was aimed at detecting GVE and getting a general indication of its prevalence in a South African vineyard. The

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seasonal titer of GVE was monitored in grapevine using quantitative reverse transcription-PCR (qRT-PCR) developed for GVE detection. The Bioreba GVA DAS-ELISA kit was evaluated for possible cross-reactivity to GVE. Another aim was to biologically characterize GVE by determining the herbaceous host spectrum for GVE. The functions of ORF2 and ORF5 of GVE were also investigated in *N. benthamiana* by constructing GVA-GVE chimeric viral vectors.

The aims will be achieved through the following objectives:

- To determine the incidence of GVE with RT-PCR in a field survey of the vineyard where GVE was first identified in South Africa.
- To determine cross-reactivity with GVA in a GVA DAS-ELISA using GVE positive plant material.
- To determine possible seasonal fluctuation in GVE virus titer with qRT-PCR for the growing season of 2010/2011.
- To determine the herbaceous host plant spectrum of GVE using different buffers and a range of herbaceous plant species.
- To evaluate GVE ORF2 and ORF5 in *N. benthamiana* plants by constructing GVA-GVE chimeric viral vectors.
- To evaluate the GVA-GVE chimeric clones in *N. benthamiana* by performing post-infection analyses, including: TPIA, visual symptom examination, RT-PCR and DAS-ELISA.

1.3 Breakdown of chapters

This thesis is divided into 5 chapters with a general introduction and literature review as the first two chapters, followed by three research chapters with their own introduction and a final conclusion as the last chapter.

Chapter 1: Introduction

In this chapter a general introduction is given with the aims and objectives of this study and a breakdown of the thesis into different chapters.

Chapter 2: Literature review

This chapter gives a general overview of literature related to this study including: viral diseases of grapevine, vitiviruses, GVE, detection techniques and infectious clones.

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Chapter 3: Detection of Grapevine virus E in a South African vineyard

In this chapter a survey is described in which the incidence of GVE was determined in the vineyard where GVE was first identified in South Africa. The industry standard GVA DAS-ELISA is evaluated for cross reactivity to GVE and the seasonal virus titer of GVE in infected grapevine is determined over the growing season of 2010/2011 for 15 weeks.

Chapter 4: Biological characterization of GVE

In this chapter different transmission buffers and several herbaceous plants are screened in attempts to mechanically transfer GVE to a potential alternative host species. The construction of chimeric infectious clones is also described. This was performed by cloning GVE ORF2 and ORF5 into GVA exchange vectors (previously constructed) and agroinfiltrating *N. benthamiana* plants. This was done in an attempt to evaluate the function(s) of these ORFs.

Chapter 5: Final conclusion

This chapter concludes the thesis and discusses future prospects.

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Chapter 2

Literature review

2.1 Introduction

Grapevine (*Vitis vinifera*) is an important agricultural crop that contributes greatly to the South African economy. However, there are a variety of pathogenic agents that have a negative impact on the yield and quality of grapes. Grapevines are susceptible to more than 60 different virus species, some of which that have been associated with a number of grapevine diseases (Martelli and Boudon-Padieu, 2006). The most common viral diseases affecting grapevine in South Africa are Grapevine leafroll disease (LRD), Rugose wood complex (RWC), Shiraz disease (SD) and Fanleaf decline. Accurate and early diagnosis of disease infections is the first important aspect of disease management. Currently, there are no treatments for viral infection in vineyards and no natural resistance in grapevine has been identified (Espinoza *et al*., 2007). Viruses are spread through the use of infected propagation material, as well as by insect vectors such as nematodes, aphids and mealybugs (Sforza et al., 2003; Goszczynski and Jooste 2003). Sanitation and pest control is important in vineyards to control the spread of viruses throughout the vineyard. Removal of infected material and the up-keep of proper quarantine are necessary to prevent planting of infected material.

2.2 Grapevine viral diseases

2.2.1 Grapevine leafroll disease (LRD)

Grapevine leafroll disease is the most prevalent viral disease affecting grapevines worldwide (Martelli and Boudon-Padieu, 2006). Symptoms of LRD include downward rolling of leaf margins, in red cultivars the leaves turn red while the main veins remain green, and in white cultivars the interveinal regions turn yellow (Figure 2.1). Viruses associated with LRD are phloem-limited and infection causes the degeneration of the vascular system, poor pigmentation, delayed ripening of fruit and a reduction in grape yield (Pietersen, 2004; Uyemoto *et al*., 2009). Nine serologically diverse grapevine leafroll-associated viruses (GLRaVs) have been identified with GLRaV-3 being the most prevalent virus associated with LRD (Martelli *et al*., 2002). Through evolutionary studies of the viruses within the genus *Ampelovirus,* two new species were identified, GLRaV-Pr and GLRaV-De (Maliogka *et al*., 2008). Most of the GLRaVs belong to the genus *Ampelovirus* with

GLRaV-2 a member of the genus *Closterovirus*. These genera, along with a third genus, *Crinivirus*, are included in the family *Closteroviridae*.

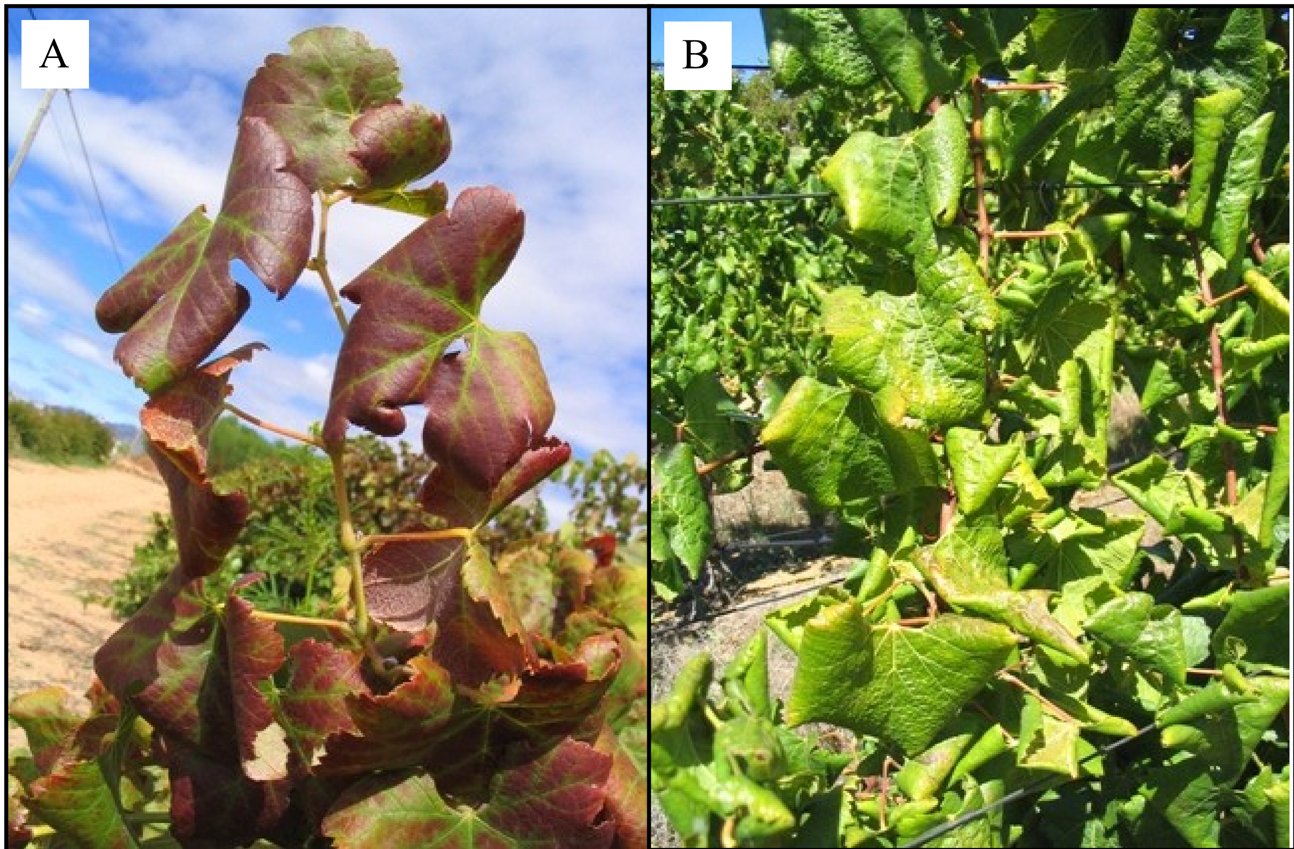

Figure 2.1: Grapevine displaying typical leafroll disease symptoms A) in red cultivars leaves turn red while the main vein remains green and B) down rolling of leaf margins in white cultivar (Maree, 2010).

2.2.2 Rugose wood complex (RWC)

Another important disease complex affecting grapevine in SA since the 1970s is the RWC. This complex includes four major diseases namely Rupestris stem pitting (RSP) (Figure 2.2 A), LN33 stem grooving (Figure 2.2 B), Corky bark and Kober stem grooving (Rosa and Rowhani, 2007; Constable and Rodoni, 2011). The movement of water and nutrients through the vascular system are affected in infected vines (Gribauda *et al.*, 2006). As a result, it may cause graft union incompatibility, bud bursting delay, yield and vigor reduction and overall decline (Gribauda *et al.*, 2006). Most of the viruses found to be associated with this disorder belong to the family *Betaflexiviridae*, and specifically the genera *Vitivirus* and *Foveavirus* (Rosa and Rowhani, 2007). Several viruses are involved in this complex and infections of vines with different viruses result in diverse disease states. Grapevine virus A (GVA) is associated with Kober stem grooving, grapevine Rupestris stem pitting associated-virus (GRSPaV) with RSP (Meng *et al.*, 1999), the most common disease in this complex. Vines only infected with GRSPaV show little or no symptoms compared to those infected with GRSPaV together with other viruses. This indicates that GRSPaV-infected vines

carry the risk of developing RSP if simultaneously infected with other viruses, such as GLRaV-2 or a combination of viruses involved in the RWC (Rosa and Rowhani, 2007; Rosa *et al*., 2011). Symptoms include swelling at the graft union and these affected crops fail to thrive. Grapevine virus B (GVB) is associated with corky bark, a disease that only affects grafted vines. The severity is more pronounced in vines also infected with other viruses. The role of grapevine virus D (GVD) in RWC is still unclear and no viruses have as yet been found associated with LN33 stem grooving (Monis, 2005). The etiology of the RWC is still largely unknown even though viruses such as GVA, GVB and GRSaPV have been associated with disease symptoms in this complex.

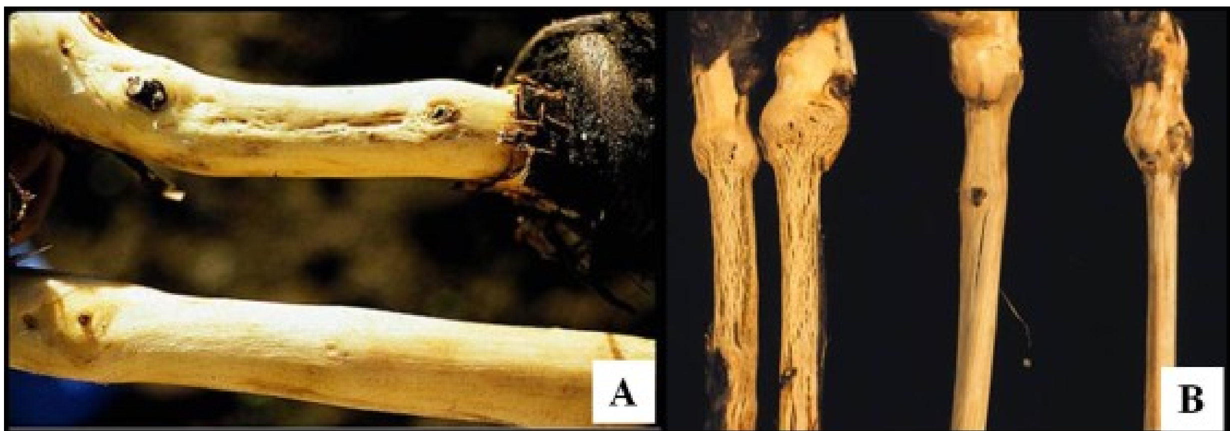

Figure 2.2: Symptoms associated with the RWC, A) Rupestris stem pitting infected grapevine cylinder compared to a healthy plant (http://www.agf.gov.bc.ca/cropprot/grapeipm/virus.htm) and B) comparison of a healthy stems (right) with LN33 infected stem grooving (left) (http://wine.wsu.edu/research-extension/grape-growing/plant-health/virology/symptoms).

2.2.3 Shiraz disease (SD)

Grapevine virus A has been associated with SD (Goszczynski and Jooste, 2003), which affects the grapevine cultivars Shiraz, Merlot, Gamay, Malbec and Viognier (Goszczynski *et al.*, 2008). Infected vines never reach full maturation and die within 3-5 years. Symptoms of SD include the typical leaf reddening from the margins (Figure 2.3 A); non-lignified shoots (Figure 2.3 B), appearing green and rubbery, delayed bud burst and buds dying off during the winter seasons (Goussard and Bakker, 2006). Symptoms are due to the absence of secondary phloem fibre and the formation of cork layers that limit the ability of infected vines to transport photosynthetic products, which are important for storage in areas such as the roots and stems (Goussard and Bakker, 2005). Lower sugar concentrations are observed in infected grapes (Goussard and Bakker, 2005). Grapevine leafroll associated virus-3 has also been identified in vines displaying symptoms of SD (Burger and Spreeth, 1993).

Figure 2.3: Symptoms typically associated with SD includes A) redding of leaves and B) non-lignified shoots (Goussard and Bakker, 2006).

2.2.4 Grapevine fanleaf disease

Another devastating disease affecting grapevine worldwide is fanleaf decline. This disease is caused by grapevine fanleaf virus (GFLV), a member of the genus *Nepovirus*, family *Secoviridae*, (Hewit *et al*., 1962; Quacquarelli *et al*., 1976). The name fanleaf is derived from the malformation in leaf appearance, taking an open fan-like shape, in infected grapevine. Other symptoms typically associated with fanleaf disease include: yellowing of leaves, vein banding, abnormal branching and short internodes (Figure 2.4) (Andret-Link *et al*., 2004; Monis, 2005). This disease reduces the quality and yield of grapes. In severely infected grapevine up to 80% yield loss has been observed (Monis, 2005).

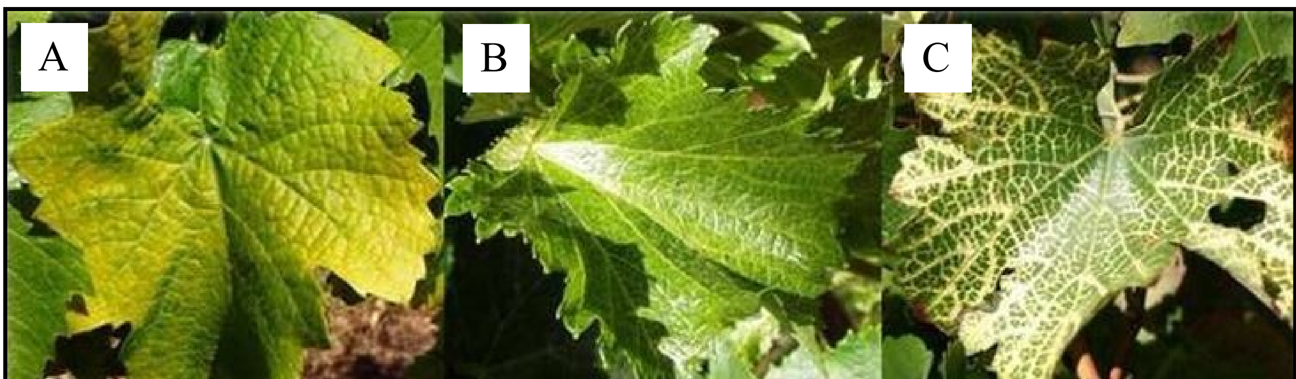

Figure 2.4: Symptoms associated with grapevine fanleaf disease A) yellow mosaic, B) open fan-like shape and C) vein banding of GFLV in infected grapevine (Liebenberg et al., 2009).

2.3 Vitiviruses

Viruses in the genus *Vitivirus* has been associated with disease complexes such as RWC, SD and Shiraz decline. Currently, there are six virus species grouped in this genus, namely GVA, GVB, GVD, Heracleum latent virus (HLV), mint virus 2 (MV2) and the recently identified GVE

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(http://www.dpvweb.net/notes/showgenus.php?genus=Vitivirus). *Agave tequilana* leaf virus, Actinidia virus A and Actinidia virus B is tentative members of this genus. Grapevine virus C (GVC) has been excluded from this genus, as it has been suggested that GVC is the same virus as GLRaV-2 from the genus *Ampelovirus* family *Closteroviridae* (Masri *et al.*, 2006). Grapevine virus A is the type member of this genus and is associated with several destructive grapevine diseases in South Africa (Goszczynski and Jooste, 2003; Goszczynski, 2007).

Until recently, the genus *Vitivirus* was included in the family *Flexiviridae*. Members of this family have flexuous filamentous particles (Adams *et al.*, 2004). The family *Flexiviridae* has undergone taxonomic re-arrangement and has been divided into three new families, *Alphaflexiviridae*, *Betaflexiviridae* and *Gammaflexiviridae*. The family *Betaflexiviridae* contains the genera *Foveavirus*, *Trichovirus* and *Vitivirus* (http://www.dpvweb.net/notes/showgenus.php?order=Tymovirales).

2.3.1 General properties

Members of the genus *Vitivirus* are between 725-825 nm in length with a diameter of 12nm and virions are not encapsulated in an envelope (Figure 2.5). The genomes of these viruses are linear, +ss RNA of approximately 7.5 kb in size and are translated by means of 5 ORFs (Figure 2.6). These ORFs overlap and putative functions have been ascribed (Galiakparov *et al.*, 2003). Open reading frame 1 encodes a 194 kDa protein product, which correspond to a methyl tranferase domain, a helicase motif and an RdRp domain (Minafra *et al.*, 1997). These are replication-related conserved regions. An AlkB domain has also been identified, encoded by this ORF (Martelli *et al*., 2007) and has been associated with RNA repair (Van den Born *et al*., 2008). Open reading frame 1 is directly translated by a genomic RdRp and spliced into functional peptides of which the viral RdRp recognizes sgRNA for the production of downstream ORFs (Martelli *et al*., 2007). Two sets of sgRNA were characterized at the 5' and 3' ends and no sgRNAs detected for ORF5 which is probably transcribed by bi- or polycistronic mRNA (Galiakparov *et al*., 2003). The ORF2 encodes a 19 kDa protein of which the function is still unknown (Galiakparov et al., 2003). The protein product for ORF2 is speculated to be involved in the transmission of the virus through mealybugs (Galiakparov *et al*., 2003). ORFs 3, 4 and 5 encode a 13 kDa movement protein, a 22 kDa coat protein and a 10 kDa RNA binding protein, p10, respectively (Minafra *et al*., 1997). The p10 protein product of ORF5 has been identified as a weak silencing suppressor (Zhou *et al*., 2006), of which the activity increases up to 1000X in the presence of other factors (Mawassi *et al*., 2007). The 3'end of the genomic RNA is polyadenylated and the 5' end contains a methylated nucleotide cap (Minafra *et al*., 1997).

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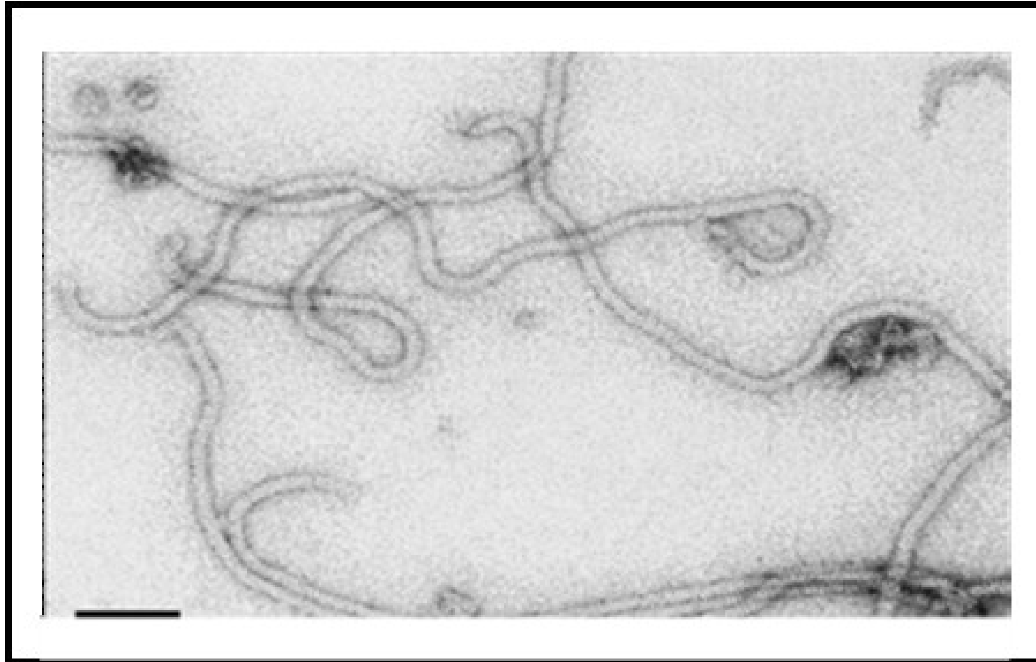

Figure 2.5: Electron microscopic picture of GVA virus particles (Bar = 100nm) (http://www.dpvweb.net/notes/showem.php?genus=Vitivirus).

2.3.2 Transmission and spread

Viruses of the genus *Vitivirus* are phloem-limited and transmitted through the use of infected propagation material as well as insects (nematodes, aphids and mealybugs), which serve as vectors for viruses. Grapevine virus A and GVB are naturally transmitted in a semi-persistent manner by pseudococcid mealybugs, in particular by members of the genus *Pseudococcus* and *Planococcus* (Engelbrecht and Kasdorf, 1987; Goszczynski and Jooste, 2003), while MV2 and HLV are transmitted by aphids. Grapevine virus A can also be transmitted by the insect *Neopulvinaria innumerapilis* and *Parthenolecanium corni* (Hommay *et al*., 2008). With the use of mechanical inoculation, GVA, GVB and GVD have been transmitted from their natural host to herbaceous plants. Grapevine virus A has been transmitted to the herbaceous plants *N. benthamiana*, *N. clevelandii*, *Chenopodium amaranticolor* and *C. quinoa* with phosphate and nicotine buffers (Monette and James., 1990; Conti *et al*., 1980; Hommay *et al*., 2008). Grapevine virus B was transferred to *N. occidentalis* (Boscia *et al*., 1993) with potassium phosphate buffer. Heracleum latent trichovirus was mechanically transmitted to herbaceous plant species that included *C. quinoa* and *C. amaranticolor* (Bem and Murant, 1979). Attempts of transferring MV2 to herbaceous hosts, which included *N. tabacum*, *C. amaranticolor* and *C. quinoa* have been unsuccessful (Tzanetakis *et al*., 2007). Attempts by Nakaune *et al*., (2008) to mechanically transfer GVE to *N. benthamiana*, *N. clevelandii*, *N. glutinosa* and *N. occidentalis* were also unsuccessful.

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2.4 Grapevine virus E (GVE)

In 2008 Nakaune *et al*. described a new virus, Grapevine virus E (GVE) in *Vitis labrusca*. Two sequence variants of GVE were identified, TvAQ7 and TvP15 sharing 73% nt and 84% aa identity. The GVE-TvAQ7 variant was identified in a plant displaying stem pitting, while TvP15 was identified in a plant not displaying any apparent disease symptoms. The genome organization and phylogenetic analysis of the coat protein, group these two variants of GVE as members of the genus *Vitivirus*, family *Betaflexiviridae* (Figure 2.6) (Nakaune *et al*., 2008). Grapevine virus E shares approximately 60% nt and aa identity with other members of the genus *Vitivirus* (GVA, GVB, GVD and MV2). Partial nucleotide sequences for the two variants are available. For TvP15, a 3.2kb sequence stretch near the 3' end is available, Genbank accession number AB432911. For TvAQ7, a near complete sequence is available lacking only the exact 5' terminal, Genbank accession number AB432910. Nakaune *et al*. (2008) also conducted transmission experiments and identified the mealybug, *P. comstoki* as a vector for GVE in the presence of GLRaV-3, while mechanical inoculation of GVE to herbaceous plants was unsuccessful. Double antibody sandwich ELISA determined that GVE is not serological related to GVA or GVB (Nakaune *et al*., 2008).

As part of a metagenomic study of a diseased vineyard (*V. vinifera* cv. Merlot) in South Africa, a partial GVE sequence was reported. From the data generated, 0.9% was identified as GVE sequences (Coetzee *et al*., 2010a). This was only the second report of GVE and the first in South Africa. The sequences obtained had homology to the partial sequence available for GVE-TvP15 (Nakaune *et al*., 2008). The metagenomic data generated two GVE scaffolds, the largest, Node 3404 (Genbank accession number GU903011), being 5172 bp in length.

The first complete nucleotide sequence for a GVE isolate, SA94 (Genbank accession number GU903012), was reported by Coetzee *et al*. (2010b). Grapevine virus E isolate SA94 was detected in a grapevine plant (*V. vinifera* cv. Shiraz), displaying symptoms of SD. Sequencing of RT-PCR products, poly-A tailing for the 3' end and RLM-RACE for the 5' end was used to determine the complete genome sequence of isolate SA94. This virus isolate has a genome size of 7568 nt and shares 98.1%, 69.6% and 98.2% nt identity to TvP15, TvAQ7 and Node 3404, respectively. This suggests that the variants SA94 and TvP15 are members of the same strain. The 3' end of SA94 is identical to that of TvP15 and the 5' end extending that of TvQA7 by 8 nts. Grapevine virus E-SA94 has a genome organization that is similar to that described for TvAQ7 and TvP15 (Figure 2.6).

Interestingly, for GVE-SA94 the AlkB domain is located within the helicase domain as opposed to up-stream, as seen in other members of the genus *Vitivirus* (Figure 2.6). The AlkB domain has been associated with the repair of methylation damage (Bratlie and Drablos, 2005). Another observation

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is that the ORF1 in SA94 does not overlap with ORF2 as it does in the variant TVaQ7 and other vitiviruses. The implications of these observations have not been determined. Re-assembly of the data generated through the metagenomic study indicated high sequence homology between the GVE sequences, suggesting low sequence variation within variants (Coetzee *et al*., 2010b). This is different from what is observed in GVA and GVB where high sequence variation is observed between variants (Goszczynski and Jooste, 2003; Shi *et al*., 2004).

2.5 Virus detection techniques

Grapevine is an important agricultural crop that is susceptible to a range of pathogens. Detection of these pathogenic agents is of great importance to control the spread of the disease in vineyards. For the detection of plant viruses four techniques are generally used and these are based on: biological indexing (symptomology), serology, electron microscopy and nucleic acid binding methods.

Biological indexing is one of the oldest techniques used for virus detection. This technique makes use of symptom development for virus identification (Martelli, 1979). The virus is transferred from an infectious (test) vine to an indicator plant, which can be an herbaceous or woody plant. Plants are then left to grow in glasshouse conditions for symptom development.

For grapevine viruses hard-wood indexing has been used for the detection of viruses on indicator plants such as *V. rupestris* St george, *V. vinifera* cv Cabernet Franc and Kober 5BB (Gambino *et al*., 2010, Meng *et al*., 1999). Here, the chip buds of infected material are grafted onto indicator plants (Martelli, 1979). These plants are then left to grow and monitored for symptom development. The use of hard-wood indexing as a technique for viral detection in grapevine can be a time consuming and labour-intensive process (Weber *et al*., 2002).

The use of symptomology on its own as a detection technique is not sufficient for virus identification as the development of diseases symptoms are influenced by several factors. Symptom development can be the result of infection with more than one virus, different viruses can cause similar symptoms in the same host and the different strains of the same viruses may cause different disease symptoms. Viral infection can also be latent were no disease symptoms will be observed and various environmental conditions, such as temperature, can also influence symptom development (Weber *et al*., 2002).

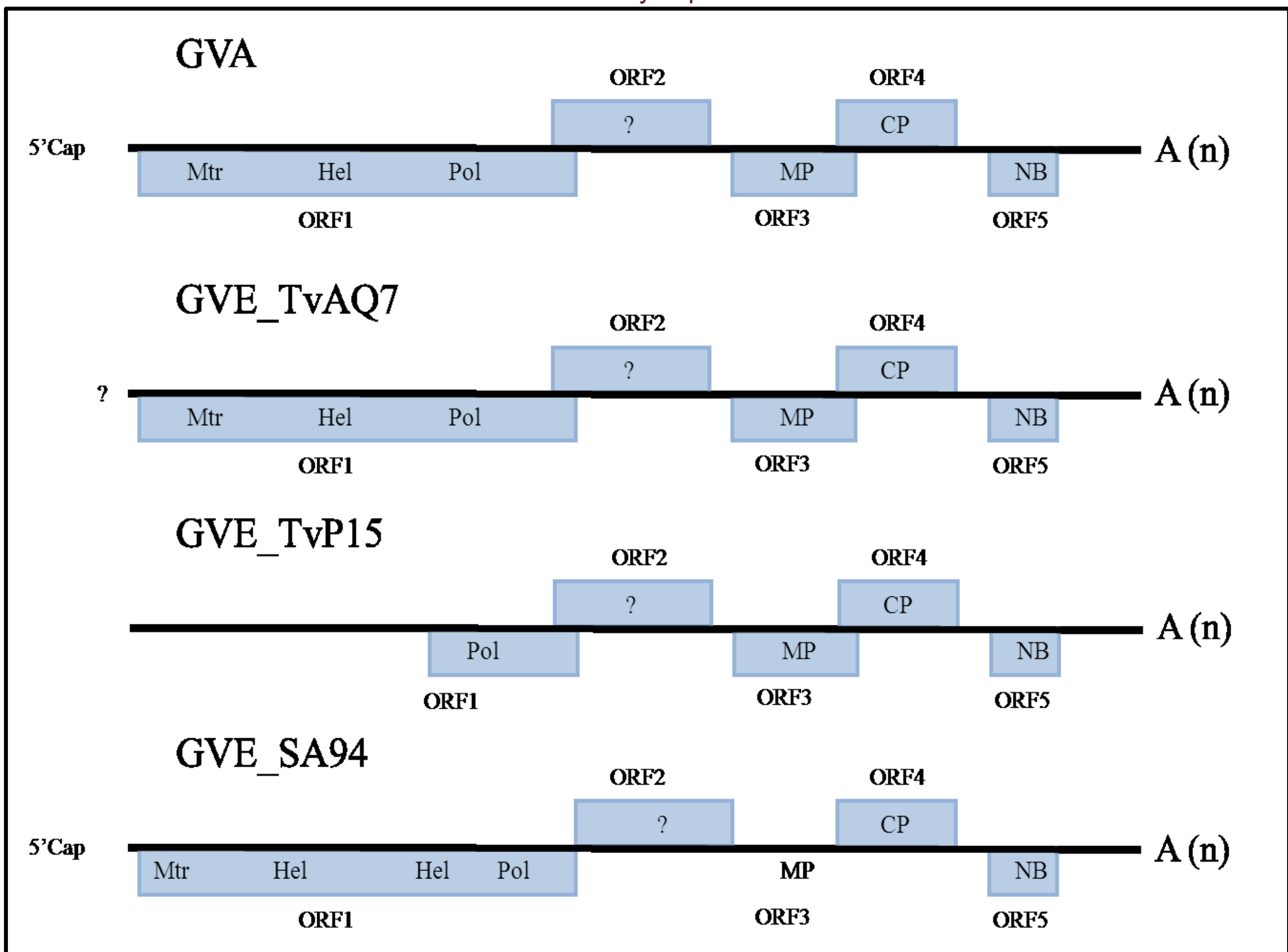

Figure 2.6: Genome organization of the GVE variants: TvAQ7, TvP15 and SA94 compared to GVA, the type member of the genus *Vitivirus*, indicating ORFs encoded in these viruses (modified from Nakaune *et al*., 2008). Mtr= methyl transferase, Hel= helicase, Pol= polymerase, MP= movement protein, CP= coat protein, NB= nucleotide binding protein.

Serological techniques make use of antibodies for the detection of an antigen, here the antibodies are coupled to an enzyme-mediated colour reaction that occurs upon detection. Several serological techniques have been developed for plant virus detection, these include techniques such as the Enzyme-linked immunosorbent assay (ELISA) of which several variations are available, including the double antibody sandwich (DAS) ELISA, triple antibody sandwich (TAS) ELISA and direct antigen coating (DAC) ELISA (Clark and Adams, 1977; Naidu and Hughes, 2001). Other techniques including the tissue-blot immunoassay (TBIA), western blot and dot blot assay. In South Africa, DAS-ELISA is the most popular serological test routinely used for the detection of grapevine viruses. In DAS-ELISA, specific antibodies are used for the detection of the viral coat protein (or viral particle). The ELISA is an inexpensive technique, suitable for viral detection in large sample numbers and can be use for the semi-quantification of the viral pathogen, without the need for viral purification (Gugerli and Gehringer, 1980; Reddy, 1981; Crowther *et al*., 1995). Limitations of the ELISA include the availability of antibodies, as production of antibodies is an expensive and labour-intensive process. It also has a lower sensitivity as compared to some nucleic

acid based detection techniques and is dependent on the presence (abundance) of the target for detection to occur (Reddy, 1981; Rowhani *et al*., 1998).

Electron microscopy is a specialized application used to determine the structure of the virus particle. This is a rapid procedure, for which plant crude extract, containing a high virus concentration is needed. High virus titers are usually obtained from an herbaceous plant, to which the virus was transferred, as the virus titer in their natural woody host are often too low (Reddy, 1981). The virus particles are visualized under a microscope, which makes use of a beam of electrons to produce a magnified image of the virus particle. Electron microscopy is a powerful tool, from which information such as topography and morphology can be obtained as well as the composition of the virus particles and crystallographic information (Voutou *et al*., 2008).

With the advances in molecular techniques, hybridization of nucleic acid is more popular for viral detection. This technique is based on the homology between nucleic acid strands, detecting the viral genome directly in RNA or DNA extracted from infected plant material. The dot-blot assay, RT-PCR, qRT-PCR, multiplex PCR detection and microarrays are some of the techniques based on nucleic acid hybridization currently used for plant virus detection.

For nucleic acid hybridization-based detection, nucleic acid has to be extracted from the infected plant tissue. Several nucleic acid extraction methods have already been described, which include total RNA (White *et al*., 2008) or dsRNA extractions (Valverde *et al*., 1990). The quality of the extracted nucleic acid is very important and the extraction process can be time consuming.

Probes and primers are used in molecular hybridization techniques for the detection of viruses (Rouhiainen *et al*., 1991). These probes and primers are single stranded nucleic acid molecules complementary to the virus genome sequence. Probes are reporter molecules and can be labelled as radioactive or non-radioactive for signal transmission (Sharma *et al*., 2009).

The use of RT-PCR has become more popular for the detection of RNA viruses because of the sensitivity, allowing detection even at low virus titer (Lievens *et al*., 2005). In RT-PCR, complimentary primers hybridize to specific positions on the viral genome and a thermostable enzyme amplifies that specific part of the genome. The RT-PCR products are separated with gel electrophoresis and visualized under UV light.

Another form of RT-PCR is the use of multiplexing. This technique allows for the simultaneous detection of different viruses in a single reaction (Dovas and Katis, 2003; Gambino and Gribaudo, 2006). Several primers are designed to amplify different pathogens in the same reaction. Some technical difficulties have been associated with the use of multiplexing, of which the compatibility of the primers is one of the most important aspects (Dovas and Katis, 2003). Extensive optimization is therefore needed for the efficient detection of the pathogens. The amplicons have to be

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distinguishable for each pathogen, for identification to occur after gel electrophoresis (Lievens *et al*., 2005).

Quantitative reverse transcription PCR (qRT-PCR) offers an enhanced sensitivity compared to conventional PCR (Dorak, 2006). With qRT-PCR an intercalating fluorescent dye is added that binds to dsDNA during the elongation phase. Upon amplification of the DNA, the amount of bound dye increases, emitting a fluorescent signal that is recorded in time. This eliminates the need for post-amplification analysis. The accumulation of fluorescence is recorded in real time as amplification occurs. In qRT-PCR amplification, detection and quantification of the pathogen in the starting material is possible (James *et al*, 2006). After amplification, melting curve analysis can be performed, making detection of different strains within a sample possible, as amplicons with sequence variation will melt at different temperatures (Farrar *et al*., 2010). High resolution melt analysis, which is an extension of the melt analysis, can also be performed by using a high saturation dye making detection up to strain variants possible (Corbett research, 2006). Detection specific fluorescent chemistries can also be used in qRT-PCR instead of adding a fluorescence dye, by adding labelled probes and oligonucleotide primers, increasing the sensitivity of the assay when detection occurs (James *et al*., 2006).

Combinations of the four mentioned detection techniques are also available. These include immunocapture PCR, which combine the use of serology with PCR, and immunosorbent electron microscopy, combining serology with electron microscopy (Candresse, 1995; Chevalier *et al*., 1995).

Newer detection techniques that are currently at the forfront of technology are also available. These include the use of microarrays and metagenomic sequencing. These technologies are still expensive to perform and extensive data analysis is needed as large amount of data can be generated (Lievens *et al*., 2005; James *et al*., 2006, Coetzee *et al*. 2010a; Giampetruzzi *et al*., 2012).

2.6 Infectious clones

The construction and manipulation of full-length infectious clones have proven to be a useful tool to investigate RNA viruses on a molecular level (Galiakparov *et al*., 2003; Lico *et al*., 2008). These infectious clones have been used in deciphering gene functions, understanding virus-host interactions and as vector systems for the expression of foreign genes. Reasons for their popular use are the ability to replicate and produce high copy numbers rapidly.

Infectious clones are constructed by reverse transcribing and amplifying the viral RNA genome into a cDNA copy. Infectious RNA is generated by cloning the viral genome under the control of a bacteriophage RNA polymerase promoter such as T7, T3 or SP6 (Chapman, 2008). The RNA transcripts can subsequently be generated *in vitro* from the bacteriophage RNA polymerase.

Alternatively, cDNA infectious clones are constructed from cDNA cloned into a bacterial plasmid under the control of a constitutive promoter such as CaMV 35S (Vives *et al.*, 2008). The viral infectious RNA is generated *in vivo* from cDNA in the bacterial vector with the help of host RNA polymerase.

Several approaches can be used to deliver infectious clones into plants. For the inoculation of whole plants; mechanical inoculation, agroinfiltration or biolistics are the methods commonly used while for protoplasts; electroporation, microinjection and liposome-mediated inoculations are used (Nagyova and Subr, 2007). During mechanical inoculation the plant or tissue of an herbaceous host are damaged with an abrasive material, which allows for direct inoculation of the nucleic acid. Agroinfiltration uses the natural ability of *Agrobacterium tumefaciens* to infect plants and transfer the DNA to the cell nucleus (Bevan, 1984) while with the biolistic approach, the DNA is shot directly into the nucleus on gold or tungsten microcarriers. Electroporation is where a high voltage pulse in an electroporator is used to make the cell membrane permeable to the nucleic acid, with micro-injection the nucleic acid is directly injected into the nucleus and liposomes are used as a non-invasive method to introduce the nucleic acid into protoplasts (Nagyova and Subr, 2007).

2.6.1 Grapevine virus A-based vector systems/ infectious clones

The first full-length cDNA infectious clone constructed for GVA is pGVAN3 (Galiakparov *et al.*, 1999). This infectious clone was constructed with a cDNA copy of the PA3 isolates' genome, cloned downstream of a T7 promoter. The pGVAN3 clone was shown to be infectious in herbaceous hosts *N. benthamiana* and *N. clevelandii*. Symptoms in *N. benthamiana* included vein clearing, leaf curling and mottling appeared 7-8 dpi (Galiakparov *et al.*, 1999). These symptoms were indistinguishable from the native virus. Galiakparov *et al*. (2003) used this infectious clone to determine the functions of 4 ORFs by site-directed mutagenesis.

In 2006, Haviv *et al*. reported a viral vector, pGVA118, for the expression of foreign genes in *N. benthamiana*. The vector was engineered with a duplicated movement protein sgRNA promoters. This viral vector was able to express the reporter gene GUS as well as the coat protein of the citrus tristeza virus in *N. benthamiana*. Even though the pGVA118 vector could express foreign genes, there were limitations associated with this vector as it was less efficient than expression vectors constructed from other plant viruses such as TMV and PVX.

To overcome the limitations associated with the pGVA118 vector, Muruganantham *et al*., (2009) cloned the cDNA under a duplicated CaMV 35S promoter and 35S termination signal. *Nicotiana benthamiana* and *in vitro* cultured *V. vinifera* were successfully infected by this viral vector.

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In 2010, Du Preez constructed an infectious clone for the GTR1-2 variant of GVA with the use of population cloning strategies and mutation correction. This infectious clone was able to infect *N. benthamiana* plants.

A cDNA infectious clone for GVB was constructed by Saldarelli *et al*., (2000). This clone was infectious in *N*. *benthamiana* but unstable in *E. coli* cells. Later, a stable clone was generated from a GVB isolate obtained from a *V. vinifera* plant displaying symptoms of corky bark (Moskovits *et al*., 2008).

2.6.1.1 GVA ORF2 and ORF5 deletion mutation vectors

The function of ORF2 in vitiviruses is still unknown, as there is no significant sequence homology or similarity to any known proteins in the protein databank. Mutation studies in ORF2 of the pGVAN3 infectious clone did not affect viral expression or movement in *N. benthamiana* (Galiakparov *et al*., 2003). It is suggested that the protein product could be involved in viral infection or transmissions of the virus by mealybugs. An exchange vector, pGVA-GR5-ΔORF2, containing a 35S promoter, a sgMP promoter and ORFs of GVA isolate GR5 with ORF2 deleted, was constructed (Du Preez, 2010) and evaluated for its use for gene expression and as a VIGS vector in herbaceous hosts. The vector was able to infect *N. benthamiana* plants and express the GUS reporter gene, successfully. This confirmed that ORF2 is not essential for viral replication or movement in *N. benthamiana*. The role of ORF2 in vitiviruses is still unknown.

Studies of the GVA genome suggest that the protein encoded by ORF5 could play a role in the pathogenicity of these viruses (Galiakparov *et al*., 2003). In *N. benthamiana*, infiltrated with the PA3 infectious clone containing mutations in ORF5, cell to cell movement was reduced and plants stayed asymptomatic. To further investigate the functions of this ORF, Blignaut (2009) made a ORF5 deletion-mutated infectious clone and inserted restriction enzyme sites in the GVA 118 infectious clone, creating pGVA118-ΔORF5. The ORF5-deleted clone was unable to infect *N. benthamiana* plants after infiltration. The ORF5 of three different South African GVA variants: GTR1-1, GTR1-2 and GTR11-1 were substituted in the pGVA118-ΔORF5 construct to study symptom development in *N. benthamiana*. Mild symptom development was observed in plants infiltrated with the GTR1-1 substitution compared to the severe symptoms observed for GTR1-2 and GTR11-1 substitutions (Blignaut, 2009). A recent study conducted by Haviv *et al*. (2012) revealed that swapping of the ORF5 from a virulent GVA strain to a mild stain resulted in severe symptom development and swapping ORF5 from the mild strain with the ORF5 of the virulent strain resulted in mild symptom development. This indicates that ORF5 is a determinant of the symptom development in *N. benthamiana*. Amino acid residue changes of the eight amino acids at the N-terminus were responsible for the change in symptom development. If the aa at this position

is a Ala or Ser severe symptoms develop in *N. benthamiana* whiles a Thr at this position resulted in mild symptoms development.

2.7 Conclusion

Grapevine virus E was first identified in Japanese vineyards (Nakauna *et al*., 2008) and more recently in South Africa (Coetzee *et al*,. 2010a), respectively. No disease association has been determined for GVE, although GVE-TvAQ7 was identified in a plant that displayed symptoms of RSP, while isolate SA94 was identified in a Shiraz plant displaying symptoms of SD. Grapevine virus E is a member of the genus *Vitivirus*, family *Betaflexiviridae* and members of this genus are associated with diseases such as the RWC and SD. These diseases are known to cause devastating losses in grapevine.

Though GVE has been characterized genetically, little is still known about this virus. Investigating biological and molecular properties of GVE could bring insight into how these viruses interact with their host and determine the possible impact it may have on vine health. Currently, there is no cure for viral infection and vines have no known natural resistance against viruses. It is important to study these viruses, to understand how they infected and cause disease. This information can be important in developing methods of control for viral infection.

2.8 References

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Internet resources:

(http://www.dpvweb.net/notes/showgenus.php?genus=Vitivirus)
(http://www.dpvweb.net/notes/showgenus.php?order=Tymoviriales)
(http://www.agf.gov.bc.ca/cropprot/grapeipm/virus.htm)
(http://wine.wsu.edu/research-extension/grape-growing/plant-health/virology/symptoms)

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Chapter 3

Detection and incidence of grapevine virus E in a South African vineyard

3.1 Introduction

Grapevine is susceptible to a range of pathogens such as fungi, bacteria and viruses. These pathogens reduce the quality of grapes by lowering berry weight and sugar content as well as decreasing the general health of the vine. The potential economic impact of these pathogens on the grapevine industry warrants research into the determination of the disease status of vines and the elucidation of the pathogenic agents associated with specific diseases. Results from these research projects form the basis upon which decision makers and farmers can introduce strategies to control or prevent the spread of pathogens and diseases. Reliable and sensitive detection techniques are therefore important for the certification of clean plant material (Rowhani *et al*., 2005).

Viruses are important pathogens infecting grapevine and so far, no natural resistance has been found against viruses (Liamer *et al*., 2009). Virus diseases of grapevine can spread rapidly through planting and grafting of infected propagation material as well as insect vectors. More than 60 different virus species have been found to infect grapevine, which is the most known for any agricultural crop (Martelli and Boudon-Padieu, 2006). Viruses that infect grapevine are commonly found in complexes, with reports of up to 9 different viruses being identified in a single vine (Prosser *et al*., 2007).

Detection of viruses is the first important step in viral disease control. Over the years many techniques have been developed for the detection and identification of viruses in plants. Currently, the ELISA and RT-PCR are the diagnostic tools of choice for the detection of viruses in grapevine (Monis, 2005).

In South Africa, ELISA is the industry standard for the detection of known viruses in grapevine. It is relatively inexpensive and robust to use. Enzyme-linked immunosorbent assay (ELISA) was developed in the late 1970’s (Clark and Adams, 1977) and is still routinely used for grapevine virus detection, with DAS-ELISA being the most frequently used.

More recently, the use of RT-PCR has become more popular (Constable *et al*., 2009) and is mostly used for high value samples or small sample sets (Monis, 2005). Reverse transcription PCR is a sensitive technique that can detect viral RNA at very low concentrations (Rowhani *et al*., 2000).

Detection is very specific as the primers are developed to hybridize to a specific sequence of the viral genome. However, this technique requires sequence information of the virus target.

Quantitative reverse transcription PCR (qRT-PCR) is more sensitive than conventional RT-PCR and has become the preferred technique for PCR based detection (Dorak, 2006). The advantages of qRT-PCR are i) the enhanced sensitivity, ii) reproducibility and iii) increased turnaround, due to a reduction in cycling time and the removal of post-PCR detection to evaluate the product (Mackey *et al*., 2002). The use of qRT-PCR also can provide additional information such as viral titer and discrimination between different strain variants. However, the running cost of qRT-PCR including acquisition and maintenance of qRT-PCR machinery is more expensive than the conventional RT-PCR.

The advantages associated with qRT-PCR makes it suitable to use for calculation of virus titer with absolute or relative quantification. Different methods and tools are available for relative quantification such as the two standard curve method, relative expression software tool (REST) (Pfaffl *et al*., 2002) and the delta delta ct method (Livak and Schmittgen, 2001). For accurate quantification, some important factors need to be considered, such as the quality of RNA and primers used in qRT-PCR reactions for optimal detection. Another factor is the use of an appropriate reference gene needed to normalize the data, compensating for experimental errors. Various studies have been performed to validate the use of certain genes as reference genes, as it is dependent on the organism and tissue that is used. Suitable reference genes for grapevine that are constitutively expressed in most tissues include actin, GAPDH and SAND family protein (Cottage *et al*., 2001; Reid *et al*., 2006 and Selim *et al*., 2012).

High resolution melt (HRM) analysis is an extension of the melting curve analysis performed during qRT-PCR. It uses the dissociation behaviour of dsDNA to ssDNA with increased temperature to distinguish between strands with different nucleotide compositions. A standard melt profile is created according to which unknown samples can be analysed. When analysing melt curves of different samples with HRM, it is possible to detect and identify different variants present within the samples (Farrar *et al*., 2010).

The above-mentioned techniques are all reliable and specific for the detection of known grapevine viruses. These diagnostic tests do not address the possibility of infection with new or unknown viruses. Next generation sequencing or deep sequencing is being used to determine the aetiology of diseased samples without prior sequence knowledge. A metagenomic study, performed in South Africa, used the sequencing-by-synthesis technology on an Illumina Genome Analyser II to determine the viral infection profile of an environmental sample that consisted of 44 randomly

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selected vines from a severely diseased vineyard. In this study, Grapevine virus E (GVE), a newly identified virus, was detected (Coetzee *et al*., 2010a).
Grapevine virus E was first identified by researchers in Japan, in *Vitis labrusca* and has a similar genome organization as members of the genus *Vitivirus* family *Betaflexiviridae* (Nakaune *et al*., 2008). Members of this family are known to infect grapevine and are associated with disease complexes such as the Rugose wood complex (RWC). It is therefore important to study newly identified viruses, like GVE, to determine the possible impact they may have on vine health.

Surveys have been performed all over the world where grapevine is grown, providing valuable information in deciphering the aetiologies of diseased vineyards (Constable *et al*., 2009; Fiore *et al*., 2008; Rayapati *et al*., 2009; Afsharifar *et al*., 2009; Vonicina *et al*., 2009; Fuchs *et al*., 2009 and Jooste *et al*., 2011). The ELISA and RT-PCR or a combination of both techniques, are generally used for viral detection in grapevine. Therefore, as an initial study on GVE distribution, a survey was performed to determine the incidence of GVE in the vineyard where GVE was first identified in South Africa. For the survey, RT-PCR was the only method of detection used for GVE, as an ELISA cannot be performed, due to the lack of anti-GVE antibody availability. With GVE being a member of the genus *Vitivirus*, family *Betaflexiviridae,* there is a possibility that the GVA DAS-ELISA could detect GVE. If the source plants used for the production of GVA antisera was infected with GVE, it is possible that GVE would be detected with a GVA ELISA. The cross reactivity with GVA, will be evaluated using a commercially available GVA DAS-ELISA kit. The relative virus titer of GVE in selected grapevine plants over the growing season of 2010/2011 was also determined with qRT-PCR.

3.2 Material and Methods

3.2.1.1 Sample collection

A survey to determine the incidence of GVE was conducted in a *Vitis vinifera* cv. Merlot vineyard (Kanonkop, Stellenbosch), where GVE was first identified in South Africa. Sample size was calculated for a 95% confidence, that the amount of samples is a true representative of the vineyard, with approximately 1320 vines. The sample size (n) is calculated is as follows:

$$n = \frac{\left(Z\left(1-\frac{\alpha}{2}\right)\right)^2 \cdot Pa\left(1-Pa\right)}{e^2}$$

Equation 1

In equation 1, Z $\mathbf{1}-\frac{\alpha}{2}$, is a quantile from normal distribution, were α was chosen as 0.05 for a 95% confidence, so that Z $\mathbf{1}-\frac{\alpha}{2}$ = 1.96. During an initial screening, 3 out of 30 samples gave a positive result (these GVE positive plants were used as positive controls for the survey), therefore a Pα, which is the initial estimate of the probability of a positive result, of 0.1 was used. For this study, equation 1 can be simplified to:

$$n = \frac{1.96^2 \cdot 0{,}1 \cdot \;\; 1 - 0{,}1}{e^2}$$

Equation 2

In equation 2, if $\boldsymbol{e}$, the sampling error, is made smaller, the more accurate the estimation of positive results. This also means that the number of samples increases. Figure 3.1 represents the relationship between sample size, n, and sampling error, $\boldsymbol{e}$.

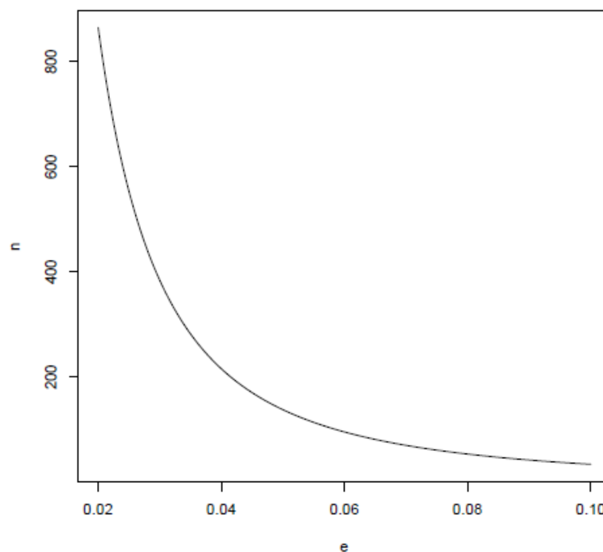

Figure 3.1: Graph indicating required sample size (n) as a function of sampling error (e).

For equation 2, e value of 0.05 was selected, implicating that the final result will be within 0.05% with a 95% confidence. The number of samples needed for e = 0.05, is 139.

Plants were randomly selected throughout the block and petioles from different canes of each plant were collected to compensate for possible uneven distribution of the virus within the vine. The symptoms of these plants were documented and collected plant material was immediately stored at -80°C. If positive plants were found, the plants flanking these plants were also collected and tested for GVE infection.

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For the detection and quantification with qRT-PCR petioles of five GVE infected grapevine plants were randomly collected every second week from 15 November 2010 – 30 May 2011.

3.2.1.2 Total RNA extraction

Total RNA extractions were performed according to White *et al*. (2009). Two grams of petioles were ground to a fine powder in liquid nitrogen and 10 ml CTAB extraction buffer [2% CTAB, 2.5% PVP-40, 2M NaCl, 100 mM Tris-HCl (pH8), 25 mM ETDA (pH8) (preheated at 65°C)] and 3% β-ME were added just before use. Samples were vortexed and incubated at 65°C for 30 min. Samples were centrifuged at 10 000 g for 10 min at 4°C. The supernatant was transferred to a new tube and subjected to two chloroform: isoamyl alcohol, (C:I), extractions where an equal volume C:I (24:1) was added to the samples. Samples were mixed and centrifuged at 10 000 g for 10 min at 4°C. The supernatant was transferred to a new tube and LiCl was added to a final concentration of 2 M, in order to allow precipitation of RNA for a minimum of 16 h at 4°C. A centrifugation step followed at 10 000 g for 1 h at 4°C to recover the RNA. The supernatant was removed and the pellet washed by centrifugation at 10 000 g for 15 min with 70% EtOH. The pellet was air dried and resuspended in 50 µl DEPC-treated water.

Agarose gel electrophoresis was used to evaluate the quality and integrity of RNA samples. RNA samples and RNase free loading dye (Fermentas) were loaded onto a 1% agarose gel [1% w/v agarose and 1X TAE buffer (40 mM Tris-acetate and 10 nM ETDA pH 8)] containing 0.5 µg/µl EtBr for visualization under UV light. Electrophoresis was performed at 80-100 V for 30-45 min in 1X TAE buffer. Agarose gels were visualized under UV light in a Multi Genius Bio-imaging system (Syngene™). Spectrophotometric absorbance at 230, 260 and 280 nm was used to evaluate the concentration and purity of RNA samples with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc), as per manufacturer's instructions. RNA samples were considered as suitable for qRT-PCRs when an A_{260}/A_{280} ratio of 1.8 – 2 and A_{260}/A_{230} ratio of ~2.0 for RNA was measured.

For the qRT-PCR analysis, DNase treatment was performed on all extracted RNA before the RNA was evaluated with agarose gel electrophoresis and with the NanoDrop™ 1000 spectrophotometer. Twenty µl 1X RQ1 RNase free DNase buffer (Promega) with 5 µl RQ1 RNase free DNase (Promega) was added to the extracted RNA. Reactions were performed in a final volume of 200 µl and incubated for 30 min at 37°C.

Following DNase treatment of RNA samples, phenol and chloroform clean-up was performed. An equal volume phenol (pH 4.3) was added to the samples, which were mixed by inverting. Samples were then centrifuged for 10 min at 13 000 rpm at 4°C. The supernatant was removed and placed in

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a new tube. An equal volume of chloroform was added to the supernatant and the centrifugation step was repeated. After centrifugation the supernatant was transferred to a new tube and 1/3 volume of 8 M LiCl was added to the samples. Samples were incubated at 4°C for a minimum of 16 h for RNA precipitation to occur. The samples were centrifuged for 1 h at 13 000 rpm (4°C) to pellet RNA and washed by centrifuging the samples for 15 min at 13 000 rpm at 4°C with 70% EtOH. The RNA was air dried in a BioCap (Captair™ from Labotec) for 15 min to remove excess EtOH and resuspended in 25 µl DEPC-H_2O.

3.2.2 Detection and incidence using RT-PCR

All 139 samples collected for the survey were screened for the presence of GVE. Primers that were used in the RT-PCR detection of GVE were designed on the consensus sequence of all the available sequences for GVE variants, which included GVE-TvAQ7 (Genbank accession number AB432910), -TvP15 (Genbank acc AB432911), -SA94 (Genbank acc GU903012) and Node3404 (Genbank acc GU903011). The GVE positive samples were subsequently screened for the following grapevine viruses: GLRaV-1, 2, 3, 5, 9, and GLRaV-3 isolate GH11, GVA, Grapevine virus B (GVB), GRSPaV and Grapevine fanleaf virus (GFLV), known to infect grapevine. The primers used are listed in Table 3.1.

Primer annealing was performed by adding 0.25 µl of 20 µM reverse primer to 200 ng RNA in a 5 µl reaction. The primer annealing cocktail was incubated at 70°C for 10 min, followed by 2 min in ice water.

Complementary DNA was synthesized by using AMV reverse transcriptase (Fermentas Life Sciences). The RNA and reverse primer was added to a master mixture containing 1X RT-Buffer [50 mM Tris-HCl, (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl and 1 mM DTT], 1 mM of each dNTP, 0.25 U RNaseOUT (Fermentas)] and 1U AMV in a 20 µl reaction. The reaction was incubated at 48°C for 60 min.

PCR's were performed using KapaTaq DNA Polymerase (KAPA Biosystems) in an AB 2720 Thermal Cycler (Applied Biosystems). The PCR reaction contained 1X Kapa buffer A (+Mg), 0.4 µM forward and reverse primer, 0.2 µM dNTP's, 1X cresol loading dye (20% w/v sucrose, 1 mM cresol), 1U KapaTaq DNA polymerase and 2.5 µl cDNA in a 25 µl reaction. The cycling conditions of the PCR were 94°C for 5 min for the initial denaturation, 35 cycles of 94°C for 30 s, T_A°C (as shown in Table 1) for 30 s and 72°C for 30 s for the amplification followed by a final elongation step for 7 min at 72°C. The PCR products were separated on a 1% (w/v) agarose gel (containing 0.5 µg/µl EtBr) in 1X TAE buffer. Electrophoresis was carried out at 100 V for approximately 45 min. Fragments were visualized with a UV transilluminator Multi Genus Bio-imaging system (Syngene™).

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Table 3.1: Primers used in RT-PCR for screening of survey samples, to test for viral infection.

Virus	Primer	Primer sequence	Amplicon length (bp)	T_A (°C)	Reference
GVE	GVE-1 For GVE-Rev	AATGGAGTCAAAAGCGATCC GTAGGGTCAATCAACCAACA	991	55	Coetzee *et al.*, 2010b
GVA	GVA-P-F-7038 GVA-P-F-7273	AGGTCCACGTTTGCTAAG CATCGTCTGAGGTTTCTACTA	238	56	MacKenzie, 1997
GVB	GVB-CP-F GVB-CP-R	TGACCTTCGTAACTGATGCT GCTGTGAAGACGTTCTTAGCAC	498	56	Shi *et al.*, 2004
GLRaV-1	LQVI-H47 LEVI-C-447	GTTACGGCCCTTTGTTTATTATGG CGACCCCTTTATTGTTTGAGTATG	397	58	Osman and Rowhani, 2006
GLRaV-2	Rooi-F Rooi-R	TATGAGTTCCAACACAAGCGTGC ACACCGTGCTTAGTACCTCC	681	58	Engelbrecht (unpublished)
GLRaV-3	LC1 LC2	CGCTAGGGCTGTGGAAGTATT GTTGTCCCGGGTACCAGATAT	546	58	Osman and Rowhani, 2006
GLRaV-3 GH11	LR3'SANZ'_26F LR3'SANZ'_775R	TAAATGCTCTAGTAGGTATCGAACAC CGAATGTAATCCATGACCTTAGG	750	55	Bester *et al.*, 2012
GLRaV-5	GR5HSP_V_F GR5HSP_C_R	AACACTCTGCTTTCTGCTGGCA TCTCCAGAAGACGGACCAATGTAA	272	58	Osman *et al.*, 2007
GLRaV-9	LR9-F LR9-R	CGGCATAAGAAAAGATGGCAC TCATTCACCACTGCTTGAAC	393	58	Alkowani *et al.*, 2004
GRSPaV	RSP-21F RSP-22R	GAGGATTATAGAGAATGCAC GCACTCTCATCTGTGACTCC	440	58	Meng *et al.*, 2003
GFLV	GFLV1-02b-2772 GFLV1-03b-3521	GCGAGTTCTATGATTGATG CTACCTTGCTTTGTCCT	750	55	Lamprecht (unpublished)

3.2.3 Detection by ELISA

Double antibody sandwich enzyme-linked imunosorbent assay (DAS-ELISA) developed for GVA was used to evaluate cross reactivity to GVE. Grapevine virus E positive samples were used and incubated with a GVA DAS-ELISA kit (BioReba), to evaluate the DAS-ELISA kit. The samples include those that test positive for GVE infection in the survey conducted (section 3.2.1) as well as other grapevine samples that tested positive for GVE, with RT-PCR, from another vineyard on the same farm (Kanonkop, Stellenbosch) *V. vinifera* cv. Cabernet Sauvignon. A GVA positive sample was included as a positive control and GVA negative grapevine material as negative control. The GVA DAS-ELISA was carried out according to the manufacturer's instructions and performed in NUNC maxiSorp® microtiter plates. A 5:1 ratio plant material with extraction buffer (200 mM Tris pH 8, 137 mM NaCl, 2% PVP 24 kD, 1% PEG and 0.05% Tween) was used and 100µl was analysed in triplicate. The change in colour development was measured with a Bio Rad xMark™ Microplate Spectrophotometer at 405nm between 30 and 120 min after incubation. By using the

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statistical equations for the t- and f-tests, significant difference in absorption for all the GVE positive samples were calculated.

3.2.4 Virus titer using qRT-PCR

Diagnostic primers were designed for detection of all known isolates of GVE (GVE_Diag_1F and GVE_Diag_1R, Table 4.1). Primer sequences for GVE are based on the multiple sequence alignment of the sequences available for GVE TvAQ7 (Genbank accession number AB432910), GVE TvP15 (Genbank acc AB432911), GVE SA94 (Genbank acc GU903012) and Node 3404 (Genbank acc GU903011). Primers were designed on Vector NTI Advance 11.0 (Invitrogen). Primers for grapevine viruses that were used in the study are listed in Table 3.2. Actin was chosen as reference gene as the primers amplify a region which contains an intron. This primer set could therefore distinguish between RNA amplification and DNA contamination and an optimized qPCR reaction already existed (Reid *et al.*, 2006). If RNA is the substrate for cDNA synthesis and subsequent amplification, an 82 bp fragment is amplified and if DNA including the intron is the substrate for PCR amplification, a larger 166 bp fragment can be detected.

Table 3.2: GVE and actin primers used in qRT-PCR amplification

Origin	Primer name	Primer sequence	Amplicon length (bp)	T_A°C	Reference
GVE	GVE-1 For GVE-R	AATGGAGTCAAAAGCCATCC GTAGGGTCAATCAACCAACA	991	55	Maree, (unpublished)
GVE	GVE_Diag_1F GVE_Diag_1R	AGTATTTGATGCTCAGTCACAGG GGGTTCTTATGGCCTGCTTA	216	58	This study
actin	*V. vinefera* actin F *V. vinefera* actin R	CTTGCATCCCTCAGCACCTT TCCTGTGGACAATGGATGGA	82	58	Reid *et al.*, 2006

A qRT-PCR protocol was developed for the detection of GVE and actin on the Qiagen Rotor-Gene Q. The standard qRT-PCR cycles used are described in Table 3.3.

Table 3.3: Cycling conditions used in qRT-PCR for amplifying GVE (GOI) and actin (reference gene).

Process	Temperature (°C)	Time	
Reverse transcription	48	45 min	
Initial denaturation	95	5 min	
Denaturation	95	20s	45 X
Primer annealing	58	20 s	45 X
Elongation	72	20 s	45 X
Melt	70-90	2 s for each steps	

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Standard curves were constructed for GVE and actin using a total RNA sample that tested positive for GVE with RT-PCR, during an initial screening for GVE. A dilution series using 200, 50, 12.5, 3.125 and 0.78 ng (a 4-fold dilution series) of total RNA was used to set up the standard curves. Ten µl diluted RNA was added to 2.5 µl 10X KapaTaq buffer A (+Mg) buffer, 0.4 µM forward and reverse primers, 0.2 mM dNTP's, 1 µM Syto®9 (Invitrogen™), 0.5 U AMV (Fermentas) and 1 U KapaTaq DNA polymerase in a 25 µl reaction. Samples were then amplified in duplicate for both the GVE and actin standard curve reactions.

The protocol and temperature cycles were set up as described for the construction of standard curves (Table 3.3). A standard concentration of 50 ng total RNA was used for qRT-PCR quantification reaction and samples were tested in duplicate. A calibrator sample (sample amplified in the construction of the standard curves) was also included into the amplification to normalize the virus titer runs with those of the standard curves. Sample 2 in the standard curves was used as calibrator sample.

3.3 Results

3.3.1 Detection and incidence of GVE in a South African vineyard

An initial screening of the selected vineyard was performed, and 30 plants were randomly selected and screened for GVE infection. Of these 30 plants, three tested positive for GVE infection, giving an estimated probability that 10% (0.1) of the plants in the vineyard are GVE infected. For the survey petioles from 139 randomly selected plants were collected throughout the vineyard (Figure 3.2). Samples were named according to the plant position in the vineyard, e.g. sample 1.19 was collected from plant number 19 in row 1.

The symptoms of all sampled plants were documented. This was done to possibly correlate GVE infection with a specific symptomatology. For the 139 samples collected a range of symptoms was observed, from samples that displayed no apparent disease symptoms to severely diseased. Samples were arranged into 4 groups: no symptoms, mild, medium and severely diseased, depending on the severity of disease symptoms observed (Figure 3.2).

	Rows																					
Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1																						
2																						
3																						
4																						
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Figure 3.2: Table view of the vineyard block surveyed at Kanonkop, Stellenbosch. The139 samples screened in this survey were randomly collected and are indicated in the colour boxes. Different colour boxes symbolise the severity of typical leafroll symptoms observed with yellow = mild leafroll symptoms, orange = intermediate leafroll symptoms, red = severe leafroll symptoms and grey = displays no apparent leafroll disease symptoms. Vines that tested positive for GVE with RT-PCR are indicated with a + in the green circles.

RNA was extracted from all 139 samples and visualized under UV light after separation in a 1% agarose gel to determine the quality and integrity of the RNA (Figure 3.3). The expected fragment sizes for 28S and 18S ribosomal RNAs (rRNA) migrates at approximately 1400 bp and 800 bp, respectively, compared to the molecular DNA marker. The intensity of the two rRNA bands is an indication of the concentration of the sample. Large molecular weight bands above 10000 bp are an indication of DNA contamination in RNA samples and smearing on the gel is indicative of degraded RNA. RNA that was extracted had no visible DNA contamination, but for some samples slight degradation of RNA was detected. RNA concentrations obtained ranged between 50–500 ng/µl.

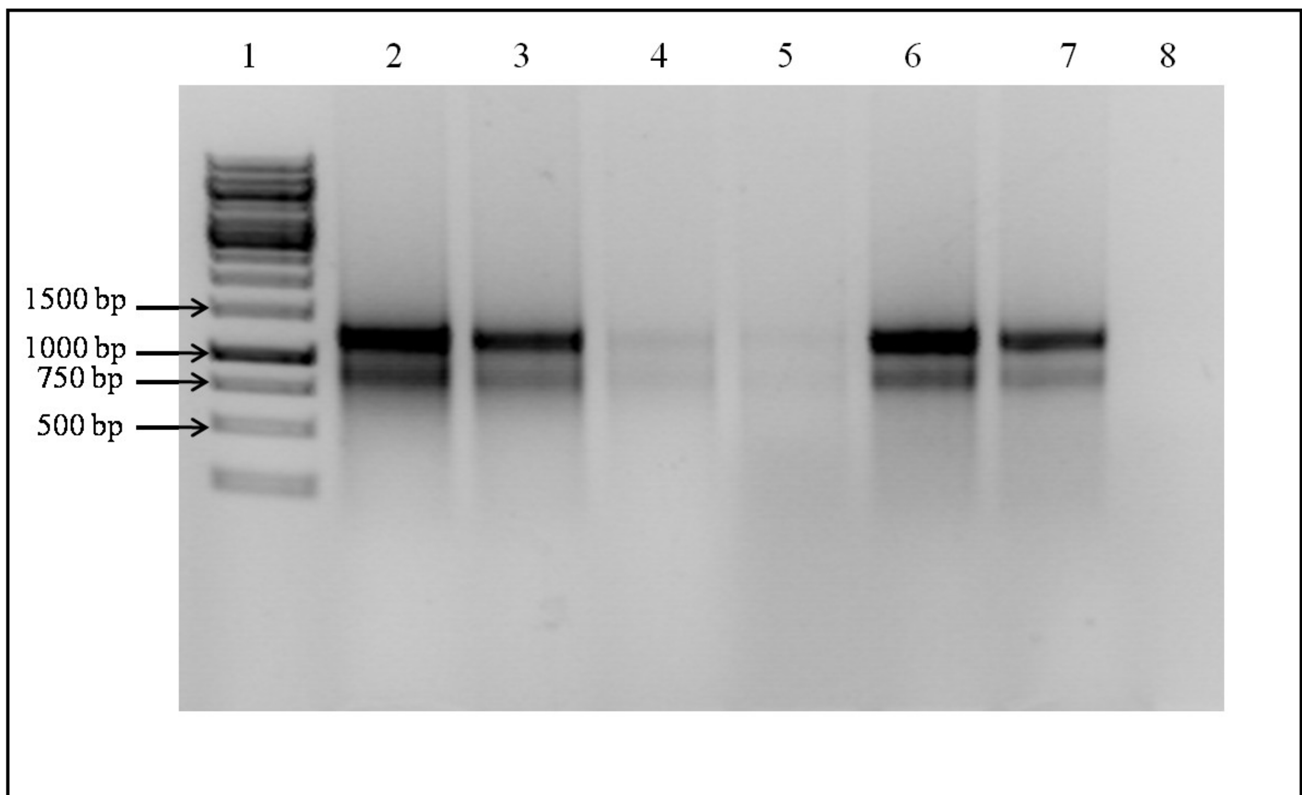

Figure 3.3: 1% Agarose gel indicating 28s and 18s rRNA from total extracted RNA. In lane 1) Gene Ruler™ 1kb DNA ladder (Fermentas) and Lanes 2 – 7) different RNA samples from the survey with different concentrations as determined by agarose gel electrophoresis. Lane 8) Water control.

A primer set (Table 3.1) amplifying a 991 bp fragment on the 3' end in the GVE genome was used to screen the 139 samples with RT-PCR for the presence of GVE. In this survey only four plants tested positive for GVE with RT-PCR (Figure 3.4 A), indicating an incidence below 3% (2.78%). Non-specific amplification was observed with the GVE 1F/rev primer set at ~700 and 250 bp. Sequencing of these fragments identified these as grapevine genome background. Therefore, with

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the statistical analysis that was performed, we are 95% confident that less than 3% of the vineyard block in which the survey was conducted, was infected with GVE.
As three of the four GVE positive plants were in close proximity to each other the adjacent plants were also screened for GVE infection, as this could be a possible hot spot for viral infection. Reverse transcription-PCR indicated that these plants were not infected with GVE. The virus has therefore not spread to any of the adjacent plants in this block.

The four GVE positive plants were screened for the presence of other viruses. Amplification using the LC1/LC2 primers detecting GLRaV-3, indicated that one GVE positive plant, namely 5.19 (Figure 3.4 B) was co-infected with GLRaV-3. The GVE positive samples were further screened to determine if a newly identified GLRaV-3 isolate, namely GLRaV-3 GH11, was present, which cannot be detected by the standard GLRaV-3 primer set LC1/LC2. By using the LR3L1 primers a 750 bp fragment is amplified, detecting GLRaV-3 GH11. All GVE positive plants were infected with GLRaV-3 isolate GH11 (Figure 3.4 C). This indicates that all of the GVE positive plants are co-infected with the GLRaV-3 GH11 isolate and plant 5.19 is co-infected with an additional GLRaV-3 isolate.
The symptoms observed in grapevine plants used in this survey were documented to determine if there is a correlation between GVE infection and disease symptoms. All four GVE positive grapevine plants displayed typical LRD symptoms, which includes down rolling of leaf margins and interveinal reddening. Samples 1.19, 1.20 and 5.19 were severely diseased, while sample 11.51 displayed medium to mild symptom development.

A summary of the survey results are given in Table 3.4. Out of the 139 samples screened, four samples tested positive for GVE infection. These samples were screened for other viruses known to infect grapevine to correlate with disease symptoms that are observed. The GVE-positive samples were also screened for the presence of other members of the genus *Vitivirus,* which included GVA and GVB. All of the GVE-positive samples tested negative for these two viruses. The GVE-positive samples were screened for viruses that are, besides GLRaV-3, associated with LRD, which included: GLRaV-1, GLRaV-2, GLRaV-5 and GLRaV-9. Nevertheless, no co-infections with any other leafroll associated virus other than GLRaV-3 were detected. Screening for the presence of GRSPaV and GFLV were also negative.

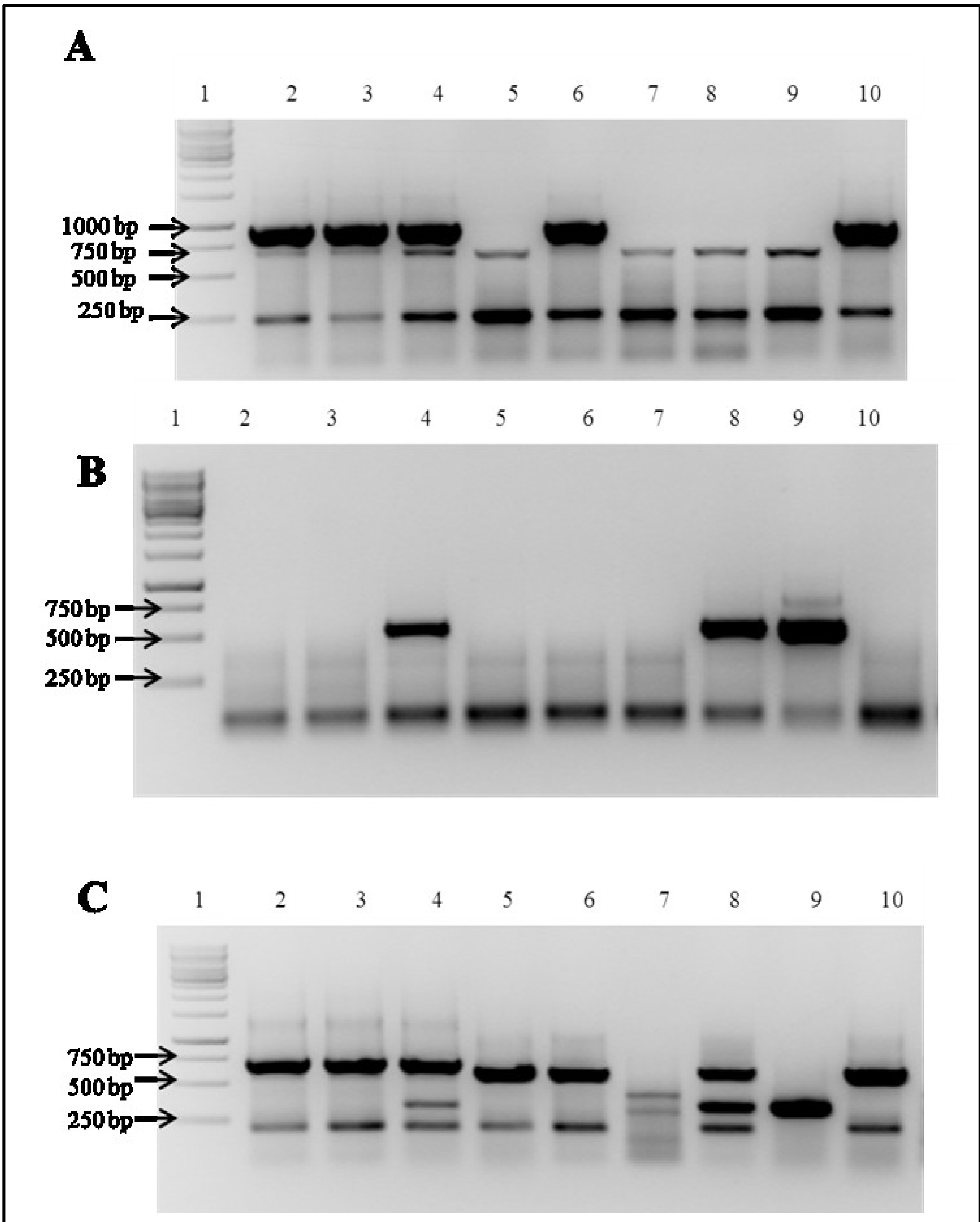

Figure 3.4: 1% Agarose gel photos indicating positive samples for A) GVE, B) GLRaV-3 and C) GLRaV-3 GH11. Lane 1) 1kb molecular marker, lane 2) survey plants 1.19, lane 3) survey plant 1.20, lane 4) survey plant 5.19, lane 5) survey plants 7.20, lane 6) survey plant 11.51, lane 7) survey plant 22.25, lane 8) GVE positive control (GVE +), lane 9) GLRaV-3 LC1/LC2 positive control (GLRaV-3 +) and lane 10) GLRaV-3 GH11 positive control (GLRaV3 GH11 +).

Table 3.4: Summary of survey results.

GVE positive samples	Symptoms*	GVE	GVA	GVB	GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-3 GH11	GLRaV-5	GLRaV-9	GRSPaV	GFLV
1.19	+++	√	-	-	-	-	-	√	-	-	-	-
1.20	+++	√	-	-	-	-	-	√	-	-	-	-
5.19	+++	√	-	-	-	-	√	√	-	-	-	-
11.51	++	√	-	-	-	-	-	√	-	-	-	-

*Typical LR disease symptoms:

+++ Serverly diseased

++ Medium symptom development

+ Mild symptom display

3.3.2 Screening by ELISA

Grapevine virus A specific DAS-ELISA was used to screen 10 GVE-positive plants (Table 3.5). The four samples that tested positive for GVE infection in Table 3.4 and six other GVE positive samples from another study in the same vineyard (*Vitis vinifera* cv Cabernet Sauvignon). Absorbance values for all the samples evaluated with the GVA DAS-ELISA are indicated in Figure 3.5. All GVE-positive samples, except for 22.25, had no differences in absorbance values between these samples and the negative control.

Reverse transcription-PCRs were performed with GVA diagnostic primers (GVA-P-F-7038 and GVA-P-R-7273, Bertin *et al*., 2010) on samples used in the GVA DAS-ELISA. Sample 22.25, was the only sample that tested positive for GVA by RT-PCR, confirming DAS-ELISA results and mixed-infection of that plant with GVE and GVA.

Table 3.5: The average GVA DAS-ELISA absorbance for the GVE positive samples at 405 nm. With the standard error and P-values indicating significant difference relative to mock inoculated plants.

Sample	Average absorbance (405 nm)	Standard error	P-value	Result
1.19	0.184	0.005	0.532	Negative
1.2	0.176	0.001	0.268	Negative
5.19	0.174	0.007	0.267	Negative
11.51	0.187	0.002	0.633	Negative
20.31	0.218	0.034	0.571	Negative
21.28	0.195	0.003	0.972	Negative
22.2	0.195	0.001	0.996	Negative
22.6	0.196	0.004	0.948	Negative
22.25	0.310	0.002	0.002	Positive
26.34	0.206	0.008	0.961	Negative
GVA positive	2.322	0.205	0.000	Positive
GVA positive	2.528	0.042	0.000	Negative
Resuspension buffer	0.195	0.113	-	-

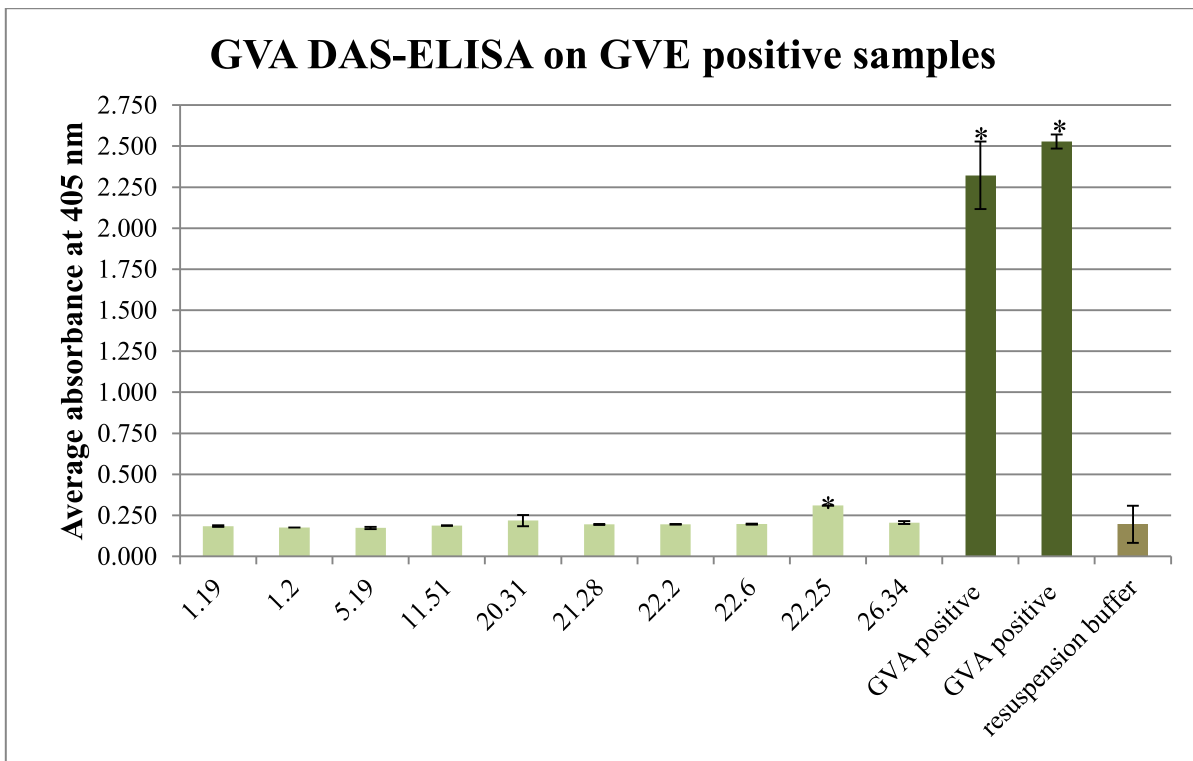

Figure 3.5: Bar chart with average absorption of 3 replicates per plant extract at 405 nm for DAS-ELISA of GVE positive samples.

* Statistical significant difference to the resuspension buffer.

3.3.3 Seasonal virus titer

3.3.3.1 Construction of standard curves

Grapevine material from 5 different GVE positive plants (3 Merlot and 2 Carbernet) was used to determine the relative GVE titer over the growing season. Plant material was collected every two weeks, for 15 time points, between 15th November 2010 and 30st May 2011. The RNA purity and concentration were determined as between 1.8 and 2 for A260/280 and ~2 for A260/230, indicating good quality RNA for qRT-PCR with the NanoDrop™ 1000 spectrophotometer. Two clear bands at ~1400 and ~800bp indicating 28S and 18S ribosomal RNA were observed on a 1% (w/v) agarose gel, indicated intact RNA. The RNA samples were used to optimize the qRT-PCR protocol on the Qiagen Rotor-Gene Q, detecting GVE as well as actin mRNA in *V. vinifera*, using the primers in Table 3.2.

For relative quantification, standard curves were constructed for both GVE (Figure 3.6) and actin (Figure 3.7) to determine the reaction efficiencies. The reaction efficiencies were obtained by plotting the ct values of the standards against the logarithm of the concentrations. For GVE (Figure 3.6) a reaction efficiency (e) of 0.89, a slope (M) of -3.607 and a regression correlation coefficient (R^2) of 0.967 was obtained. For actin (Figure 3.7) a reaction efficiency (e) of 0.89, a slope (M) of -

3.66 and a regression correlation coefficient (R^2) of 0.987 was obtained. These results indicate that the reactions were sufficient for relative quantifications with reproducible results.

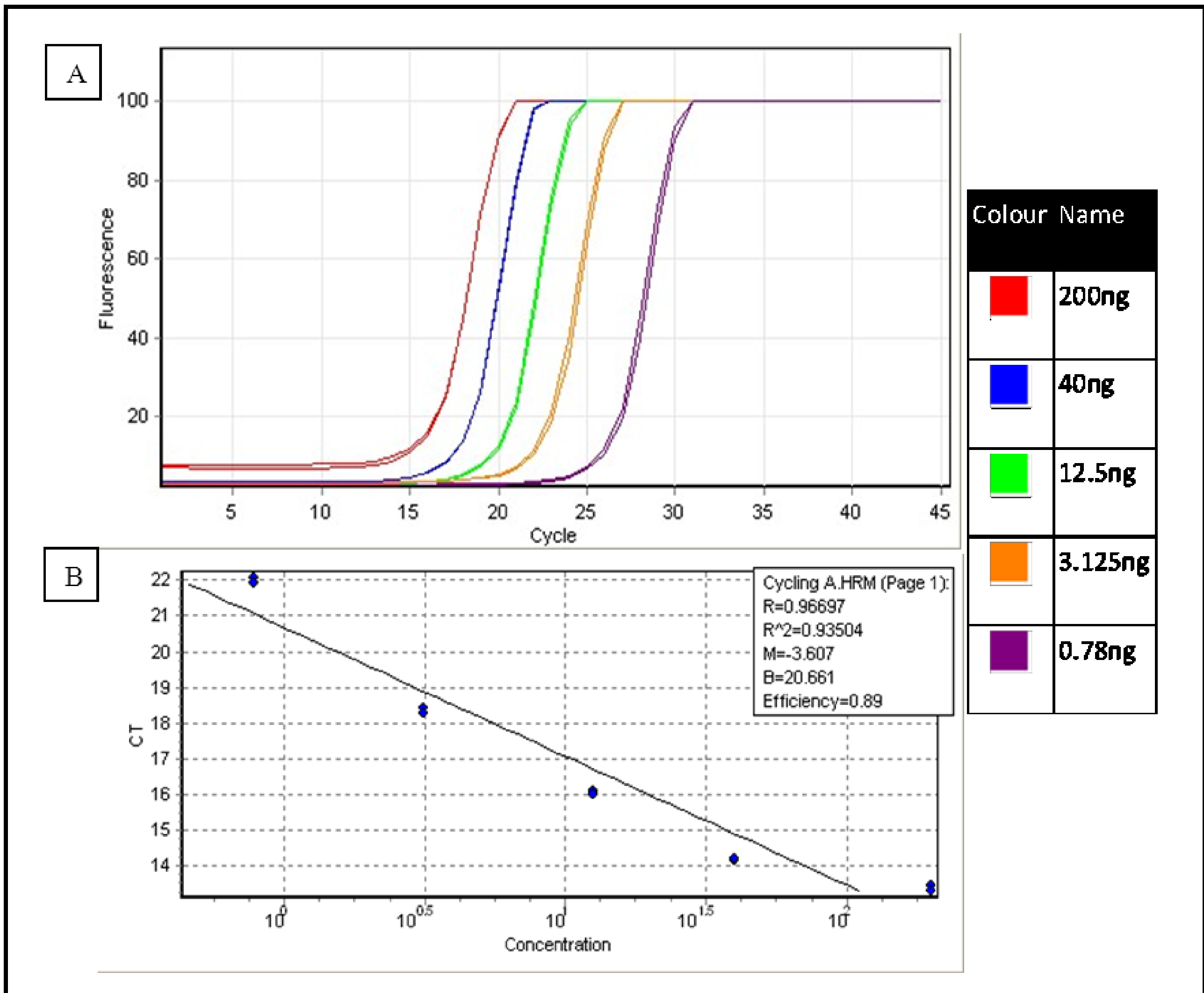

Figure 3.6: Standard curve amplification in qRT-PCR for GVE with primers (GVE_Giag_1F and GVE_Giag_1R). A) The amplification curve with increase in fluorescence against increase in cycles, with a dilution series of 200, 50, 12.5, 3.256 and 0.98 ng total RNA in duplicate and B) the standard curve with ct values plotted against the concentration series with e=0.893, M=-3.607 and R^2=0.935.

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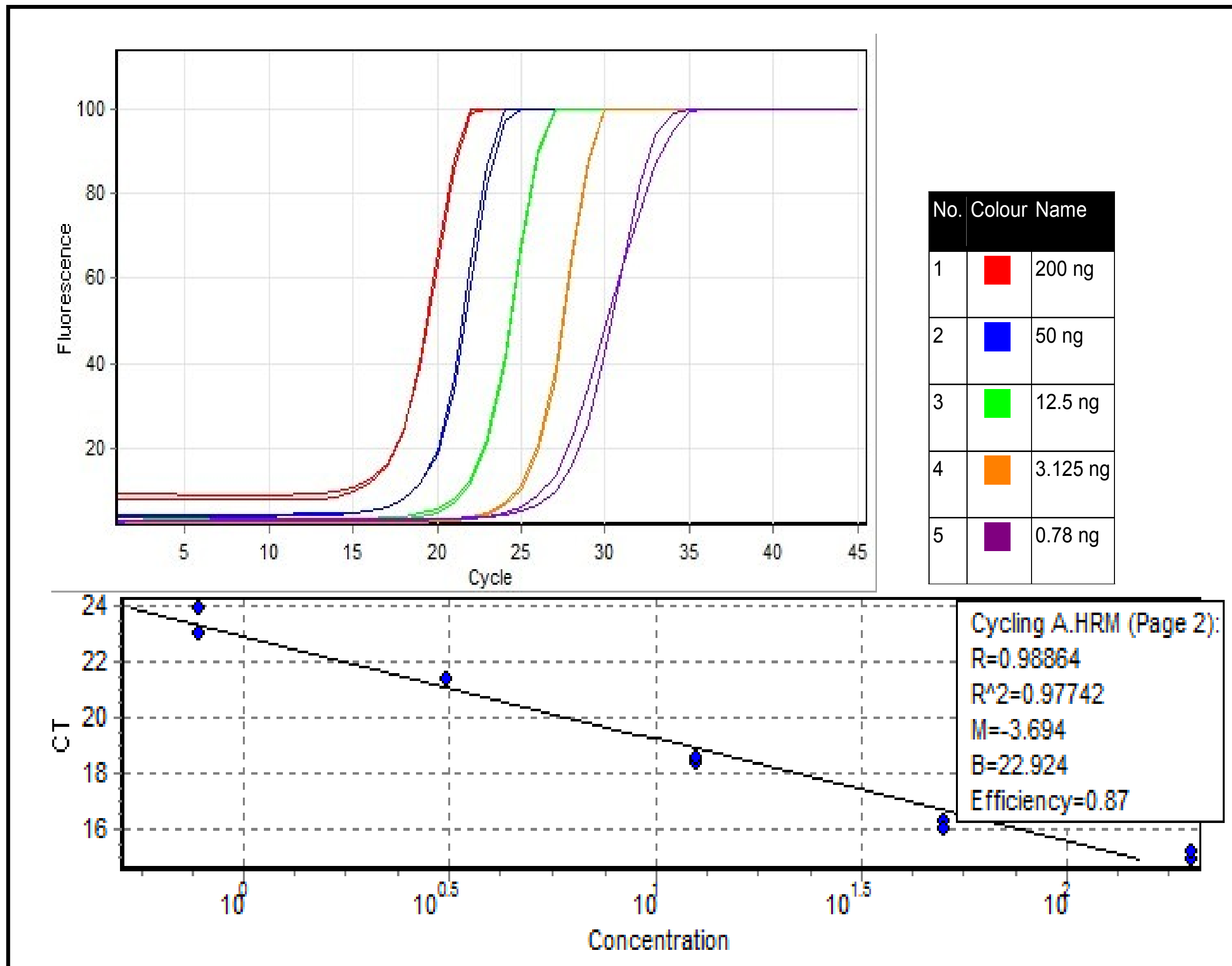

Figure 3.7: Standard curve amplification for actin with primers with A) the amplification curve with increase in fluorescence against increase in cycles, with a dilution series in duplicate of 200, 50, 12.5, 3.256 and 0.98 ng total RNA and B) the standard curve with ct values plotted against the concentration series with e=0.87, M=-3.694 and R^2=0.988.

3.3.3.2 Relative quantification of GVE

After standard curves were constructed and reaction efficiencies were obtained for GVE and actin, detection and relative quantifications of GVE for seasonal virus titer was performed for five GVE positive plants. Sample 2 from the standard curves, with 50 ng RNA, was used in relative quantification runs as calibrator sample to compensate for variation between runs and 50 ng RNA was amplified for all samples to simplify downstream calculations.

The Qiagen Rotor-Gene software was used to obtain ct values for GVE, actin as well as the calibrator sample from the amplification curves for 5 GVE positive samples for 15 time points throughout the season. The reaction efficiencies for GVE and actin obtained from the standard curves (Figures 3.6 and 3.7) and the ct values of the GVE positive samples throughout the season was calculated according to the standard curve method for relative quantification, to calculate the relative virus titers (Table 3.6). The calibrator sample negative control stayed relatively constant.

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When plotting the relative virus titer against the week of sample collection, the titer stayed more or less constant over the season. The titer for sample 5.19 is slightly higher than that of the other for samples, but no significant fluctuation in GVE titer was observed throughout the season (Figure 3.8).

Table 3.6: Results for the relative virus titer calculation over the growing season of 2010/2011, with GVE/actin ratio for relative virus titer of 5 GVE positive samples. Ratios were calculated for 15 time points of samples collected every other week.

Sample	om1.19	om1.20	om5.19	c22.25	c26.34
2010/11/15	0.825	0.945	0.949	1.107	1.037
2010/11/29	0.913	0.809	1.102	0.910	0.939
2010/12/13	0.949	0.889	1.018	0.856	1.012
2010/12/27	0.906	0.909	1.090	1.012	0.880
2011/01/10	0.905	0.969	1.050	0.970	0.977
2011/01/24	0.933	0.970	1.050	0.924	0.924
2011/02/07	0.929	0.927	1.100	0.956	0.946
2011/02/21	0.956	0.797	1.064	0.943	0.952
2011/03/07	0.944	0.915	1.116	0.977	1.016
2011/03/21	0.958	0.930	1.096	0.981	0.987
2011/04/04	1.030	1.058	1.100	0.995	0.928
2011/04/18	0.972	0.906	0.959	1.033	1.026
2011/05/02	0.942	1.031	1.070	0.975	1.057
2011/05/16	0.915	0.994	1.014	0.955	1.001
2011/05/30	0.901	0.928	0.983	0.891	0.952
calibrator	0.933	0.930	1.035	0.998	1.133
negative control	0.883	1.070	1.224	1.085	1.196

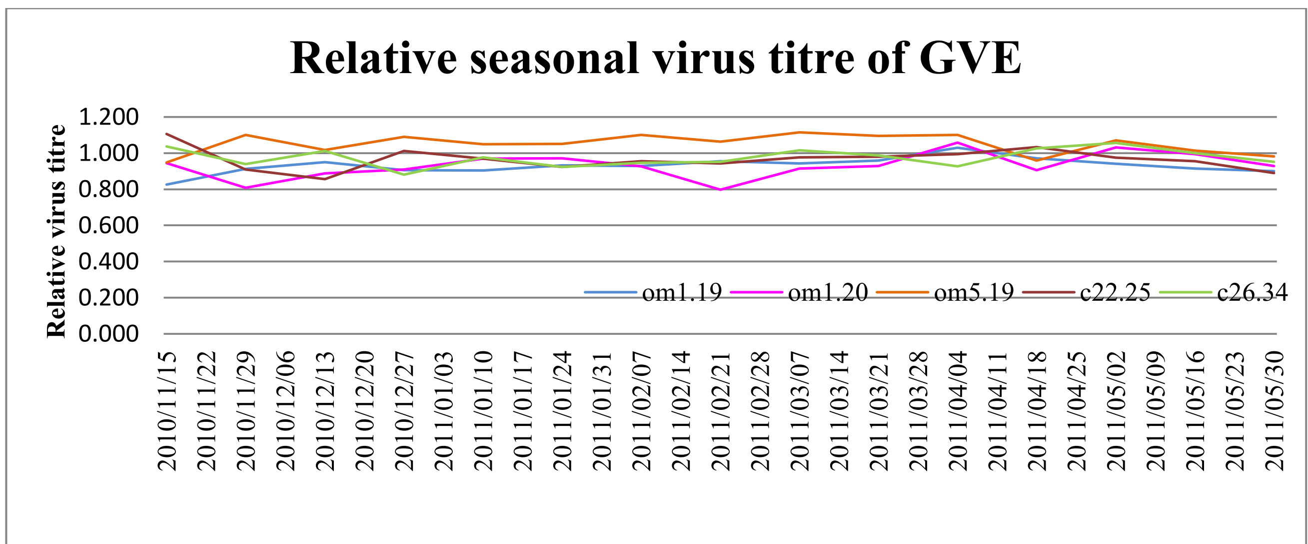

Figure 3.8: Graph results for the relative virus titer calculation over the growing season of 2010/2011 with GVE/actin ratio for relative virus titer of 5 GVE positive samples. Ratios were calculated for 15 time points of samples collected every other week.

3.4 Discussion and conclusion

Viral diseases have a negative impact on the grapevine industry and research is needed to better understand what impact specific viruses have on the grapevine host. This is to establish, if preventative measures are required to control the spread of viruses especially those causing disease. With the identification of new viruses, like GVE, research is required to determine the possible impact these viruses may have on vine health. A survey was conducted to determine the incidence of GVE in the vineyard where it was first identified in South Africa. Little is known about GVE as it has only been characterized genetically. Performing a survey will give an indication of the spread and prevalence of this virus and determine if there is any disease association or distribution clusters within the vineyard.

An RT-PCR was performed for the detection of GVE with primers that allowed for the detection of all GVE variants known to date, minimizing the risk of false negative results. Unspecific amplification also occurred during these amplifications, indicating that further optimization is still required. This warranted the development of new primers, for downstream detection. Here, the sequencing and BLAST analysis of these unspecific amplicons showed that it aligns with the grapevine genome. Other surveys, determining virus incidence in a vineyard used RT-PCR and additionally ELISA-test for viral detection (Fiore *et al*., 2008; Milkus *et al*., 2009; Padilla *et al*., 2009). This is done to confirm results and minimize the possibility of false negative or false positive results. However, for GVE, anti-GVE antibodies are not yet available and only RT-PCR was performed for virus detection.

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The RT-PCR results indicated a low incidence of GVE in the surveyed vineyard. Out of the 139 vines collected, only four vines tested positive for GVE. With 95% accuracy, it was determined that only 2.78% of plants in the vineyard block are infected with GVE. In the metagenomic study, in which GVE was first identified and which was performed in the same vineyard, 0.9% of the data obtained represented GVE (Coetzee *et al*., 2010a). As in this survey only four GVE-positive plants were found in the specific vineyard and no clear distribution pattern can be described. The GVE infected plants seem to be concentrated in one area of the vineyard where 3 out of 4 infected plants in close proximity to each other were detected. This might indicate a hot spot for infection at the border of the vineyard block, nevertheless, more samples need to be tested in that area to test the hypothesis, especially as the general incidence was low and screening of the adjacent plants did not indicate GVE infection.

All GVE positive vines displayed typical LRD symptoms, this includes downward rolling of leaf margins and the interveinal leaf areas turning red. Three of the GVE-positive plants displayed severely diseased symptoms while one showed medium disease symptoms. The South African variant GVE-SA94 was detected in a Shiraz plant that displayed typical SD symptoms that included: non-lignified canes, delayed leaf fall and reduced vigor. The infection status of this plant did reveal the presence of GLRaV-3, GRSPaV and GVA with GVE infection (Coetzee *et al*., 2010b). The GVE_AQ7 variant was detected in a plant which displayed symptoms of stem pitting, this plant was also infected with GRSPaV (Nakaune *et al*., 2008). The causative agent of the disease symptoms observed in these plants could therefore not be determined since any of the detected viruses, a combination of viruses or another pathogen could be involved in the disease aetiology. Grapevine is often infected with several viruses at the same time and up to nine different viruses have been found infecting a single vine (Prosser *et al*., 2007). This adds to the complicated disease aetiology seen in grapevine.

Vines infected with only one virus are rare in established vineyards and since GVE-SA94 was identified in plants containing mixed viral infection, the GVE-positive plants from this survey were screened for GVE as well as other viruses known to infect grapevine, to indicate if mix infections are present in these plants. All of the GVE-positive plants tested positive for GLRaV-3.

This study could not determine if GVE is associated with any specific disease symptoms. Co-infection with GLRaV-3 could mask any symptoms resulting from GVE infection due to the dominance of the leafroll disease symptoms. No GVE single-infected plant grapevine was identified in this survey.

The GVA DAS-ELISA (BioReba) kit, was evaluated for cross reactivity with GVE. The ELISA is a serological test that is widely used for the detection of viruses infecting grapevine (Afsharifar *et al*., 2009; Marterazzi *et al*., 2009). Grapevine virus E is a newly identified virus and an ELISA has not

been developed for this virus. If GVE was present in the plant source material from which anti GVA antisera was produced, the GVA DAS-ELISA (BioReba) kit would potentially be able to detected GVE.

Nine plants that tested positive for GVE infection with RT-PCR were also used to evaluate their infection status using a commercially available GVA DAS-ELISA kit. The ELISA results indicated that nine of the GVE RT-PCR positive samples tested negative for GVA with only one of the GVE positive samples, namely 22.25, testing positive for GVA infection using DAS-ELISA. The DAS-ELISA results for plant 22.25 were confirmed with RT-PCR that indicated co-infection with GVE and GVA. These results indicated, that the used of the GVA DAS-ELISA kit specifically detects GVA, and not GVE.

The GVA DAS-ELISA detects the epitopes of the viral coat protein, the sub-units of viral particles. The coat protein of GVA is encoded by ORF4. The amino acid similarity between GVA and GVE for the ORF4 encoded protein is between 41% and 46% (Nakaune *et al*., 2008). The amino acid similarity is low and even though the GVA DAS-ELISA makes use of both mono- and polyclonal antibodies, no cross reactivity were observed with GVE. In Nakaune *et al*. (2008) GVA and GVB DAS-ELISA were also performed on GVE positive samples using commercial kits from Agritest, Valenzano Italy and in both tests, GVE were not detected. These results indicated that the coat protein epitopes of GVE and GVA are different enough from each other, so that GVE is not detected with GVA DAS-ELISA, but also showed that a GVE specific serological detection kit is needed.

The relative virus titer of GVE was calculated with qRT-PCR over the growing season from 15th November 2010 until 30st March 2011. To determine the relative virus titer of GVE, two standard curves were created, one for GVE and one for the reference gene actin, to which the data was normalised. Actin is one of the first genes used for normalization in qRT-PCR and specifically in grapevine and has proven to be suitable for quantification in several studies involving grapevine (Gamm *et al*., 2011). The reaction efficiencies obtained from the standard curves for GVE and actin was 0.893 and 0.87, respectively. The 'two standard curves method' was used to calculate the relative virus titer as it does not assume 100% efficiency, such as some of the other methods and takes the actual efficiency into account. The results obtained from the relative GVE titer calculation did not indicate a clear fluctuation throughout the season. The titer in one of the plants, 5.19, was slightly higher than that of the other four samples. This could be a result of the co-infection status of the plant. This is the only plant that tested positive for GLRaV-3 isolate GH11 as well as for GLRaV-3 with the LC1/LC2 primers that does not detect GLaV-3 isolate GH1. Two GLRaV-3 variants were present in this sample, while in the other GVE positive samples only the GLRaV-3 GH11 isolate was detected. The relative virus titer for GVE positive plants stayed relatively

constant over the period of sample collection. Higher virus titer could allow for an easier detection of infection in plants and knowledge about seasonal variations in titer can be used to select periods in which detection is more efficient.

In grapevine, disease symptoms are most pronounced during late autumn making it the obvious time to screen for viral infection. But, viral disease symptoms are not always a direct correlation of viral infection as the virus can be dormant and display no apparent disease symptoms. TaqMan qRT-PCR assay was recently used to determine virus titer fluctuations of GFLV during the growing season of 2008/2009 in Slovenia (Cepin *et al*., 2010) and in 2007/2008 in Taiwan for GLRaV-3 (Tsai *et al*., 2011), respectively. Findings indicated that GFLV titer was at its lowest during the summer seasons. This could be the result of environmental conditions such as the warm temperatures during this season, which does not favor viral replication. Similar results were obtained for the virus titer of a GLRaV-3 with the virus titer at the highest during late autumn (Tsai *et al*., 2011).

The reason for the difference in expression of virus titer for GVE in comparison with the clear fluctuation for GFLV and GLRaV-3 are unknown. As suggested by Tsai *et al*. (2011) co-infection may lower virus titer, as all of the GVE positive plants are also infected with GLRaV-3. Another reason no clear fluctuation in the virus titer of GVE is seen could be because GVE is a newly identified virus, infection could be recent and virus replication is not very efficient in the host plant. Nevertheless, as the GVE titer seems to stay constant throughout the season, no recommendation could be drawn for a specific period of sampling to detect GVE infection in grapevine. The relative virus titers calculated during these experimental procedures were performed for samples from the same vineyard for only one growing season as an initial indication of GVE virus titer. To confirm these results, more data from additional growing seasons are needed.

In conclusion, the survey performed to detect GVE indicated that 2.78% of the vineyard is infected with GVE. No disease association has been determined for GVE. In the serological test, GVE could not be detected with the Bioreba GVA DAS-ELISA kit indicating the industry standard serological test for GVA detection in South Africa, is still valid. The relative virus titer calculation over one growing season indicated that there was no fluctuation in relative virus concentration for GVE. The results of the seasonal virus titer need to be confirmed including more growing seasons. Further analysis is therefore still needed to determine the possible impact this virus might have on grapevine and its distribution in South African vineyards.

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Chapter 4

Biological characterization of GVE

4.1 Introduction

The vitivirus GVE is a newly identified virus that has only been characterized partially. Studying virus-host interactions is important to understand disease associated with viral infection. The transmission of viruses to alternative hosts has proven to be useful to study viruses. Herbaceous plants are sustainable under glasshouse conditions, grow faster and can produce a high virus titer (Fulton, 1966). Transferring viruses to herbaceous plants is dependent on the susceptibility of the plant and cannot be predicted. Previous studies have shown that some herbaceous plants are susceptible to grapevine viruses; these plants include *N. benthamiana* (Monette and James, 1990), *N. clevelandii* (Conti *et al*., 1980), *Chenopodium amaranticolor* (Dias, 1963) and *C. quinoa* (Vigne *et al*., 2005).

Transferring viruses from woody plants can be challenging, as the viruses can be sensitive to denaturation that will affect the virus's ability to infect. The oxidation of phenolic compounds in the plant extract can inactivate viruses; the inoculation sap therefore has to be either kept on ice, diluted or an anti-oxidant added to slow down the oxidation process (Fulton, 1966). The acidity of grapevine plant sap can also reduce the efficiency of transmission to the herbaceous host; as pH may lead to necrotic reactions. This can be overcome by using a transmission buffer that neutralizes the acidity such as phosphate buffers at pH8 (Fulton, 1966). It is therefore important to choose the correct buffer that will facilitate successful virus transmission. In previous studies, the successful transmission of grapevine viruses with nicotine (Baldacci *et al*., 1962 and Hewit *et al*., 1962), phosphate (Fulton, 1966) and bentonite buffers has been demonstrated. These buffers can neutralize some of the acidity in grapevine sap inoculum and facilitate movement of the virus into the herbaceous plant.

Grapevine viruses from the genus *Vitivirus* that have successfully been transferred to herbaceous plants included GVA that was transferred to *N. benthamiana* (Monette and James., 1990) and *N. clevelandii* (Conti *et al*., 1980); and GVB that was transferred to *N. occidentalis* (Boscia *et al*., 1993) by sap inoculation. Earlier attempts to transfer GVE by mechanical inoculation to *N. benthamiana*, *N. clevelandii*, *N. glutinosa* and *N. occidentalis*, were unsuccessful (Nakaune *et al*., 2008). The transfer of GVE to a herbaceous host can be a valuable tool for other experiments. Virus concentrations in their natural grapevine hosts can be low and localized in certain parts of the plant

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only, while in alternative hosts higher titers are often observed (Fulton, 1966), which can be useful in attempts to purify GVE from the herbaceous host in order to produce anti-GVE antibodies which can be used in ELISA applications.

The construction of infectious viral clones provides a useful tool to study viral genomes on a molecular and also biological level. Full-length cDNA clones are constructed from a copy of the viral RNA genome (cDNA), which is obtained through reverse transcription.

The first full-length infectious clone for a vitivirus was successfully constructed by Galiakporov *et al*. (1999), for grapevine virus A (GVA). Through mutational analysis of different ORFs in the GVA infectious clone, the gene functions for four of the five ORFs could be elucidated (Galiakporov *et al*., 2003). In 2006, Haviv *et al*. presented a full-length infectious clone, pGVA118, for the expression of foreign genes in a herbaceous host. The pGVA118 clone comprised genomic regions of different GVA isolates and included a multiple cloning site (MCS) for the insertion of foreign genes.

In 2010, Du Preez, constructed a 35S-GVA-GR5-ΔORF2+sgMP gene exchange vector, by modifying the GVA cDNA clone that was constructed by Haviv *et al*. (2006). The vector lacks ORF2 but it is still replication competent. This vector was constructed to study its potential use as a transient expression vector or as a VIGS vector in *V. vinifera* and *N. benthamiana*. The 35S-GVA-GR5-ΔORF2+sgMP gene exchange vector (Figure 5.1) was later used to study the possible function of the protein encoded by the ORF2 from three different GVA variants (GTR1-1, GTR1-2 and GTR11-1). The function of ORF2 in vitiviruses is still unknown but it has been speculated to play a role in insect vector transmission and might not be essential for systemic movement (Galiakparov *et al*., 2003).

Blignaut (2009) modified the pGVA118 full-length infectious clone, by deleting ORF5, to create the exchange vector, pGVA118ΔORF5 (Figure 5.2). This vector is not replication competent (Blignaut, 2009). This modification was done to study the infectivity and symptom development in *N. benthamiana* created by the presence of the ORFs5 from different GVA variants, GTR1-1, GTR1-2 and GTR11-1. The 10kD protein encoded by ORF5, functions as a weak silencing suppressor and was shown to be a determinant of the pathogenicity of GVA (Chiba *et al*., 2003; Galiakarov *et al*., 2003; Goszczynski *et al*., 2008 and Zhou *et al*., 2006). Haviv *et al*. (2012) revealed that the product of ORF5 play a role in symptom development, which is due to amino-acid residue changes of the eight amino acids at the N-terminus.

This chapter focuses on biological aspects of GVE. These include transferring GVE to herbaceous hosts using a wide range of herbaceous plants and different transmission buffers to facilitate transmission. Grapevine virus A-GVE chimeric viral vectors were also constructed with GVE

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ORF2 and ORF5 cloned into GVA gene exchange vectors, 35S-GVA-GR5-ΔORF2 (Du Preez, 2010) and 35S-GVA118ΔORF5 (Blignaut, 2009), respectively. These GVA-GVE chimeric viral vectors were assembled to assist in elucidating the viral-host interaction and compatibility of GVE ORFs 2 and 5 with GVA in *N. benthamiana* plants.

4.2 Materials and methods

4.2.1 Host plant spectrum determination

Bentonite buffer [30 mM K_2HPO_4, 50 mM Glycine, 1% Bentonite and 1% Celite, pH9.2], nicotine water [1% v/v] and phosphate buffer [0.01 M KH_2PO_4 and Na_2HPO_4, pH7] were used in transmission experiments of GVE from GVE-infected grapevine plant material (GH_11) to *N. benthamiana*. Transmission to other plant species: *Capsicum, C. amaranticolor, C. murale, C. quinoa, Datura stramonium, N. benthamiana*, *N. glutinosa, N. rustica, N.tabacum* cv. *Samsun* and *N. tabacum* cv. *Xanthi* were also attempted with the nicotine buffer. Transmissions were repeated three times with 11 plants per experiment, of which two plants were used for buffer controls.

Plants were grown under glasshouse conditions of 20-28°C and 50-70% humidity in natural light. At the 6-leaf stage transmission experiments were performed. Grapevine virus E infected grapevine material was ground to a smooth pulp in the different buffers (1:4 ratio approximately) and used in the transmission experiment to the herbaceous plants. The plant extract was gently rubbed on 2 - 3 leaves per plant and leaves were rinsed with water to remove the excess buffer mixture. Leaves rubbed with nicotine and phosphate buffer were first dusted with the abrasive powder celite or carborandum was added to the buffer. Plants were tested for GVE with RT-PCR (Chapter 3, sections 3.2.1.2 and 3.2.1.3) at 7 and 14 dpi with two GVE primer sets (Chapter3, Table 3.2.)

4.2.2 Construction and agroinoculation of GVA-GVE chimeric vectors

Primers were designed for the amplification of GVE ORF2 and GVE ORF5 with Vector NTI Advance 11.0. To assist the cloning of these ORFs into constructs: 35S-GVA-GR5-ΔORF2 (Du Preez, 2010) and 35S-GVA118-ΔORF5 (Blignaut, 2009), restriction enzyme recognition sequences and two blocking nucleotides were added to the 5' end of each primer (Table 4.1).

Table 4.1: Primers used in RT-PCRs amplifying GVE ORF2 and GVE ORF5 to clone into 35S-GVA-GR5-ΔORF2 and 35S-GVA-118-ΔORF5 respectively, for the construction of GVA-GVE chimeric viral vectors. Restriction enzyme recognition sequences are underlined and blocking sequences are indicated in red.

	Primer name	Primer sequence	Fragment size	T_A°C
ORF2	GVE-ORF2-SnaBI_5172F	AATACGTAATGCAGGTAAGGCAGCTAGTTAGGA	576	60
	GVE-ORF2-Kpn2I_5719R	AATCCGGATTAGCCAAAGGGTAAAGGAG		
ORF5	GVE-ORF5-NgoMIV_7112F	AAGCCGGCATGGGTAGTGCTTATCTAGGT	351	60
	GVE-ORF5-Mph1103I_7425R	CCATGCATTCAAGTTGCGTTGAAATCACTATTAT		

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Grapevine virus E ORF2 and ORF5 were amplified from total RNA, extracted from GVE infected grapevine, by means of RT-PCR (Chapter 3, sections 3.2.1.2 and 3.2.1.3) using primers in Table 4.1. The PCR products were separated on a 1.2% (w/v) agarose gel in TAE buffer and recovered from the gel using a Zymoclean DNA recovery kit (Zymo Research Corp) as per manufacturer's instructions. The recovered fragments were then ligated into the pGEM®-T Easy vector system (Promega) according to the manufacturer's instructions, creating pGEM-GVE-ORF2 and pGEM-GVE-ORF5 intermediate constructs.

The plasmids (pGEM-GVE-ORF2 and pGEM-GVE-ORF5) were extracted from saturated overnight cultures with the GeneJET™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer's instructions and digested with relevant restriction enzymes (all restriction enzymes supplied by Fermentas, unless otherwise stated). The plasmid, pGEM-GVE-ORF2 was digested with *Sna*BI and *Kpn*2I and the resulting fragment, containing ORF2, ligated into 35S-GVA-GR5-ΔORF2 (Figure 4.1), digested with the same enzymes. The pGEM-GVE-ORF5 plasmid was digested with *Ngo*MIV (NEB) and *Mph*11031 and the resulting fragment, containing ORF5, ligated into 35S-GVA118ΔORF5 (Figure 4.2) digested with the same enzymes. Fragments were ligated into their respective vectors with T4 DNA ligase (Fermentas) and T4 DNA ligase buffer (Fermentas), which resulted in two constructs namely 35S-GVA-GR5-ΔORF2-GVE-ORF2 and 35S-GVA118-ΔORF5-GVE-ORF5. The intermediate constructs were transformed into chemically competent *Escherichia coli* DH5α or JM109 cells and recovered from the bacterial cells with the GeneJET™ Plasmid Miniprep Kit (Fermentas). Colony PCRs were performed to identify the correct constructs in the bacterial cells, which were confirmed by sequencing of recovered plasmids.

The 35S-GVA-GR5-ΔORF2-GVE-ORF2 construct was digested with *Sal*I and *Not*I and transferred to pBinSN, digested with *Sal*I and *XhoI*I, to create pBinSN-35S-GVA-GR5-ΔORF2-GVE-ORF2. The 35S-GVA118ΔORF5-GVE-ORF5 construct was digested with *Sna*BI and *Sal*I and transferred pBinSN, digested with the same restriction enzymes, to create pBinSN-35S-GVA118-ΔORF5-GVE-ORF5.

The viral vectors, pBinSN-35S-GVA-GR5-ΔORF2-GVE-ORF2 (GVA-GVE-ORF2) and pBinSN-35S-GVA118-ΔORF5-GVE-ORF5 (GVA-GVE-ORF5) were electroporated into electro-competent *A. tumefaciens* (strain C58CI) with helper plasmid pCH35 (prepared according to Annamalia and Rao, 2006). Electroporation was carried out at 25µF, 200Ω and 1.5kV.

Agrobacterium cells that contain these vectors were infiltrated into *N. benthamiana* plants, using the method described by Llave *et al*. (2000). The cell suspension (OD_{600} = 0.1-0.5) was delivered to lower leaf surface, using a 2 ml syringe (without needle) and applying low pressure against the leaf.

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Agroinfiltrated plants were grown under glasshouse conditions of 20-25°C natural light and 40-60% humidity.

The control plasmids used for this study: pBin-35S-GVA-GR5, pBin-35S-GVA-GR5-ΔORF2, pBin-35S-GVA-GR5-ΔORF2-GTR1-1, pBin-35S-GVA118 and pBin-35S-GVA118-ΔORF5-GTR1-2 in *Agrobacterium* cells, were supplied by Dr J. du Preez and Marguerite Blignaut (Department of Genetics, Stellenbosch University). All the experiments were repeated twice and six *N. benthamiana* plants per construct were agroinfiltrated.

Standard molecular techniques i.e. restriction enzyme digests, ligation reactions, transformations and colony PCRs were used.

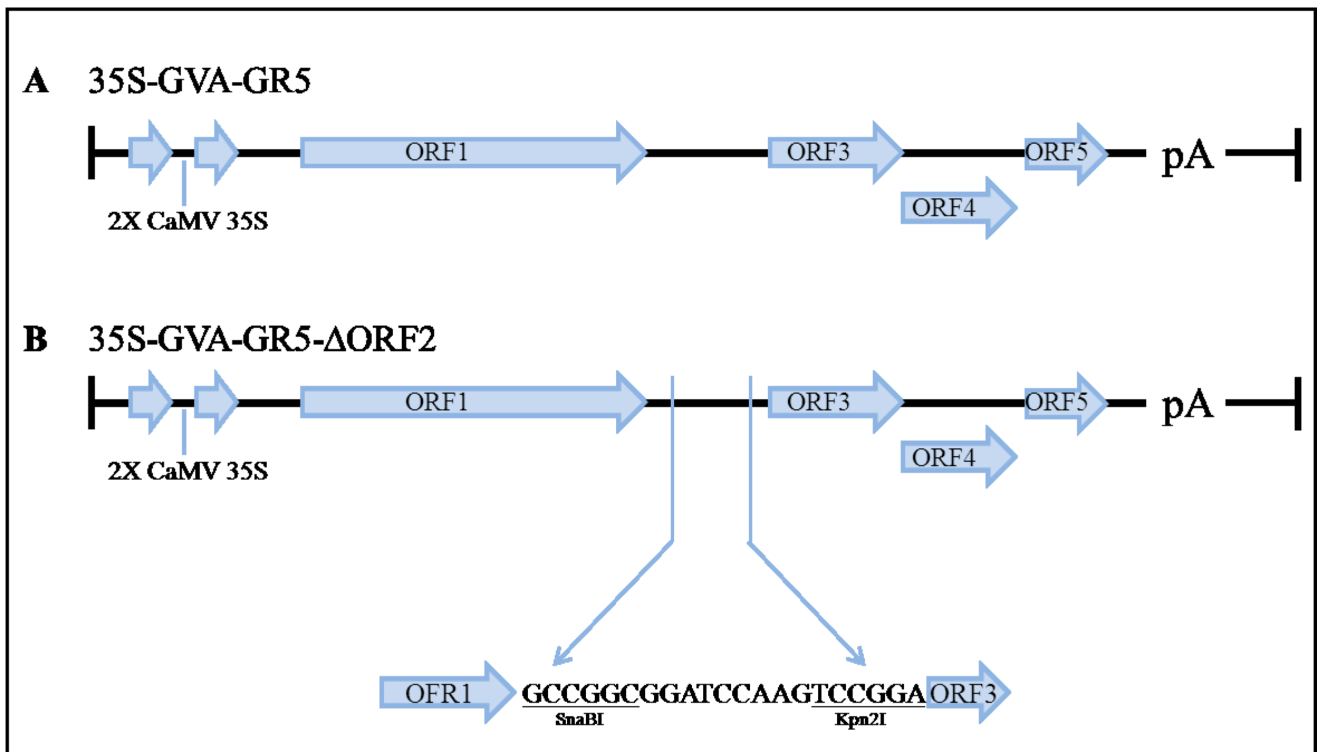

Figure 4.1: Graphical representation of the 35S-GVA-GR5 constructs, A) with all GVA ORFs and B) with ΔORF2, where ORF2 is replaced with restriction enzyme sites for *Sna*BI and *Knp*2I (adapted from Du Preez, 2010).

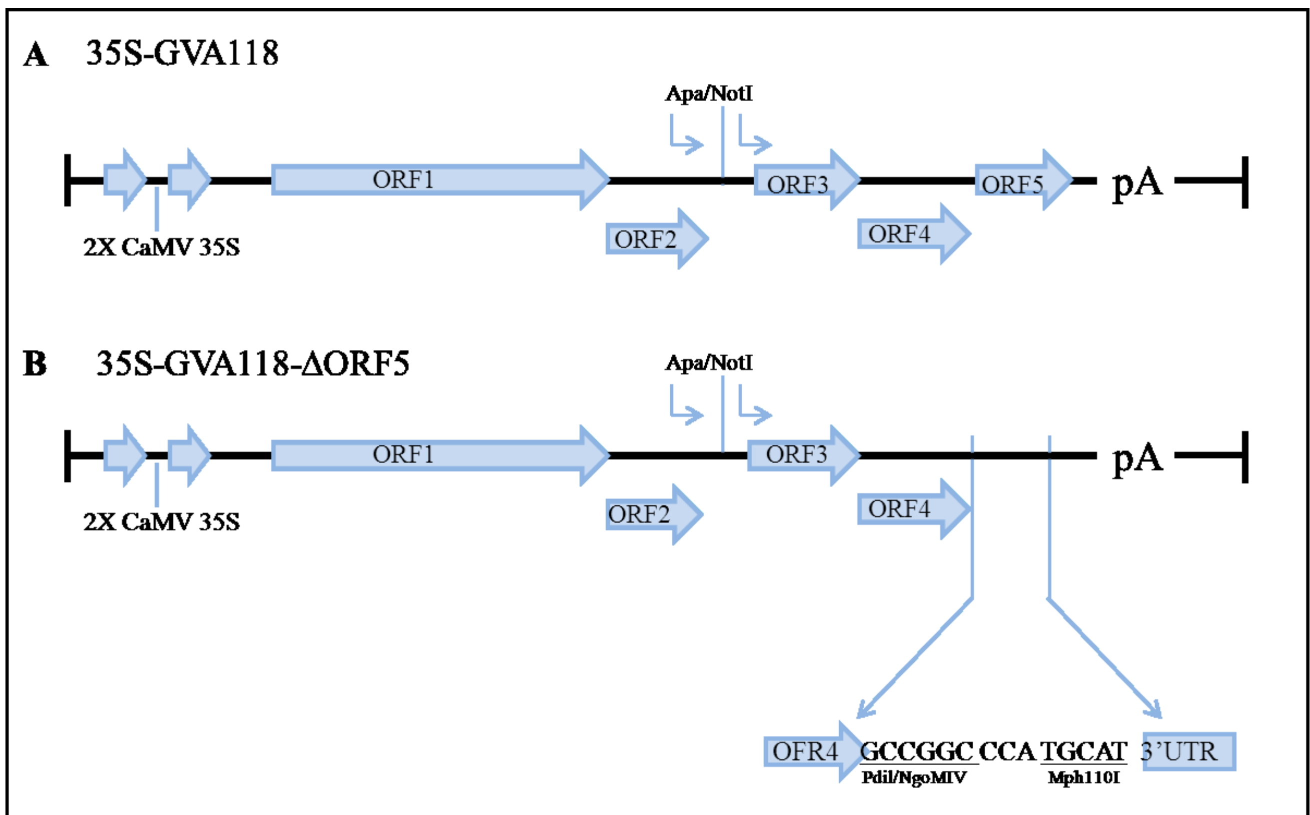

Figure 4.2: Graphical representation of the 35S-GVA-118 constructs, A) with all GVA ORFs and B) with ΔORF5, where ORF5 is replaced with restriction enzyme sites for *Ngo*MIV and *Mph*1103I (adapted from Blignaut, 2009).

4.2.3 Serological analysis: Tissue print immunoassay (TPIA) and Double antibody immunosorbent assay (DAS-ELISA)

Tissue print immunoassay (adapted from Franco-Lara *et al*, 1999) was used as initial screening on *N. benthamiana* plants agroinfiltrated with GVA-GVE chimeras and control full-length clones at 7dpi. Methanol was used to wet a Hybond PVDF membrane for 2 sec. The membrane was rinsed with an excess of water for 15 min and equilibrated in 1X TBS buffer (0.02 M Tris base and 0.05 M NaCl, pH7.5) for 15 min. The membrane was left to dry while the lower epidermis of infiltrated leaves was carefully removed with a forceps. The leaf area without epidermis was then cut with a scalpel and pressed for 2 s onto the membrane. Blocking solution (4.5% skimmed milk powder in 1X TBS) was added to the membrane and incubated at room temperature for 1 h on a shaker with gentle swirling. After incubation, the blocking solution was discarded and the membrane was washed three times in TBS-T (0.05% Tween-20 in 1X TBS) for 5 min. Following the last washing step, the membrane was incubated with the first antibody solution [400X diluted GVA-CP-antisera in 1X TBS-TPO (2% PVP-40 and 0.2% BSA in TBS-T)] for 1 h on a shaker. The first antibody solution was removed and the membrane washed three times in TBS-T for 5 min. The membrane was incubated in secondary antibody [10 000X diluted goat anti-rabbit alkaline phosphatase in 1X

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TBS-T] for 1 h on a shaker, with gentle swirling. The secondary antibody was removed, followed by another wash step and then left for a 30 min incubation step in the dark, in substrate buffer [AP buffer (100 mM Tris pH 9.5, 100 mM NaCl and 5 mM MgCl), containing 0.5 mg/ml NBT and 0.165 mg/ml BCIP]. After incubation, the membrane was rinsed in water and left to dry between filter papers. Purple spots on the membrane indicated the positive detection of GVA coat protein. Images of the tissue print were photographed with a BestScope Microscope BS-3040 microscope (Bestscope International Ltd).

ELISA detection was performed on *N. benthamiana* plants infiltrated with GVA-GVE chimera viral vectors as well as control constructs, as described in (Chapter 3, section 3.2.2) at 14dpi.

4.2.4 RNA extractions and RT-PCR

RNA extractions were performed as described in Chapter 3, sections 3.2.1.2 and RT-PCRs in section 3.2.1.3 using primers indicated in Table 4.2 on 7, 14 and 21dpi.

Table 4.2: Primer sequences used in RT-PCR for the detection of ORF2 of GVA and GVE and ORF5 of GVA and GVE.

Name	Primer sequence	Fragment size	T_A°C
GVA-GR5-ORF2_F	CTGTCACTCTGTCTCGTCAAC	1008*	55
GVA-GR5-ORF2_R	GCGGGTATTCTGTCTCATC		
GV118-ORF5_allF	AGCCGGCATGGATGACCCATCGTTTC	287	58
GV118-ORF5_1-2R	AATGCATTTATTCCTCATCATCTGAGG		

*Fragment obtained for GVE-ORF2. Different fragment sizes were obtained for the different constructs used. Expected fragment sizes for other constructs: pBin-35S-GVA-GR5 = 969bp, pBin-35S-GVA-GR5-ΔORF2 = 547bp and pBin-35S-GVA-GR5-ΔORF2-GTR1-1= 1067.

4.3 Results

4.3.1 Transmission of GVE to herbaceous host plants

Three different buffers; bentonite, nicotine and phosphate, were used in attempts to mechanically transfer GVE to *N. benthamiana* plants. No disease symptoms were observed by visual examination of the 27 inoculated plants per transmission experiment performed. Other transmission experiments were performed by inoculating the nine different herbaceous plant species *N. benthamiana, N. glutinosa, N. rustica* and *N. tabacum.* cv. Xanthi or cv. Samsun*, C. amaranticolor, C. murale, C. quinoa*, *D. stramonicum* and *Capsicum spp.*, respectively, with nicotine buffer. No apparent disease symptoms were observed on any of the 90 mechanically inoculated plants or any control plant. Plants used in these transmission experiments were monitored for 30 dpi.

RNA was extracted from all herbaceous plants and RT-PCRs were performed on 7, 14 and 21 dpi, respectively, using the GVE1F/Rev (Chapter 3, Table 3.1) and GVE_diag1F/1R diagnostic primers (Chapter 3, Table 3.2). RNA extracted from a GVE positive grapevine plant served as a positive

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control and three herbaceous plants per species, inoculated with buffer only, as negative controls. For all three time points tested, the herbaceous plants tested negative for infection with GVE.

4.3.2 Construction of GVA-GVE chimera viral vectors

To construct the GVA-GVE chimeras, ORF2 and ORF5 of GVE were amplified out of total RNA extracted from GVE-infected grapevine plant material. Restriction enzyme sites were incorporated in the amplicon with the primers to facilitate cloning into the 35S-GVA constructs. Amplifying the ORFs with the 5' restriction enzyme recognition sequence overhangs, produced a 592 bp fragment for ORF2 (Figure 4.3 lanes 2 and 3) and a 367 bp fragment for ORF5 (Figure 4.3 lanes 5 and 6), respectively.

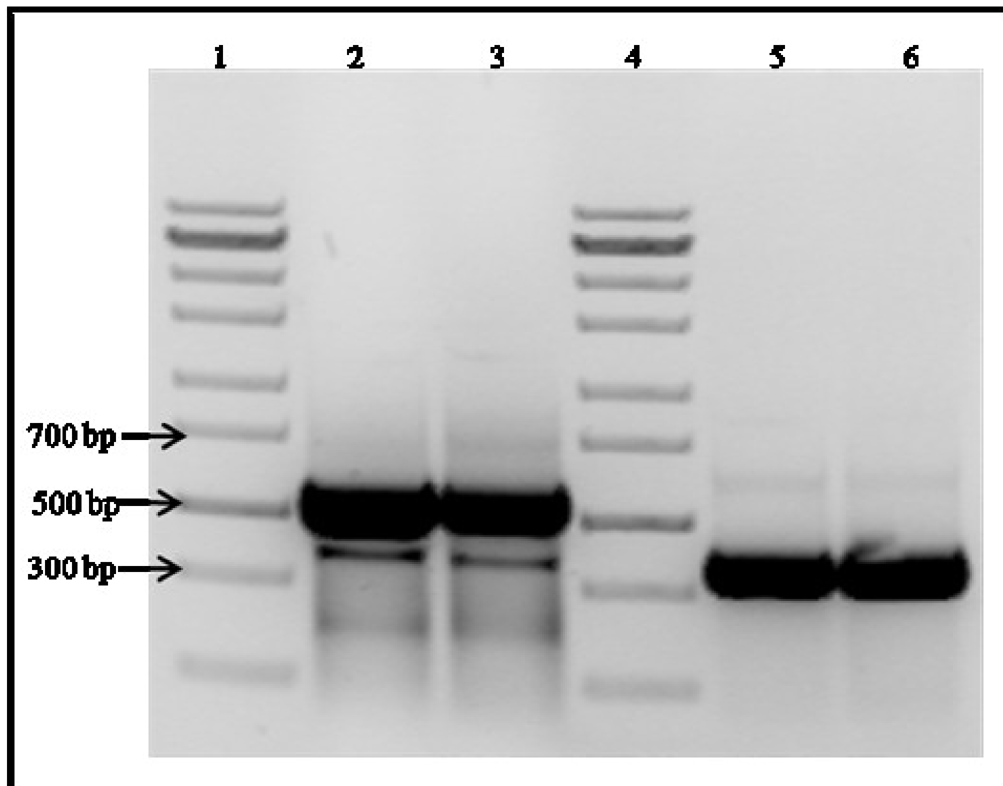

Figure 4.3: 1% Agarose gel with amplification products out of total RNA for GVE ORF2 and ORF5 with 5' restriction recognition site overhangs. Lane 1 and 4) ZipRuler™ express DNA ladder 1, lanes 2 and 3 GVE ORF2 and lane 5 and 6) GVE ORF5.

Following the recovery of the amplicons for GVEs ORF2 and ORF5, the GVA-GVE full-length clone chimeras were successfully constructed as viral expression vectors, creating the pBinSN-35S-GVA-GR5-ΔORF2-GVE-ORF2 (Figure 4.4) and pBinSN-35S-GVA-118-ΔORF5-GVE-ORF5 (Figure 4.5), respectively. The integration of the GVE ORFs into GVA viral vectors was confirmed with restriction enzyme digests and sequencing analysis.

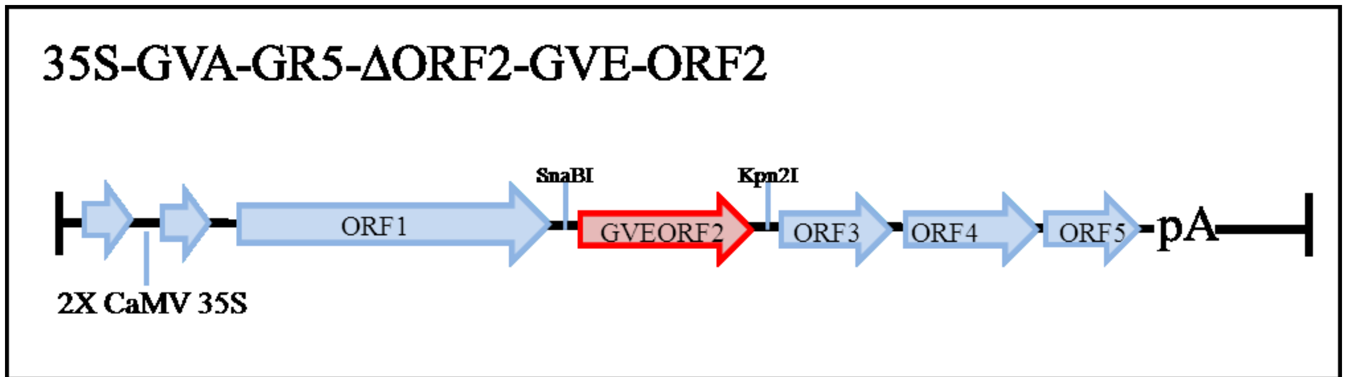

Figure 4.4: A schematic representation of the ORF2 viral expression vector 35S-GVA-GR5-ΔORF2-GVE-ORF2 between the right and left borders of pBinSN and under the control of CaMV 35S promoter. The GVE ORF2 is indicated in red.

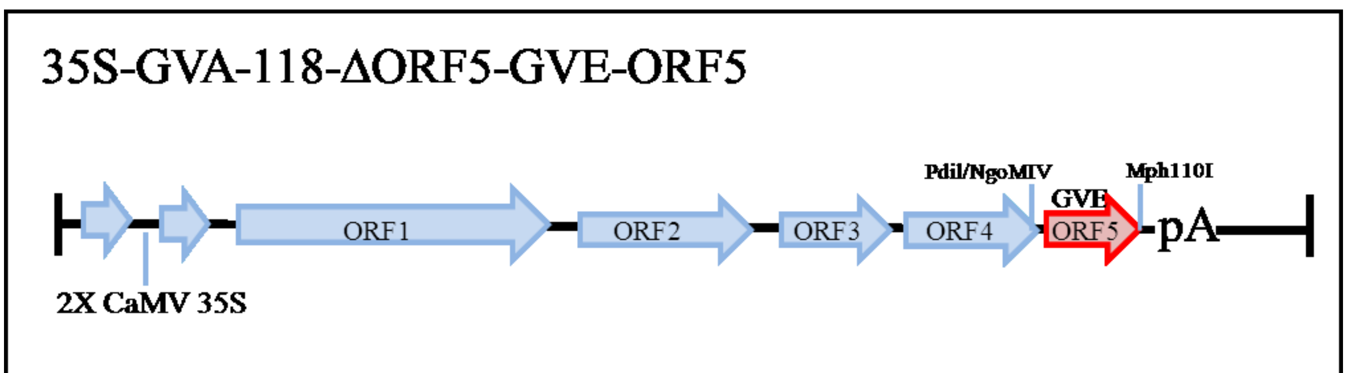

Figure 4.5: A schematic representation of the ORF5 viral expression vector 35S-GVA-118-ΔORF5-GVE-ORF5 between the right and left borders of pBinSN and under the control of CaMV 35S promoter. The GVE ORF5 is indicated in red.

4.3.3 Infectivity testing of GVA-GVE chimeric viral vectors

4.3.3.1 Tissue print immunoassay (TPIA)

To evaluate the infectivity of GVA-GVE chimeras in *N. benthamiana* plants, TPIA was performed. Tissue print immunoassays of the agroinfiltrated tissues will detect the GVA coat protein expressed from sgRNA. Expression of coat protein from a sgRNA is indicative of viral replication as sgRNAs will only be produced by an active viral RdRp. To test for local infection, agro-inoculated leaf tissue was used for the TPIA. Mock-inoculated plant material that does not contain any vectors, only inoculation buffer, was used as negative controls. Purple dark stained spots on the TPIA membrane indicate presence of GVA coat protein whereas purely greenish tissue on the membrane reflects absence of viral replication. Negative TPIA control tissues never showed any purple staining. Plants agroinfiltrated with 35S-GVA-GR5-ΔORF2 were used as a positive control as this construct (not containing an ORF2) was used to clone GVE ORF2 into and 35S-GVA-GR5-ΔORF2-GTR1-1 as the replacement controls for ORF2 and was shown in earlier experiments to be

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infectious. In agroinfiltrated tissues of both clones, purple spots on the membrane in TPIA indicated the presence of GVA coat protein and therefore replication. The 35S-GVA-GR5 (Figure 4.6 D) agroinfiltrated leaf tissue was used as additional positive control. Tissue print immunosorbent assay of leaves agroinfiltrated with the chimera construct 35S-GVA-GR5-ΔORF2-GVE-ORF2, in which the GVA ORF2 was replaced with the GVE ORF2, tested positive, indicating successful replication of that GVA-GVE chimera (Figure 4.6 E).

For ORF5 replacement experiments, plants agroinfiltrated with 35S-GVA-118-ORFΔ5-GTR1-2 construct (Figure 4.7 B) and 35S-GVA-118 (Figure 4.7 C) served as positive controls. In TPIA of leaves tissues agroinfiltrated with the chimera vector 35S-GVA-118-ΔORF5-GVE-ORF5 (Figure 4.7 D) no GVA coat protein accumulation could be detected (no purple spots) indicating a reduced or abolished replication.

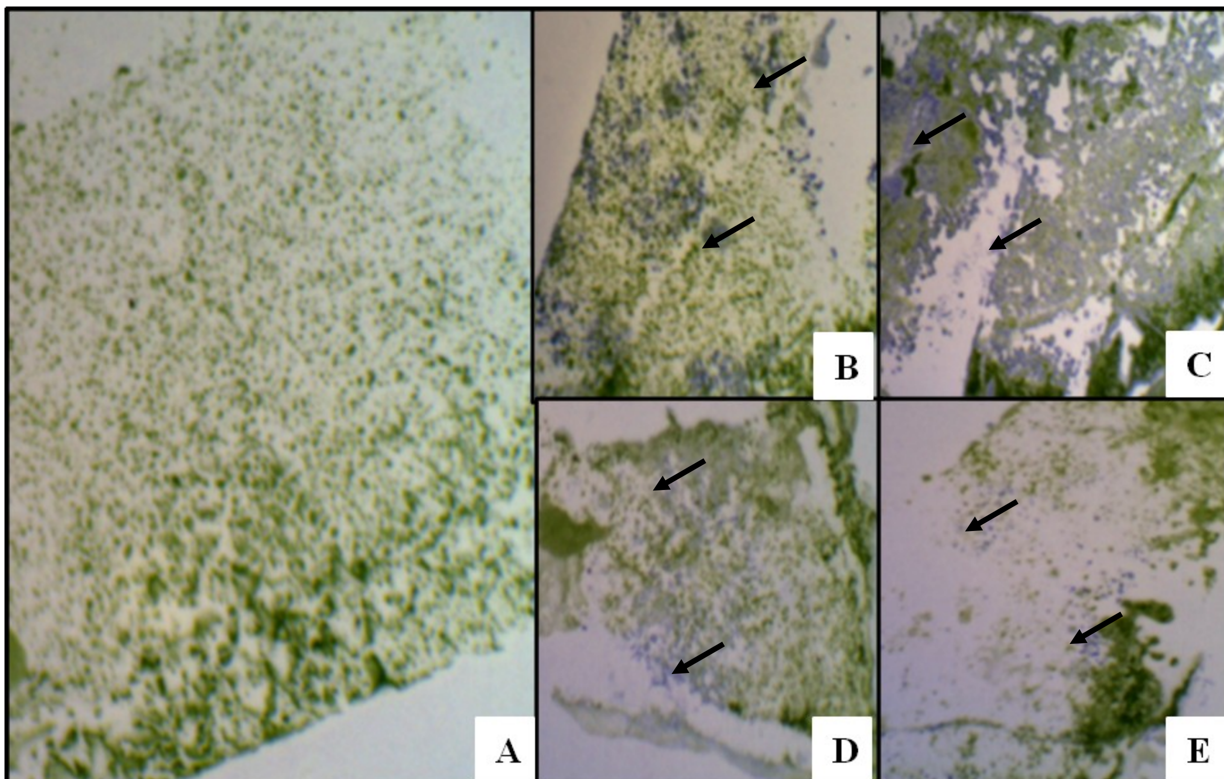

Figure 4.6: TPIA, at 7 dpi, of leaves from *N. benthamiana* plants infiltrated with the different GVA (GVA-GVE-ORF2) viral expression vectors for ORF2, reacting with the coat protein of the virus. A) Buffer control plants, B) 35S-GVA-GR5-ΔORF2, C) 35S-GVA-GR5-ΔORF2-GTR1-1, D) 35S-GVA-GR5 and E) the GVA-GVE hybrid viral vector 35S-GVA-GR5-ΔORF2-GVE-ORF2. The arrows indicate points of viral infection.

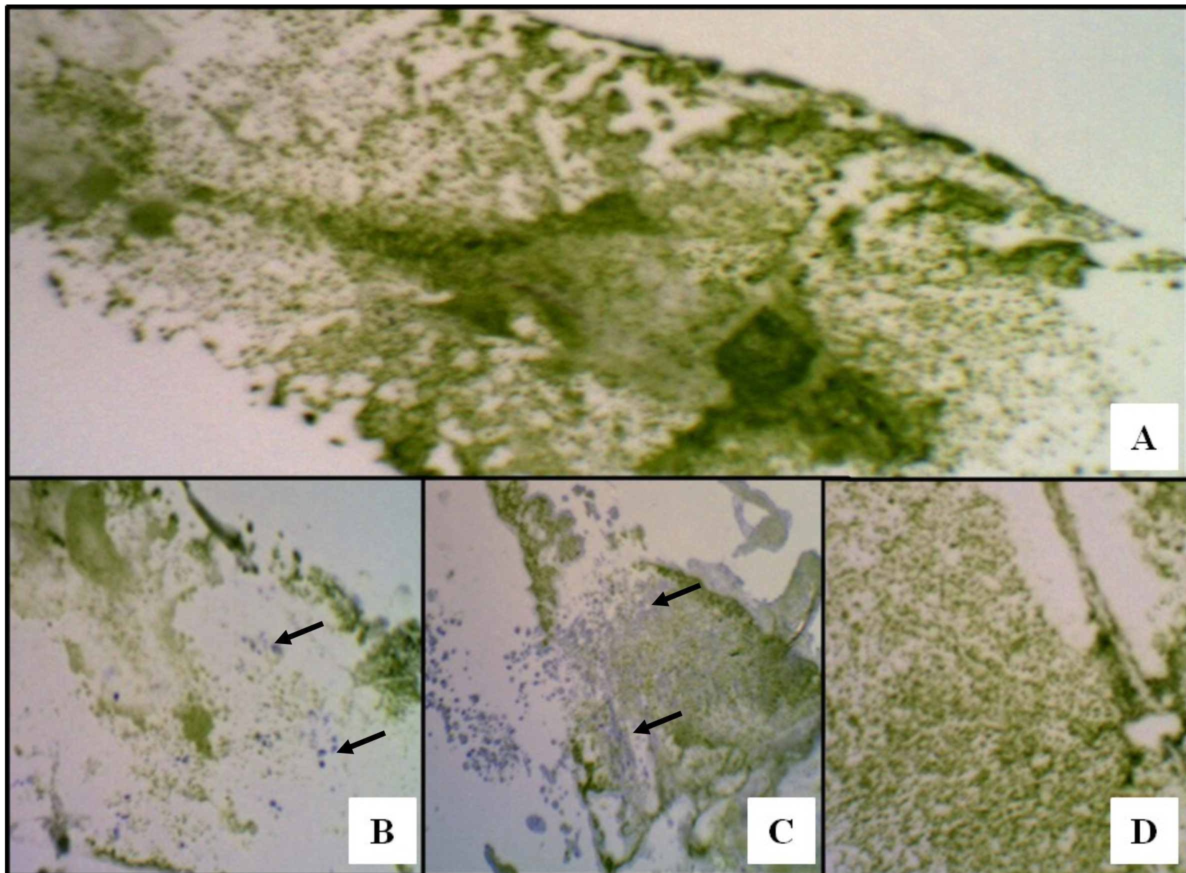

Figure 4.7: TPIA, at 7 dpi, of leaves from *N. benthamiana* plants infiltrated with the different GVA (GVA-GVE) viral expression vectors for ORF5, reacting with the coat protein of the virus. A) Buffer control plants, B) 35S-GVA118-ΔORF5-GTR1-2, C) 35S-GVA-118 and D) the GVA-GVE hybrid viral vector 35S-GVA118-ΔORF5-GVE-ORF5. The arrows indicate points of viral detection.

4.3.3.2 Biological characterization of the GVA-GVE chimera viral vectors

After testing the agroinfiltrated leaf tissues by TPIA for local infection, the plants were visually assessed for systemic spread and replication of the viral vectors by means of symptom development as well as RT-PCR in tissues adaxial of the site of agro-inoculation.

Symptom development was monitored for all 44 *N. benthamiana* plants (6 plants per construct) infiltrated with the different infectious clones with ORF2 and ORF5 substitutions. Symptom development was observed at 7-8 dpi and became more severe over the 30 dpi.

Leaves of mock-inoculated plants did not show any symptoms (Figure 4.8 A). The control infectious clone, 35S-GVA-GR5-ΔORF2 (Figure 4.8 B), that does not contain an ORF2, showed mild symptom development. Plants infiltrated with the positive control infectious clone 35S-GVA-GR5 (Figure 4.8 C) and the ORF2 replacement control 35S-GVA-GR5-ΔORF2-GTR1-2 (Figure 4.8 D), displayed severe disease symptoms compared to mock inoculated plants. In the positive control plants, whole leaves were covered by yellow streaks, downward rolling of leaf margins and stunted plants were observed. The plants infiltrated with the chimeric infectious clone, 35S-GVA-

GR5-ΔORF2-GVE-ORF2 (Figure 4.8 E) also displayed severe symptoms, as those observed in plants infiltrated with the positive controls when compared to mock-inoculated plants.

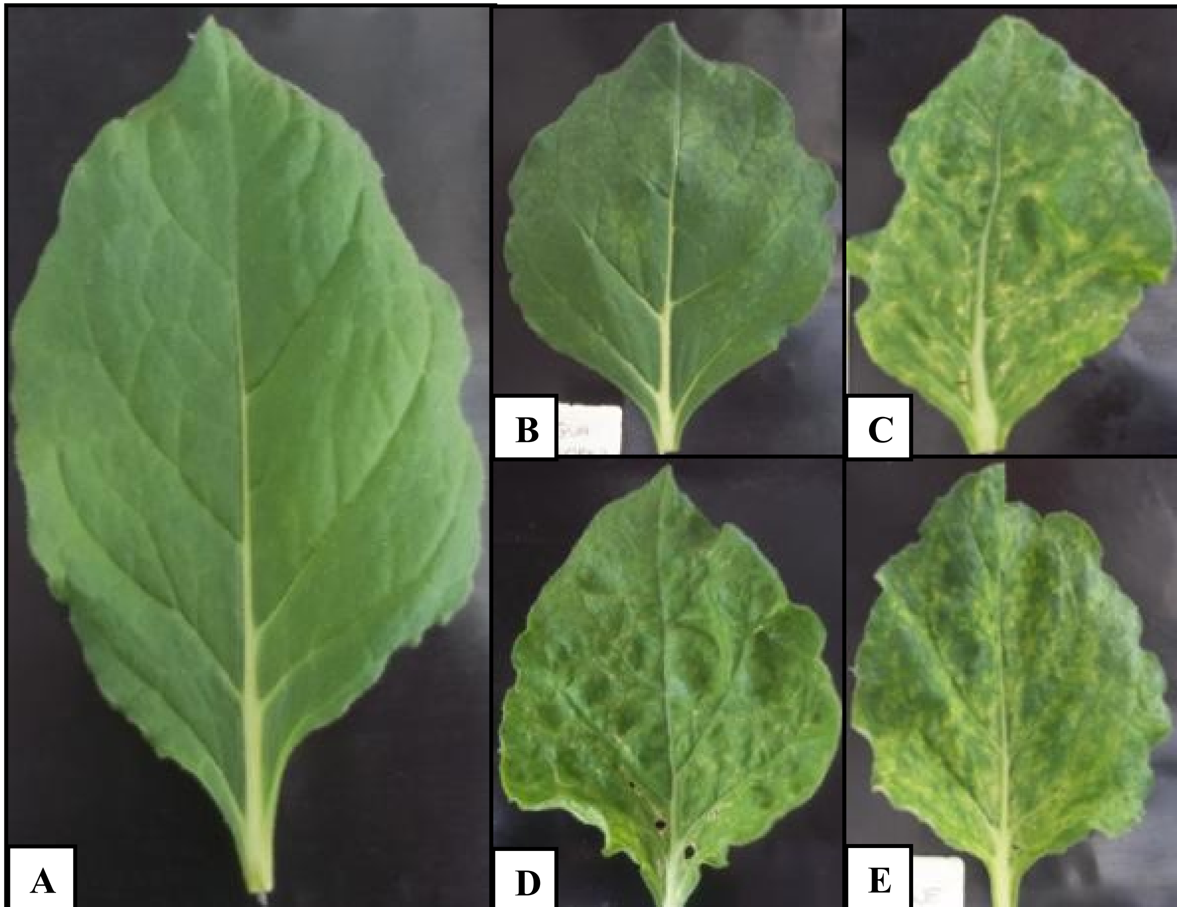

Figure 4.8: Newly developed leaves of *N. benthamiana* plants infiltrated with the different ORF2 viral expression vectors, at 14dpi, indicating symptom development associated with GVA as the vector spread throughout the plants. A) Buffer plants, B) 35S-GVA-GR5-ΔORF2, C) 35S-GVA-GR5-ΔORF2-GTR1-1, D) 35S-GVA-GR5-ΔORF2, D) 35S-GVA-GR5 and E) the hybrid vector 35S-GVA-GR5-ΔORF2-GVE-ORF2.

The symptoms of plants infiltrated with the infectious clone constructs in which ORF5 was substituted can be seen in Figure 4.9. No symptoms were observed on leaves of mock-inoculated plants (Figure 4.9 A). Disease symptoms were observed in leaves infiltrated with the positive control infectious clones; 35S-GVA-118 (Figure 4.9 B) and 35S-GVA-118-ΔORF5-GTR1-2 (Figure 4.9 C). In these leaves, clear yellowing of veins was observed, indicating the systemic spread of these viruses within the plants. In leaves of plants infiltrated with the 35S-GVA-118-ΔORF5-GVE-ORF5 chimeric construct no disease symptoms were observed (Figure 4.9 D) and they were indistinguishable from mock-inoculated plant leaves.

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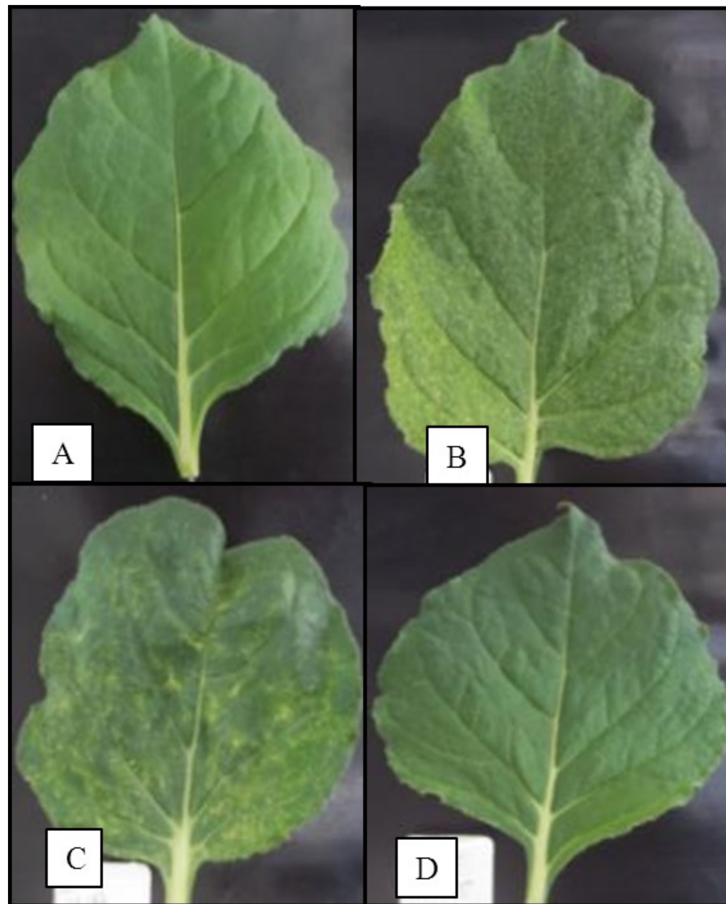

Figure 4.9: Newly developed leaves of *N. benthamiana* plants, at 14 dpi, infiltrated with the different ORF5 viral expression vectors indicating symptom development associated with GVA as the vector spread throughout the plants. A) Buffer plants, B) 35S-GVA-118-ΔORF5-GTR1-2, C) 35S-GVA-118 and D) the chimera vector 35S-GVA-118-ΔORF5-GVE-ORF5.

Total RNA was extracted from systemic leaves of agroinfiltrated *N. benthamiana* plants and screened with RT-PCR amplifying ORF2 and ORF5, respectively, in the 35S-GVA-GR5-ΔORF2-GVE-ORF2 and 35S-GVA-118-ΔORF5-GVE-ORF5 constructs to detect chimera viruses and systemic movement derived from these constructs.

As expected, in the mock-inoculated plants (Figure 4.10, lanes 2-7), no virus movement was detected, and only non-specific amplification was observed. Using RNA extracted from plants agroinfiltrated with the 35S-GVA-GR5-ΔORF2 control construct that lacks GVA ORF2 (Figure 4.10, lanes 8-12 and 14), a 546 bp fragment could be amplified, indicating systematic movement of 35S-GVA-GR5-ΔORF2-derived virus and confirming that GVA ORF2 is not needed for systemic movement. Virus containing GVA ORF2 was detected in *N. benthamiana* plants agroinfiltrated with the ORF2 replacement control constructs 35S-GVA-GR5-ΔORF2-GTR1-1 and 35S-GVA-GR5, the positive control construct. For 35S-GVA-GR5-ΔORF2-GTR1-1 a fragment of 1167 bp was detected (Figure 4.10, lanes 13, 15 and 17-19) and for 35S-GVA-GR5 a 969 bp fragment was amplified (Figure 4.10, lanes 20-25) representing ORF2 in both these constructs. Open reading

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frame 2 was amplified as a 1118 bp fragment in *N. benthamiana,* agroinfiltrated with the 35S-GVA-GR5-ΔORF2-GVE-ORF2 construct (Figure 4.11, lanes 1-5). This indicates that a virus resulted from this chimeric construct and was moving systematically through infiltrated plants.

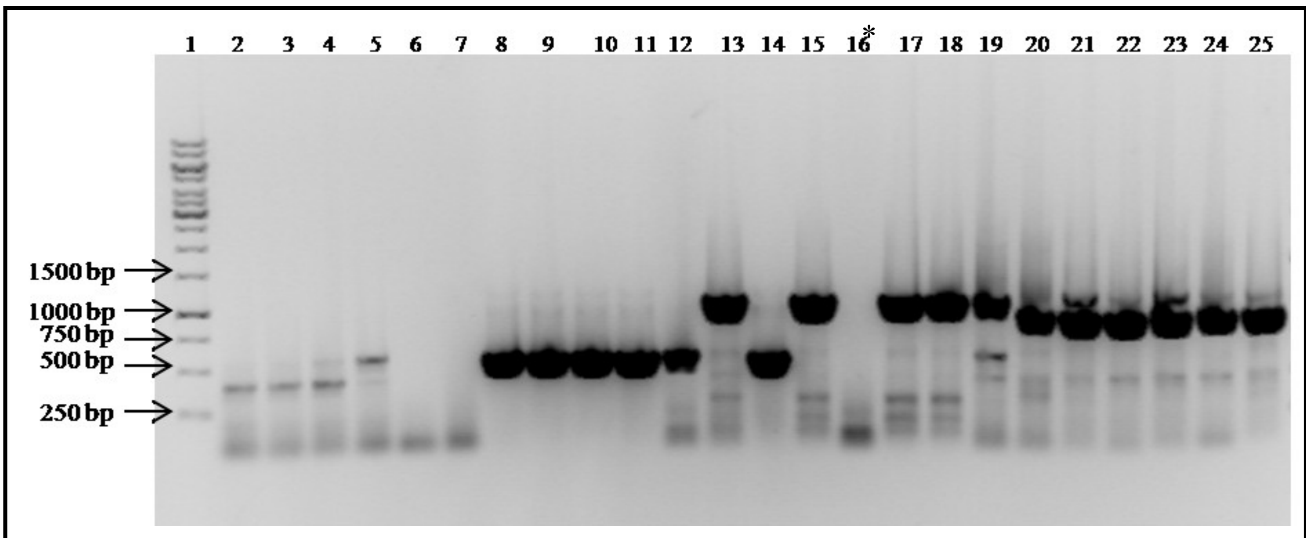

Figure 4.10: A 1% Agarose gel with the RT-PCR products of the viral expression vectors for ORF2 out of total extracted RNA from *N. benthamiana* plants. Lane 1) GeneRuler™ 1kb DNA ladder, 2-7) buffer control plants with non-specific amplification, 8-12 and 14) 35S-GVA-GR5-ΔORF2 with a 546 bp amplified product, lanes 13, 15 and 17-19) 35S-GVA-GR5-ΔORF2-GTR1-1 with a 1167 bp amplified product and 20-25) 35S-GVA-GR5 with a 969 bp amplified product. The RT-PCR products were confirmed with sequencing analysis.

*In lane 16, amplification of one the plants infiltrated with 35S-GVA-GR5-ΔORF2 failed, the PCR was repeated in as subsequent reaction in which amplification was observed (results not shown).

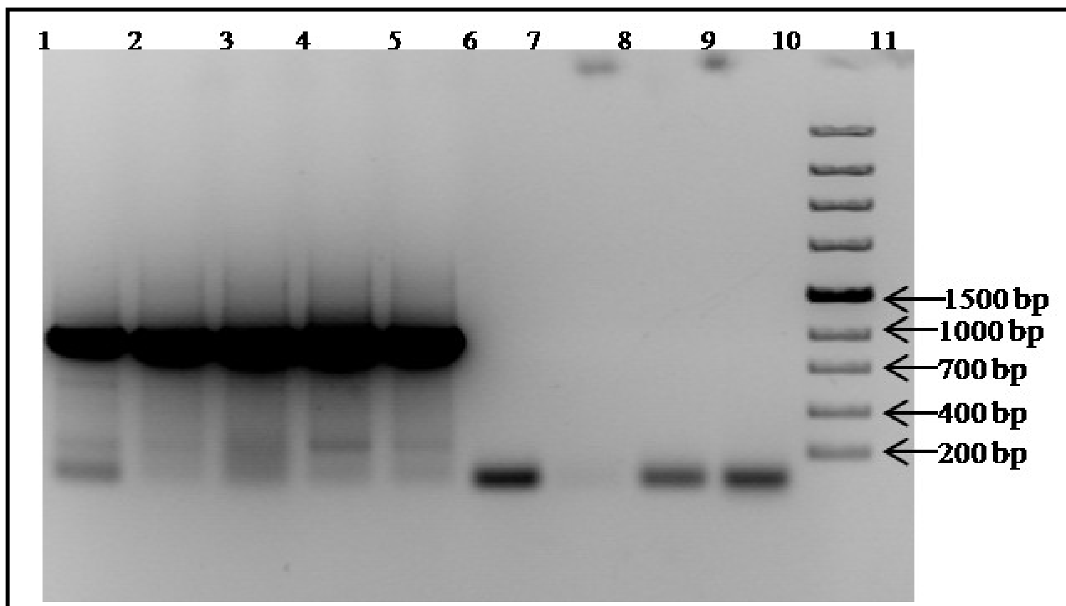

Figure 4.11: A 1% Agarose gel with the RT-PCR products of the viral expression vectors for ORF2 out of total extracted RNA from *N. benthamiana* plants. Lane 1-6) 35S-GVA-GR5-ΔORF2-GVE-ORF2 chimera viral vectors, with 1118 bp amplified product, lanes 6-9) RT-PCR non-template controls with visible primer dimer formation and lane 10) ZipRuler™ Express DNA ladder2. The RT-PCR products were confirmed by sequencing.

For the ORF5 substitution constructs, no virus was detected in any of the mock infiltrated plants (Figure 4.12, lanes 2-7). In plants infiltrated with the positive control constructs 35S-GVA-118-ΔORF5-GTR1-2 (Figure 4.12, lanes 8-12) and 35S-GVA-118 (Figure 4.12, lanes 19-24), a 287 bp fragment amplifying ORF5 could be amplified, indicating systemic movement of virus derived from these constructs. Some primer-dimers were also observed for these two constructs. In plants infiltrated with the chimeric construct, 35S-GVA-118-ΔORF5-GVE-ORF5 (Figure 4.12, lanes 13-18), ORF5 was not amplified, indicating the absence of virus derived from this construct.

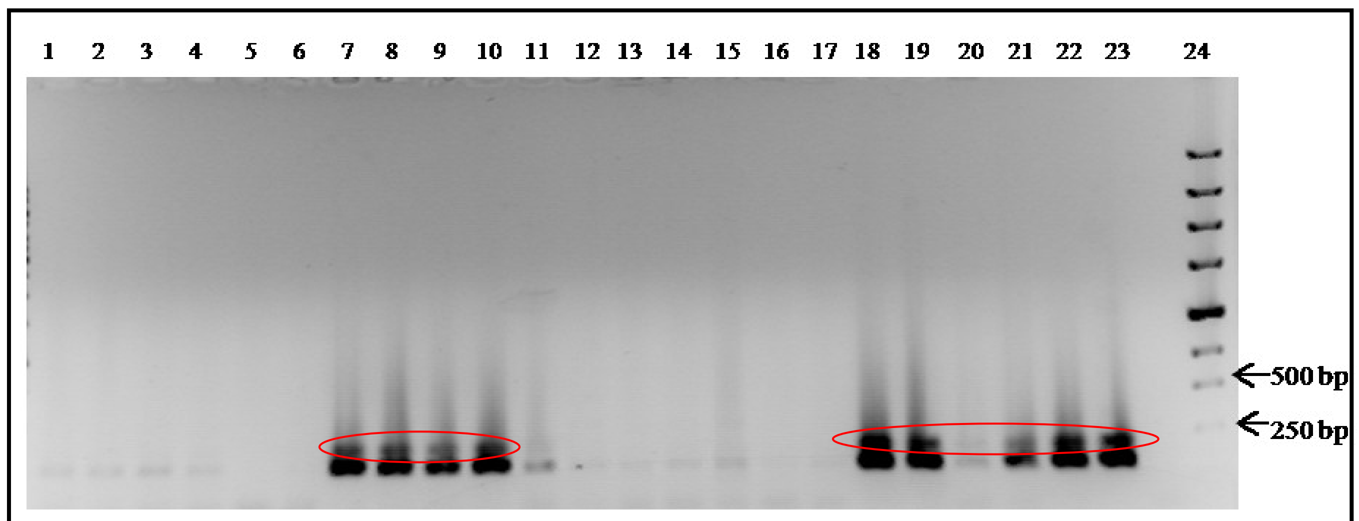

Figure 4.12: A 1% Agarose gel with the RT-PCR products of the viral expression vectors for ORF5 out of total extracted RNA from *N. benthamiana* plants. Amplification of ORF5, 278 bp product, are circled in red on the gel, primer dimer formation is also present on the gel. Lane 25) GeneRuler™ 1kb DNA ladder, 1-6) buffer control plants, 7-11) 35S-GVA-118-ΔORF5-GTR1-2, lanes 12-17) 35S-GVA-118-ΔORF5-GVE-ORF5 chimeric construct and lanes 19-23) 35S-GVA-118. The RT-PCR products were confirmed by sequencing.

4.3.3.3 Enzyme-linked immunosorbent assay (ELISA)

In addition to testing for systemic movement by RT-PCR, DAS-ELISA was performed, at 14dpi, on systemic leaves of all 44 *N. benthamiana* plants infiltrated with the infectious clones in which ORF2 (Table 4.3) and ORF5 (Table 4.4) were substituted. The GVA DAS-ELISA was positive for all plants agroinfiltrated with the four different ORF2 chimeric constructs; 35S-GVA-GR5-ΔORF2, 35S-GVA-GR5-ΔORF2-GTR1-1, 35S-GVA-GR5 and 35S-GVA-GR5-ΔORF2-GVE-ORF2 (Figure 4.13), which indicates that the coat protein is detected in systemically infected tissues of these plants. The GVA titer measurement indicated that the titers in these plants are different for each construct. Plants infiltrated with the control construct 35S-GVA-GR5-ΔORF2, lacking ORF2, had low viral titers compared to mock-inoculated plants. This construct was also detected with RT-PCR and displayed mild symptom development. The positive control constructs 35S-GVA-GR5-ΔORF2-GTR1-1 and 35S-GVA-GR5, had higher virus titers. This correlates with the RT-PCR results and

the severe symptom development in plants infiltrated with these two constructs. The virus titer for the chimeric infectious clones, 35S-GVA-GR5-ΔORF2-GVE-ORF2 was lower than that of the positive controls, even though severe disease symptoms were observed in plants infiltrated with the hybrid infectious clone. The GVE ORF2 was also detected in these plants with RT-PCR.

Table 4.3: The average GVA DAS-ELISA absorbance for the ORF2 chimera constructs at 405 nm. With the standard error and P-values indicating significant difference to mock inoculated plants.

Constructs for ORF2	Average absorbance (405 nm)	Standard error	P-value	Result
Mock	0.134	0.003	-	-
35S-GVA-GR5-ΔORF2	0.187	0.005	1.5E-06	Positive
35S-GVA-GR5-ΔORF2-GTR1-1	0.674	0.063	4.8E-06	Positive
35S-GVA-GR5-ΔORF2-GVE-ORF2	0.235	0.017	7.4E-05	Positive
35S-GVA-GR5	0.698	0.067	4.4E-06	Positive
Negative control	0.162	0.011	-	-

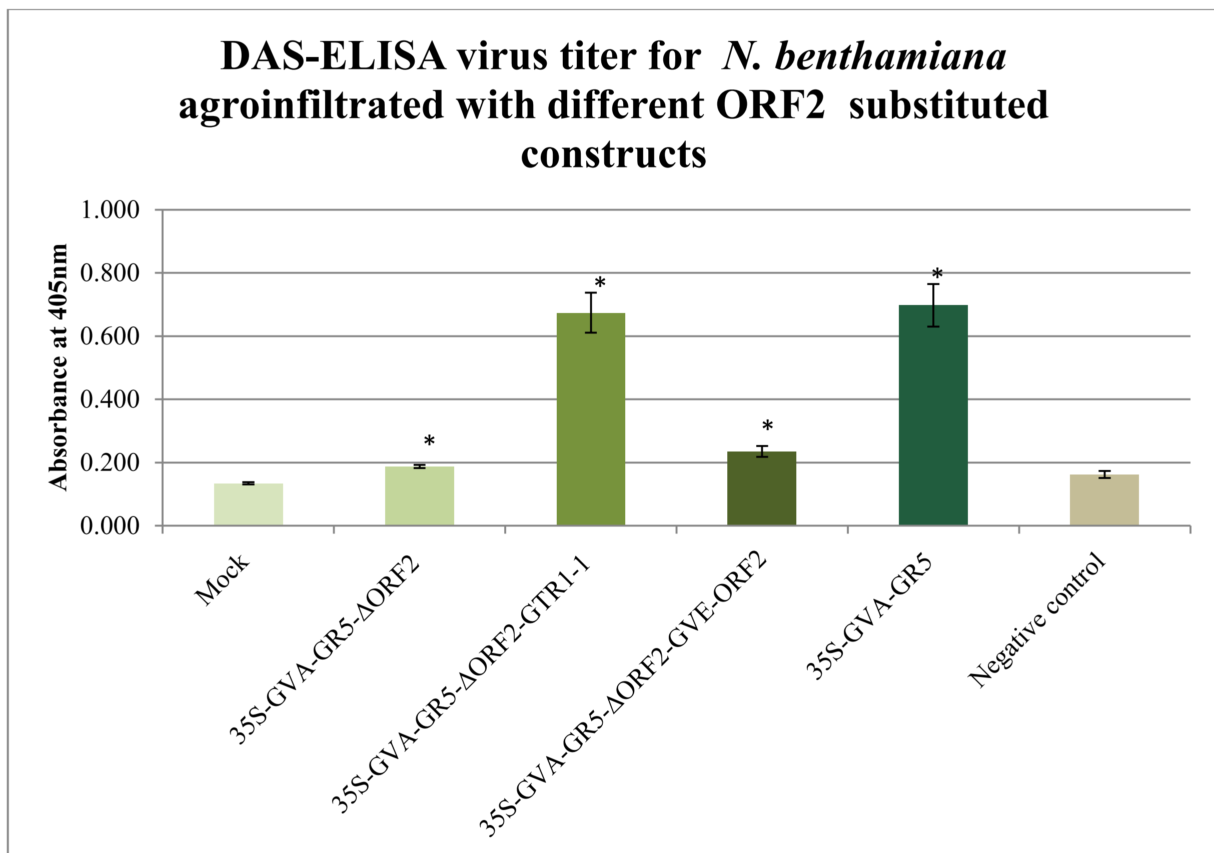

Figure 4.13: Bar chart with average absorption of three replicates per plant extract at 405 nm for GVA DAS-ELISA results of the different ORF2 constructs.

*indicate significant difference to mock inoculated plants.

Table 4.4: The average GVA DAS-ELISA absorbance for the ORF5 chimera constructs at 405 nm. With the standard error and P-values indicating significant difference to mock inoculated plants.

Constructs for ORF5	Average absorbance	Standard error	P-value	Result
Mock	0.115	0.002	-	-
35S-GVA118ΔORF5-GTR1-2	0.120	0.003	0.32315	Negative
35S-GVA118ΔORF5-GVE-ORF5	0.116	0.003	0.77703	Negative
35S-GVA118	0.173	0.012	0.00179	Positive
Negative control	0.112	0.000	-	-

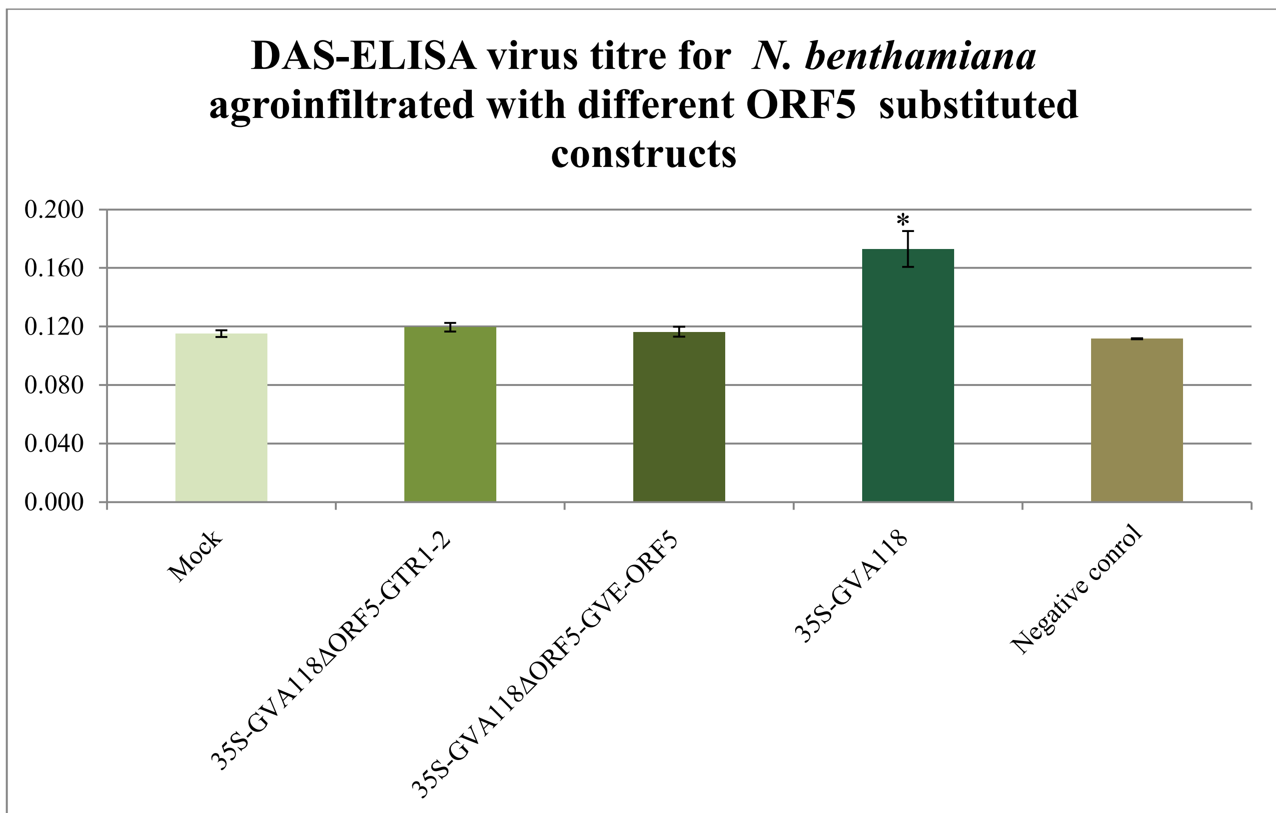

Figure 4.14: Bar chart with average absorption of three replicates per plant extract at 405 nm for GVA DAS-ELISA results of the different ORF5 constructs.

*indicate significant difference to the mock inoculated plants.

The GVA DAS-ELISA for clones in which ORF5 was substituted (Figure 4.14), only detected the GVA coat protein in the plants agroinfiltrated with the positive control construct 35S-GVA118. This is expected, as RT-PCR detected the virus and severe disease symptoms were observed for these plants. The DAS-ELISA for plants infiltrated with the replacement control, 35S-GVA-118-ΔORF5-GTR1-2 and the chimeric construct 35S-GVA-118-ΔORF5-GVE-ORF5, did not detected viral coat protein. This is contradicting the RT-PCR results for the 35S-GVA-118-ΔORF5-GTR1-2

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agroinfiltrated plants, as the virus was detected with RT-PCR and disease symptoms were observed in these plants. This could possibly be the result of low virus titer in these plants that could not be detected with the DAS-ELISA but which was detectable with the more sensitive RT-PCR procedure. The results for plants agroinfiltrated with the GVA-GVE chimeric construct 35S-GVA-118-ΔORF5-GVE-ORF5 confirmed the RT-PCR results, that did not detect the virus in any of the plants and no disease symptoms were observed. A summary of the results of the biological characterization of the GVA-GVE chimeric viral clones is shown in Table 4.3.

Table 4.5: Summary of agroinfiltration results.

Chimeras	TPIA	Symptom development	ELISA	PCR
Buffer controls	-	-	-	-
ΔORF2	+	+	+	+
ΔORF2-GTR1-1	+	+	+	+
ΔORF2-GVE ORF2	+	+	+	+
GVA-GR5	+	+	+	+
ΔORF5-GVE ORF5	-	-	-	-
ΔORF5-GTR1-2	+	+	-	+
GVA118	+	+	+	+

4.4 Discussion and Conclusion

GVE transmission experiments were conducted to transfer GVE to herbaceous hosts. Different herbaceous plants and transmission buffers were tested in experiments to mechanically transmit GVE.

Different buffers, including bentonite, nicotine and phosphate buffers, were used to facilitate the transfer of GVE from *V. Vinifera* to *N. benthamiana*. These buffers have previously been used for transmission of grapevine viruses to herbaceous hosts (Baldacci *et al*., 1962; Hewit *et al*., 1962; Fulton., 1966). The transmission of GVE to *N. benthamiana* plants were unsuccessful using the three different buffers. Reverse transcription-PCR on RNA extracted from the *N. benthamiana* plants, were negative for GVE at 7, 14 and 21 dpi. No symptom development was observed up to 30 dpi after inoculation on any of the nine plants used in all three experiments. In the study of Monette and James (1990), where *in vitro* shoot tip cultures were used in successful transmission of GVA to *N. benthamiana* plants, the onset of vein clearing was observed at 15-19 dpi.

The experiment was repeated using different herbaceous plants, previously found susceptible to plant viruses. Nevertheless, our attempts to mechanically transfer GVE using nicotine buffer, which were used in previous studies to transfer grapevine viruses (Cadman *et al*., 1960), were not successful. Different *Nicotiana* and *Chenopodium* species as well as *D. stramonium* and *Capsicum spp.* were used in our experiments, species which were earlier found to be susceptible to some grapevine viruses like GFLV, GVA and GVB (Fulton, 1966, Conti *et al*., 1990, Bocia *et al*., 1993). In total, 90 plants were used, nine experimental plants per herbaceous host. No symptoms developed during the 21 dpi observation period and RT-PCR did not detect GVE on 21 dpi. Plants were evaluated for symptom development up to 30 dpi after inoculation and no disease symptoms developed during this period. In Conti *et al*. (1990), vein clearing in young *N. clevelandii* leaves were observed 3-4 weeks after inoculation with GVA-infected plant material. In the study by Nakaune *et al*. (2008), the transmission experiments of GVE to herbaceous plants were also unsuccessful, although other phloem limited viruses and members of the genus *Vitivirus*, such as GVA and GVB were transferred successfully to *N. benthamiana* and *N. occidentalis*. Various factors contribute to the infectivity of viruses. Grapevine sap is acidic, with a pH of 3.4 (Cadman *et al*., 1960) which needs to be neutralised and components such as phenolic compounds influence virus stability, reducing their ability to infect. This could be part of the reason why infectivity was not achieved. Transferring viruses from woody plants to herbaceous hosts can facilitate downstream experiments such as the production of antibodies for ELISA, which is a difficult process in their natural, woody plant host and virus purification (Minafra *et al*., 1997). But transferring the virus to the herbaceous plants can be a challenging because of host range limitations as viral infection cannot be predicted.

To further characterize GVE, GVA-GVE chimeric clones were constructed with GVE ORF2 and ORF5 replaced in the available GVA exchange vectors, 35S-GVA-GR5-ΔORF2 and 35S-GVA-118-ΔORF5. These vectors were agroinfiltrated into *N. benthamiana* plants for replication evaluation by TPIA, visual inspection for symptom development and confirmation of presence and possible systemic movement by DAS-ELISA and RT-PCR.

The TPIA detected virus coat protein in plants infiltrated with the 35S-GVA-GR5-ΔORF2-GVE-chimeric infectious clone. This indicates that the substitution of GVE-ORF2 into 35S-GVA-GR5-ΔORF2 to form a chimeric infectious clone was able to infect and replicate in *N. benthamiana* plants. In plants infiltrated with the 35S-GVA-GR5-ΔORF2 that does not contain ORF2, coat protein was detected, indicating that the clone is still infectious without an ORF2. The ORF2 is therefore not essential for virus infection and replication in *N. benthamiana*. Infectivity of 35S-GVA-GR5-ΔORF2 agroinfiltrated *N. benthamiana* was also observed by Du Preez, (2010), these results confirm that the ORF2 of vitiviruses is not essential for infection of *N. benthamiana*. Earlier

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mutation studies of ORF2 in the PA3 infectious clone revealed that the ORF was not essential for viral movement or replication in *N. benthamiana* (Galiakparov *et al*., 2003). The use of the 35S-GVA-GR5-ΔORF2 infectious clone as an exchange vector was evaluated by Du Preez (2010), by substitution of the reporter gene GUS and GFP. Both vectors were able to move and replicate in *N. benthamiana*.

When comparing plants agroinfiltrated with the chimeric infectious clone, GVA-GVE-ORF2 and the exchange vector, 35S-GVA-GR5-ΔORF2, symptoms in *N. benthamiana* that are associated with GVA infection such as the yellowing of veins and leaf curling in non infiltrated leaves (Galiakparov *et al*., 2003; Bilgnaut, 2009; Du Preez, 2010) were observed. This is an indication that the virus is actively spreading through the plants, from the site of inoculation to other plant tissues.

Severe disease symptoms were observed in *N. benthamiana* plants agroinfiltrated with the 35S-GVA-GR5-ΔORF2-GVE-ORF2 chimeric infectious clone, compared to plants infiltrated with the ORF2 deletion mutant, 35S-GVA-GR5-ΔORF2, which displayed mild disease symptoms. The symptoms observed in plants agroinfiltrated with the positive control constructs 35S-GVA-GR5 and 35S-GVA-GR5-ΔORF2-GTR1-1, containing an ORF2, were similar to that observed by Du Preez (2010) and in the chimeric infectious clone, 35S-GVA-GR5-ΔORF2-GVE. Symptom development in plants agroinfiltrated with constructs containing an ORF2 displayed more profound disease symptoms than those, which does not contain ORF2. However, the virus titer in the plants infiltrated with the chimera, 35S-GVA-GR5-ΔORF2-GVE, was not as high as that in the positive control plants, infiltrated with 35S-GVA-GR5 and 35S-GVA-GR5-ΔORF2-GTR1-2, though the symptoms observed were just as severe.

Even though the infectious clones lacking ORF2 can infect and replicate successfully in plants, the presence of an ORF2 enhances the development of disease symptoms but is not essential for movement or symptom development. This could also be a result of lower MP production, as the ORF3 was elongated during the construction of 35S-GVA-GR5-ΔORF2 (Du Preez, 2010). The titers of the infectious clones that contain an ORF2 are higher in plants, indicating that infectious clones with an ORF2 replicates and spread more efficiently. Reverse transcription PCR confirmed the presence of the GVE ORF2 in *N. benthamiana* plants infiltrated with the 35S-GVA-GR5-ΔORF2-GVE-ORF2 chimeric infectious clone.

Plants infiltrated with the 35S-GVA118-ΔORF5-GVE-ORF5 chimeric construct did not display any disease symptoms during visual inspection of the plants. The TPIA and DAS-ELISA did not detect coat protein in any of the *N. benthamiana* plants agroinfiltrated with the 35S-GVA118-ΔORF5-GVE-ORF5 chimeric clones. This indicates that the substitution of ORF5 from GVE in the 35S-GVA118-ΔORF5, ORF5 deletion mutant, abolished infectivity. Previous studies indicated that the

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lack of, or mutations in, ORF5 of GVA effects the ability of the clone to be infectious or is not replication competent in *N. benthamiana* (Galiakparov *et al*., 2003; Blignaut, 2009). The presence of ORF5 was confirmed with sequencing analysis of the plasmid extracted from *Agrobacterium* cells before infiltration. Results obtained by TPIA and DAS-ELISA were confirmed by RT-PCR. The ORF5 in vitiviruses is possibly the determinant of symptom development (Haviv *et al*., 2012). The amino acid similarity between GVA and GVE for ORF5 is more than 90% (Nakaune *et al*., 2008). Because of this high amino-acid similarity for ORF5, it was expected that the chimeric exchange vector would be infectious and replicate in plants infiltrated with the 35S-GVA-118-ΔORF5-GVE-ORF5 construct, but with restricted movement and probably asymptomatic (Galiakparov *et al*., 2003). However, the 35S-GVA-118-ΔORF5-GVE-ORF5 was not able to produce a virus, able to infect and replicate in *N. benthamiana*, as virus was not detected in any of the infiltrated plants. Substitutions of ORF5 in 35S-GVA118-ΔORF5 with GUS and GFP genes did not result in gene expression either (Blignaut, 2009). This indicated loss of infectivity due to the lack of ORF5 showing the specificity of ORF5 in vitiviruses for infection and replication in plants. The 35S-GVA118-ΔORF5, lacking ORF5 is not replication competent in plants (Blignaut, 2009). In a study performed by Haviv *et al*. (2012), an amino acid change in the eight residues at the N-terminus was identified as a possible determinant for symptom development in GVA, when swapping ORF5 from the mild to severe strains and vice versa. Further analysis is still needed to determine the role and functions of the protein encoded by ORF5 in GVE. With the high aa similarity between the ORF5 of GVE and that of GVA, it is expected to have a similar function. Interestingly, the GVA DAS-ELISA did not detect GVA in the plants infiltrated with the positive control for the replacement vector 35S-GVA-118-ΔORF5-GTR1-2. This could be the result of low virus titers in these plants, that is not detectable with ELISA as disease symptoms and RT-PCR did indicate that these plants are positive for virus resulting from the 35S-GVA-118-ΔORF5-GTR1-1 construct. The results from the TPIA, as well as the symptom development did give an indication that the titers in these plants are low. In the study by Blignaut, (2009) low titer was also observed for the 35S-GVA-118-ΔORF5-GTR1-2 infectious clone compared to the 35S-GVA-118 positive control with low counts of virus particles observed in infiltrated plants, with a electron microscope. This indicates that ORF5 is very specific and might determine the infectivity of these viruses.

In conclusion, transmission experiments were performed, but all attempts in transferring GVE to 10 different herbaceous plants species were unsuccessful. Transferring GVE to an herbaceous plant can be a valuable tool for downstream experiments such as the production of anti-GVE antisera for the development of a GVE ELISA as well as to elucidate the virus structure via electron microscopy. The GVA-GVE chimeric clones were successfully constructed and evaluated in *N.*

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benthamiana plants. The 35S-GVA-GR5-ΔORF2-GVE-ORF2 chimeric infectious clone was infectious and detectable with TPIA, DAS-ELISA and RT-PCR in infiltrated as well as systemic leaves. The *N. benthamiana* plants agroinfiltrated with the ORF2 chimera clone developed severe disease symptoms, similar to what is seen with GVA infection. However, the virus titer of this chimeric viral vector was still lower than that of the positive control plants, agroinfiltrated with 35S-GVA-GR5 and 35S-GVA-GR5-ΔORF2-GTR1-1. The 35S-GVA-118-ΔORF5-GVE-ORF5 chimeric clone did not infect *N. benthamiana* plants. No disease symptoms were observed for plants infiltrated with this construct and the viral vector was not detected with TPIA, DAS-ELISA or RT-PCR. This indicates that either ORF5 is sequence specific or suppressor activity is very specific in vitiviruses.

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Chapter 5

Final conclusion

The grapevine and the wine industry contribute greatly to the economy of South Africa. Unfortunately, the grapevine industry is threatened by various pathogenic agents that reduce the yield and quality of grapes, which in return negatively affects the wine industry. These pathogens include fungi, bacteria, insects and viruses. Investigating these pathogenic agents can give great insight into grapevine diseases that result from infections with these pathogens.

Viruses are important pathogens of grapevine as little to no resistance have been found against these pathogenic agents. This study focused on investigating molecular and biological aspects of the recently identified virus, GVE. The first report of this virus was in 2008, when it was first identified in Japan, in *Vitis librusca* cultivars. More recently GVE was identified in South African vineyards *Vitis vinifera* cv Merlot and Shiraz. With GVE being a recently identified virus, little is still known about the possible impact it may have on vine health.

The first aim in this study was to conduct a survey of the vineyard where GVE was first detected in South Africa. The incidence of GVE was determined and a possible correlation between GVE and any disease symptoms was investigated. The survey was conducted in the vineyard where GVE was first identified in South Africa. Sample sizes were statistically calculated such that samples collected were predicted to be a true representation of the vineyard surveyed with a 95% confidence level. It was therefore calculated that 139 plant samples were needed for the survey of which randomly petioles collected throughout the vineyard. RNA was successfully extracted from all 139 plant samples and RT-PCR performed to detect GVE. The primers in this study were designed from all four GVE sequences available (TvAQ7, TvP15, SA94 and Node 3404) to detect all possible variants of GVE that may be present. From this survey the incidence of GVE was determined as ~ 3% in this vineyard, since only four plants tested positive for GVE infection with RT-PCR. With the incidence of GVE being low, a clear cluster of GVE infections was not observed, but three of the four GVE positive plants were in close proximity to one another, which possibly suggest a hot spot for infection or point where initial infection occurred. But screening of the adjacent plants did not indicate GVE infection. It could be that these plants are the initial infection of the vineyard and that spread of this virus throughout the vineyard could still occur. The monitoring and testing of this vineyard in coming years could determine whether infection is spread from these points, where infection is currently observed. The GVE positive plants did display medium to severe LRD symptoms, which included down rolling of leaf margins and reddening of interveinal leaf areas. The

GVE positive plants were subsequently screened, with RT-PCR for nine other viruses that are known to infected grapevine. It was determined that all four GVE positive plants were co-infected with GLRaV-3. With the low incidence of GVE and the presence of GLRaV-3 it was not possible to determine whether GVE is responsible for any of the disease symptoms observed. It is most likely that the presence of the GLRaV-3 in these plants are responsible for the disease symptoms, as several studies have found GLRaV-3 as the most abundant virus associated with LRD. However, GVE cannot be ruled out as a contributing agent to the disease symptoms observed as it is known that viruses are often found in complexes, were they cause disease, while single infections in established vineyards are uncommon. The results obtained here were determined from a single vineyard at a certain time point and serves only as an initial insight into the occurrence of GVE. Surveying and monitoring additional vineyards for GVE infection could reveal more information on the impact of this virus on vine health as well as incidence in South Africa.

The serological test, ELISA is used as the industry standard for detection of grapevine viruses. Grapevine virus E is a newly identified virus and an ELISA detecting GVE does not exists. The GVA DAS-ELISA from Bioreba was evaluated with nine GVE positive plants to determine if cross reactivity with GVE occurs. Out of the 10 different GVE positive plants used only one tested positive with the ELISA kit. The results of the ELISA were confirmed with RT-PCR for GVA, were the one ELISA positive plant was co-infected with GVA. The ELISA did not detect GVE.

The relative seasonal virus titers for five GVE infected plants were calculated over the growing season from 15 November 2010 – 30 May 2011. This was performed to be an indication of when during the season the virus titer is at its highest, making it the most appropriate time to test for viral infection. The relative GVE titer in these plants, for the before mentioned period, did not indicate a clear fluctuation throughout the season. This indicated that the GVE titer relative to actin stayed constant during this growing season and does not indicate a clear peak indicating higher titer or a dip indicating lower virus titer throughout the season. As disease symptoms in grapevine are at their most prominent during the autumn season, it was expected that the virus titer in plants would also be at its highest during this time. From the results obtained here, this is not the case for this particular growing season. These results are only indicated for one growing season and additional data are needed for confirmation.

This study was also aimed at biologically characterizing GVE, as little information on this virus is available. Transmission experiments were carried out to transfer GVE to nine different herbaceous host plants. Different herbaceous plants as well as three different buffers were used to facilitate the transfer of GVE. The transmissions were unsuccessful with the transmission buffers and herbaceous plants used. Inoculated plants were monitored for a period of 30 dpi and RT-PCR was performed on extracted RNA to detect GVE on regular intervals. No infection occurred in any of the plants and no

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disease symptoms were observed. Transmission experiments are known to be a difficult process as infection cannot be predicted and various factors can prevent viral infection. These factors include the acidity of the grape extract, which can reduce the ability of the virus to infect. Choosing the correct transmission buffer is therefore very important. Using a phosphate buffer for grapevine viruses will neutralize the acid minimizing the effect grape sap has on the virus. Other factors such as oxidation reactions are also important to consider, since it can also reduce the ability of viruses to infect. Though the effects of these factors can be minimized to an extent, it does not guarantee that infection will occur. Transmitting newly identified viruses to herbaceous host plants are important when studying viruses, as the virus titer is often higher in these plants, they grow faster and can be maintained under glasshouse conditions. This can subsequently be used to purify the virus and for the production of antibodies for ELISA. The successful transmissions can therefore be a valuable tool for the above-mentioned experiments as well as others. Repeating the transmission is therefore important. Using different herbaceous plants, increasing the number of plants and monitoring it for longer periods could yield the successful transmission of GVE.

Further attempts were made at characterizing GVE by the construction and evaluation of GVA-GVE chimera clones. The chimera clones were successfully constructed by cloning GVE ORF2 and ORF5 in the 35S-GVA-GR5-ΔORF2 and 35S-GVA-118-ΔORF5 deleted mutants, respectively to create 35S-GVA-GR5-ΔORF2-GVE-ORF2 and 35S-GVA-118-ΔORF5-GVE-ORF5. These constructs were evaluated in *N. benthamiana* plants. Results obtained indicated that only the 35S-GVA-GR5-ΔORF2-GVE-ORF2 infectious clone was able to infect and replicate in plants. Disease symptoms were observed in plants infiltrated with this construct and the virus was detected with TPIA, RT-PCR as well as DAS-ELISA. The virus titers obtained in these plants were lower than that observed for the positive control plants even though the disease symptoms observed was just as severe. The 35S-GVA-GR5-ΔORF2-GVE-ORF2 construct was not infectious in *N. benthamiana* as virus was not detected via TPIA, RT-PCR or ELISA in plants infiltrated with this construct. This indicated that the GVE ORF5 was not compatible with the ORFs in the 35S-GVA-118-ΔORF5 deletion mutant. This indicated that ORF5 of vitiviruses are very specific for infection to occur, correlating with previous studies performed on ORF5. This showed that ORF5 plays a role in the infectivity of these viruses and is very specific for infection to occur.