The development and characterisation of grapevine virus-based expression vectors

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

_____   Date: _____

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Abstract

Grapevine (*Vitis vinifera* L.) is a very important agricultural commodity that needs to be protected. To achieve this several *in vivo* tools are needed for the study of this crop and the pathogens that infect it. Recently the grapevine genome has been sequenced and the next important step will be gene annotation and function using these *in vivo* tools. In this study the use of *Grapevine virus A* (GVA), genus *Vitivirus*, family *Flexiviridae*, as transient expression and VIGS vector for heterologous protein expression and functional genomics in *Nicotiana benthamiana* and V. *vinifera* were evaluated. Full-length genomic sequences of three South African variants of the virus (GTR1-1, GTG11-1 and GTR1-2) were generated and used in a molecular sequence comparison study. Results confirmed the separation of GVA variants into three groups, with group III (mild variants) being the most distantly related. It showed the high molecular heterogeneity of the virus and that ORF 2 was the most diverse. The GVA variants GTG11-1, GTR1-2 and GTR1-1 were placed in molecular groups I, II and III respectively. A collaboration study investigating the molecular divergence of GVA variants linked to Shiraz disease (SD), described two interesting GVA variants of group II, namely GTR1-2 and P163-M5 (Goszczynski et al., 2008). The group II variants were found to be closely linked to the expression of SD. GTR1-2 was isolated from a susceptible grapevine plant that never showed SD symptoms (Goszczynski 2007). The P163-M5 variant that resulted in exceedingly severe symptoms in *N. benthamiana* and is that used as SD positive control by the grapevine industry, was found to contain a 119 nt insert within the native ORF2. Comparative analysis performed on the complete nt and aa sequences of group II GVA variants suggested that the components in the GVA genome that cause pathogenicity in *V. vinifera* are more complex (or different) to those that cause pathogenicity in *N. benthamiana*. The three South African variants (GTR1-1, GTG11-1 and GTR1-2) were assembled into full-length cDNA clones under control of CaMV 35S promoters. After several strategies were attempted, including a population cloning strategy for GTR1-2, none of the clones generated were able to replicate in *N. benthamiana* plants. A single amino acid substitution at position 13 (Tyr/Y→Cys/C) in ORF 5 of the GTR1-2 cDNA clone was shown to abolish or reduce replication of the virus to below a detectable level. Two infectious clones of Israeli variants of GVA (T7-GVA-GR5 and T7-GVA118, obtained from M. Mawassi) were brought under control of a CaMV 35S promoter (35S-GVA-GR5 and 35S-GVA118). Both clones were infectious, able to replicate, move systemically and induce typical GVA symptoms after agroinfiltration in *N. benthamiana*. These Israeli clones served as backbone for further

experiments in characterisation of transient expression and VIGS vectors. The use of GVA as gene insertion vector (35S-GVA118) and gene exchange vector (35S-GVA-GR5-ΔORF2+sgMP) in *N. benthamiana* and *V. vinifera* was compared. The gene insertion vector, 35S-GVA118 was based on the full-length GVA genome. The gene exchange vector, 35S-GVA-GR5-ΔORF2+sgMP, was constructed in this study by elimination of ORF 2 and insertion of a sgMP and unique restriction sites to facilitate transgene insertion. In *N. benthamiana* both vectors showed similar GUS expression levels and photobleaching symptoms upon virus-induced NbPDS silencing. In *V. vinifera* limited GUS expression levels and VIGS photobleaching symptoms were observed for the gene insertion vector, 35S-GVA118. No GUS expression was observed for the gene exchange vector 35S-GVA-GR5-ΔORF2+sgMP in this host. As for silencing, one plant, agroinfiltrated with 35S-GVA-GR5-ΔORF2-VvPDS+sgMP, developed photobleaching symptoms in 3 systemic infected leaves after 4 months. This study showed that GVA can be used as gene insertion and gene exchange vector for expression and VIGS in *N. benthamiana*, but in grapevine its use is limited to expression and silencing of genes in the phloem tissue. It is also the first report that ORF 2 of GVA is not needed for long distance movement in grapevine.

To investigate the possible role of the P163-M5 119 nt insertion and the GVA ORF 2 (of unknown function), in expression of symptoms in plants, ORF 2 of a 35S-GVA-GR5 cDNA clone was removed and subsequently substituted by the corresponding ORFs of four South African GVA variants. Upon agro-infiltration into *N. benthamiana* leaves, all chimaeric GVA constructs were able to move systemically through the plant. At this stage no correlation could be found between severity of symptoms, the presence of the P163-M5 insert and the specific GVA ORF 2 present in the chimaeras, indicating that other factors in the viral genome or the host plant probably play a crucial role.

This study contributed to the pool of available *in vivo* tools for study and improvement of the valuable grapevine crop. It also opened several exciting research avenues to pursue in the near future.

Opsomming

Wingerd (*Vitis vinifera* L.) is 'n baie belangrike landboukundige gewas wat beskerm moet word. Om die rede word verskeie *in vivo* gereedskap vir die bestudering van die wingerdplant, en die patogene wat dit infekteer benodig. Die wingerd genoom se volgorde is bepaal en dus is die volgende logiese stap om die gene te annoteer en funksie daaraan toe te skryf. In hierdie studie is die gebruik van *Grapevine virus A* (GVA), genus *Vitivirus*, familie *Flexiviridae*, as tydelike uitdrukking- en virus-geinduseerde geenuitdowingsvektor vir heteroloë proteïen uitdrukking en funksionele genoomstudies in *Nicotiana benthamiana* en *V. Vinifera* getoets. Vollengte genoomvolgordes van drie Suid-Afrikaanse variante van die virus (GTR1-1, GTG11-1 en GTR1-2) is gegenereer en in 'n molekulêre volgorde vergelyking studie gebruik. Resultate het die verdeling van GVA variante in drie groepe, waar groep III die verste verwant is, bevestig. Dit het ook gewys dat die virus 'n baie hoë molekulêre heterogeniteit het en dat oopleesraam 2 (ORF 2) die mees divers is. 'n Samewerking studie waar die molekulêre diversiteit van GVA variante, gekoppel aan Shiraz siekte (SD), ondersoek is, is twee interessante variante van groep II beskryf, naamlik GTR1-2 en P163-M5 (Goszczynski et al., 2008). Groep II variante is vooraf gevind om nou verwant te wees aan die ontwikkeling van SD in wingerd. Die GTR1-2 variant is uit 'n vatbare wingerd plant, wat nooit SD-simptome vertoon het nie, geïsoleer (Goszczynski et al., 2007). In die ORF 2 van die P163-M5 variant, wat simptome van die ergste graad in *N. benthamiana* geïnduseer het, en ook deur die industrie as betroubare SD-positiewe kontrole gebruik word, is 'n 119 nt invoeging gevind. Die vergelykende analise wat uitgevoer is, het daarop gedui dat die determinante van patogenisiteit in die GVA genoom moontlik meer kompleks kan wees in *V. vinifera* as in *N. benthamiana*. Die drie Suid-Afrikaanse variante (GTR1-1, GTG11-1 en GTR1-2) is in afsonderlike vollengte cDNA klone, onder beheer van CaMV 35S promotors, aanmekaargesit. Nadat verskeie kloneringstrategieë, insluitend 'n populasie kloneringstrategie vir die GTR1-2 kloon, gebruik is, het geen een van die cDNA klone die vermoë besit om in *N. benthamiana* te repliseer nie. 'n Enkele aminosuur substitusie in posisie 13 (Tyr/Y→Cys/C) in ORF 5 van die GTR1-2 kloon, het die replisering van die virus tot laer as 'n opspoorbare vlak verlaag. Twee infektiewe klone van Israeliese GVA variante (T7-GVA-GR5 en T7-GVA118, verkry van M. Mawassi) is onder beheer van 'n CaMV 35S promotor geplaas (35S-GVA-GR5 and 35S-GVA118). Beide klone het na agro-infiltrasie in *N. benthamiana* plante gerepliseer, sistemies beweeg en tipiese GVA simptome geinduseer. Hierdie twee klone het as raamwerk gedien vir verdere eksperimente in karakterisering van

tydelike uitdrukkings- en VIGS vektore. Die gebruik van GVA as geen-insvoegingsvektor (35S-GVA118) en geen-vervangingsvektor (35S-GVA-GR5-ΔORF2+sgMP) is in *N. benthamiana* en *V. vinifera* vergelyk. Die geen-invoegingsvektor 35S-GVA118, was op die vollengte GVA genoom gebasseer. Die geen-vervangingsvektor 35S-GVA-GR5-ΔORF2+sgMP, was in hierdie studie gekonstrueer. Dit is gemaak eerstens deur eliminasie van ORF 2 in die 35S-GVA-GR5 kloon, en tweedens deur die invoeging van 'n subgenomiese promotor van die beweginsproteïen (sgMP) en unieke beperkings-ensiemsetels om klonering van transgene te fasiliteer. Beide vektore het in *N. benthamiana* vergelykbare GUS uitdrukkingsvlakke en fotobleikende simptome getoon na virus-geinduseerde NbPDS uitdowing. In *V. Vinifera* is beperkte GUS uitdrukkingsvlakke en VIGS fotobleikende simptome opgemerk met die geen-invoegingsvektor, 35S-GVA118. Geen GUS uitdrukking is in hierdie gasheerplant met die geen-vervangingsvektor opgemerk nie. Slegs een wingerdplant het fotobleikende simptome, na 4 maande in 3 sistemies geïnfekteerde blare gewys, na agro-infiltrasie van die 35S-GVA-GR5-ΔORF2-VvPDS+sgMP konstruk. Hierdie studie het bevestig dat GVA as geen-invoeging en geen-vervangingsvektor, vir heteroloë proteïen-uitdrukking en VIGS, in *N. benthamiana* gebruik kan word, maar dit blyk of die gebruik daarvan in wingerd meer tot die floeëm weefsel beperk is. Hierdie studie wys vir die eerste keer dat ORF 2 nie nodig is vir langafstand beweging van die virus in wingerd nie.

Om die moontlike rol van die P163-M5 119 nt invoeging en die GVA ORF 2 (met onbekende funksie), in die uitdrukking van simptome in plante te ondersoek, is ORF 2 van die 35S-GVA-GR5 cDNA kloon verwyder en daaropvolgens vervang met die ooreenstemmende ORFs van vier Suid-Afrikaanse GVA variante. Na agro-infiltrasie in *N. benthamiana* blare, het al die chimeras die vermoë gehad om te repliseer, sistemies te beweeg en simptome te induseer. Geen korrelasie kon gevind word tussen die graad van simptome, die teenwoordigheid van die P163-M5 insersie en die spesifieke GVA ORF 2 teenwoordig in die chimeras nie, wat dus daarop dui dat ander faktore in die virusgenoom of die gasheerplant `n moontlike belangrike rol kan speel.

Hierdie studie het bygedrae tot die beskikbare poel van *in vivo* gereedskap vir die bestudering en verbetering van die kosbare wingerdgewas. Dit het ook talle interessante navorsingsgeleenthede oopgemaak om in die nabye toekoms te betree.

Preface

This thesis is divided into 7 chapters:

Chapter 1: General Introduction and Project Aims

Chapter 2: Literature Review

Chapter 3: Complete nucleotide sequences and molecular characterisation of three South African *Grapevine virus A* variants

Chapter 4: Construction of infectious clones of three South African GVA variants

Chapter 5: The characterisation of GVA vectors for transient expression and virus-induced gene silencing in *N. benthamiana* and *V. vinifera*

Chapter 6: Towards the elucidation of *Grapevine virus A* ORF 2 gene function

Chapter 7: Conclusion and future prospects

Abbreviations

μF	microfarad
μg	microgram(s)
μL	microliter(s)
μM	micromolar
A	Adenine
aa	amino acid(s)
AGO	Argonaute protein
Ala	Alanine
AlkB	alkylated DNA repair protein
Asn	Asparagine
Asp	Aspartate
bp	base pair(s)
C	Cytosine
CaMV	*Cauliflower mosaic virus*
cDNA	Complementary deoxyribonucleic acid
ChlH	H subunit of magnesium chelatase
CI	Consistency index
CP	Coat protein
CTAB	N-Cetyl-N,N,N-trimethyl Ammonium Bromide
Cys	Cysteine
Asp	Aspartic acid
ddH$_2$O	Double distilled water
DI RNA	Defective interfering ribonucleic acid
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate(s)
dpi	days post infiltration (inoculation)
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra-acetic Acid di-sodium salt
EmGFP	enhanced green fluorescent protein
EST	expressed sequence tags
Gly	Glycine
g	Gram(s)
G	Guanine
GFP	Green Fluorescent Protein
Glu	Glutamic acid
Gly	Glycine
GOI	gene of interest
GUS	β-glucoronidase
GVA	*Grapevine virus A*
h	Hour(s)
Hel	Helicase
ICTV	International Committee on Taxonomy of Viruses
kb	Kilobase(s)
kDA	Kilo Dalton
kPa	kilopascal
KSG	Kober Stem Grooving
kV	kilovolt(s)
Leu	Leucine
M	Molar
MCS	multiple cloning site
min	minute(s)
miRNA	micro RNA
mL	millilitre(s)
mM	millimolar
MP	Movement protein
mRNA	Messenger ribonucleic acid

mRNA	messenger RNA
MS	Murashige and Skoog
Mtr	Methyl-transferase
mV	millivolt(s)
NbPDS	*Nicotiana benthamiana* phytoene desaturase
ng	nanogram(s)
nm	nanometer(s)
nt	nucleotide(s)
ºC	degrees Celcius
OD	optical density
OE-PCR	overlap extension PCR
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PDS	phytoene desaturase
pH	potential of Hydrogen
Phe	Phenyl-alanine
pmol	picomole(s)
Pro	Proline
PTGS	Post-transcriptional gene silencing
qRT-PCR	quantitative reverse transcription real-time PCR
RBCS	RuBisCo small subunit
RCA	rolling circle amplification
RdRP	RNA-dependant RNA polymerase
REST	Relative expression software tool
RI	Retention index
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
rpm	Revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RT-PCR-RFLP	Reverse transcription polymerase chain reaction restriction fragment length polymorphisms
RW	Rugose Wood
SD	Shiraz disease
Sec	second(s)
Ser	Serine
sgMP	sub-genomic promoter of the movement protein
sgORF	sub-genomic promoter of open reading frame
sgRNA	Sub-genomic ribonucleic acid
siRNA	small-interfering RNA
SSCP	Single-strand conformational polymorphisms
ssRNA	Single stranded ribonucleic acid
T	Thymine
TAE	Tris/acetic acid/EDTA
TBR	Tree bisection reconnection
T-DNA	Transfer DNA
Thr	Threonine
TPIA	tissue-print immuno-assay
Tris	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
U	Unit(s)
U	Uracil
UTR	Un-translated region
UV	Ultra Violet
v\v	Volume per volume
VIGG	virus-induced grapevine protein
VIGS	Virus-induced gene silencing
vRNA	viral RNA
VvPDS	*Vitis vinifera* phytoene desaturase
w\v	Weight per volume

Ω ohm(s)

x

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Dedicated to my beautiful wife Helen.

Contents

Chapter 1: General Introduction and Project Aims

Grapevine (*Vitis vinifera* L.) has through the years been measured as a very valuable agricultural crop and has been grown internationally for production of grapes for winemaking, spirits, juice, table grapes and raisins. In South Africa, the wine industry is a very important contributor to the economic wellbeing of the country and it is an essential resource that needs to be protected. According to the "South African Wine Industry Statistics Report" in May 2009, South Africa is the 7th largest wine producing country in the world, which adds up to 3.6 % of the world's production. There are currently 112 700 hectares of South African terrain covered by vines, which is 1.5 % of the total world vineyard surface. In 2008, South African grape producers delivered a production of 1 425 612 tons of grapes that were crushed to yield 1089 million litres of wine, brandy, distilling wine, grape juice concentrate and grape juice. This amounted to a significant total producers' income of ZAR 3 319.9 million (www.sawis.co.za). In order to protect this resource, studies need to be undertaken to prevent or control disease and to improve the grapevine plant.

New generation sequencing technologies like 454 (Roche) and Illumina® (Solexa) are fast producing an immense amount of sequence information. Several plant genomes have been sequenced and made available, including the genome of the grapevine cultivar Pinot Noir (Jaillon et al., 2007; Velasco et al., 2007). A number of papers were also published reporting the deep sequencing analysis of grapevine disease complexes giving insight on the specific pathogens that are present (Al Rwahnih et al., 2009; Sabanadzovic et al., 2009; Coetzee et al., 2009). With the wealth of sequence data that is being generated, there is an ever growing demand for the development of *in vivo* tools to explore this data and perform functional analysis on it.

A very attractive approach to perform functional genomics in plants is by the method of virus-induced gene silencing (VIGS). This method makes use of a vector derived from a viral genome that has been engineered to carry a sequence of an endogenous plant gene. By delivery of the vector and replication of the recombinant virus within the plant, the natural plant defence known as post-transcriptional gene silencing (PTGS) is activated against the virus resulting in silencing of the plant gene. *Grapevine virus A* (GVA), genus *Vitivirus*, family *Flexiviridae* is a regularly detected virus in vineyards all over the world (Boscia et al., 1997a). It is a well characterised virus and it is a good candidate for consideration in the

development of virus-based vectors for grapevine as it can use both *V. vinifera* and *Nicotiana benthamiana* as hosts.

The aim of this study was to evaluate the use of GVA as expression and VIGS vector for transient heterologous protein expression and functional genomics in grapevine. In order to achieve this purpose the following objectives were pursued:

- Full-length sequencing of three South African GVA variants (GTR1-1, GTG11-1 and GTR1-2) representing each of the molecular groups.
- Construction and characterisation of full-length infectious clones from South African GVA sequence variants, capable of systemic infection in *N. benthamiana* plants. Such clones could be used as a molecular tool in the unraveling of the aetiology of disease and gene expression studies on South African isolates of GVA.
- The development and characterisation of GVA-based expression vectors. Evaluate the use of the GVA genome as a transient expression vector that could serve as a tool for transient foreign protein expression in *N. benthamiana* and *V. vinifera* plants. Evaluate the use of the GVA genome as a VIGS vector that could serve as a tool for functional genomics studies in grapevine.
- The molecular and biological characterisation of ORF 2 GVA hybrids in *N. benthamiana*.
- Evaluation of a protocol for infiltration of GVA-based constructs into *N. benthamiana* and *V. vinifera* plants. *[This objective was initially to develop an infiltration protocol for grapevine, but during progression of this research the technique was developed by Dirk Stephan (Department of Genetics, Stellenbosch University) and was only evaluated in the current study]*

The thesis is divided into 7 chapters of which each will be introduced briefly in the following sections.

Chapter 1: General Introduction and Project Aims
This chapter gives a general introduction about the thesis and describes the aims of the study.

Chapter 2: Literature review
This chapter gives a broad overview of the current literature and state of affairs regarding GVA, infectious clones, virus-based expression vectors, VIGS vectors and suppressors of gene silencing.

Chapter 3: Complete nucleotide sequences and molecular characterisation of three South African *Grapevine virus A* variants

Previous studies performed on the variability of GVA in South African vineyards have been based on short genomic regions of the virus (Goszczynski & Jooste, 2002, 2003b, c; Goszczynski, 2007). These studies revealed that the virus had a very heterogenous population structure and that variants phylogenetically divided into three different molecular groups. This chapter describes the full-length genome sequencing and molecular comparison of three South African GVA variants (GTR1-1, GTR1-2 and GTG11-1), representing each of the molecular groups.

Chapter 4: Construction of infectious clones of three South African GVA variants

Three GVA variants, representing each of the molecular groups, were selected in order to establish full-length infectious cDNA clones of South African variants. These three variants were fully sequenced (chapter 3) and the sequence data used to devise strategies for assembly. This chapter describes the construction of cDNA clones of South African GVA variants GTR1-1, GTR1-2 and GTG11-1. Strategies and pitfalls for making infectious clones are discussed. Two infectious GVA clones under T7-promoter control (T7-GVA-GR5 and T7-GVA118) were obtained from Munir Mawassi (The S. Tolkowsky Laboratory, Department of Plant Pathology-The Virology Unit, Agricultural Research Organization, Volcani Center, Bet Dagan, Israel) and their use as positive controls in this study is also described.

Chapter 5: The characterisation of GVA vectors for transient expression and virus-induced gene silencing in *N. benthamiana* and *V. vinifera*

Once a full-length cDNA clone of a virus is established, which is able to replicate and induce a systemic infection in a plant, the modification thereof into an expression or VIGS vector can follow suit. This chapter describes how an infectious GVA cDNA clone can be utilised for transient protein expression or mediation of VIGS in N. benthamiana *and* V. vinifera. *It also compares the gene exchange and gene insertion vector strategies with each other.*

Chapter 6: Towards the elucidation of *Grapevine virus A* ORF 2 gene function

The function of the open reading frame 2 protein product is still not known for vitiviruses. This chapter describes early attempts to elucidate the function of the gene by the characterisation of GVA ORF 2 chimaeras in N. benthamiana.

Chapter 7: Conclusion and future prospects

This chapter concludes the thesis and discusses future prospects and avenues opened by this study.

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Chapter 2: Literature Review

2.1. INTRODUCTION

From ancient times Grapevine (*Vitis vinifera* L.) has been considered a very valuable crop and has been grown globally for the production of several products. As with most vegetatively propagated crops grapevine is prone to attacks of many kinds of infectious agents that shorten the productive lifespan of vines and cause heavy losses worldwide. Viruses are among the most significant of these pathogens because there are currently no cures, treatments or natural resistance. In fact, until now 60 different grapevine-infecting viruses have been noted, representing the most ever identified in a single agricultural product (Martelli & Boudon-Padieu, 2006). Recently, two new viruses have been added to the number namely Grapevine virus E (GVE), genus *Vitivirus*, family *Flexiviridae* (Nakaune et al., 2008) and Grapevine Syrah virus 1 (GSyV-1), genus *Marafivirus*, family *Tymoviridae* (Al Rwahnih et al., 2009). Interestingly, GSyV-1 is the first virus that has been identified with a new generation deep sequencing technology. The same virus, named Grapevine virus Q (GVQ) was identified in an independent study (Sabanadzovic et al., 2009). The vineyards of South Africa are plagued by three important destructive disease complexes in which viruses are thought to be involved namely grapevine leafroll disease, Shiraz disease (SD) and Shiraz decline. With the identification of new viruses and disease complexes, new research challenges arise. In order to progress in the understanding of grapevine disease, the host plant and the viruses involved need to be extensively studied. The sequence of the grapevine cultivar Pinot Noir has been determined and was made available recently (Jaillon et al., 2007; Velasco et al., 2007). This leads to an ever-growing requirement for functional genomic studies in this crop. The remarkable *in silico* advances made in grapevine genomics over the last ten years, have not been marvelled by the development of *in vivo* tools to execute proficient functional genetic studies (Santos-Rosa et al., 2008).

Infectious clones of several plant viruses are available and most of these have been engineered into transient expression vectors and VIGS vectors for recombinant protein expression and silencing of target genes in major crop plant species. Most of these have been developed for utilisation in herbaceous and solanaceous plants (Igarashi et al., 2009). These viral constructs aid in the study of viruses and the plants which they infect. It has been shown that grapevine is susceptible to infection by *Agrobacterium* species (Mezzetti et al., 2002). The stable transformation and regeneration of transgenic grapevine plants was achieved by

both biolistic and *Agrobacterium*-mediated systems (Santos-Rosa et al., 2008). Stable transformation is a time-consuming, inefficient process and is not amenable to high-throughput technologies. As an attractive substitute for stable transformation, transient expression is a fast, simple and reproducible technique to examine gene function and disease resistance in plants. Transient gene expression methods (non-viral based) have been established for grapevine over the recent years, these include: (1) cell suspensions, (2) particle bombardment and agroinfiltration of leaves (Torregrosa et al., 2002; Vidal et al., 2003; Santos-Rosa et al., 2008; Zottini et al., 2008). The development of efficient viral-based transient expression and VIGS systems for grapevine has not been established as yet. Once established, these powerful tools will greatly benefit functional genomic studies for the analysis of gene functions in this valuable crop.

2.2. *GRAPEVINE VIRUS A*

In South African vineyards GVA is thought to be the second most significant virus of importance due to the involvement of viral variants in SD (Goszczynski, 2007). It is second only to *Grapevine leafroll-associated virus 3* (GLRaV-3), genus *Ampelovirus*, family *Closteroviridae*, which is the pathogenic agent in the economically important grapevine leafroll disease (Gerhard Pietersen, Department of Plant Pathology, University of Pretoria, South Africa, pers. Comm.). In the following section an overview of GVA will be presented.

2.2.1. Taxonomy

Grapevine virus A is a constituent of the genus *Vitivirus* which is incorporated in the family *Flexiviridae*. The taxonomic re-arrangement of the family *Flexiviridae* was recommended in a recent phylogenetic and evolution study (Martelli et al., 2007). Martelli et al. suggested that this family should be divided into three new families *Alphaflexiviridae*, *Betaflexiviridae* and *Gammaflexiviridae* and that these three families should be included with the family *Tymoviridae*, in a new order *Tymovirales*. The family *Betaflexiviridae* now hold the genera *Foveavirus*, *Trichovirus* and *Vitivirus*. These taxonomic proposals are pending authorization from the Executive Committee of the International Committee on Taxonomy of Viruses (ICTV) (Martelli, 2009). The genus *Vitivirus* includes five definite species: GVA, *Grapevine virus B* (GVB) (Goszczynski et al., 1996), *Grapevine virus D* (GVD) (Choueiri *et al.*, 1997), *Heracleum latent virus* (HLV) (Murant et al., 1985), and *Mint virus 2* (MV2) (Tzanetakis et al., 2007). Grapevine virus E (GVE) (Nakaune et al., 2008), and Agave Tequilana leaf virus

are   provisional   species   of   this   genus.
(http://www.dpvweb.net/notes/showgenus.php?genus=Vitivirus).

2.2.2. Morphology

The virus consists of a flexuous, non-enveloped, filamentous particle with length and diameter of 800 nm by 12 nm. The nucleocapsid has a rope-like characteristic, is diagonally striated and crossbanded from corner to corner (figure 1) (Conti et al., 1980). The virions include more or less 5 % nucleic acid (Boccardo & d'Aquilio, 1981).
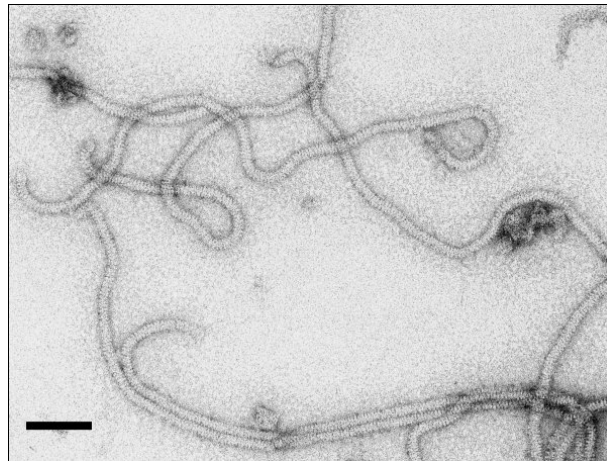
Figure 1. Electron micrograph of GVA showing the rope-like features of the viral particles. The bar represents 100 nm. (www.dpvweb.net /dpv/showfig.php?dpvno=383&figno=06).

2.2.3. Genome, genomic organisation and replication mechanism

The complete genomic sequence of GVA has been generated. The monopartite, positive sense single stranded (+ss) RNA, linear genome was found to be ~7.3-7.4 kb in length. The genome possesses a methylated nucleotide cap at the 5' end, a 3' poly-A tail (Minafra et al., 1994; 1997) and is organised into five open reading frames (ORFs) that overlap to some extent (figure 2). Putative functions were ascribed to translation products of all ORFs, by utilisation of an infectious GVA cDNA clone, except for the 19.8 kDa [~177 amino acids (aa)] protein product of ORF 2 (Galiakparov et al., 2003a), which is speculated to play a role in mealybug transmission (Galiakparov et al., 2003a). It was found that the ORF 1 encoded polypeptide (1707 aa) contains conserved motifs similar to replication associated proteins [methyl-transferase, RNA-helicase and RNA-dependent RNA polymerase (RdRP)] (Minafra et al., 1994; 1997). Intriguingly, it was recently reported that the replicase protein of members of the family *Flexiviridae* also contain an alkylated DNA repair protein (AlkB) domain (Aravind &

Koonin, 2001; Martelli et al., 2007). This domain was also identified in a sadwavirus (Halgren et al., 2007) a potyvirus (Susaimuthu et al., 2008) and several ampeloviruses (Maliogka et al., 2009). This protein is suggested to have evolved to permit viral infection of perennial or woody hosts (Dolja, 2009). The AlkB is an enzyme that is implicated in DNA repair and is prevalent in eukaryotes (Bratlie & Drablos, 2005). When taking into consideration the small size and restricted coding capacity of the GVA genome, it is astonishing that such a domain is present. Recently the AlkB protein of GVA and two other family *Flexiviridae* members were functionally characterised (van den Born et al., 2008). It was observed that viral AlkB proteins had substrate specificity and favoured RNA over DNA substrates. These viral AlkBs showed robust iron(II)- and 2-oxoglutarate-dependent demethylase activity *in vitro* and were able to efficiently reactivate methylated bacteriophage genomes when expressed in *Escherichia coli.* These results advocate that viral AlkBs retain viral RNA genome integrity by repair of methylation damage and support the biological relevance of AlkB-mediated RNA repair (van den Born et al., 2008). The 31 kDa (278 aa) movement protein (MP) and the 22 kDa (198 aa) coat protein (CP) are encoded by ORF 3 and 4, respectively. ORF 5 codes for a 10 kDa protein (P10, 90 aa) with homology to RNA binding proteins (Minafra et al., 1997; Galiakparov et al., 2003b). The P10 of GVA has recently been shown to act as a weak RNA silencing suppressor (Zhou et al., 2006; Chiba et al., 2006). It appears that the activity of P10 is increased ~1000 fold by another factor in the GVA genome (Mawassi, 2007).

Grapevine virus A is a phloem-associated virus (Tanne et al., 1989) and replicates in the cytoplasm of host cells in conjunction with membranous vesicles. The virus utilises a sub-genomic (sgRNA) RNA replication strategy in which ORF 1 (coding for the RdRP among others) is translated from genomic RNA into a polyprotein and then spliced into functional peptides (Buck, 1996). The viral RdRP subsequently recognises subgenomic promoters in the viral genomic RNA to produce sgRNAs. These sgRNAs have the same 3' ends as genomic RNA, but are shorter at their 5' ends in order to bring this end closer to the initiation codon of downstream ORFs. These ORFs normally code for products needed during later stages of infection, such as structural or movement proteins (Miller et al., 2000). Two nested sets of sgRNAs were characterised for GVA following the exploration of viral RNA production in GVA-infected *N. benthamiana* (Galiakparov et al., 2003c). These included one set of three 5' terminal sgRNAs of 5.1, 5.5 and 6.0 kb and another set of three 3' terminal sgRNAs of 2.2, 1.8 and 1.0 kb. The latter could possibly serve as template for expression of ORFs 2-4.

Interestingly, no sgRNA that corresponded to ORF 5 was detected and it is suggested that expression of this ORF occurs through bi-or polycistronic mRNA. The presence of both the minus and plus strands of 5' and 3' terminal sgRNAs in different levels of accumulation was observed in *N. benthamiana* (Galiakparov et al., 2003c).
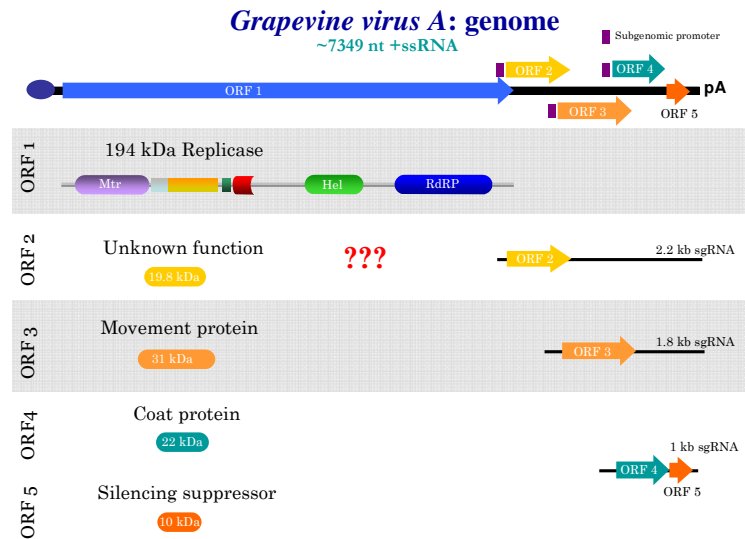
Figure 2. Graphic representation of the genome organisation, gene expression and replication strategy of GVA. The GVA genome consists of a +ssRNA genome of ~7.3-7.4kb in length. It possesses a 5' methylated CAP and a 3' poly-A tail and is organised into 5 overlapping ORFs. The functions of all the gene products are known except for ORF 2. GVA utilises a subgenomic RNA replication strategy. ORF 1 is translated directly from genomic RNA into a 194 kDa polyprotein that drives replication of the virus. ORFs 2-5 are translated from three 3' co-terminal subgenomic RNAs. Mtr = methyl-transferase, AlkB = Alkylated DNA repair protein domain, Hel = helicase, RdRP = RNA dependent RNA polymerase (Minafra et al., 1997; Galiakparov et al., 2003a; Galiakparov et al., 2003b; Chiba et al., 2006; Zhou et al., 2006).

2.2.4. Molecular diversity

Due to the inaccurate replication and short generation times, RNA viruses have the likelihood to set up large population diversity. This is an advantage as studies have shown a correlation between mutation frequency and virus host range. A virus with a higher mutation rate is more likely to become accustomed to a variety of plant hosts, upon insect transmission and could mean survival in a natural surrounding (Schneider et al., 2000; Schneider et al., 2001; Roossinck, 2003). It has been observed that RNA viruses persevere as a population of non-identical, closely related mutant and recombinant variants, known as viral quasispecies. This is due to the subjection of a virus to incessant genetic variation, competition and selection and allows viral populations to survive, adapt and cause disease (Martel et al., 1992; Domingo *et al.*, 1998; Forns et al., 1999)

In South African vineyards it was observed that GVA has a broad molecular heterogeneity. Three distinctly different molecular groups (I, II and III) were acknowledged based upon single-strand conformational polymorphism (SSCP) investigation of various short genomic regions of GVA sequence variants (Goszczynski & Jooste, 2003b). Each group gave rise to a different symptomology in the herbaceous host *N. benthamiana* that ranged from mild vein clearing to widespread patchy necrosis. Mild variants (group III) shared only 78 – 79.6 % nt sequence identity with other variants in the 3'terminal part of the viral genome (part of ORF 3, entire ORF 4, ORF 5 and part of the 3' UTR) (Goszczynski & Jooste 2003b). A recent study performed on grapevines in Italy, confirmed the high molecular diversity of the virus. Thirty seven GVA isolates were subjected to comparative RT-PCR-RFLP analysis of the CP gene. These were shown to cluster into 4 molecular groups, the three previously identified by Goszczynski and Jooste (2002) and a fourth (IV) putative group (Murolo et al., 2008).

2.2.5. Transmission

Grapevine virus A naturally proliferates in grapevine from which it can be transmitted by sap inoculation to a limited variety of herbaceous plant species. It is the first phloem-associated virus to be successfully transmitted to herbaceous plants (Conti et al., 1980). Natural vectors of the virus include species of the pseudococcid mealy bug genera *Planococcus* and *Pseudococcus* (Rosciglione et al., 1983; Garau et al., 1995; Engelbrecht & Kasdorf, 1987). Recently, GVA was successfully transmitted experimentally by the scale insect *Parthenolecanium corni* at the same time with the Ampelovirus *Grapevine leafroll-associated virus 1* (GLRaV-1, Hommay et al., 2008). This suggests that there could be a possible interaction involving these two viruses for transmission.

2.2.6. Diseases and geographical distribution

Grapevine virus A is one of the most regularly detected viruses and it is most likely present wherever *V. vinifera* is cultivated (Boscia et al., 1997a). Plants infected with GVA, generally hold a population of different sequence variants of the virus (Goszczynski & Jooste, 2003b). *Grapevine virus A* is implicated in the aetiology of Kober stem grooving (KSG; Digiaro et al., 1994; Chevalier et al., 1995; Garau et al., 1995), which is included in the four economically significant diseases of the grapevine rugose wood complex (RW; Martelli, 1993). When grafted from infected grapevines, the virus induces distinct longitudinal grooves on the stem of the American rootstock hybrid Kober 5BB (Garau et al., 1994). The virus causes harvest losses of up to 22 % in wine grape varieties in Italy (Garau et al., 1994) and it was found that

GVA can proliferate in grapevines without presenting symptoms (Garau et al., 1991). Interestingly, in Germany a study indicated that GVA infection had a very low impact on vines grown in this country even though the virus showed a high incidence of infection (46.9%) (Ipach & Kling, 2008).

In South Africa GVA has been found to be associated with a deadly disease of grafted and own rooted grapevine cultivars including Gamay, Malbec, Merlot, Shiraz and Voignier (Goszczynski & Jooste, 2003a). The disease, known as Shiraz disease, causes the vines of affected plants to stay green for extended periods in the growing season and to remain immature (Goussard & Bakker, 2006). Cross sections show excessive phloem and cambium growth and feebly developed non-lignified xylem causing the shoots to have a rubbery consistency. Affected vines show postponed budding and fruit production is diminished. SD vines never recuperate and always die in a period of five years. The disease is dormant in non-susceptible grapevine cultivars, from which it can effortlessly be transmitted by grafting and by the mealybug *Planococcus ficus* to SD-susceptible grapevine cultivars (Goszczynski & Jooste, 2003a). It was shown that variants of molecular group II are closely linked with SD, and variants of molecular group III are present in GVA-infected SD-susceptible grapevine that do not show symptoms of the disease (Goszczynski, 2007). Group II variants show a strong association with SD, but captivatingly a variant of this group, GTR1-2, was recovered in *N. benthamiana* from a consistently symptomless Shiraz plant (Goszczynski, 2007). In a recent study, a new virus-induced grapevine protein (VIGG) was identified and characterised in GVA-infected grapevine (Katoh et al., 2009). It was found that VIGG expression was constitutively expressed in GVA-infected grapevine and induced by GVA, but not other viruses. Grape berries that were harvested from grapevines, expressing VIGG, showed a higher content of phenolic substances and organic acid. This study suggested that the expression of VIGG increases the phenol content in berries by suppression of a decrease in organic acid (Katoh et al., 2009). Future functional characterisation of VIGG could prove invaluable in the understanding of grapevine fruit quality. A recent study described an attempt to develop GVA resistance in plants (Brumin et al., 2008). The authors developed a GVA-minireplicon, tagged with green fluorescent protein (GFP) that was used to activate RNA silencing consistently. A strong silencing response was found after delivery of this minireplicon via agro-infiltration in *N. benthamiana* plants. The authors subsequently generated transgenic *N. benthamiana* plants that constitutively expressed the minireplicon of GVA. These plants showed phenotypes that could be standardised and reproduced in order to

activate PTGS consistently. It was found that the minireplicon-derived transgene accumulated to low levels, that GFP expression was increased after delivery of viral silencing suppressors and that the plants showed resistance to GVA infection. The authors also suggested transmission of the RNA silencing signal from silenced rootstocks to non-silenced scions using a grafting assay. It was observed that the GVA-resistant transgenic plants were susceptible to GVB and that the GVA-specific resistance was suppressed after infection with GVB or *Potato virus Y* (PVY). The authors concluded that the consistent activation of PTGS by the GVA-minireplicon will provide an efficient approach for control of grapevine-infecting viruses (Brumin et al., 2008).

2.3. THE ESTABLISHMENT OF INFECTIOUS CLONES OF PLANT VIRUSES, THEIR INTRODUCTION INTO PLANTS AND THEIR USE AS TRANSIENT EXPRESSION VECTORS

The use and development of plants as bioreactors for foreign protein production has flourished in the last few years. Many of these proteins are produced through the generation of transgenic plants, which is a time-consuming and tedious process. As an attractive alternative to stable transformation, transient expression through a viral vector is a fast and efficient method of choice. The first step towards the development of a plant viral vector is the generation of an infectious clone of the virus able to infect and replicate in the desired host plant. For the purpose of this review, only infectious clones of plant RNA viruses will be presented in section (2.3.1). Several methods, discussed in section (2.3.2) have been developed to introduce an infectious clone (and viral vectors) into a plant. The next step is to convert this viral clone into a vector for transient expression of heterologous proteins or silencing of endogenous host genes. This will be presented in section (2.3.3).

2.3.1. The development of infectious clones of plant RNA viruses

Plant RNA viruses are among the smallest known viruses and cause significant damage to crop quality and yield worldwide. There are currently no cures for viral diseases and very few resistance genes against plant viruses exist. It is therefore essential to study viral pathogens in depth to acquire knowledge into their role in disease. The construction of full-length infectious clones establishes imperative tools for mutational and functional analysis studies of gene expression and replication of plant RNA viruses that can aid in the study of natural or induced RNA recombination, mechanisms of plant-virus movement and pathogen host

interactions. Over the years, the construction of infectious clones has become a standard protocol in laboratories worldwide. However, there are limitations and pitfalls when it comes to the assembly of such clones. It is often a long and tedious process and the infectivity of the clone is strongly influenced by cDNA synthesis, the cloning strategy used and the design of sequences bordering the viral insert (Boyer & Haenni, 1994). Generally, viral RNA genomes are reverse transcribed and PCR-amplified into cDNA. The resulting cDNA is then cloned into bacterial plasmids for manipulation, propagation and multiplication. In prokaryotic systems, some complications may arise due to toxicity of the viral insert. This may lead to instability and may result in random rearrangements and mutations in *Escherichia coli* (Yamshchikov et al., 2001). Shifting to another cloning vector or bacterial strain may correct these problems (Boyer & Haenni, 1994). More sophisticated procedures have been described to circumvent the instability problem, methods include the use of a nonbacterial cloning system (Polo et al., 1997), long high-fidelity PCR (Campbell & Pletnev, 2000), the incorporation of short introns into toxic genomic areas (Yamshchikov et al., 2001), and the inclusion of frameshifts in cDNA clones (Satyanarayana et al., 2003). According to the place of transcription, infectious clones of plant RNA viruses can be divided into two types: (1) *Infectious RNA* - The cloning of a viral genome under control of a bacteriophage (T7, T3 or SP6) RNA polymerase promoter from which *in vitro* RNA transcripts can be generated (Ryabov, 2008; Chapman, 2008), and (2) *Infectious cDNA* - the cloning of a viral genome under control of a CaMV 35S promoter from which infectious viral RNAs can be produced *in vivo* from cDNA containing vectors, delivered to the plant via several different methods (Dagless et al., 1997; Vives et al., 2008). When generating infectious RNA transcripts from a bacteriophage promoter, two critical factors play a part in infectivity of the *in vitro* transcript, namely the transcription itself and the distance between the bacterial promoter and the 5'-end of the virus. The *in vitro* transcription needs to be optimised and standardised for RNA of high quality and yield. Non-viral sequences between the promoter and the 5'end of the viral genome have been recognized to decrease infectivity of RNA transcripts (Nagyova & Subr, 2007). When expression of infectious viral RNAs are driven by a CaMV 35S promoter through the *in vivo* transcription of cDNA-containing vectors there are a number of advantages. The RNA transcripts are synthesised within living cells, making the infectivity of the clone less reliant on RNA degradation and no costly *in vitro* transcription step is required (Boyer & Haenni, 1994). Viral replication and expression of viral genes are rendered independent of each other facilitating studies of the role and/or localization of proteins expressed by mutant viral RNAs unable to replicate in cells. *In vivo*-produced viral transcripts

would then behave similar to messenger RNAs produced by a host RNA polymerase, still able to express native or mutant proteins without being replicated (Van Bakoven et al.*, 1993; Boyer & Haenni, 1994). Furthermore, the clones are very stable *in vitro* as isolated plasmid DNA. The introduction of the construct into the nucleus to allow transcription is a prerequisite, and this decreases the efficacy of some transfection methods (Nagyova & Subr, 2007). Problems may come up in transport of the transcript out of the nucleus and some AU-rich regions may induce incorrect splicing, resulting in non-infectious transcripts (Gleba et al., 2004).

Quite a few factors play a role in the infectivity of an infectious clone. These include: the heterogeneity of transcript population generated from a bacteriophage promoter, the incidence of point mutations, and the incidence of non-viral nucleotides at the 5' and 3' ends (Boyer & Haenni, 1994; Nagyova & Subr, 2007). Most of the problems can be circumvented by using high-fidelity enzymes and driving expression from a CaMV 35S promoter. Another method to minimize mutations and obtain viable clones is by using a population cloning strategy (Yu & Wong, 1998). The effect of short non-viral nucleotide sequences at the termini of viral transcripts, have been studied extensively. In general, it is known that extensions at the 5' end of viral transcripts, result in a reduction of infectivity, whereas 3'-extensions don't have such a huge effect (Boyer & Haenni, 1994). The addition of a poly-A tail or poly-adenylation signal at the 3' end significantly increases infectivity. The number of adenosyl residues however seems to be essential for infection of viruses that have a poly-adenylated genome (Holy & Abouhaidar, 1993; Viry et al., 1993; Boyer & Haenni, 1994).

Infectious clones of many plant RNA viruses have been reported in recent years (reviewed in Nagyova & Subr, 2007). For vitiviruses, an infectious T7-promoter driven cDNA clone of GVA was reported to be stable and infectious in *N. benthamiana* plants (Galiakparov et al, 1999). This clone was used for functional and genomic analysis of the virus (Galiakparov et al., 2003a, b, c). For GVB, a cDNA clone derived from an Italian isolate was reported to be infectious in *N. benthamiana* plants (Saldarelli et al., 2000). This clone was shown to be unstable in *Escherichia coli* cells resulting to arbitrary mutations in the cDNA clone. A stable clone of a South African isolate 94/971 of GVB was described and was shown to be infectious in *N. benthamiana* plants (Moskovitz et al., 2007). This isolate was obtained from corky bark diseased grapevine and the development of infectious clones for viruses associated

with RW will facilitate unravelling of the aetiology of these key disease complexes in the future (Moskovitz et al., 2007).

2.3.2. Introduction of infectious clones into plants

There are several ways by which infectious clones (and viral vectors) can be introduced into a plant. Both whole plants and protoplasts can be inoculated. Mechanical inoculation, agroinfection and biolistics are generally used to inoculate complete plants, while electroporation, microinjection and liposome-mediated inoculation are used for protoplast transformation as a rule (reviewed in Nagyova & Subr, 2007).

2.3.2.1. Inoculation of whole plants or plant tissue

Mechanical inoculation (or DNA abrasion for DNA) is commonly used when *in vitro* RNA transcripts are to be introduced into plants, mainly members of the herbaceous or solanaceous species. This method entails the damaging of the leaf exterior with an abrasive material, such as carborundum or celite, which allows direct introduction of nucleic acid into the injured cells (Hull, 2002; Ding et al., 2006; Ascencio-Ibanez & Settlage, 2007). Agroinfection is a very efficient technique that utilises the natural capability of members of the *Agrobacterium* genus to infect plants and launch transfer DNA (T-DNA) into the cell nucleus. This T-DNA is incorporated erratically into the plant genome (Ziemienowicz et al., 2000). When the T-DNA is substituted with a cDNA clone of a virus, the virus will be transcribed, transported from the nucleus to the cytoplasm, where it will replicate and induce a systemic infection in the plant. This method is particularly helpful for the delivery of phloem-limited viruses to permit functional genomics studies in the viral host plants (Grimsley et al., 1987; Leiser et al., 1992). Agroinfection can be applied in stable transformation or transient procedures such as infiltration with a syringe, vacuum or agrodrenching (Ekengren et al., 2003; Vaghchhipawala & Mysore, 2008; Brigneti et al., 2004; Liu et al., 2002a, b). In the biolistic approach, nucleic acids are layered onto gold or tungsten microcarriers that are shot directly into plant tissues with the assistance of a gene gun. The shot is facilitated by a force of compressed Helium. This method is normally used for plant species that are not hosts of *Agrobacterium* (Turnage et al., 2002).

2.3.2.2. Transformation of protoplasts

During electroporation, a suspension of protoplasts and recombinant nucleic acid is subjected to a high voltage pulse in an electroporator. This momentarily makes the cell permeable to the

nucleic acid, resulting in transformed protoplasts. Another protoplast transformation method is the expensive and lengthy microinjection of exogenous nucleic acids straight into an individual cell (Kost et al., 1995; Reich et al., 1986). A non-invasive, reproducible technique for introduction of nucleic acids into isolated cells is through the use of liposomes (Lurquin and Rollo, 1993).

2.3.3. Transient expression vectors based on plant viruses

Conversion of infectious clones into transient expression vectors in general includes the introduction of a multiple cloning site (MCS) for transgene addition into the viral genome. When this viral vector, containing a gene of interest, is introduced into the plant, it will replicate and produce considerable quantities of heterologous protein. This method has a number of advantages over stable transformation procedures: very high amounts of foreign protein expression are obtained in a very short time and the transgene is not passed on to the progeny, because no stable transformation of the plant occurs (Gleba et al., 2007). Since the 1980's foreign genes have been introduced into plants using plant viruses. Many improvements have been made to expression systems through recent advances in the fields of plant virology and molecular biology. Advantages include extremely fast, high-yield of protein expression and enhanced transgene containment. The first plant virus expression systems were based on the double stranded DNA virus *Cauliflower mosaic virus* (CaMV) of the *Caulimoviridae* family. Recent advances have expanded these to the ssRNA viruses which are the most abundant type of plant viruses in nature (Lico et al., 2008). Viral expression vectors can be generally divided into: (1) gene exchange vectors, (2) gene insertion vectors, (3) deconstructed vectors, and (4) peptide display vectors (Gleba et al., 2007; Nagyova & Subr, 2007; Lico et al., 2008). For the purpose of this review only gene exchange, gene insertion, and deconstructed vectors will be presented briefly.

2.3.3.1. Gene exchange vectors

Gene exchange or substitution vectors are normally based on a full-length virus where an endogenous viral gene has been substituted with a heterologous transgene (Nagyova & Subr, 2007). In early experiments, the coat protein was often exchanged, but these vectors had the tendency to lose the ability of cell-to-cell movement (Dawson et al., 1989; French et al., 1986). Viral encoded genes that direct the transmission of the virus by natural vectors are normally disposable for replication and systemic movement. These genes are therefore useful targets for exchange in construction of substitution vectors. These vectors disable the

horizontal transfer of the virus to other than experimental hosts, leading to increased control over recombinant viruses (Scholthof et al., 1996).

2.3.3.2. Gene insertion vectors

Gene insertion vectors are normally based on a full-length viral genome that has been engineered to include an extra ORF for expression of a transgene (Lico et al., 2008). Insertion vectors still preserve the capability to replicate and induce a systemic infection in a plant. Vectors developed from rod-shaped and spherical viruses have been reported, although the rod-shaped viruses have transgene size constraints. The most well-known gene insertion vectors are based on plant viruses that have a +ssRNA genome. These have been derived from *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) and transgene expression is driven from a native subgenomic promoter (Lico et al., 2008). Problems due to homologous recombination resulting in instability of the vectors have been reported (Dawson et al., 1989). Incorporation of a subgenomic promoter of a related virus to circumvent recombination has solved a number of these problems (Lico et al., 2008). In viruses where the genome is translated into a single polyprotein, such as the potyviruses, the open reading frame must be kept uninterrupted. In these cases the transgenic protein product can be expressed as a fusion with other viral products or a new viral protease recognition sequence can be engineered for correct cleavage after translation (Nagyova & Subr, 2007). In 2006, the genome of the Israeli GVA variant Is151 was engineered into a T7 promoter-driven gene insertion vector for transient protein expression in herbaceous hosts (Haviv et al., 2006). A cloning site and a duplicated subgenomic promoter of the movement protein MP were inserted to facilitate protein expression from the MP sgRNA. The vector was proficient to replicate and move throughout *N. benthamiana* plants, it was however not stable and loss of the foreign sequence (transgene) was reported. Improved stability was found after substitution of the duplicated promoter with a promoter derived from a distantly related isolate of GVA. This vector was subsequently shown to successfully express the CP of *Citrus tristeza virus* (CTV) and the beta-glucuronidase (GUS) gene in *N. benthamiana* plants (Haviv et al., 2006), but is still to be adapted for perennial plants such as grapevine (see chapter 5 of this thesis).

2.3.3.3. Deconstructed vectors

The development of new generation deconstructed vectors was driven by several limitations to the "full virus" strategy (Gleba et al., 2007). Vectors based on the full viral genome had restrictions to the insert size, were not very stable, did not infect all harvestable parts of the

plant and the infection progressed at different speeds (Gleba et al., 2007). These shortcomings and the knowledge that not all viral genes are required in an expression vector (Lico et al., 2008) prompted the development of improved vectors. Deconstructed vectors are normally made out of different genomic parts of a virus that can operate together during infection, similar to multipartite genome viruses. During the method of agroinfection, different regions of a viral genome can be independently cloned into binary vectors and co-infiltrated into plants. In this strategy, the replicon portion of the virus can be reduced down to a minimal, to facilitate accommodation of the transgene. Other components of the virus, necessary for functionality of the vector can be provided *in trans* during the co-infection process (Lico et al., 2008). The most widely used deconstructed vector system is based on TMV. This vector has been shown to be very versatile and robust, allowing high levels of transgenic protein expression i.e. antibodies, interferons, hormones, viral antigens and enzymes (Gleba et al., 2005). In this vector, the TMV genome was divided into two cDNA modules: the viral RdRP and the MP was included in a larger 5' module and the gene of interest (GOI) and the 3' UTR was included in a smaller 3' module. These two modules assemble together *in vivo* by a recombinase provided *in trans* by a third recombinant *Agrobacterium* suspension co-infiltrated with the 5' and 3' modules. The incorporation of introns in coding regions significantly improved recombinant protein expression levels (López-Moya & García, 2000; Marillonnet et al., 2004, 2005; Lico et al., 2008).

2.4. VIGS AND FUNCTIONAL GENOMICS

New generation sequencing technologies are producing an infinite quantity of sequence data for analysis (Morozova & Marra, 2008). The genomes of several plant species have been made available over the last years. The genome of the grapevine cv Pinot Noir was determined, recently (Jaillon et al., 2007; Velasco et al., 2007). *In silico* analysis of sequence data allows the comparison of new plant genomes to existing ones. To carry out *in vivo* functional analysis on the large quantity of sequence information, tools have to be developed. Virus-induced gene silencing is a rapidly emerging technique of preference for the investigation of plant gene function through the process of PTGS. For effective VIGS, the alteration of an infectious clone (DNA or cDNA) of a virus into a silencing vector is needed as well as a method for delivery (mechanical, biolistic or Agrobacterium-mediated) of the vector into the plant. An overview of VIGS and functional genomics will be presented in the following sections.

2.4.1. Virus-induced gene silencing to study gene function in plants

Virus-induced gene silencing is a very effective method for elucidation of gene function in plants (Waterhouse et al., 2001; Vance and Vaucheret 2001; Voinnet 2005) and is based on the ubiquitous biological process known as RNA-interference (RNAi) where the sequence-specific degradation of endogenous RNA is triggered by double-stranded RNA (dsRNA) (Fire et al., 1998). RNAi (in animals) is referred to as PTGS in plants and quelling in fungi (Romano & Macino, 1992). During RNA-induced gene silencing, dsRNA is cleaved by an RNAse-like enzyme, known as DICER (in animals) or DICER-like (DCL1-4, in plants), into 21-25mer small interfering RNAs (siRNAs) or microRNA (miRNA). These small RNAs are subsequently integrated into the RNA-induced silencing complex (RISC) which targets specific mRNA transcripts, complementary to the small RNAs, for degradation (Bartel, 2004). The RISC complex consists of several interacting proteins that bind target sequences and small RNAs, including the *Argonaute* (AGO) protein (Verchot-Lubicz & Carr, 2008; figure 3).
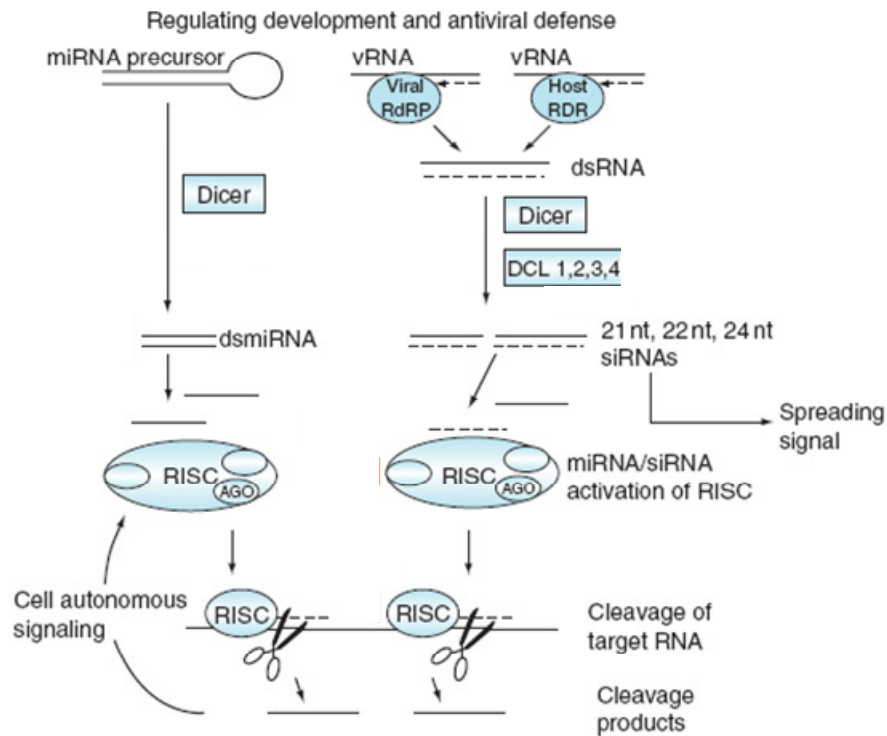
Figure 3. Simplified model of RNA silencing adapted from Verchot-Lubicz & Carr, 2008. Viral or host RdRPs and hairpin interactions generate dsRNA from viral RNA (vRNA) or mRNA templates. These dsRNA molecules are recognised by Dicer or Dicer-like enzymes and cleaved into small RNAs (siRNA & miRNA) which associate with the RISC complex and guide sequence specific degradation of target RNAs. Short RNAs resulting from target RNA cleavage can interact with the RISC complex and continue the RNA degradation cycle. The silencing signal can be spread systemically by cell-to-cell or long distance movement of some of the siRNAs.

When a virus infects a plant, the natural PTGS defence reaction is triggered leading to silencing of the virus in a sequence-specific manner. Double-stranded RNA or RNA with a high level of secondary structure formed during viral replication elicits this defence response (Hamilton & Baulcombe, 1999; Moissiard & Voinnet, 2006; Pantaleo et al., 2007). This process has been exploited during the recent years as a potent reverse genetics tool for the investigation of plant gene function (Burch-Smith et al., 2004; Talon & Gmitter Jr., 2008). In VIGS a viral genome is engineered to carry a sequence of a plant gene (figure 4), upon viral infection the PTGS pathway is triggered leading to the homology-dependent RNA degradation of viral RNA as well as endogenous mRNA homologous to the plant gene (Lu et al., 2003a, b; Waterhouse and Helliwell, 2002). Other factors, in addition to sequence homology, are necessary for launch of RNAi in VIGS. The length of the DNA (or RNA) fragment seems to be important for efficient silencing and it was reported that a minimum length of 23 nucleotides with 100% homology is adequate for induction of silencing (Thomas et al., 2001) however other studies state that longer fragments are needed (Ekengren et al., 2003). The orientation of the insert was also reported to influence efficacy of VIGS (Lacomme et al., 2003; Hein et al., 2005). A study performed by Igarashi et al., (2009) indicated that the length and/or positions of inserted phytoene desaturase (PDS) sequences affected the efficiencies of VIGS in tobacco mediated by *Apple latent spherical virus* (ALSV), genus *Cheravirus*, vectors. VIGS has numerous advantages, which makes it the technique of preference for swift deciphering of plant gene function. VIGS has the potential to silence multi-copy genes and conserved genes belonging to families, thus circumventing redundancy. During VIGS there is no need for time-consuming stable transformation of plants, which makes it applicable to species recalcitrant to transformation. The methods used are easy and simple and often entail the use of biolistics or agroinfiltration as delivery methods. Results are generated in a very short time period and a loss-of-function phenotype for a specific gene can be obtained within 2-3 weeks depending on the plant species studied. Furthermore, VIGS can be applied to high-throughput functional genomics in plants (Benedito et al. 2004; Burch-Smith et al., 2004; Lu et al., 2003a, b; Godge et al., 2008).

Van Kammen et al. 1997, first pioneered the use of the term VIGS to described the phenomenon of recovery from virus infection in plants. Nowadays, the term is synonymous with the technique used to knock-down the expression of endogenous genes by deployment of a recombinant viral vector (Ruiz et al., 1998; Baulcombe, 1999). This viral vector can be delivered to plants using methods described in section 2.3.2. Several viruses have been
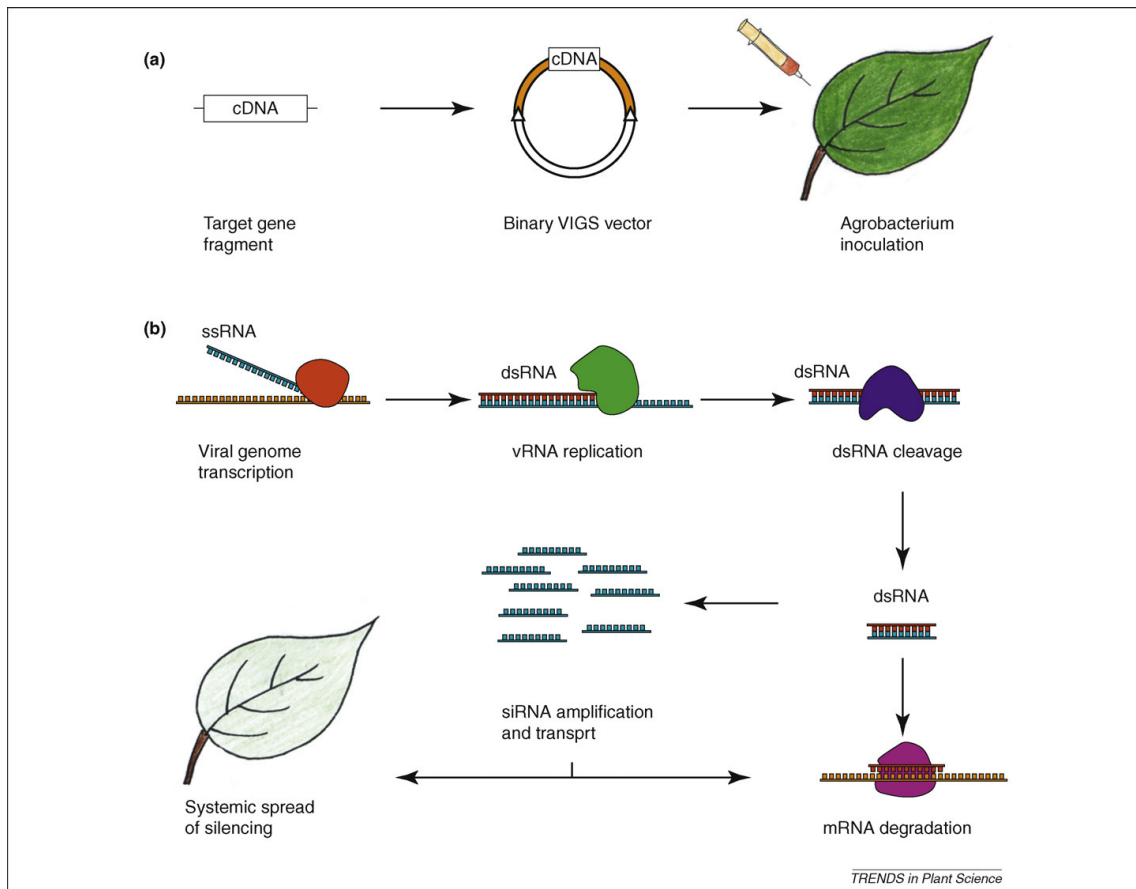
Figure 4. Graphic representation of the molecular mechanism of VIGS. (a) cDNA of the target gene is inserted into a viral cDNA clone contained in the T-DNA of a binary vector. This binary vector is transformed into *Agrobacterium* and the recombinant cell suspensions infiltrated into plants. (b) After infiltration, the T-DNA containing the viral cDNA clone is integrated into the genome and transcribed by the plant's RNA polymerase (shown in red). dsRNA is produced by the RdRP (shown in green) and acts as elicitor for PTGS. DICER-like enzymes (shown in blue) recognises this dsRNA and cleaves it into siRNAs. These siRNAs are subsequently incorporated into the RISC (shown in purple) which targets specific mRNA transcripts, complementary to the siRNAs, for degradation. Amplification of the single stranded siRNAs result in the mobile systemic spread of silencing signals that lead to target gene silencing at sites distant from the point of infection (figure taken from Becker & Lange, 2009).

converted into VIGS vectors for functional genomics studies in plants (Godge et al., 2008). Most of these have been developed from viruses infecting *Solanum* species such as tomato, potato, *N. benthamiana* and *Arabidopsis thaliana* (table 1). These plants, especially *N. benthamiana* (Goodin et al., 2008), are very susceptible to viral infection and normally exhibit effective silencing due to well established infection (Godge et al., 2008). The first RNA virus to be utilised as a silencing vector was TMV. A short sequence of the PDS gene was integrated into the TMV genome and the resulting *in vitro* transcripts, inoculated into *N. benthamiana*, effectively led to silencing (Kumagai et al., 1995). Normally, genes that result in an observable phenotype when silenced are used in VIGS to establish the protocol. These

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include PDS, the H subunit of magnesium chelatase (ChlH) and the RuBisCo small subunit (RBCS) (Senthil-Kumar et al., 2008). Vectors based on PVX (Chapman et al., 1992; Ruiz et al., 1998) and *Tomato golden mosaic virus* (TGMV, Peele et al., 2001) were soon to follow, but these vectors, together with TMV, had limitations in severity of symptoms induced, host range or meristem exclusion. These limitations were soon overcome by a vector based on *Tobacco rattle virus* (TRV, Liu et al. 2002b; Ratcliff et al. 2001) that had a broad host range, induced milder symptoms and spread more vigorously throughout the whole plant. In further studies, TRV vectors were produced that were more efficient in silencing of endogenous genes (Liu et al. 2002a, b). These vectors were modified to be under control of a duplicated CaMV 35S promoter, contained a ribozyme at the C-terminus, and included a number of amino acid changes in the viral genome sequence (Liu et al. 2002b). These vectors were also engineered to be GATEWAY compatible and allowed large-scale functional genomic screening (Liu et al. 2002a). Other viruses that have been developed into VIGS vectors for Solanum species are *Cabbage leaf curl virus* (CbLCV, Turnage et al., 2002) and *Turnip yellow mosaic virus* (TYMV) for VIGS in *A. thaliana* (Pflieger et al., 2008) and satellite TMV (STMV) in *N. tabacum* (Gossele et al., 2002). VIGS technologies are becoming available for more and more plant species (Reviewed in Lu et al., 2003; Roberson, 2004; Burch-Smith et al., 2004; Godge et al., 2007; Senthil-Kumar et al., 2008; Unver et al., 2009; Purkayastha et al., 2009). Recent examples of vectors reported for other hosts are *Barley stripe mosaic virus* (BSMV) for VIGS in barley (Holzberg et al., 2002; Lacomme et al., 2003), *Pea early browning virus* (PEBV), *Cucumber mosaic virus* and *Bean pod mottle virus* in legume species (Constantin et al., 2004; Zhang and Ghabrial, 2006; Nagamatsu et al., 2007), *African cassava mosaic virus* (ACMV) in cassava (Fofana et al., 2004), and *Brome mosaic virus* in monocotyledonous plants (Ding et al., 2006). Satellite-virus based vectors have also efficiently been used for VIGS in plants with help from other viruses. These include *Tomato yellow leaf curl china virus* (Tao & Zhou, 2004) and *Tobacco curly shoot virus* (Huang et al., 2009) for VIGS in *N. benthamiana*. Vectors based on tree-infecting viruses have also been described namely *Poplar mosaic virus* (PopMV) for VIGS in *N. benthamiana* (Naylor et al., 2005) and ALSV for tobacco, tomato, Arabidopsis, cucurbits and legumes (Yamagashi et al., 2009, Igarashi et al., 2009). Functional genomics of tree species is yet to be demonstrated, however, with established ALSV- and PopMV-VIGS vectors.

Table 1. Table showing characteristics of different VIGS vectors and the relevant plant species that serve as hosts (Adapted from Senthil-kumar et al., 2008 and Purkayastha et al., 2009).

Virus/Satellite	Genus	Nature of genome	Important natural hosts	Silenced hosts	References
African cassava mosaic virus (ACMV)	Begamovirus	ssDNA, bipartite	*Manihot esculenta*	*N. benthamiana, M. esculenta*	Fofana et al., 2004
Apple latent spherical virus (ALSV)	Cheravirus	Positive-strand RNA, bipartite	Apple	*N. tabacum, N. occidentalis, N. benthamiana, N. glutinosa, Solanum lycopersicon, A. thaliana* Cucurbit species, several legume species	Igarashi et al., 2009
Barley stripe mosaic virus (BSMV)	Hordeivirus	Positive-strand RNA, tripartite	Barley, wheat, oat, maize, spinach	*Hordeum vulgare*	Holzberg et al., 2002
Bean pod mottle virus (BPMV)	Cucumovirus	Positive-strand RNA, bipartite	*Phaseolus vulgaris, Glycine max*	*G. max*	Zhang & Ghabriel, 2006
Brome mosaic virus (BMV)	Bromovirus	Positive-strand RNA, tripartite	Barley	*Hordeum vulgare, Oryza sativa* and *Zea mays*	Ding et al., 2006
Cabbage leaf curl virus (CaLCuV)	Begomovirus	ssDNA, bipartite	Cabbage, broccoli, cauliflower	*A. thaliana*	Turnage et al., 2002
Cucumber mosaic virus (CMV)	Cucumovirus	Positive-strand RNA, tripartite	Cucurbits, *S. lycopersicon, Spinacia oleracea*	*G. max*	Nagamatsu et al., 2007
Cymbidium mosaic virus (CymMV)	Potexvirus	Positive-strand RNA, monopartite	Orchids	Orchids, tomato	Lu et al., 2007; Gilliberto et al., 2005
Pea early browning virus (PEBV)	Tobravirus	Positive-strand RNA, bipartite	*Pisum sativum, Phaseolus vulgaris*	*P. sativum*	Constantin et al., 2004
Pea seed-borne mosaic virus (PSbMV)	Potyvirus	Positive-strand RNA, monopartite	*Pisum species*	*Pea*	Voinnet, 2001
Pepper huasteco yellow vein virus (PHYVV)	Begomovirus	ssDNA, bipartite	*Capsicum*	*Capsicum, tobacco, tomato*	Carrillo-Tripp et al., 2006; Abraham-Juarez et al., 2008
Plum pox virus (PPV)	Potyvirus	Positive-strand RNA, monopartite	*Prunus*	*N. benthamiana*	Voinnet, 2001
Poplar mosaic virus (PopMV)	Carlavirus	Positive-strand RNA, monopartite	Poplar	*N. benthamiana*	Naylor et al., 2005
Potato virus X (PVX)	Potexvirus	Positive-strand RNA, monopartite	*Solanum tuberosum, Brassica campestris* ssp. *rapa*	*N. benthamiana, A. thaliana*	Ruiz et al., 1998
Potato virus A (PVA)	Potyvirus	Positive-strand RNA, monopartite	*Solanaceae*	*N. benthamiana*	Gammelgard et al., 2007
Satellite tobacco mosaic virus (STMV)	RNA satellite virus	RNA, satellite	*Nicotiana glauca*	*N. tabacum*	Gossele et al., 2002
Sweet potato feathery mottle (SPFMV)	Potyvirus	Positive-strand RNA, monopartite	*Solanaceae*	*Sweet potato*	Sonoda & Nishiguchi, 2000a, b
Tobacco curly shoot virus DNA1 component	DNA satellite-like virus	DNA, satellite	*N. tabacum*	*N. tabacum, Solanum lycopersicon, Petunia hybrida, N benthamiana*	Huang et al., 2009
Tobacco etch virus (TEV)	Potyvirus	Positive-strand RNA, monopartite	Wide host range (dicots)	*N. benthamiana*	Voinnet, 2001
Tobacco mosaic virus (TMV)	Tobamovirus	Positive-strand RNA, monopartite	*N. tabacum*	*N. benthamiana, N. tabacum*	Kumagai et al., 1995
Tobacco rattle virus (TRV)	Tobravirus	Positive-strand RNA, bipartite	Wide host range	*N. benthamiana, A. thaliana, S. lycopersicon*	Liu et al., 2002; Ratcliff et al., 2001
Tobacco yellow dwarf virus (TYDV)	Mastrevirus	ssDNA, monopartite	Dicots	*Petunia hybrida*	Voinnet, 2001
Tomato bushy stunt virus (TBSV)	Tombusvirus	Positve-strand RNA, monopartite	Wide host range	*N. benthamiana*	Pignatta et al., 2007
Tomato golden mosaic virus (TGMV)	Begomovirus	ssDNA, bipartite	*S. lycopersicon*	*N. benthamiana*	Peele et al., 2001
Tomato yellow leaf curl China virus– associated b DNA satellite	Begomovirus	ssDNA, satellite	*S. lycopersicon*	*N. benthamiana S. lycopersicon, N. glutinosa, N. tabacum*	Tao & Zhou, 2004
Turnip yellow mosaic virus (TYMV)	Tymovirus	Positive-strand RNA, monopartite	Brassicaceae	*A. thaliana*	Pflieger et al., 2008

An ideal VIGS vector should induce very mild symptoms in infected plants and infect a large number of cells and tissues leading to silencing of target genes in plant meristems. The unavailability of vectors with these characteristics has weighed down the application of VIGS in functional genomics. Development and improvement of TRV-based vectors have resolved some of the issues, and functional studies in plant tissues other than leaves, are now very possible (Burch-Smith et al., 2004; Ratcliff et al., 2001). VIGS has been successfully applied to study gene functions in several different plant organs which will not be presented here as two recent reviews have discussed these extensively (Senthil-Kumar et al., 2008; Purkayastha et al., 2009). Examples presented in these two reviews include genes involved in flower development, flower senescence, flower morphology, fruits, and roots. VIGS has also been successfully applied to study genes involved in cellular functions, plant development and metabolic pathways. The elucidation of mechanisms involved in plant responses, disease resistance and plant-microbe interactions have also been studied using VIGS.

Besides all the advantages, VIGS has several restrictions. VIGS is a transient system and the silencing phenotype or effect cannot be passed on to the plant progeny. VIGS efficiency depends on the plant species, the viral vector used and the expression levels of the gene to be silenced. For example, certain crop varieties such as cucumber, pea, bean, tomato, etc. (Kang et al., 2005), possess several resistance genes against certain viruses. A VIGS vector derived from a virus that a certain plant has resistance to will not be effective (Purkayastha et al., 2009). Furthermore, complete silencing of the target gene is often not possible, but in most cases the partial knock-down of a gene may result in a significant phenotype that can support functional characterisation. Empirical factors, such as plant age, light and temperature may affect the efficiency of silencing and therefore experiments need to be standardised for each specific viral vector and host plant combination. Due to the high extent of homology of some genes, in some cases off-target gene silencing can take place that can skew the interpretation of a resulting phenotype. This can be circumvented by designing improved VIGS constructs through the use of software that can predict possible off-targets of the genes to be silenced (Xu et al., 2006). When using recombinant viruses, as is the case in VIGS, special precautions and biosafety regulations have to be met (Senthil-Kumar et al., 2008).

There is no doubt that VIGS is a fast and efficient method for characterisation of gene functions and discovery of novel genes and has become a very popular tool for functional genomics in plants. New and improved VIGS tools are being developed and new generation sequencing technologies and EST databases will greatly assist the application of this

technique. The main challenge is still to establish VIGS for functional genomics in woody or perennial plants such as citrus, plums, apples, pears, berries and grapevine. Grapevine as perennial plant has a long reproductive cycle and therefore its use as a model organism is limited (Zottini et al., 2008). In plant virology, the most widely used model plant, due to it's susceptibility to numerous plant viruses, is *N. benthamiana*. This weed can be genetically transformed and regenerated with ease and is very open to to transient expression and VIGS methodology. A comprehensive review about the history and future of this molecularly well-characterised plant was written recently (Goodin et al., 2008). Other model plants include tomato, potato and *A. thaliana*. The use of alternative herbaceous hosts, such as these is very useful in the study of plant viruses. To date, the tools to study viruses that infect woody, perennial plants in their natural hosts are limited. Expression and VIGS vectors developed for these purposes need to be more stable, due to the long time required for systemic infection in such host plants. Earlier, a CTV-based vector was reported to be stable in citrus trees and resulted in GFP expression after more than 4 years (Folimonov et al., 2007). The vectors based on the tree-infecting viruses ALSV and PopMV were described earlier, but have only been implemented in alternative herbaceous and solanaceous plants. For grapevine, similar technologies to induce a systemic, persistent viral infection are limited. Non-viral based transgenic gene expression methods have been developed for grapevine over the recent years, which include cell suspensions, particle bombardment and agroinfiltration of leaves (Torregrosa et al., 2002; Vidal et al., 2003; Santos-Rosa et al., 2008; Zottini et al., 2008). The adaptation of these techniques to viral-based transient expression and VIGS systems for grapevine, and other woody plants will greatly benefit functional genomic studies for the analysis of gene functions in these valuable crops.

2.5. SUPPRESSORS OF RNA GENE SILENCING ENCODED BY VIRAL GENOMES

As a counter-defence to PTGS in plants, viruses encode suppressors of RNA gene silencing in their genomes. These suppressors function in such a way that it counteracts PTGS to allow efficient systemic infection of the virus in the plant (Qu & Morris, 2005). Many of these suppressors have recently been identified as pathogenicity factors to assist in cell-to-cell or long-distance movement of the virus throughout the plant (Siddiqui et al., 2008). To date, there is no obvious sequence or structural similarity between most of the characterised plant viral suppressor groups or families and there seems to be no correlation between viral protein function and it's evolution to function as silencing suppressor. Structural and non-structural proteins have been identified to possess suppressor activity and this shows how complex the

mechanisms involved are to unravel (Qu & Morris, 2005). Each suppressor differs in the mode of suppression and at the point where it influences, affects or disrupts the normal PTGS pathway (figure 5). Silencing suppressors can either, inhibit the production of siRNAs, prevent their incorporation into the RISC complex or put a stop to local or systemic spread of the silencing signal (Verchot-Lubicz & Carr, 2008). Several well-characterised viral-encoded silencing suppressors have been reported and are summarized in table 2. For the purpose of this study, no in depth discussion of viral encoded silencing suppressors will be presented. Most research on plant virus suppressors have been focussed on herbaceous and solanaceous plant-infecting viruses and therefore only a limited number of suppressors, encoded by viruses that infect woody plant species are known and highlights the need for functional characterisation of some viruses in their natural woody host plants/trees. This short list include the three suppressors encoded by CTV (P20, P23 & CP) for citrus trees (Lu et al., 2004); the CP of *Hibiscus chlorotic ringspot virus*, for *Malvaceae* (Verchot-Lubicz & Carr, 2008); the P50 of *Apple chlorotic leafspot virus* (Yeagashi et al., 2007) and the Vp20 of ALSV (Yeagashi et al., 2007) for apple; the HC-Pro of *Plum pox virus* (Sáenz et al., 2001); the ORF 2 gene product of Poplar mosaic virus for poplar (Naylor et al., 2005); and the P10 of GVA (Chiba et al., 2006) and the P24 of *Grapevine leafroll-associated virus-2* for grapevine (Chiba et al., 2006).

Table 2. Table showing respective viral silencing suppressors that have been identified (adapted from Li & Ding, 2006; Verchot-Lubicz & Carr, 2008)

Genome	Genus	Virus	Natural Host	Suppressor	Reference
DNA	Begomovirus	*Tomato leaf curl virus* (ToLCV)	Tomato, bean, petunia	C2	Chellappan et al., 2005; Cui et al., 2005; Trinks et al., 2005; van et al., 2002; Vanitharani et al., 2004; Voinnet et al., 1999; Wang et al., 2005
		Tomato yellow leaf curl virus (TYLCV)	Tomato, bean, petunia	C2	
		African cassava mosaic virus (ACMV)	*Manihot esculenta*	AC2, AC4	
		Mungbean yellow mosaic virus (MYMV)	*Leguminosae*	AC2	
		Tomato golden mosaic virus (TGMV)	Solanaceae	AL2	
	Curtovirus	*Beet curly top virus* (BCTV)	Dicotyledonous plants	L2	Wang et al., 2005
+ssRNA	Aureusvirus	*Pothos latent virus* (PoLV)	Pothos	P14	Merai et al., 2005
	Benyvirus	*Beet necrotic yellow vein virus* (BNYVV)	*Chenopodiaceae, Tetragonia expansa, Gomphrena globosa*	P14	
				P31	
	Carmovirus	*Turnip crinkle virus* (TCV)	Wide host range (dicots)	P38	Qi et al., 2004; Qu et al., 2003
		Hibiscus chlorotic ringspot virus (HCRSV)	Malvaceae	CP	
	Closterovirus	*Beet yellows virus* (BYV)	Chenopodiaceae	P21	Reed et al., 2003
		Beet yellow stunt virus (BYSV)	Dicotyledonous families Chenopodiaceae, Compositae, Geraniaceae, Portulacaceae and Solanaceae	P22	Chapman et al., 2004
		Citrus tristeza virus (CTV)	*Citrus*	P20	Lu et al., 2004
				P23	
				CP	
		Grapevine leafroll-associated virus-2 (GLRaV-2)	Grapevine	P24	Chiba et al., 2006
	Crinivirus	*Sweet potato chlorotic stunt virus* (SPCSV)	Sweet potato	P22	Kreuze et al., 2005
				RNAse 3	
		Tomato chlorosis virus (ToCV)		P22	Canizares et al., 2008
				CP	
				CPm	
	Comovirus	*Cowpea mosaic virus* (CPMV)	Limited host range (dicots)	Small CP	Liu et al., 2004
	Cucumovirus	*Cucumber mosaic virus* (CMV)	Wide host range	2b	Brigneti et al., 1998; Li et al., 1999; Qi et al., 2004
		Tomato aspermy virus (TAV)	Wide host range	2b	
	Furovirus	*Soil-borne wheat mosaic virus* (SBWMV)	Wheat, barley, rye	19K	Te et al., 2005
	Hordeivirus	*Barley stripe mosaic virus* (BSMV)	*Graminae*	γb	Donald & Jackson, 1996; Yelina et al., 2002
	Ipomovirus	*Cucumber vein yellowing virus* (CVYV)		P1	
	Pecluvirus	*Peanut clump virus* (PCV)	Izoaceae, Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Gramineae, Leguminosae, Scrophulariaceae and Solanaceae.	P15	Dunoyer et al., 2002; 2004
	Polerovirus	*Beet western yellows virus* (BWYV)	Dicotyledonous families	P0	Pfeffer et al., 2002
		Beet mild yellows virus (BMYV)		P0	
		Cucurbit aphid-borne yellows Virus (CABYV)		P0	
		Potato leafroll virus (PLRV)	Solanaceae	P0	
		Sugarcane yellow leaf virus (SCYLV)		P0	Mangwende et al., 2009
	Potexvirus	*Potato virus X* (PVX)	Solanum tuberosum, Brassica campestris ssp. rapa	TGBp1	Voinnet et al., 2000
	Potyvirus	*Plum pox virus* (PPV)	Prunus	HC-Pro	Sáenz et al., 2001
		Potato virus Y (PVY)	Solanaceae	HC-Pro	Brigneti et al., 1998; Anandalakshmi et al., 1998; Kasschau & Carrington, 1998; Kasschau et al., 2003
		Tobacco etch virus (TEV)		HC-Pro	
		Turnip mosaic virus (TuMV)	Dicots	HC-Pro	
		Zucchini yellow mosaic virus (ZYMV)	Aizoaceae, Amaranthaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Labiatae, Leguminosae, Ranunculaceae, Scrophulariaceae, Solanaceae and Umbelliferae	HC-Pro	
	Sobemovirus	*Rice yellow mottle virus* (RYMV)	Gramineae	P1	Voinnet et al., 1999
		Cocksfoot mottle virus (CfMV)	Gramineae	P1	Siddiqui et al., 2008
	Tobamovirus	*Tobacco mosaic virus* (TMV)	Wide host range	126K	Kubota et al., 2003
		Tomato mosaic virus (ToMV)	Solanaceae	126K	
	Tobravirus	*Tobacco rattle virus* (TRV)	Wide host range	16K	Liu et al., 2002
	Tombusvirus	*Artichoke mottled crinkle virus* (AMCV)		P19	
		Carnation Italian ringspot virus (CIRV)		P19	
		Cymbidium ringspot virus (CymRSV)	Wide host range	P19	Qi et al., 2004; Silhavy et al., 2004; Voinnet et al., 1999
		Tomato bushy stunt virus (TBSV)	Wide host range	P19	
	Trichovirus	*Apple chlorotic leafspot virus* (ACLSV)	Apple	P50	Yeagashi et al., 2007
	Tymovirus	*Turnip yellow mosaic virus* (TYMV)	Cruciferae	P69	Chen et al., 2004
	Vitivirus	*Grapevine virus A* (GVA)	Grapevine	P10	Chiba et al., 2006
-ssRNA	Carlavirus	Poplar mosaic virus (PopMV)	Poplar	ORF2 product	Naylor et al., 2005
	Cheravirus	*Apple latent spherical virus* (ALSV)	Apple	Vp20	Yeagashi et al., 2007
	Tenuivirus	*Rice hoja blanca virus* (RHBV)	Gramineae	NS3	Bucher et al., 2003
	Tospovirus	*Tomato spotted wilt virus* (TSWV)	Wide host range	NSS	Bucher et al., 2003; Takeda et al., 2002
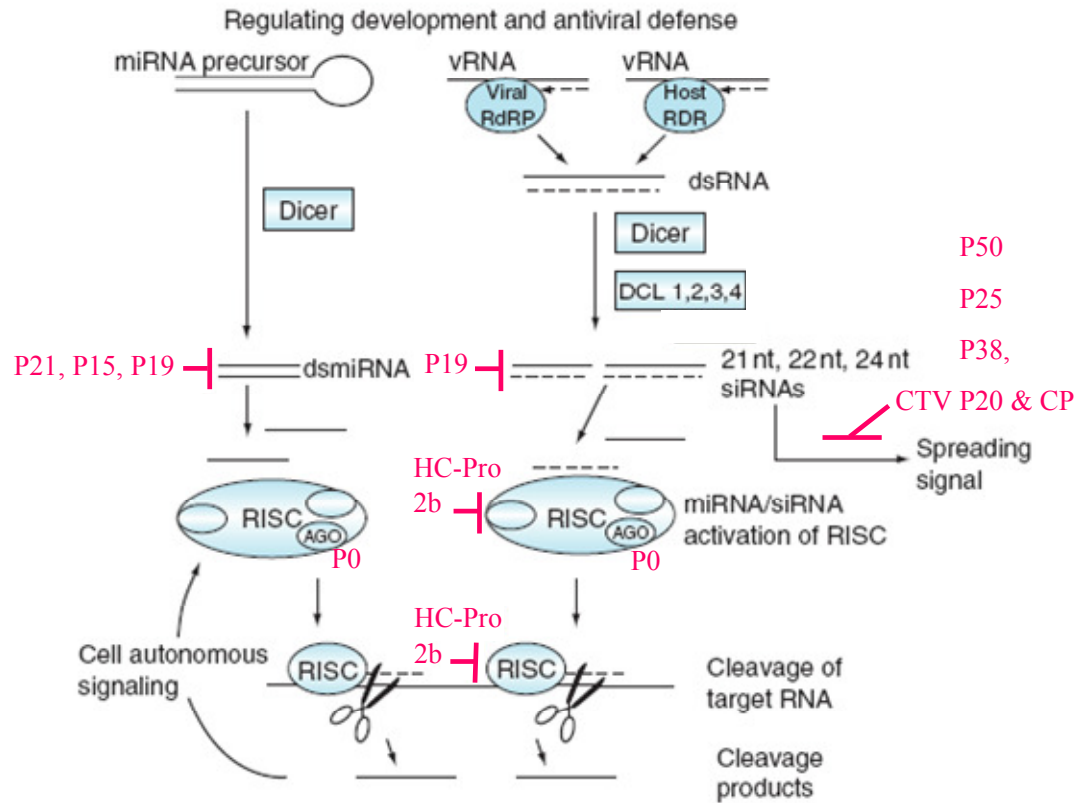
Figure 5. Simplified model of RNA silencing adapted from Verchot-Lubicz & Carr, 2008, showing examples of silencing suppressors (shown in red) and the point in the pathway where silencing is blocked. HC-Pro and 2b proteins block silencing by acting on the RISC complex (Verchot-Lubicz & Carr, 2008). The P19, P15 and P21 block silencing by inhibition of the miRNA-mediated cleavage of their target mRNAs (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003). P25, P38, P50 and the CTV P20 and CP primarily prevent the short- or long-distance spread of the silencing signal (Bayne et al. 2005; Deleris et al. 2006; Dunoyer et al. 2004; Yaegashi et al. 2007; Lu et al., 2004). P0 of the poleroviruses suppress PTGS by destabilizing the Argonaute protein (Baumberger et al., 2007; Bortolamiol et al., 2007).

2.6. CONCLUSION

Grapevine is a very important agricultural product internationally and needs to be protected. Through the years grapevine has been subjected to constant selection of traits that humanity have found appealing. Currently, new technologies are being developed at a rapid tempo that can hasten the breeding and selection of better-quality grapevines. For the grapevine plant, a vast majority of sequence information is being generated. The tools for *in silico* analysis of sequence data are in place. The progress made in the field of bioinformatics has not been marvelled by the development of *in vivo* tools for functional analysis of sequence information to attribute function to genes. Virus-induced gene silencing is an attractive method for fast, high-throughput functional genomic analysis of plant genomes. Most VIGS vectors have been developed from viruses that infect herbaceous and solanaceous plants that are easy to

manipulate and have been well characterised. For viruses infecting woody plants, a limited number have been utilised as VIGS vectors in model plants, but the majority are still to be implemented in their natural perennial plant hosts. For grapevine, the challenge is to select the ideal virus for development of virus-based expression and VIGS vectors for transient expression and functional genomics in this valuable crop plant. Furthermore the development of an efficient transformation system for delivery of these constructs to grapevine will greatly benefit the grapevine genomics community.

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Chapter 3: Complete nucleotide sequences and molecular characterisation of three South African *Grapevine virus A* variants

The work presented in this chapter contributed to the following publication in collaboration with D.E. Goszczynski (Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa):"Molecular divergence of GVA variants associated with Shiraz disease in South Africa", 2008, Virus Research 138, 105-110. Major findings of this paper will be discussed briefly at the end of this chapter.

3.1. ABSTRACT

Recent studies performed on short genomic regions of GVA, showed that three diverse molecular groups of the virus are present in South African vineyards. These variants were found to induce different symptoms in *N. benthamiana* (Goszczynki & Jooste 2003b). The pathogenicity determinants in the GVA genome associated with disease expression in grapevine have not been characterised. Variants of Group II were found to be linked with a very destructive disease of grapevine, known as SD. A variant of group II, GTR1-2, was found to be present in a consistently SD-negative plant (Goszczynski 2007). Three GVA sequence variants (GTR1-1, GTR1-2 and GTG11-1), representing each of the molecular groups, were completely sequenced and compared to 7 other GVA variants in a phylogenetic analysis. Results of the comparative analysis of these genomes are presented in this chapter. The mild variants out of Group III were found to be the most divergent of all groups and were distantly related to other GVA variants. Amino acid alignments of all ORFs revealed that ORF 2, of which the function is not known, was the most heterogenic. The CP (ORF 4) and the P10 (ORF 5) expression products were found to be fairly conserved among GVA variants. Furthermore, comparative analysis performed on group II GVA variants (Goszczynski et al., 2008) suggest that the components in the GVA genome that cause pathogenicity in *V. vinifera* are more complex (or different) to those that cause pathogenicity in *N. benthamiana*. A variant P163-M5, which induced extremely severe symptoms in *N. benthamiana* and is used as SD positive by the grapevine industry, was identified and contained a 119 nt insert within the native ORF2. The role of this insert, and other genomic pathogenicity factors of GVA, in expression of disease in both plants, remains to be determined.

3.2. INTRODUCTION

In South Africa GVA has been found to be connected with an extremely crippling disease of grafted and self rooted grapevine cultivars like Gamay, Malbec, Merlot, Shiraz and Voignier (Goszczynski & Jooste, 2003a). This disease, named Shiraz disease, was described in section 2.2.6. The GVA genome, genomic organisation and replication mechanism was described in section 2.2.3. In South Africa it was observed that GVA has a broad molecular heterogeneity. Three molecular groups (I, II and III) were recognised based upon single-strand conformational polymorphism (SSCP) analysis of different short genomic regions of GVA sequence variants (Goszczynski & Jooste, 2003b). Each group induced different symptoms in the herbaceous host *N. benthamiana,* ranging from mild vein clearing to extensive patchy necrosis (figure 1A-B). Mild variants (group III) shared only ~79 % nt sequence identity with other variants in the 3' terminal part of the viral genome (part of ORF 3, entire ORF 4, ORF5

and part of the 3' UTR) (Goszczynski & Jooste 2003b). Recently, it was shown that variants of molecular group II are strongly associated with SD, and variants of molecular group III are present in GVA-infected SD-susceptible grapevine that do not present symptoms of the disease (Goszczynski, 2007). Group II variants show a strong association with SD, but captivatingly a variant of this group, GTR1-2, was recovered in *N. benthamiana* from a Shiraz plant that persistently did not present symptoms (Goszczynski, 2007). Furthermore, another variant of group II, P163-M5, used by the South African grapevine industry as dependable positive control of SD in woody indexing, induces exceptionally severe symptoms in *N. benthamiana* (figure 1C) when compared to other variants of this group (Goszczynski & Jooste, 2003a). These symptoms include severely deformed leaves, disruption of leaf lamina, stunting and severe chlorosis. The P10 has been linked to pathogenicity in *N. benthamiana* (Zhou et al., 2006; Chiba et al., 2006), but the genomic pathogenicity determinants of GVA in the grapevine host, are still unclear. It was shown previously for *Potato virus Y* (PVY), genus *Potyvirus*, family *Potyviridae*, that symptom determinants may be different even between strains of the same virus species in a particular host (Bukovinski et al., 2007). This suggests that the pathogenecity determinants in the GVA genome could differ in *N. benthamiana* and *Vitis vinifera*.

Over the years, specific motives within a viral genome, and specifically, single nt or aa changes, have been linked to pathogenicity, symptom expression and systemic infection of viruses in their hosts. Recently, it was shown that a single nucleotide change in the "cachexia expression motif" of *Hop stunt viroid* (HSVd), genus *Hostuviroid*, family *Pospiviroidae*, modulates cachexia symptoms in citrus trees (Serra et al., 2008). In another study, it was shown that a single nucleotide change (A-1,627 to G-1,627) resulting in a single amino acid change (Asp-205 to Gly-205) in the HC-Pro cistron of the PVY correlates with a loss of vein necrosis phenotype in tobacco (Hu et al., 2009). For GVA, one such study was undertaken by Haviv et al., 2006. They showed that ORF 5 was not needed for replication in protoplasts, but was necessary for efficient infection in plants. They also found that symptomatology observed in *N. benthamiana* is linked to ORF 5 and/or the 3' UTR and not to the viral load. Experiments performed with GVA-ORF 5 hybrids, suggested that the first 5-20 % of the ORF 5 sequence determines pathogenicity in *N. benthamiana* and that the 8th amino acid residue affected symptoms. Their sequence alignments of ORF 5 protein products revealed that mild variants contained a threonine (Thr) at position 8 and severe variants an alanine (Ala). This

was tested *in vivo* and it was found that this single amino acid affects pathogenicity (Haviv et al., 2006).

A complete nucleotide sequence comparison of different GVA sequence variants could shed light on the possible association of specific genomic regions with disease. Comparison of the GTR1-2 genome with other variants of group II, could lead to the identification of possible significant amino acid changes that are involved in expression of SD symptoms. This could pave the way for the unravelling of the aetiology of SD and could ultimately lead to elucidation of this crippling disease in South African vineyards.

The complete nucleotide sequencing and comparative analysis of three GVA variants, GTR1-1 (group III), GTR1-2 (group II) and GTG11-1 (group I), representing each of the molecular groups are discussed in this chapter. Major findings of the Goszczynski et al., (2008) paper dealing with the divergence of GVA variants associated with Shiraz disease in South Africa are also discussed briefly.

A) Group III variants
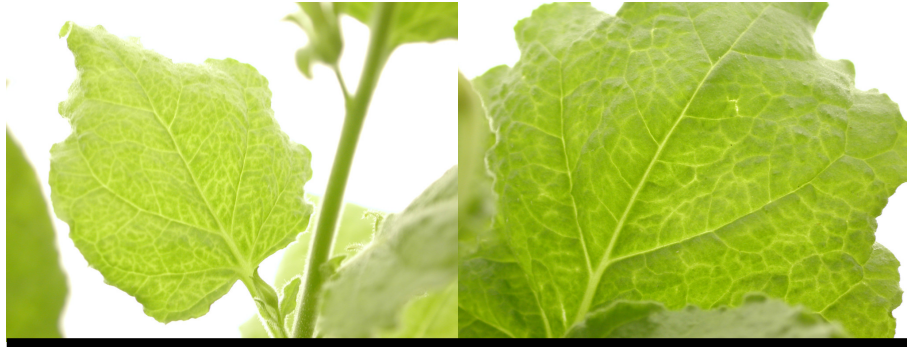
B) Group I & II variants

C) Variant P163-M5 (group II)

Figure 1. Symptoms induced on *N. benthamiana* by different GVA sequence variants (photographs courtesy of DE Goszczynski). A) Mild vein clearing induced by mild variants of molecular group III (GTR1-1 and P163-1). B) Symptoms induced by variants of molecular groups I and II. These include vein clearing, chlorosis, extensive patchy necrosis and downward rolling of the leaves. C) Extremely severe symptoms that include severe chlorosis, severe deformation of leaves, disruption of leaf lamina and stunting of plant induced by variant P163-M5 (Group II).

3.3. MATERIALS AND METHODS

During the GVA lifecycle, the genome of the virus is represented in replicative form as dsRNA. Therefore, dsRNA of GTR1-1, GTR1-2 and GTG11-1 was obtained from D.E. Goszczynski (Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa). Full-length sequences of variants GTR1-1, GTR1-2 and GTG11-1 were generated from overlapping reverse transcription Polymerase Chain Reaction (RT-PCR) fragments using the primers shown in table 1. This was performed as described by Goszczynski et al., (2008). Sequence chromatograms were edited using Chromas version 1.45 (Technelysium Pty. Ltd.). Sequence comparisons were performed using the BLAST algorithm (Altschul et al., 1990) against the NCBI GenBank database (www.ncbi.nlm.nih.gov). Overlapping clones were analysed and full-length sequence generation was performed with BioEdit Version 5.0.9 (Hall, 1999). Three clones per fragment were used for generation of full-length genome sequences that were submitted to Genbank/EMBL database. Nucleotide and amino acid identity comparisons were performed with a demo version of Vector NTI 10 (Invitrogen). Full-length GVA nucleotide sequences and amino acid sequences of respective GVA ORFs were aligned using the embedded Clustal W alignment function within the BioEdit software. Putative and conserved protein domain searches were performed using the pfam database [http://pfam.sanger.ac.uk/; (Fin et al., 2008)].

Phylogenetic analysis of aligned GVA nucleotide and amino acid sequences were performed using PAUP (4.0b10) (Swofford, 2003). GTR1-1, GTR1-2, GTG11-1 and 7 other GVA variants found in Genbank, (accession numbers shown in table 2) were used in the analysis. *Grapevine virus B* (GVB, accession # X75448.1) was used as outgroup in the phylogenetic analysis. Trees were generated from full-length nucleotide alignments and ORF 1-5 amino acid alignments. A heuristic search (1000 replicates) using tree bisection reconnection (TBR) branch swapping was performed. All characters in the analysis were weighed equally to establish the shortest trees from the data matrix. To establish clade support, a bootstrap analysis (1000 replicates) was performed. Bootstrap values below 50 % were considered weakly supported, branches with values between 50 % and 75 % as moderately supported and those with bootstrap values ≥ 75 % as well supported.

Table 1. Primers used in this study for RT-PCR amplification and sequencing of GVA sequence variants GTR1-1, GTR1-2 and GTG11-1.

GVA variant	Primer pairs	Primer sequence (5'-3')	PCR fragment size (bp)
GTR1-1	PdT[a] GTR1-1-305R	TACGATGGCTGCAG(T)17 GTGAGCATGTCCTTGGAAGGAGTG	±340
	GTR1-1-EF GTR1-1-1560R	CGAATATTTAATTTGATTCCCATCG CAGGATTAGTGCGTGTGACC	1560
	P527F[b] P1476R	GCT CGC TGT GCG GTG TAA TA TGG TTC TGG GTT TCG GTC ATT	949
	P1478F[b] P2431R	ACG GTG CGT TGG TGT TGA AT TGGCTTTCAGCGAGCACATA	953
	P2333F[b] P3187R	AGG AGG GCT CAT CTG AAT ACG CAT TGT CTC GTT TGC TGT GGT	854
	P3161F[b] P4013R	GCT CTT ACC ACA GCA AAC GA GCC GTA TCG TCT GAT GTA TGC	852
	P4627F[b] P5475R	CTC AAA CTG ATG ATG GGC AGC GGACGAGGGCTGAAACTAAGA	849
	P5096F[b] P6012R	AGC ATA AAC ACA AAC TCC CG CTA CCA CTT TGC CAC TTA GG	916
	P6014F[b] P6804R	GGA GGT CAA GGA CAC AAG A CAA CTC AGC CAT AAC GAC C	790
	PdT[a] GTR1-1-6860F	TACGATGGCTGCAG(T)17 GTCGGGCTTCAAGGAGCCACAGG	±540
GTR1-2	GVA-EF[c] GVA-1188R	GAATATTTAACTTGATTCCCATCG CCATCTTGTCATACAGAGCC	1188
	GTR1-2-989F GTR1-2-2059R	GGTAGCAAGCCATTGTATCTCCG CACCCTTGGAGTATCTTTGGA	1070
	GTR1-2-1850F GTR1-2-3066-R	GGTACGCAAACTCTGCAAGGA ATACCCAGGTGGTAGAAGACC	1216
	GTR1-2-2772-F GTR1-2-4138-R	ACAATGGGAATACGGGCGTG CTCCTTTAGCTGGTATGTGTCC	1366
	GVA-4110-F[d] GVA-5061-R	TTCAAGGACACATACCAGC GCTGTGCATCAACATCAATA	951
	GVA-4110-F GVA-6141-R	TTCAAGGACACATACCAGC GTGTATCCGTTCAGCAGATC	2031
	GTR1-2-4970F GVA-6141R	CCTTATGCCTAGTGAATTATGCG GTGTATCCGTTCAGCAGATC	1178
	GTR1-2-6062-F GVA-ER	GACTTATCCAAGCCGCTCAGC GTCTTCGTGTGACAACCTAG	1289
	GVA-6979-FN[e] GVA-ER	GGAGTATTCAACGCTCAGTC GTCTTCGTGTGACAACCTAG	392
GTG11-1	GVA-1188F GVA-2193R	GGCTCTGTATGACAAGATGG GTAGCTCCTTCTTCTCGATC	1005
	GTG11-1-2115F GTG11-1-4134R	CGTCACCGTCAATCTATTTGG CCTTCAGCTGGTATGTGTCC	2019
	GVA-4110F GVA-5467R	TTCAAGGACACATACCAGC GACTTCTTGGACTGTTGCAC	1357
	GTG11-1-5407F GVA-6979R	GATCGGAGAGGCTTTACAGC GACTGAGCCTTGAATACTCC	1572
	GTG11-1-2640F GTG11-1-3517R	CACGTGGAACCTTTCGAGG CACCACGAGAGCGGGTGAG	878
	GVA-5880F GVA-6979R	ATCTGGACATCATGGATGAG GACTGAGCCTTGAATACTCC	1099

[a]Primer designed by Meng et al. (2003).
[b]Primers designed by Goszczynski et al. (2008).
[c,d,e]Primer pairs used in RT-PCR amplifications of GTG11-1 as well.

3.4. RESULTS AND DISCUSSION

3.4.1. Full-length GVA genome organisation, comparison and phylogenetic analysis

Full-length genome sequences of three South African GVA variants were submitted to Genbank/EMBL database with the following accession numbers: DQ787959 (GTR1-1), DQ855086 (GTR1-2) and DQ855084 (GTG11-1). These sequences were included in a molecular comparison study of different GVA sequence variants associated with Shiraz disease (Goszczynski et al., 2008). The genome structure, based on complete sequence, of GTR1-1, GTG11-1, GTR1-2 and 7 other GVA variants were compared (table 2). It was found that the total genome size of GVA variants varied from 7348-7360 nucleotides (nt). The genome size of variant P163-M5 was found to be 7471 nt due to an imperfect duplication of 119 nt between ORF 1 and 2 (Goszczynski et al., 2008). This duplication seems to be tolerated and as shown in the table, the sizes of the ORF 1 and ORF 2 are not affected. This variant induces severe symptoms in *N. benthamiana* (Goszczynski et al., 2008; figure 1C), whether this is due to the presence of the duplication will have to be investigated. The genomic position and length of ORF 1 is fairly consistent among variants and amino acid lengths vary from 1706 (GVA-GR5) to 1710 aa (P163-1 and GTR1-1). The length of the ORF 2 encoded protein product was found to vary from 175-177 aa. The MP, CP and P10 protein lengths (278 aa, 198 aa and 90 aa, respectively) and genomic positions were consistent, with exception of the GTG11-1 MP, that contained an extra three amino acids at the N-terminus of the protein (281 aa). It seems that a mutation introduced a putative new start codon 9 nt upstream of the original MP start codon.

Nucleotide and amino acid sequence identity of GTR1-1, GTR1-2 and GTG11-1 were compared to the Italian type strain, GVAIs151 (table 3), and the Israeli type strain, GVA-GR5 (table 4), respectively. Analysis showed, that of all ORFs, ORF 2 was the most divergent. This was highlighted by the extreme divergence of the GTR1-1 ORF 2 compared to GVAIs151 (53,7 %) and GVA-GR5 (54,2 %). Full-length genome nucleotide sequence comparison also revealed that the GTR1-1 (variant of group III) is the most divergent when compared to the two reference strains. GTR1-1 induces very mild symptoms in *N. benthamiana* and reasons for this may be revealed by comparing different ORFs to those of severe GVA variants and by performing *in vivo* site-directed mutagenesis experiments on infectious cDNA clones.

Table 2. Genome sizes, genomic position and amino acid (aa) length of ORFs of 10 GVA sequence variants out of the three molecular groups.

Molecular group	GVA variant (Genbank accession)	Genome size (nt)	ORF 1	ORF 2	ORF 3 (MP)	ORF 4 (CP)	ORF 5 (P10)
I	GVAIs151 (X75433)	7351	87-5210 1707 aa	5179-5712 177 aa	5654-6490 278 aa	6414-7010 198 aa	7011-7283 90 aa
I	GTG11-1 (DQ855084)	7351	87-5210 1707 aa	5179-5712 177 aa	5645-6490 281 aa	6414-7010 198 aa	7011-7283 90 aa
I	GVA-GR5 (AY244516)	7348	87-5207 1706 aa	5176-5709 177 aa	5651-6487 278 aa	6411-7007 198 aa	7008-7280 90 aa
II	GTR1-2 (DQ855086)	7351	87-5210 1707 aa	5182-5712 176 aa	5654-6490 278 aa	6414-7010 198 aa	7011-7283 90 aa
II	BMO32-1 (DQ855087)	7352	88-5211 1707 aa	5183-5713 176 aa	5655-6491 278 aa	6415-7011 198 aa	7012-7284 90 aa
II	GTR1SD-1 (DQ855081)	7352	88-5211 1707 aa	5183-5713 176 aa	5655-6491 278 aa	6415-7011 198 aa	7012-7284 90 aa
II	KWVMo4-1 (DQ855083)	7351	87-5210 1707 aa	5182-5712 176 aa	5654-6490 278 aa	6414-7010 198 aa	7011-7283 90 aa
II	P163-M5 (DQ855082)	7471	88-5211 1707 aa	5302-5832 176 aa	5774-6607 278 aa	6534-7130 198 aa	7131-7403 90 aa
III	P163-1 (DQ855088)	7360	88-5220 1710 aa	5189-5716 175 aa	5664-6500 278 aa	6424-7020 198 aa	7021-7293 90 aa
III	GTR1-1 (DQ787959)	7360	88-5220 1710 aa	5189-5716 175 aa	5664-6500 278 aa	6424-7020 198 aa	7021-7293 90 aa

Table 3. Complete genome nucleotide (nt %) and amino acid sequence identity (aa %) of variants GTR1-1, GTR1-2 and GTG11-1 compared to the Italian type strain GVAIs151 (X75433).

Variant	Genome (nt %)	ORF 1 (aa %)	ORF 2 (aa %)	MP (aa %)	CP (aa %)	P10 (aa %)
GTG11-1	84,0	92,1	74,6	90,4	97,0	96,7
GTR1-1	70,8	74,8	**53,7**	75,2	84,3	90,0
GTR1-2	76,5	84,2	69,5	80,6	93,4	92,3

Table 4. Complete genome nucleotide (nt %) and amino acid sequence identity (aa %) of variants GTR1-1, GTR1-2 and GTG11-1 compared to the Israelian type strain GVA-GR5 (AY244516). ORF 2 of variant P163-M5 is included in analysis (see chapter 6).

Variant	Genome (nt %)	ORF 1 (aa %)	ORF 2 (aa %)	MP (aa %)	CP (aa %)	P10 (aa %)
GTG11-1	77,6	84,5	73,4	82,6	96,0	93,3
GTR1-1	70,2	76,6	**54,2**	76,3	82,3	86,7
GTR1-2	76,0	84,2	71,2	84,2	92,4	91,2
P163-M5			74,0			

Phylogenetic analysis performed on full-length GVA genome nucleotide sequences (figure 2) confirmed that three distinct molecular groups of GVA are present in South Africa. These findings were previously based on short genomic regions in the GVA genome (Goszczynski & Jooste, 2003b). Variant GTR1-1, which induces mild symptoms in *N. benthamiana*, is included in the most divergent group III, together with variant P163-1. This correlates with symptom expression in *N. benthamiana* and both GTR1-1 and P163-1 induce mild vein clearing in the herbaceous host. None of these variants are associated with SD of grapevine (Goszczynski, 2007). Variant GTG11-1 is closely related to the Italian type strain GVA Is151 and the Israeli type strain GVA-GR5 which can be found in group I. These variants induce severe symptoms in *N. benthamiana* and are not associated with SD. Variant GTR1-2 clusters together with GVA variants of group II, which are strongly associated with SD, but as mentioned, GTR1-2 itself is not associated with SD (Goszczynski et al., 2008).

3.4.2. Amino acid alignments and phylogenetic analysis of GVA ORF protein products

Analysis of the amino acid alignments of the ORF 1 encoded protein revealed two significant aa changes in the protein of the mild variants GTR1-1 and P163-1. This included a single aa deletion at position 118 (ΔSer^{118} when compared to group II variants or ΔAsn^{118} when compared to group I variants) in the methyl-transferase domain and a four aa insertion Glu^{563}-Ser-Glu-Gly^{566} in the pfam-B-44564 domain (figure 3). Furthermore, a single aa deletion was found at position 152 in the methyl-transferase domain of the GVA-GR5 ORF 1 encoded protein. The Clustal W alignment of ORF 2 amino acid sequences (figure 4) showed that this protein, of which the function is still unknown, is highly variable. Alignment of the ORF 3 encoded aa sequence (figure 5) revealed a mutation in the GTG11-1 genome, introducing a new putative start codon (ATG), 9 nt upstream of the native start codon of ORF 3. This could result in a MP that is 3 aa longer in length (281 aa) than those of all other GVA variants. These in-frame mutations seem to be tolerated by the specific variants, but this assumption is only based on sequence information. No insertions or deletions were detected in any of the other ORFs [ORF2 (figure 4), CP (figure 6) or P10 (figure 7)] of GVA variants used in the alignments. Alignments also revealed that the CP (ORF 4) and the P10 (ORF 5) was fairly conserved among variants used in the analysis. This was surprising for P10, as this protein has been linked to pathogenicity in *N. benthamiana* (Haviv et al., 2006). For the array of symptoms observed, one would expect a protein that is very diverse among variants. Instead, the alignments revealed only a few amino acid differences. This could imply that single amino acid differences are involved in symptom expression in *N. benthamiana* as was shown by Haviv et al., (2006b), or that other factors in the genome are involved. Haviv et al. (2006b) showed that the 8th amino acid residue in the P10 protein affects symptom expression in *N. benthamiana* plants. This residue was found to be an Ala in severe variants and a Thr in mild variants. From the results of our findings, P163-M5 induces extremely severe symptoms in *N. benthamiana*, but contradictory to the Haviv et al. results, this variant contains a Thr at position 8 in ORF 5 protein. This signifies that other factors in the genome may be involved in symptom expression as was first suggested by M. Mawassi (Mawassi, 2007).

Figure 2. Phylogenetic analysis showing the shortest tree drawn from the heuristic search performed on the full-length GVA genome (nt) data matrix. Values above branches indicate branch lengths and values (*italics)* beneath branches show bootstrap values. GVA molecular groups (I, II & III) are shown on the right hand side of the tree.

```
                370       380       390       400       410       420       430       440       450       460       470       480
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GVA-GR5   TALYDKMGNPSLRSILSESFYDVAGNLFTRLFNRPEYDARCLEKFTRACETTEIHIGRRFMEGVLRGSSYQVQKVVDWVEDEEANALSEVNLVELTRNEREPQPYGSEQLQGSGARVRFP
GVAIs151  ..........N........I..S...............S......VE..Y...IR..AFK..N.M.....DS.......FLDISW.D.VSE...I.CIH.E.S.I.V.
GTG11-1   ..........N........I.ES...............S.D...L.VE..Y...IR..APFK..NII.....DS.......FL.ISW.D.IRT..STVGSHIAESKI.V.
P163-M5   .................F.I..............R....D......A.....TR..APLK..D.I......S..V.....I.VSKDD..AS....SAMDARNK.LLV.
KWVMo4-1  .................F.I..............R....D......A.....TR..APLK..G.I......S.......FI.VSKDD..AN....SAMEAKNK.LLV.
BMO32-1   .................F.I..............D....A......TR..APFK..D.AE.IA..G..I....F..VSKDD...S...TSAMNPESK.LQV.
GTR1-2    .................F.I..............R....D....N......IR..APLK..N.AE.IG..S..SF...FL.VNKDD.V.R..DTSAMENGNRKL.V.
GTR1SD-1  .................F.I..........K........D..F..A......TR..APFK..D.A..I...G.GI....F..VSKDD....S...TSAMNSKSK.LQV.
GTR1-1    .G...R.....IK.L...A.H.I..........K..........FY.P....R.Y....IM..R........D....Q..CK.D...E..SASVSN.ENR.AYV.
P163-1    .G...R.....IK.L...A.H.I..........K..........FY.P.K..R.Y....IM..R........D....Q..CK.D.N.E...ALV.N.DNR.AYV.

                490       500       510       520       530       540       550       560       570       580       590       600
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GVA-GR5   LSRLMRAHEMVHEAQFEVNFNHIRIVCSSKALVHLRQYLIRLGRFKFMEGRAIEDIQDIQEGLELGLITEEEAESRLLPPDNI----APSEEAQEEVQQSGPIEEVSEKFMGARKLVKEN
GVAIs151  ...IL....LIAGV.TD.EI.FP.Y....R..I.F....K....S....S...K.E....A...E.V......L....TTKP----KIT.IHMDDDTPGTSG.SDV...KSV.S.CR.E
GTG11-1   IT.IL....LITG..ED.EI.LP.F....AR..M.F.R....K....R....S...K.E....A...E.V......L....SATP----NVTVALI..TAPGPSE.MGK.R.L...N.CR.E
P163-M5   .P.IL..QSLIMDM.AA.EI.V......P...I.F.C...K....R...SK.LC..E.LA...E..L..D.V.A...SSESE----GEV..CVVKPAPVPID.KEEI.YS.V...C..S
KWVMo4-1  ...IL...SLIMDM.AP.EI.V......P...A.F.C........R...S..LC..E.LA...E..L..V.A....SSESE----SEV..SVV.PAPVPID.KEEI.YS.V...CR..
BMO32-1   .T.IL...QSLIM.M.VP.EI.LV.....P...I..C........R...S...C..E.LS...E..L..D.V.A....SSESE----GEC..SIV.IAPERVD.KGEV.YS.V...CQ.S
GTR1-2    ...IL....LIM...AH.KI.TVHV..PR..INF.C...K....R...S.LC..E.LS...E...V.A...SS.DE----EVA..SEIKTVPREEESGKEMEYT.V...C..
GTR1SD-1  .T.IL...QNLIM.M.AP.EI.QV.V..FR...Y..C........R...S...C..ENL.S...E..L....V.A....SSESE----GEC..SIVKMAPERVE.REEV.YS.V...CQ.S
GTR1-1    IT.I..S.ALIL.SAKS.EI.RVVV....ARS.LN..LF.......RY..S.GPT.V..M..A..D..LS...V.A..FTSQDTESEGSTN..TGHI.NE.RND.VGTPR.SKV.E.I..G
P163-1    IT.I..S.ALIL.SARS.EI.RVVV....ARS.LN..MF.......RY..S.GLT.V..M..A..D..LS...V.A..FTSQDTESEGST...TGHI.DEPRND.AGTPR.SKV.N.I..G
                                                                                          ___
                                                                                          ☆
                610       620       630       640       650       660       670       680       690       700       710       720
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GVA-GR5   IYSEKLHNREVAFYSRYSKQYSYKGGSHHSLGWDKALDELIQELGLDESYDHCLIQRYSEGGSINFHADDEPCYLPGGTVVTVNLNGEAIFELKENTSGKTESKRLKDGDVFTMGAGMQQ
GVAIs151  ......KG........H..E.K.N....R.....E..N..T....D.........TA...G..........S....H.D.T..V...Q...I.K.E.H....YV..P....
GTG11-1   ......KG........NH.RE.K.N..A.R.....R..T..R....D.........TA...G..........S....F.D.T..V...K.E.I.K.E.H....YV..PRK..
P163-M5   .TDT.KGG.A.....G...E...T....R.............VA..........K...G....R....S..L.H.....V...AT..IQQIKMR....Y...P....
KWVMo4-1  .TDT.KG..A.........E...T....R.............VA..........K...G....S....S..L.H.....V...AT..I.QIK.....Y...P....
BMO32-1   .TDT.KG........H..E...T....R...........V..........K...G....R....S..L.H.....V...T..IKKIK.G....Y...P....
GTR1-2    .T...KG........KH..E...N....R...........LA..........TK...G....R....S..L.H.D...V..V...IQEIK.A....Y...P....
GTR1SD-1  .TDT.KG........H..E...TS...R...........V..........K...G....R....S..L.H.....V...AT..VQKIK.G....Y...P....
GTR1-1    .FT.Q.KG..........D.K.A..H.R.....D...S.VA..........VKD.A.G..........S........L..V...S..AIKQIHM.....Y...P....
P163-1    .FT.Q.KG..........D.K.A..H.R...D....A..A..........MKD.A.G..........S........L..V...S..VIKKIHM.....Y...P....
```
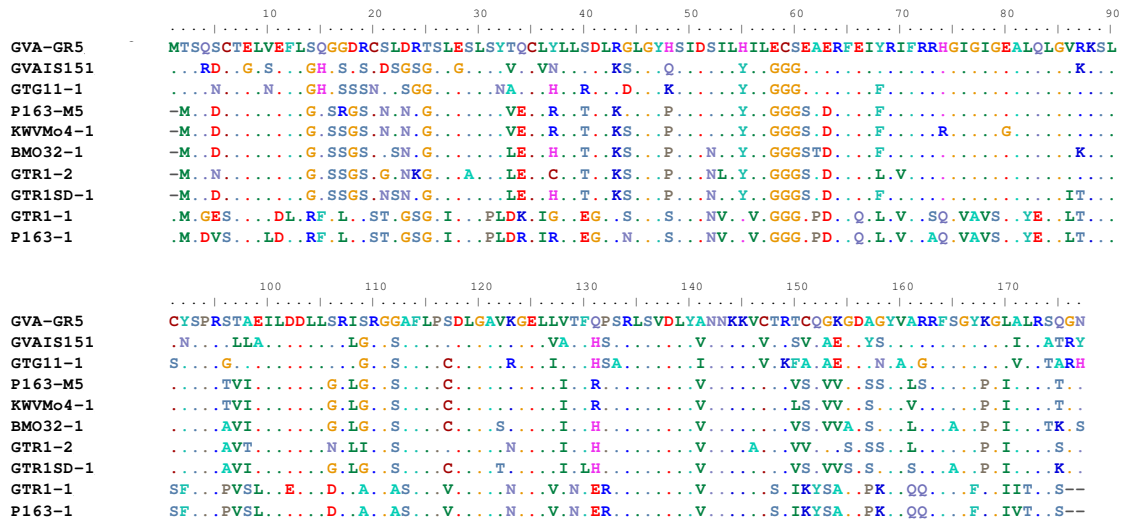
Figure 3. Amino acid sequence alignment of the ORF 1 encoded protein of GVA variants showing, part of the pfam-B-44564 domain. The four aa insertion at position 563 in mild variants of group III (GTR1-1 and P163-1) is indicated with an asterisk.


```
                10        20        30        40        50        60        70        80        90
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GVA-GR5   MTSQSCTELVEFLSQGGDRCSLDRTSLESLSYTQCLYLLSDLRGLGYHSIDSILHILECSEAERFEIYRIFRRHGIGIGEALQLGVRKSL
GVAIS151  ...RD..G.S...GH.S.S.DSGSG..G....V..VN.....KS...Q......Y..GGG......................K...
GTG11-1   ....N....N...GH.SSSN..SGG......NA...H..R...D...K......Y..GGG......F...............
P163-M5   -M..D........G.SRGS.N.N.G......VE..R..T..K....P......Y..GGGS.D....F...............
KWVMo4-1  -M..D........G.SSGS.N.N.G......VE..R..T..KS...P......Y..GGGS.D...F.....R....G.......
BMO32-1   -M..D........G.SSGS..SN.G......LE..H..T..KS...P...N..Y..GGGSTD....F.............K...
GTR1-2    -M..N........G.SSGS.G.NKG...A...LE..C..T..KS...P...NL.Y..GGGS.D....L.V...........
GTR1SD-1  -M..D........G.SSGS.NSN.G......LE..H..T..KS...P...N..Y..GGGS.D....F.............IT...
GTR1-1    .M.GES....DL.RF.L...ST.GSG.I...PLDK.IG..EG..S...S...NV..V.GGG.PD...Q.L.V..SQ.VAVS..YE..LT...
P163-1    .M.DVS...LD..RF.L...ST.GSG.I...PLDR.IR..EG..N...S...NV..V.GGG.PD...Q.L.V..AQ.VAVS..YE..LT...

                100       110       120       130       140       150       160       170
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GVA-GR5   CYSPRSTAEILDDLLSRISRGGAFLPSDLGAVKGELLVTFQPSRLSVDLYANNKKVCTRTCQGKGDAGYVARRFSGYKGLALRSQGN
GVAIS151  .N....LLA........LG..S..........VA..HS......V.....V..SV.AE...YS...........I..ATRY
GTG11-1   S....G...........G..S....C.....R...I...HSA......I....V.KFA.AE...N.A.G......V..TARH
P163-M5   .....TVI.......G.LG..S....C..........I..R........V......VS.VV..SS..LS....P.I....T..
KWVMo4-1  .....TVI.......G.LG..S....C.......I..R........V......LS.VV..S...V......P.I....T..
BMO32-1   .....AVI.......G.LG..S....C....S...I..H........V......VS.VVA.S...L...A..P.I....TK.S
GTR1-2    .....AVT.......N.LI..S.......N....I..H........V..A...VV...S.SS..L.....P.I....S..
GTR1SD-1  .....AVI.......G.LG..S....C...T....I.LH........V......VS.VVS.S...S..A..P.I...K..
GTR1-1    SF...PVSL..E...D..A..AS...V.....N...V.N.ER.........V........S.IKYSA..PK..QQ....F..IIT..S--
P163-1    SF...PVSL......D..A..AS...V.....N...V.N.ER.........V........S.IKYSA..PK..QQ....F..IVT..S--
```

Figure 4. Amino acid sequence alignment of ORF 2. The alignment reveals the diversity of this protein among variants and also shows that mild variants P163-1 and GTR1-1 are most divergent from other variants.

Phylogenetic analysis performed on the respective GVA ORF protein products strengthened the fact that GVA variants can be divided into three molecular groups in South Africa. The shortest tree resulting from each analysis is presented in Appendix A. The tree statistics for the phylogenetic analyses are shown in table 5. For all the trees, the consistency index (CI)

and the retention index (RI) were above 0.72 (except for the full-length genome with an RI value of 0.629). This showed that the topographical layout of the trees can be trusted due to an indication of confidence. The three molecular groups of GVA can be observed in the trees of full-length nt (figure 1), ORF 1 protein (Appendix A, figure 1), ORF 2 protein (Appendix A, figure 2), and MP (Appendix A, figure 3). The CP tree (Appendix A, figure 4) only supported the grouping of P163-1 and GTR1-1 of Group III and the P10 (Appendix A, figure 5) tree did not support any grouping of variants. From the analysis it was quite evident that mild variants of group III are the most divergent group of all. It was also found that the CP gene was fairly conserved among variants of Group I and II, and that the P10 was fairly conserved among variants of all groups. This implies that grouping of variants into 3 distinct molecular groups is based on full-length genome sequence and ORF 1 – 3 aa sequences.
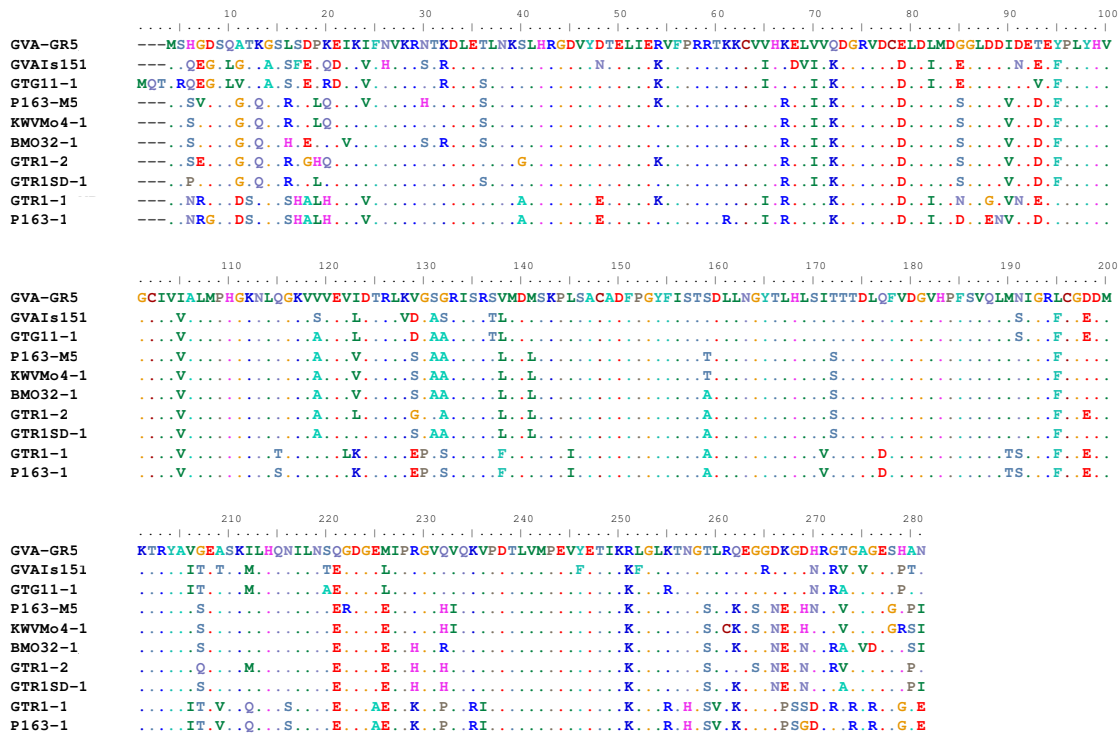
```
                 10        20        30        40        50        60        70        80        90       100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GVA-GR5   ---MSHGDSQATKGSLSDPKEIKIFNVKRNTKDLETLNKSLHRGDVYDTELIERVFPRRTKKCVVHKELVVQDGRVDCELDLMDGGLDDIDETEYPLYHV
GVAIs151  ---..QEG.LG..A.SFE.QD..V.H...S.R.............N....K..........I..DVI.K......D..I..E......N.E.F.....
GTG11-1   MQT.RQEG.LV..A.S.E.RD..V.......R...S.............K..........I....I.K......D..I..E......V.F.....
P163-M5   ---..SV...G.Q..R..LQ...V....H.....S..............K.........R..I.K.....D.....S...V..D.F.....
KWVMo4-1  ---..S....G.Q..R..LQ................S.............R..I.K.....D.....S...V..D.F.....
BMO32-1   ---..S....G.Q..H.E...V........S.R...S.............R..I.K.....D.....S...V..D.F.....
GTR1-2    ---..SE...G.Q..R.GHQ...................G..........K.........R..I.K.....D.....S...V..D.F.....
GTR1SD-1  ---..P....G.Q..R..L.................S.............R..I.K.....D.....S...V..D.F.....
GTR1-1    ---..NR...DS...SHALH...V................A......E....K........I.R....K.....D..I..N..G.VN.E.......
P163-1    ---..NRG..DS...SHALH...V................A......E........R...I.R....K.....D..I..D..ENV..D.......

                110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GVA-GR5   GCIVIALMPHGKNLQGKVVVEVIDTRLKVGSGRISRSVMDMSKPLSACADFPGYFISTSDLLNGYTLHLSITTTDLQFVDGVHPFSVQLMNIGRLCGDDM
GVAIs151  ....V.............S...L....VD.AS....TL.............................................S...F..E..
GTG11-1   ....V.............A...L....D.AA....TL.............................................S...F..E..
P163-M5   ....V.............A...V.....S.AA.....L..L....................T...............S...................F.....
KWVMo4-1  ....V.............A...V.....S.AA.....L..L....................T...............S...................F.....
BMO32-1   ....V.............A...V.....S.AA.....L..L...................................S...................F.....
GTR1-2    ....V.............A...L....G..A.....L..L.................A..................S...................F..E..
GTR1SD-1  ....V.............A........S.AA.....L..L.................A..................S...................F.....
GTR1-1    ....V..........T......LK.....EP.S.....F.....I.................A..........V.....D...........TS...F..E..
P163-1    ....V..........S.......K.....EP.S.....F.....I.................A..........V.....D...........TS...F..E..

                210       220       230       240       250       260       270       280
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
GVA-GR5   KTRYAVGEASKILHQNILNSQGDGEMIPRGVQVQKVPDTLVMPEVYETIKRLGLKTNGTLRQEGGDKGDHRGTGAGESHAN
GVAIs151  .....IT.T..M.......TE....L....................F....KF............R....N.RV.V...PT.
GTG11-1   .....IT....M.......AE....L.................K...R.............N.RA.....P..
P163-M5   ......S..............ER...E.....HI.................K.......S..K.S.NE.HN..V....G.PI
KWVMo4-1  ......S..............E....E.....HI.................K.......S.CK.S.NE.H...V....GRSI
BMO32-1   ......S..............E....E..H..R.................K.......S..K..NE.N..RA.VD...SI
GTR1-2    ......Q....M.........E....E..H..H.................K.......S....S.NE.N..RV......P.
GTR1SD-1  ......S..............E....E..H..H.................K.......S..K..NE.N...A......PI
GTR1-1    .....IT.V..Q...S.....E...AE..K..P..RI.............K...R.H.SV.K....PSSD.R.R.R...G.E
P163-1    .....IT.V..Q...S.....E...AE..K..P..RI.............K...R.H.SV.K....PSGD...R.R..G.E
```

Figure 5. Amino acid sequence alignment the MP (ORF 3) of GVA variants.
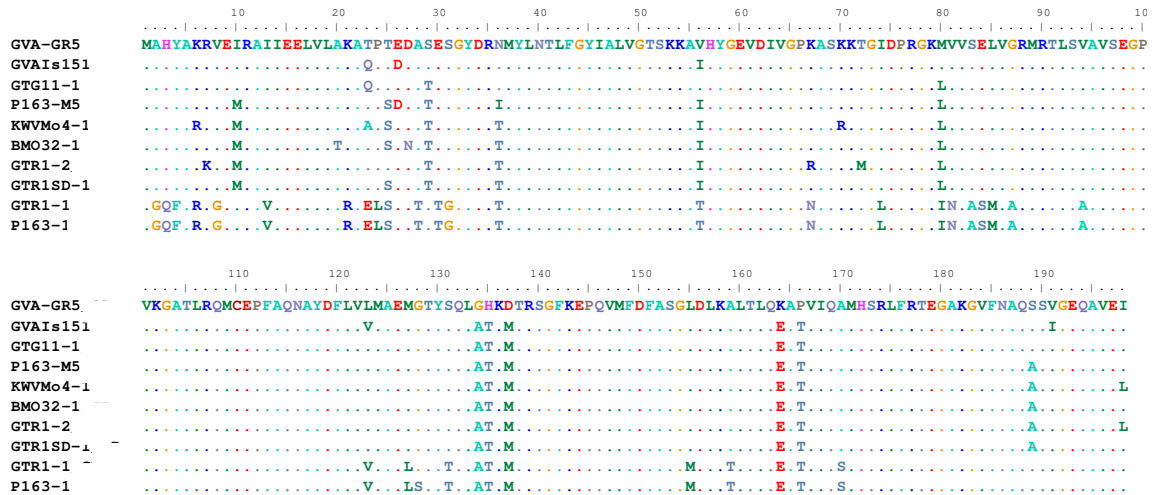
Figure 6. Amino acid sequence alignment of CP (ORF 4) of GVA variants showing that this protein is fairly conserved among variants of Groups I and II.
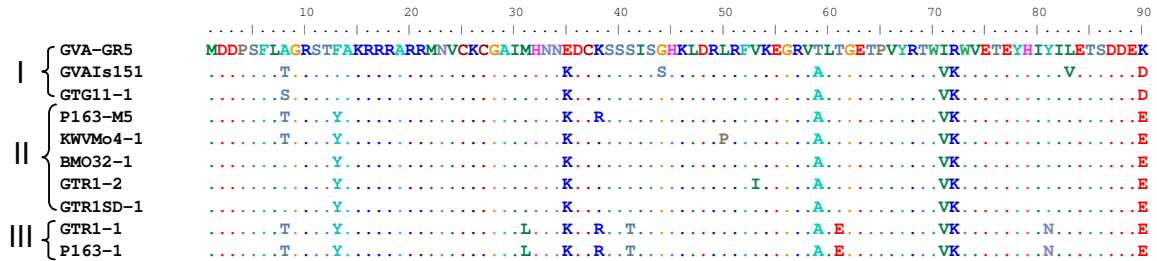
Figure 7. Amino acid sequence alignment of P10 (ORF 5) of GVA variants showing that this protein is fairly conserved among variants of each group, with Group III being the most divergent.

Table 5. Phylogenetic tree statistics

Tree	Total number of trees	Tree length	Number of characters				Consistency index	Retention index
			Parsimony-informative	Parsimony-uninformative	Constant	Total		
Genome (nt)	1	9711	3031	1667	2921	7619	0.720	0.629
ORF 1 (aa)	1	1933	474	541	705	1720	0.892	0.775
ORF 2 (aa)	2	373	88	81	16	185	0.898	0.776
MP (aa)	2	376	82	131	109	322	0.870	0.746
CP (aa)	9	140	35	69	94	198	0.940	0.860
P10 (aa)	40	82	9	57	60	126	0.976	0.857

3.4.3. Molecular divergence of GVA variants associated with SD in South Africa (Goszczynski, D.E., du Preez, J., & Burger, J.T., 2008)

In this paper, GVA variant GTR1-2 was compared to 4 other fully sequenced GVA variants of group II (GTR1SD-1, KWVM04-1, BMO32-1 and P163-M5). These variants of group II show a strong association with SD expression, with the exception of GTR1-2 that was isolated from a Shiraz plant that consistently tested negative for SD. Amino acid alignments of all ORFs revealed that the sequence of the ORF 2 protein product is clearly divergent from

other group II variants (figure 8). Divergence of GTR1-2 from other variants of group II was also observed in 5'untranslated region (UTR) and 3'UTR nt sequences as well as aa sequences of ORF 1 and ORF 3 protein products (figure 8). Predicted protein alignments revealed several aa substitutions which led to net charge changes in translation products encoded by ORF 1, 2 and 3 for variant GTR1-2 (figure 8). These substitutions are discussed in detail in Goszczynski et al., 2008. The most significant discovery reported in this paper, was the presence of a 119 nt insertion 20 nt downstream of the ORF 2 start codon of variant P163-M5 (figure 9A). This insert resulted in a putative new stopcodon for ORF 1 and a putative new initiation codon for ORF 2. Furthermore this insert could be translated into a 40 aa polypeptide that showed homology to native ORF 2 of P163-M5 and other variants of molecular group II (figure 9B). The P163-M5 variant induces extremely severe symptoms in *N. benthamiana* when compared to other variants of group II (figure 9C). The possible association of the presence of this insert with the severity of symptoms will have to be determined. Results presented in this paper suggest that pathogenicity determinants in the GVA genome differ in *N. benthamiana* and grapevine.
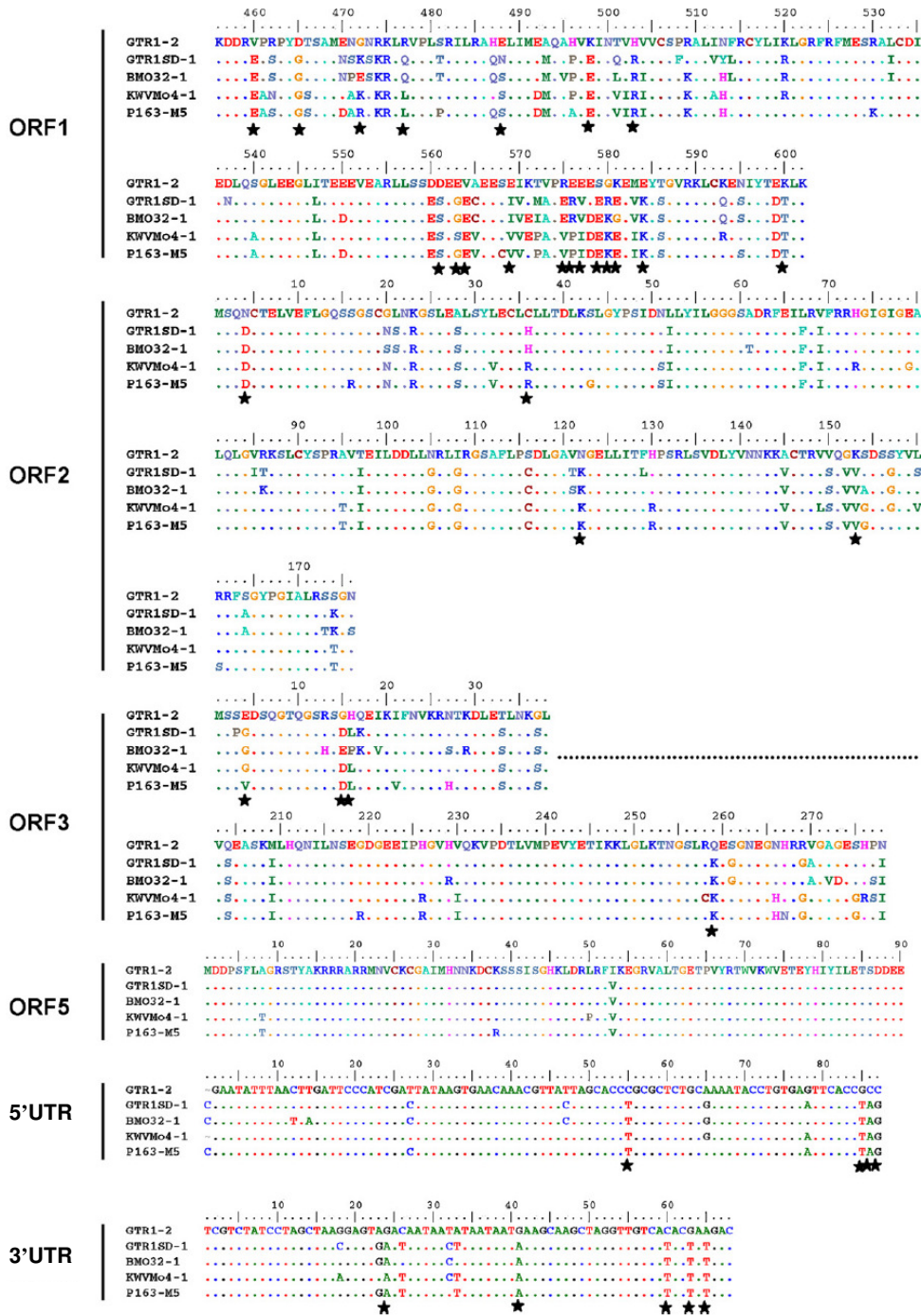
Figure 8. Figure taken from Goszczynski et al., (2008) showing alignments of complete (ORF2, 5'UTR, 3'UTR) and incomplete (ORF1, ORF3) aa and nt sequences of group II GVA variants highlighting the noticeable divergence between GTR1-2 and other members of this group. The asterisks indicate the positions of significant aa substitutions, which lead to a change in the net charge of encoded proteins, and nt divergence of the 5'UTR and 3'UTR sequences that are attributes of the GTR1-2 variant. An alignment of complete predicted amino acid sequences encoded by ORF5 of these variants is included as an example of lack of significant aa substitutions in this gene between variants.
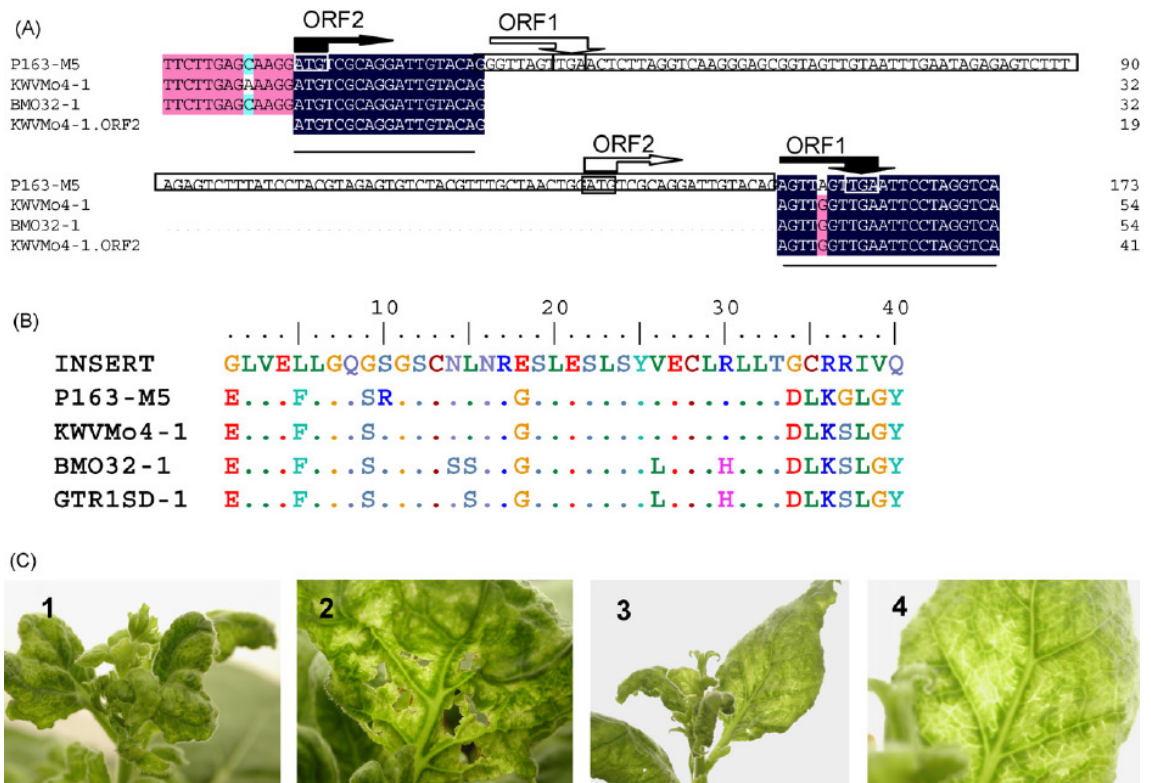
Figure 9. Figure taken from Goszczynski et al., 2008. (A) Sequence alignment of the 5' termini of ORF2 (underlined) of molecular group II GVA variants, which indicate the 119 nt insertion (boxed) in ORF2 of the P163-M5 variant. The insert shows a putative new stop codon for ORF1 and a new start codon for ORF2 of P163-M5 variant (open arrows) and it can be translated to the amino acid sequence from the native start codon of ORF2 of this variant (closed arrow). (B) The predicted aa sequence encoded by 119 nt insert shows many characteristic aa substitutions when aligned with homologous sequences encoded by native ORF2 of P163-M5 and other variants of group II. (C) P163-M5 variant containing the 119 nt insert induces clearly more severe symptoms in *N. benthamiana* (C1, 2) compared with symptoms induced in this host by other variants of molecular group II (C3, 4).

3.5. CONCLUSION

Full-length sequencing of the South African GVA variants GTR1-1, GTR1-2 and GTG11-1 confirmed the heterogenic population structure of the virus and the division into three distinct molecular groups. Based on phylogenetic analysis performed on GVA ORFs, it could be observed that the mild variants P163-1 and GTR1-1 of group III are the most divergent. These variants are not associated with SD and induce mild symptoms in the herbaceous host *N. benthamiana*. GTG11-1 was grouped in group I together with the Israeli and Italian GVA strains. This group was also found not to be associated with SD. In group II of GVA, two intriguing variants were found namely P163-M5 and GTR1-2 (Goszczynski et al., 2008). The P163-M5 variant was found to induce extremely severe symptoms in *N. benthamiana* compared to other variants in group II. It was found to contain a 119 nt insertion 20 nt

downstream of the start codon for ORF2. This variant is used in woody indexing by the grapevine industry as positive control for SD. The association of the insert to severity of symptoms in *N. benthamiana* and expression of SD in grapevines remain to be determined. The GVA genomic determinants of pathogenecity in grapevine are still not known. In *N. benthamiana* the P10 protein of GVA has been associated with pathogenicity (Haviv et al., 2006), but it seems, based on results presented here, that pathogenicity determinants are more complex in grapevine. Variant GTR1-2 of group II was isolated from a consistenly SD negative Shiraz plant. The protein sequences of the P10 of group II variants that differ in pathogenecity to grapevine, and GTR1-2 are almost identical and it seems that other factors in the genome could play a role in pathogenicity determination in this host. Extensive studies will have to be performed in the future to unravel the role that GVA plays in the aetiology of disease expression in grapevine and the possible association of the P163-M5 insert to disease.

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Chapter 4: Construction of infectious clones of three South African GVA variants

Part of the work described in this chapter was included in a presentation at the 16th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine 31 August to 4 September 2009, Dijon, France. The same work will be submitted for publication in the near future.

4.1. ABSTRACT

Infectious cDNA clones of RNA viruses are important tools for the study of gene expression, replication and pathogen-host interactions. Shiraz disease is a major problem in South African vineyards and specific GVA variants are thought to be involved. In order to unravel the contribution of different GVA variants to the aetiology of SD, infectious clones of these variants are needed. In this chapter, the construction of full-length cDNA clones of three South African GVA variants (GTR1-1, GTR1-2 and GTG11-1), representing each of the three molecular groups is discussed. After several cloning strategies were attempted, none of these clones (pBIN-e35S-Asc-GTR1-1-pA, pGREEN-e35S-Stu-GTR1-2 and pBIN-e35S-Asc-GTG11-1-pA) were infectious upon agroinfiltration in *Nicotiana benthamiana*. A population cloning strategy was used in a second attempt to assemble variant GTR1-2 into an infectious cDNA clone (pBIN-e35S-GTR1-2-pA). Twelve pBIN-e35S-GTR1-2-pA clones were infiltrated into *N. benthamiana* and none of them was found to be infectious. Two significant mutations were found after full-length sequencing of one of these clones: a 13 nt deletion (missing nucleotides 1335-1347) and a premature stopcodon at position 4125 in ORF 1. After correction of both these mutations, a full-length cDNA clone (pBIN-e35S-GTR1-2$_{fl*}$-pA) showed small clusters of infected cells in infiltrated mesophyll tissue of *N. benthamiana* plants after TPIA detection of GVA CP. This indicated that this clone was replicating locally, but as the plants stayed symptom-free, it seemed not to be able to move systemically. After alignment of protein sequences of all ORFs, an amino acid mutation at position 13 [Tyrosine (Tyr/Y)→Cysteine (Cys/C)] in the ORF 5 encoded P10 of pBIN-e35S-GTR1-2$_{fl*}$-pA was found. To test the effect of this mutation, ORF 5 of this clone was transferred into a 35S-GVA118ΔORF5 construct (Blignaut et al., 2009) and compared to other 35S-GVA118ΔORF5 constructs in *N. benthamiana*. Preliminary results suggested that this single amino acid mutation reduced replication of the virus to lower than a detectable level. Furthermore, two infectious clones of Israeli variants of GVA (T7-GVA-GR5 and T7-GVA118, obtained from M. Mawassi) were brought under control of a CaMV 35S promoter (35S-GVA-GR5 and 35S-GVA118). Both clones were infectious, able to replicate, move systemically and induce typical GVA symptoms after agroinfiltration in *N. benthamiana*.

4.2. INTRODUCTION

An important forward step in the study of RNA viruses has been made by the capacity of generating infectious clones representing a viral genome. The discovery of reverse transcriptases that convert RNA to complementary DNA or cDNA was the solitary most significant movement towards the era of infectious clones. *In vitro-* or *in vivo*-transcribed infectious RNA derived from full-length cDNA clones of RNA viral genomes are practical tools for studying viruses at a molecular level (Boyer & Haenni, 1994). By deployment and manipulation of such clones, knowledge can be gained into viral gene expression, viral replication strategy and pathogen-host interactions.

Grapevine virus A infects grapevine and a range of herbaceous hosts. Functional analysis of the GVA genome was performed on a T7-promoter driven full-length infectious cDNA clone of a GVA variant (GR5) that was able to systemically infect *N. benthamiana* plants upon introduction of capped *in vitro* RNA transcripts (Galiakparov et al., 2003). Functions were attributed to all GVA ORFs, except for ORF 2. Recently, a transient expression vector (GVA118) based on the 5' genomic region of GR5 and the 3' genomic region of the SA variant GTR1-3 (Goszczynski & Jooste 2003) was constructed (Haviv et al., 2006). A multiple cloning site was introduced downstream of a duplicated sub-genomic promoter of the GR5 movement protein (MP, ORF 3). This vector was shown to successfully express the GUS reporter gene and the CTV coat protein in *N. benthamiana* plants (Haviv et al., 2006).

Grapevine virus A has a broad molecular heterogeneity in South Africa and variants have been shown to separate into three divergent molecular groups (Goszczynski & Jooste 2002, 2003). These variants induced different symptoms in the leaves of the herbaceous host *N. benthamiana*. Pathogenicity determinants in the GVA genome of respective sequence variants are still under investigation. The P10 protein encoded by the GVA ORF 5 has been shown to be connected with symptom expression in *N. benthamiana* (Haviv et al., 2006). As of yet, for grapevine nothing is known about the occurrence of symptom expression and disease determinants in the GVA genome. Recently, it was found that variants of molecular group II are narrowly associated with a recently emerging disease of grapevine known as SD, and variants of molecular group III are present in GVA-infected SD-susceptible grapevine that do not present symptoms of the disease (Goszczynski, 2007). Group II variants show a strong association with SD, but interestingly a variant of this group, GTR1-2, was recovered in *N. benthamiana* from a constantly symptomless Shiraz plant (Goszczynski, 2007). This disease mostly affects red cultivars and diseased vines always die within a five year time period (Goszczynski & Jooste, 2003a).

Construction of infectious clones of different South African GVA variants capable of infecting *Vitis vinifera*, could pave the way in understanding and unravelling the aetiology of SD and GVA-associated diseases of grapevine.

In this chapter the assembly of CaMV 35S-promoter driven cDNA clones of three South African variants of GVA (GTR1-1, GTR1-2 & GTG11-1), representing each of the three molecular groups is discussed. The characterisation of 35S-GVA-GR5 and 35S-GVA118

clones in *N. benthamiana* as positive controls is also discussed. Unfortunately the P163-M5 variant was not available from D.E. Goszczynski (Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa) for assembly into an infectious clone.

4.3. MATERIAL AND METHODS

4.3.1. Plant cultivation

N. benthamiana plants were grown from seed in heat sterilised soil in a growth room with controlled conditions of temperature between 18ºC and 26ºC, relative humidity of approximately 70% and a 16 h – 8 h light/dark cycle.

4.3.2. Oligonucleotide primers

Primers used in this study was synthesised by either Inqaba Biotech (Pretoria, South Africa) or Integrated DNA technologies (IDT, Coralville, USA). Primers are listed in table 1. Also refer to chapter 3, table 1 for primers described earlier.

Table 1. Primers used in this study.

Primer name	Sequence (5'$\rightarrow$3')
GVA118-ORF5-allF	AGCCGGCATGGATGACCCATCGTTTC
GVA118-ORF5-1-2R	AATGCATTTATTCCTCATCGTCTGAGG
GVA118-5'-F	GGCGCGCCGAATATTTAACTTGATTCC
GVA118-5'-R	GGTTTCGCAGGCCCTGATGAACTT
GTR1-1-EF-AscI	TATGGCGCGCCCGAATATTTAATTTGATT
GTR1-1-7360-R-pA	ATTGCGGCCGC(T)$_{30}$GTCACCGTGTGACAACCTAGCTTGCTTTACC
GTG11-1-EF-AscI	TATGGCGCGCCGAATATTTAACTTGATTC
GTG11-1-7351-R-pA	ATTGCGGCCGC(T)$_{30}$GTCTTCGTGTGACAACCTAGCTTGC
GTR1-2-3763-F	GATTGAGATGGAGGAGGTCG
GTR1-2-2972-R	CCAATCGTCCTTGAGCACCTTCCT
GTR1-2-2041-F	CAAAGATACTCCAAGGGTGGG
GTR1-2-4501-F	CTGATTCTGATTATGAGGCATTTGA

4.3.3. Double stranded RNA extraction, cDNA synthesis, PCR, cloning, sequencing and sequence analysis

These procedures were performed as described in chapter 3.3.

4.3.4. Joining of overlapping RT-PCR fragments

Overlapping RT-PCR fragments were assembled by restriction digestion or overlap extension PCR (OE-PCR; Higuchi et al., 1988).

4.3.4.1. OE-PCR

Fragments to be joined were amplified from existing clones using Takara Pyrobest and the primer pairs used to generate them. A 100X dilution of the template plasmid DNA was made and 2 µL of DNA was added to 48 µL PCR cocktail (1X Pyrobest TM Buffer II, 0.2 mM dNTPs, 0.5µM each primer, 1U Pyrobest polymerase) PCR cycling conditions were 1 cycle of 94ºC for 4 min, 30 cycles of 94ºC for 30 sec, annealing temperature for 30 sec, 72ºC for 1 min/kb and 1 cycle of 72ºC for 7 min. Fragments were gel purified, quantified (as described previously) and 50 ng/µL dilutions made. Twenty five nanograms of each fragment were used in an overlap extension PCR using the external primers. PCR cycling conditions were 1 cycle of 94ºC for 4 min, 30 cycles of 94ºC for 1 min, annealing temperature for 1 min, 72ºC for 1 min/kb and 1 cycle of 72ºC for 10 min. Fragments were gel purified, quantified (as described previously) and used in subsequent reactions.

4.3.5. GVA constructs for use as positive controls

Two T7-promoter driven GVA cDNA clones (T7-GVA-GR5 & T7-GVA118) described earlier (Haviv et al., 2006), were obtained from Munir Mawassi (The S. Tolkowsky Laboratory, Department of Plant Pathology-The Virology Unit, Agricultural Research Organization, Volcani Center, Bet Dagan, Israel). T7-GVA-GR5 is a normal full-length cDNA clone of a native GVA variant GR5. T7-GVA118 is a hybrid virus, containing ORF 1 and 2 of GR5 and ORF 3–5 of GTR1-3 that was modified into an expression vector by duplication of the subgenomic promoter of the movement protein and introduction of a MCS. These clones were brought under control of an enhanced CaMV-35S promoter and the resulting 35S-cassettes were cloned into a modified pBIN19 vector (pBIN-e35S-GVA-GR5 & pBIN-e35S-GVA118, figure 1) for agroinfiltration experiments.

GVA constructs

A

B
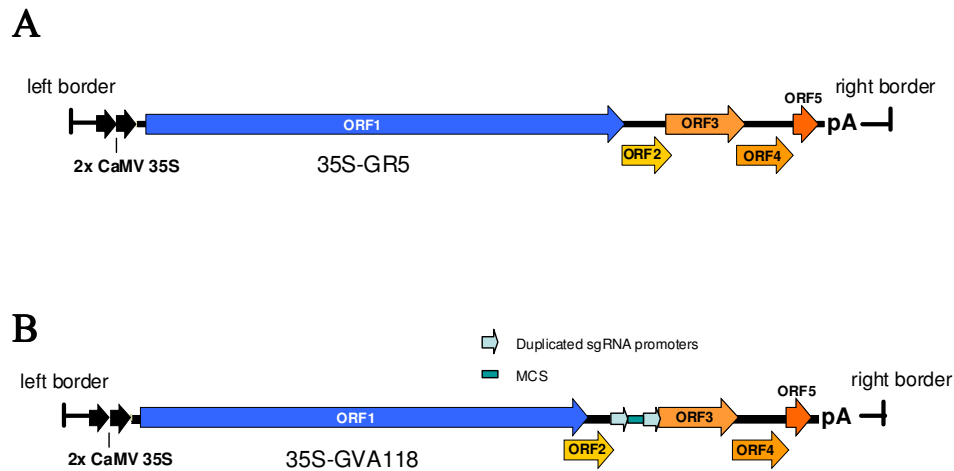
Figure 1. Graphic representation showing full-length GVA cDNA clones that were brought under control of an enhanced CaMV 35S promoter. a) 35S-GVA-GR5, is a full-length unmodified native variant of GVA. b) 35S-GVA118 is a hybrid virus consisting of ORF 1 of GR5 and ORFs 2-5 of GTR1-2 that was modified into an expression vector for herbaceous plants. It was modified to contain a duplicated subgenomic promoter of the MP and a MCS (Haviv et al., 2006).

4.3.5.1. 35S-GVA118 cloning strategy

To bring T7-GVA118 under control of a CaMV 35S promoter the 5'end of GVA was PCR amplified from this clone using primers GVA118-5'-F and GVA118-5'-R (table 1). This fragment was digested with *Asc* I and *Eco* 47III and ligated downstream of an enhanced CaMV 35S promoter (Töpfer et al., 1987) in a pBluescript II SKM backbone to generate pSKM-e35S-GVA118-partI. T7-GVA118 was digested with *Eco* 47III and *Pvu* II to generate a fragment representing the rest of the GVA118 genome, including the poly-A tail. This fragment was cloned into *Eco* 47III and *Nru* I digested pSKM-e35S-GVA118-partI to result in pSKM-e35S-GVA118-pA. The e35S-GVA118-pA cassette was digested from pSKM-e35S-GVA118-pA with *Sna* BI/*Sal* I and transferred to a *Sna* BI/*Xho* I digested pBIN_SN, an in the MCS modified pBIN19 derivative (Bevan, 1984) to generate pBIN-e35S-GVA118-pA. pBIN_SN and the CaMV 35S promoter containing plasmid were provided by Edgar Maiss and Dirk Stephan (Leibniz University Hannover, Institute of Plant Disease and Plant Protection, Germany). In the Muruganantham et al., (2009) paper GVA118 was brought under

control of a CaMV 35S promoter and cloned into a different binary vector to result in a construct named pGVA378, which is essentially the same construct as 35S-GVA118.

<u>4.3.5.2. 35S-GVA-GR5 cloning strategy</u>

As ORF1 of GVA118 and GR5 are identical only the GVA GTR1-2 part in GVA118 (including the polylinker) had to be replaced with the corresponding part of GR5. This was accomplished by digestion of T7-GVA-GR5 with *Mlu* I/*Sal* I. The resulting fragment was ligated into similarly digested pSKM-e35S-GVA118-pA to generate pSKM-e35S-GVA-GR5-pA. This e35S-GVA-GR5-pA cassette was transferred to pBIN_SN as described above for pBIN-e35S-GVA118-pA to obtain pBIN-e35S-GR5-pA.

4.3.6. Assembly of infectious clones of South African GVA variants GTR1-1, GTG11-1 and GTR1-2

Assembly strategies of GVA variants GTR1-1, GTG11-1 and GTG11-1 are presented in the next sections, and a revised population cloning strategy for the GTR1-2 variant will be presented in section 4.4.2.3.

<u>4.3.6.1. Assembly of GTR1-1</u>

In the assembly of GTR1-1 into a full-length cDNA clone unique restriction sites, spanning the whole genome, could be identified which would allow the joining of 5 overlapping RT-PCR fragments (figure 2). Several vectors and cloning strategies were evaluated in assembly of GTR1-1 (not discussed). The pART7/pART27 (Gleave, 1992) and the pGreenII 0000 (Hellens et al., 2000) vector systems were tested without success. It was decided to use the same vectors as were used for GVA-GR5 and GVA118. GTR1-1 was ligated downstream of an enhanced CaMV 35S promoter in a pBluescript II SKM backbone to generate pSKM-e35S-Asc-GTR1-1-pA. This was achieved using primers GTR1-1-EF-Asc and GTR1-1-1560-R and the restriction enzymes *Asc* I, *Nde* I and *Not* I. The e35S-Asc-GTR1-1-pA cassette was digested from pSKM-e35S-Asc-GTR1-1-pA with *Sma* I/*Not* I and transferred to a *Sna* BI/*Not* I digested pBIN_SN, to generate pBIN-e35S-Asc-GTR1-1-pA.
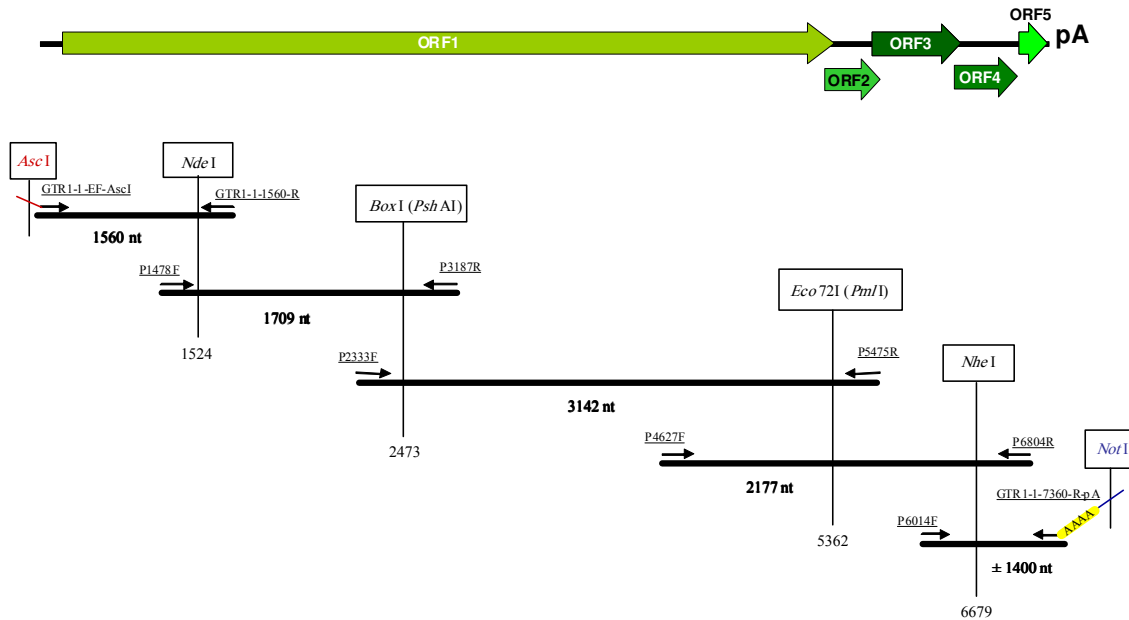
Figure 2. Graphic representation showing the cDNA full-length clone assembly strategy for the GVA variant GTR1-1. Five overlapping RT-PCR fragments were generated for assembly of the GTR1-1 variant into a cDNA clone. The size of each fragment (nt) is shown in bold underneath the respective fragment. Primers used in the generation of fragments are underlined and restriction enzymes used for assembly are boxed.

4.3.6.2. Assembly of GTG11-1

The GTG11-1 variant was assembled from 6 overlapping RT-PCR fragments into a full-length cDNA clone using restriction enzyme digestion and overlap extension PCR (figure 3). As for GTR1-1, several vectors and cloning strategies were evaluated in assembly of GTG11-1 (not discussed). The pART7/pART27 (Gleave, 1992) and the pGREEN (Hellens et al., 2000) vector systems were tested without success. It was decided to use the same vectors used for GVA-GR5 and GVA118. GTG11-1 was ligated downstream of an enhanced CaMV 35S promoter in a pBluescript II SKM backbone to generate pSKM-e35S-Asc-GTG11-1-pA. This was achieved using primers GTG11-1-EF-Asc and GVA-1188-R and the restriction enzymes *Asc* I, *Nco* I and *Not* I. The e35S-Asc-GTG11-1-pA cassette was digested from pSKM-e35S-Asc-GTG11-1-pA with *Pvu* I/*Not* I and transferred to a *Sna* BI/*Not* I digested pBIN_SN, to generate pBIN-e35S-Asc-GTG11-1-pA. The *Pvu* I site was blunted with T4 DNA polymerase according to the manufacturer's instructions.

GTG11-1 assembly strategy
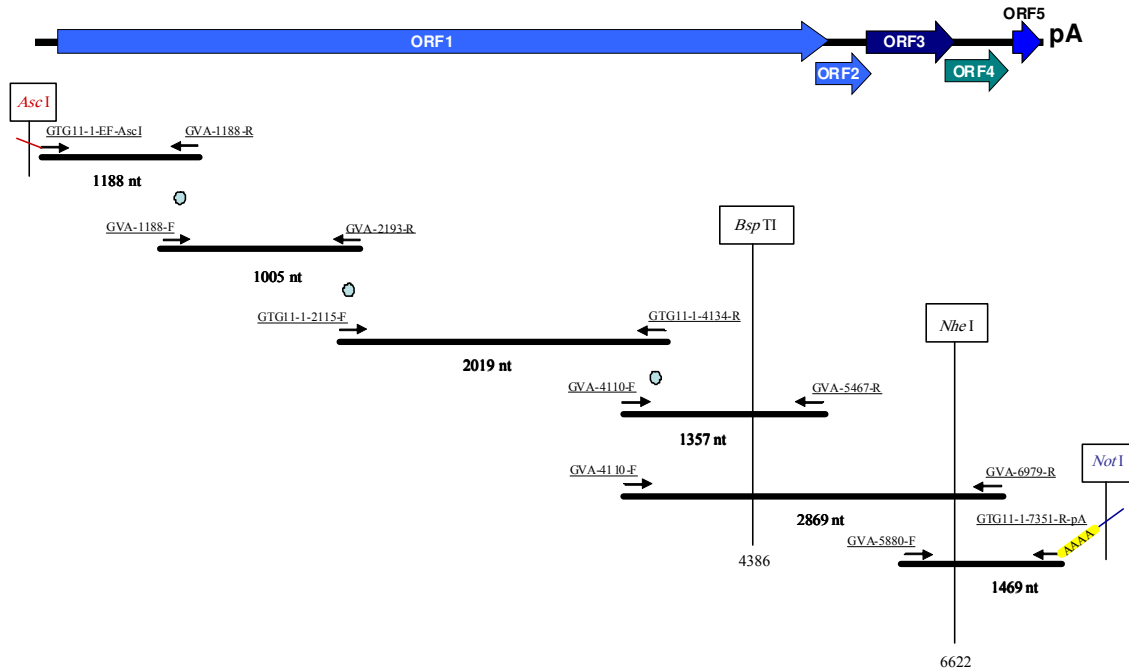
Figure 3. Graphic representation showing assembly strategy for GTG11-1 variant. Six overlapping RT-PCR fragments were generated for assembly of the GTG11-1 variant into a cDNA clone. The size of each fragment is shown in bold underneath the respective fragment. Primers used in generation of fragments are underlined and restriction enzymes used for assembly, boxed. Fragments joined with overlap extension PCR are indicated by a blue circle.

4.3.6.3. Assembly of GTR1-2

4.3.6.3.1. First assembly attempt

GTR1-2 was assembled from 7 overlapping RT-PCR fragments into a full-length cDNA clone using restriction enzyme digestion and overlap extension PCR (figure 4). Several vectors and cloning strategies were assessed in the assembly of GTR1-2 (not discussed). The assembled GTR1-2 cDNA was cloned immediately downstream of a duplicated CaMV 35S promoter in a pStu442 vector (Provided by Edgar Maiss, Leibniz University Hannover, Germany). The final 35S-GTR1-2-pA cassette was cloned into the pBIN_SN vector resulting in pBIN-e35S-stu-GTR1-2-pA.

GTR1-2 first assembly strategy
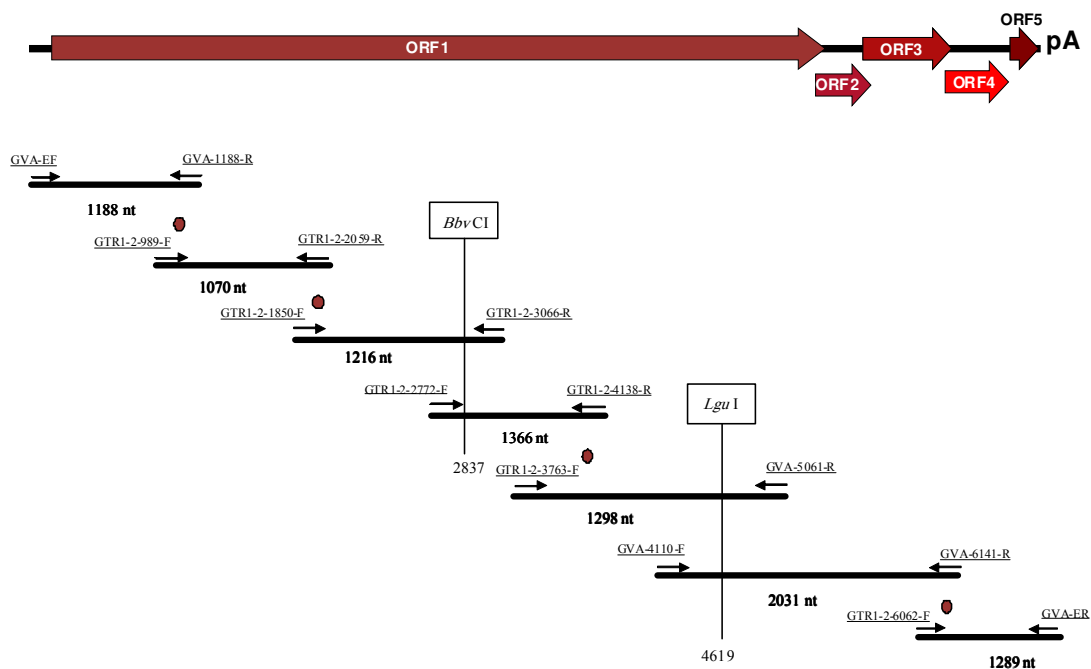
Figure 4. Graphic representation showing the first assembly strategy for the GTR1-2 variant. Seven overlapping RT-PCR fragments were generated for assembly of the GTR1-2 variant into a cDNA clone. The size of each fragment is shown in bold underneath the respective fragment. Primers used in generation of fragments are underlined and restriction enzymes used for assembly, boxed. Fragments joined with overlap extension PCR are indicated by a red circle.

4.3.7. Electrocompetent *Agrobacterium* cells

Electrocompetent *Agrobacterium* cells were prepared according to the method described by Annamalai et al., (2006).

4.3.8. Electroporation

The electroporator settings were as follows: Capacitance 25 µF, Resistance 200 Ω and Voltage 1,5 kV.

4.3.9. Agroinfiltration of *N. benthamiana* plants

Agrobacterium infiltration was performed as described by Voinnet et al., (1998).

Constructs were co-agroinfiltrated (1:1) with 35S:BMYV-P0 containing the strong P0 viral suppressor of *Beet mild yellowing virus* (BMYV), genus *Polerovirus*, family *Luteoviridae*, derived from an infectious BMYV full-length clone (Stephan & Maiss, 2006).

4.3.10. Tissue print Immuno-assay (TPIA) and visualisation

A modified version of the method described by Franco-Lara et al. (1999) was used to detect GVA-CP at 6-7 dpi in infiltrated leaves.

To improve detection the Hybond PVDF membrane was first wetted in methanol (2 s). This was followed by a rinse in excess water (15 min) and an equilibration in 1X TBS (0.02 M Tris-base, 0.5 M NaCl, pH 7.4) (15 min). The membrane was incubated (shaker, 1 h) in modified blocking solution (4.5 % milk powder dissolved in 1X TBS). A primary GVA-CP antibody solution [1 GVA-CP-antisera : 400 1X TBS-TPO (1X TBS, 2% PVP-40, 0.2% Albumin Fraction V] and a secondary conjugate solution [1 goat anti-rabbit alkaline phosphatase (GAR-AP) : 10 000 1X TBS-T] was used in the subsequent steps. Pictures of tissueprints were taken by Charlene Janion (Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University) with a Leica DFC 320 digital camera mounted on a Leica MZ 7.5 microscope.

4.3.11. Electronmicroscopy

Electronmicroscopy was performed by Ramola Chauhan (Plant Health Diagnostic Services, Directorate Plant Health, National Department of Agriculture, Stellenbosch).

4.3.12. Symptom development

The development of symptoms was monitored at daily intervals and pictures taken with a Canon IXUS 90 IS camera using the macro function.

4.3.13. RNA extraction for GVA detection in infiltrated plants

Total RNA extraction was performed according to the method described by White et al., (2008). Two hundred milligrams of leaf tissue was used as starting material. RNA was treated with 10 units (U) of DNase I for 30 min at 37ºC in 1X DNase Buffer. Integrity of RNA was assessed on 1% (w/v) agarose D1 LE gels in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA pH 8). RNA samples were quantified with the NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Inqaba Biotechnical Industries (Pty) Ltd, SA) according to the manufacturer's instructions.

4.3.14. RT-PCR to detect GVA in infiltrated plants

4.3.14.1. First strand cDNA synthesis

First strand cDNA synthesis was performed on total RNA as described in chapter 3.3. Two microlitres of DNase I treated total RNA was used instead of dsRNA. Refer to section 4.3.14.2. for primers used in detection.

4.3.14.2. PCR

PCR amplification using first strand cDNA as template was performed as described in chapter 3.3. The primer pair GVA-GR5-4344F and GVA-GR5-ER (chapter 5, table 1) was used for detection of GVA-GR5 and the primer pair GVA-GR5-5781-F and GVA-GR5-6188-R (chapter 5, table 1) was used for GVA118. For detection of GTR1-1, GTR1-2 and GTG11-1 primers pairs used in assembly were used for detection (chapter 3, table 1; chapter 4, table 1).

4.3.15. Illustra™ TempliPhi 100 Amplification Kit

Rolling circle amplification (RCA) was performed with the Illustra™ TempliPhi 100 Amplification Kit. The primer annealing cocktail was prepared in a 10 µL volume (50-500ng DNA, 0.625mM dNTPs, 250pmol Random hexamer primers), denatured (3 min, 95ºC) and chilled (2 min, ice). The amplification cocktail was prepared in a 10 µL volume (2.5 U phi29 polymerase, 1X phi29 buffer) and added to the primer annealing cocktail. PCR cycling conditions were as follows: 1 hold for 18 h @ 30ºC, followed by 1 hold of 10 min @ 65ºC.

4.4. RESULTS AND DISCUSSION

4.4.1. Characterisation of 35S-GVA118 and 35S-GVA-GR5 in *N. benthamiana*

Two T7 promoter-driven GVA cDNA clones (T7-GVA-GR5 and T7-GVA118) were brought under control of a CaMV 35S promoter to allow agroinfiltration experiments. These clones served as positive controls for optimisation of protocols and comparison of South African GVA variants to the existing Israeli GVA clones (Haviv et al., 2006). Typical GVA symptoms were observed as early as 3-4 days post-infiltration (dpi) of 35S-GVA-GR5 and 35S-GVA118 in *N. benthamiana* (figure 5B&C). These symptoms included vein clearing, interveinal chlorosis, necrosis, downward rolling of leaves and stunting of plants. TPIA performed 6-7 dpi showed replication of the constructs in the agroinfiltrated tissue. Co-infiltration (1:1) of 35S-GVA constructs with 35S:BMYV-P0 containing the strong viral

suppressor P0 of BMYV enhanced replication and improved detection of GVA-CP through TPIA (figure 6). This improvement was not measured and was only observed visually.
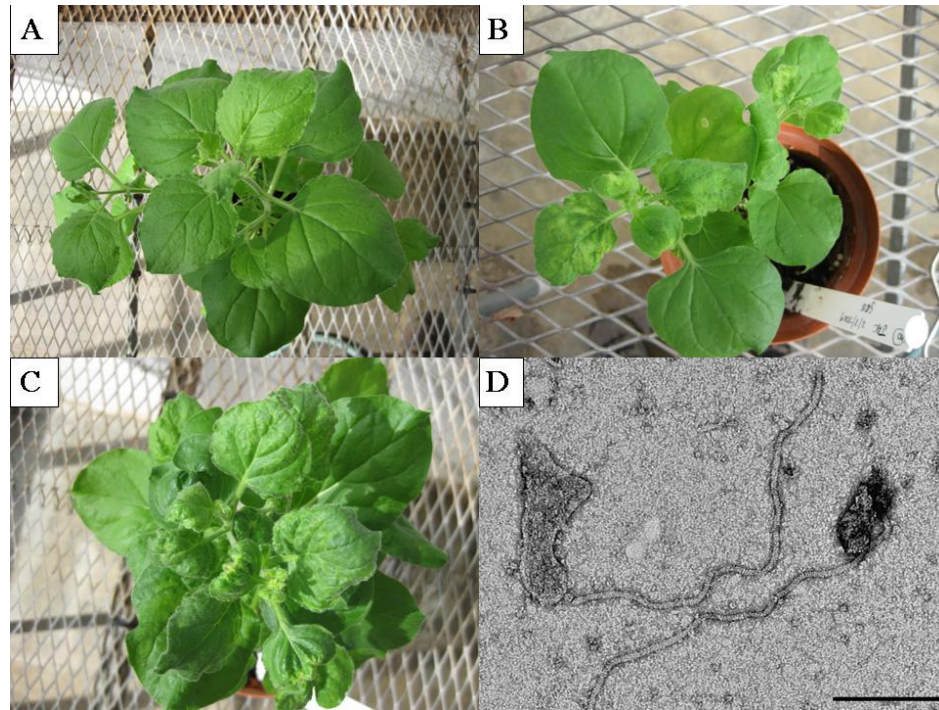
Figure 5. Characterisation of 35S-GVA-GR5 and 35S-GVA118 in *N. benthamiana*. A: Mock-inoculated *N. benthamiana* plant, B: Typical GVA symptoms on a *N. benthamiana* plant agroinfiltrated with 35S-GVA-GR5, C: Typical GVA symptoms on a *N. benthamiana* plant agroinfiltrated with 35S-GVA118, D: Electronmicrograph showing GVA particles of GVA-GR5 from *N. benthamiana*.
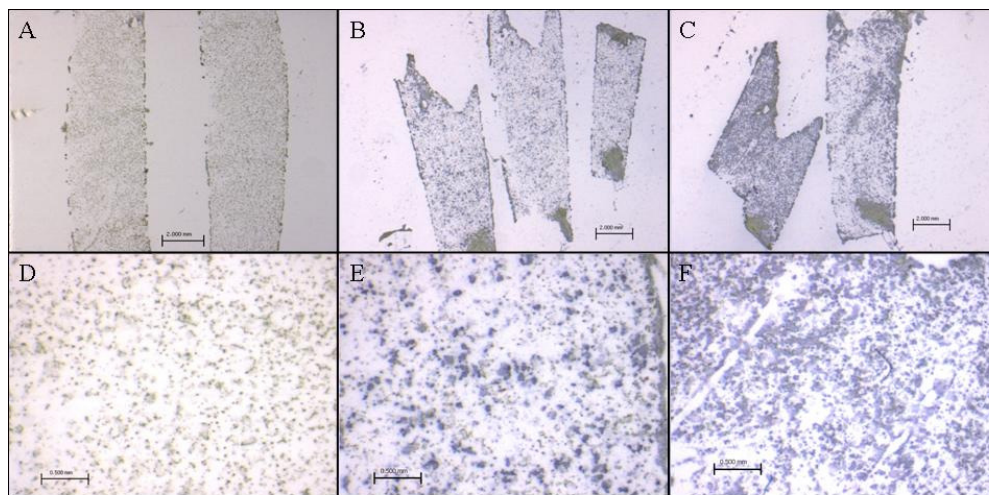
Figure 6. TPIA performed on mesophyll tissue of agroinfiltrated *N. benthamiana* plants. A&D) Mock-inoculated plant, B&E) 35S-GVA-GR5 (without P0 suppressor), C&F) GVA-GR5 (with P0 suppressor) showing enhancement of expression by at least 5 times. Bar sizes: A-C = 2 nm; D-E = 0.5 nm

Intact GVA particles were also detected by electron microscopy (figure 5D). RT-PCR performed on total RNA, extracted from systemic leaves, detected the virus at 14-21 dpi showing systemic movement of the virus throughout the plant. The cloning strategy used resulted in the incorporation of 6 foreign nucleotides, representing the *Asc* I site, between the CaMV 35S promoter and the 5'-end of the GVA genome. Previous studies have shown that 5' and 3' extensions to the GVA genome can play a significant role in infectivity of the virus (Boyer and Haenni, 1994). The general rule is that 5'-end extensions of viral transcripts, strongly reduce infectivity, and that 3'-end extensions are more easily tolerated. Extensions seem to be more easily tolerated when the transcripts are produced *in vivo* as is the case for 35S-GVA in this study. Commandeur et al. (1991) showed that in *vitro*-derived transcripts containing an extension of up to 40 extra-viral nucleotides at the 5'end of a *Beet necrotic yellow vein virus* (BNYVV), genus *Benyvirus*, cDNA clone were biologically inactive in the host plant. When cloning the same cDNA downstream of a CaMV 35S promoter, the *in vivo* produced transcripts were infectious *in planta*. In the case of GVA, it seems that 6 foreign nucleotides are easily tolerated when cDNA is delivered by agroinfiltration and transcripts are produced in *N. benthamiana*. To test whether the poly-A tail is needed for infectivity, it (containing 30 A's) was removed in the 35S-GVA118 clone (strategy not shown) and infiltrated into *N. benthamiana*. All clones that did not contain a poly-A tail were not infectious when compared to the normal 35S-GVA118 clone confirming that the poly-A tail is essential for infectivity (data not shown).

4.4.2. Assembly of infectious clones of South African GVA variants GTR1-1, GTG11-1, GTR1-2 and characterisation in *N. benthamiana*

4.4.2.1. GTR1-1, GTG11-1 and GTR1-2

Several vectors and cloning strategies resulted in different 35S-contructs carrying the full-length GTR1-1 or GTG11-1 cDNA in a binary vector backbone (strategies not discussed, shown in table 2). Constructs were agroinfiltrated into *N. benthamiana* plants and characterised with TPIA, inspection for symptom development and RT-PCR (table 2). No symptoms were observed on any of the infiltrated plants. Plants tested with TPIA did not show replication of the virus in infiltrated mesophyll cells or petioles of systemic leaves. GVA could also not be detected by RT-PCR in apical leaves of infiltrated plants. Upon infiltration of the final pBIN-e35S-Asc-GTR1-1-pA, pBIN-e35S-Asc-GTG11-1-pA and pBIN-e35S-Stu-GTR1-2-pA clones into *N. benthamiana* plants all plants stayed symptom-

free. TPIA and RT-PCR could not detect GVA (table 2) suggesting that the assembled clones were not able to replicate and move systemically throughout the plants.

Table 2. Table showing specifics of characterisation of GVA-clones (not assembled with a population cloning strategy) in *N. benthamiana*. The number (#) of plants indicated in the table is the total number for all independent rounds of infiltrations performed for the specific construct. Not all plants were analysed by TPIA and RT-PCR, but all plants that were tested, were negative as shown in these columns. Np = not performed.

Variant	Construct name	Binary vector	35S-vector	# plants	TPIA	Symptoms	RT-PCR
GTR1-1	pART27-35S-GTR1-1-pA	pART27	pART7	20	np	0/20	-
	pGreen-35S-GTR1-1-pA	pGreenII 0000	pART7	10	-	0/10	-
	pGreen-e35S-Stu-GTR1-1-pA	pGreenII 0000	pStu442	8	-	0/8	-
	pBIN-e35S-Stu-GTR1-1-pA	pBIN_SN	pStu442	8	-	0/8	-
	pBIN-e35S-Asc-GTR1-1-pA	pBIN_SN	pBluescript-35S	8	-	0/8	-
GTG11-1	pART27-35S-GTG11-1-pA	pART27	pART7	20	np	0/20	-
	pGreen-35S-GTG11-1-pA	pGreenII 0000	pART7	10	-	0/10	-
	pGreen-e35S-Stu-GTG11-1-pA	pGreenII 0000	pStu442	8	-	0/8	-
	pBIN-e35S-Stu-GTG11-1-pA	pBIN_SN	pStu442	6	-	0/6	-
	pBIN-e35S-Asc-GTG11-1-pA	pBIN_SN	pBluescript-35S	8	-	0/8	-
GTR1-2	pART27-35S-GTR1-2	pART27	pART7	20	np	0/20	-
	pGreen-35S-GTR1-2	pGreenII 0000	pStu442	10	-	0/10	-
	pBIN-e35S-Stu-GTR1-2-pA	pBIN_SN	pStu442	8	-	0/8	-

4.4.2.2. Characterisation of GVA118$_{ORF1}$/GTG11-1$_{ORF2-5}$ and GVA118$_{ORF1}$/GTR1-1$_{ORF2-5}$ hybrids in *N. benthamiana*

Open reading frame 1 is the largest in the GVA genome and codes for replication-related proteins (Galiakparov et al., 2003). The ORF 1 gene product is translated directly from genomic RNA and a significant mutation in this region could influence replication. The assembled GTG11-1 and GTR1-1 clones were not replicating in *N. benthamiana*. To test whether the ORF 1 was the problematic area in these clones, ORFs 2-5 of each clone were substituted into GVA118 to result in GVA118$_{ORF1}$/GTG11-1$_{ORF2-5}$ and GVA118$_{ORF1}$/GTR1-1$_{ORF2-5}$ hybrids (strategy not shown). These hybrids contained the native ORF 1 of GVA118 and ORFs 2-5 of the respective South African variants. Upon infiltration of these hybrids in *N. benthamiana*, none was shown to be infectious by TPIA, and no symptoms were observed indicating that possible mutations were present in ORFs 2-5. It could also be that the non-infectivity of these hybrids is due to the loss of protein-protein interactions between ORF 1 of

GVA118 and ORFs 2-5 of the respective heterologous GVA variants, however this seems unlikely as a hybrid GVA expression vector was described earlier (Haviv et al., 2006).

4.4.2.3. Assembly of GTR1-2 using a population cloning strategy

The first assembly strategies for generation of GTR1-1, GTG11-1 and GTR1-2 GVA cDNA clones had some limitations. Devised assembly strategies for South African GVA variants resulted in between 5-7 overlapping RT-PCR fragments that had to be generated. Furthermore, some parts of the GVA genomes were devoid of useful restriction enzymes and we had to revert to an overlap extension PCR strategy for assembly of these fragments. This was not optimal as every round of PCR amplification increases the chance for incorporation of mutations, even if proofreading enzymes are used. It has been reported previously, however, that infectious clones can be obtained successfully by use of *Taq* polymerase (Hayes and Buck, 1990) despite the high error rate of the enzyme (Keohavong and Thilly, 1989). As presented in chapter 3, sequence information for all three GVA variants existed, but sequencing after every single assembly step was not possible.

After none of the assembled South African GVA cDNA clones were able to replicate after agroinoculation of *N. benthamiana*, a population cloning strategy was devised for the assembly of the GTR1-2 variant only. The vectors used to transfer the infectious GVA118 under a CaMV 35S promoter yielded clones that were able to replicate and induce a systemic infection, therefore the pSKM-e35S-GVA118-pA construct was used as scaffold for substitution of the GTR1-2 genome in place of GVA118. In order to achieve this, two unique restriction sites were identified within the GTR1-2 genome namely *Nhe* I (cutting at position 2754) and *Sac* I (cutting at position 4619). Three primer sets were used to generate three overlapping RT-PCR fragments named GTR1-2-5' (2980 bp), GTR1-2-internal (2972 bp) and GTR1-2-3' (2902 bp) (figures in Appendix B). An *Asc* I site and a *Not* I site were incorporated on flaps of outermost primers to facilitate subsequent cloning into a vector downstream of a CaMV 35S promoter (Töpfer et al., 1987). RT-PCR fragments were generated from dsRNA as described previously in chapter 3.3. These fragments were ligated into the pDRIVE vector resulting in pDRIVE-GTR1-2-5', pDRIVE-GTR1-2-internal and pDRIVE-GTR1-2-3', respectively. Two clones per fragment were sequenced at the extremities for sequence confirmation. Ten to twelve clones of each fragment were pooled together in a population (pop) to give the maximum representation of quasispecies and to circumvent possible natural and PCR-induced lethal mutants (pDRIVE-GTR1-2-5' (pop)

→J131, pDRIVE-GTR1-2-internal (pop) →J132 and pDRIVE-GTR1-2-3' (pop) →J133). The 5' end of GTR1-2 was substituted from the pDRIVE-GTR1-2-5' population (J131) into the pSKM-e35S-GVA118-pA construct by *Asc* I and *Nhe* I digestion resulting in pSKM-e35S-GVA118-GTR1-2-*Asc*/*Nhe*-pA. Thirteen clones of this construct were pooled into a population and named J136. The internal and 3' parts of the GTR1-2 genome had to be joined first before substitution into J136. To achieve this, the pDRIVE-GTR1-2-internal population (J132) had to be subcloned into pDRIVE with *Nhe* I and *Sac* I to generate pDRIVE-GTR1-2-NheI-1865-*Sac*I. A *Sac* I site was eliminated during this step. Twelve clones of the pDRIVE-GTR1-2-*Nhe*I-1865-*Sac*I construct were pooled in a population (J134) and digested with *Sac* I and *Not* I to generate an acceptor vector for ligation of the GTR1-2-3' end after similar digestion of the pDRIVE-GTR1-2-3' population (J133). This resulted in pDRIVE-GTR1-2-3'-subclone. Twelve clones of this construct were pooled in a population (J135) and then substituted into J136. This was done by digestion with *Nhe* I and *Not* I to generate twelve full-length GTR1-2 clones named pSKM-e35S-GTR1-2$_{fl}$-pA (J173-J184). The e35S-GTR1-2$_{fl}$-pA construct was transferred to pBIN_SN using *Sna* BI and *Not* I to result in twelve pBIN-e35S-GTR1-2$_{fl}$-pA clones (J192-203). All twelve clones were infiltrated individually into *N. benthamiana* plants and tested for infectivity at 6-7 dpi by TPIA. No CP was detected in the infiltrated areas suggesting that the clones were not infectious. One clone from the pSKM-e35S-GTR1-2$_{fl}$-pA population was selected and sequenced completely to identify any mutations that may be present. J180 was randomly selected and sequenced with existing primers spanning the whole genome. Upon inspection of the full-length sequence, a 13 nt deletion (missing nts 1335-1347, figure 7) was identified in ORF 1, resulting in a frame shift that could abolish RNA replication.

4.4.2.4. Correction of the 13 nt deletion in clone J180

All pDRIVE-GTR1-2-5' clones were sequenced to identify a clone that did not contain the 13 nt deletion. A correct clone (J220) was identified and used to substitute into the J180 clone containing the deletion. For unknown reasons, the plasmid DNA concentration of both J180 and J220 were very low after isolation from *E. coli*. The Illustra™ TempliPhi 100 Amplification Kit was used to amplify these constructs in order to increase the plasmid DNA concentration. The correct fragment was digested from J220 with *Asc* I and *Stu* I and substituted into similarly digested J180 to result in pSKM-e35S-GTR1-2$_{fl}$-cor-pA. Five clones (J245, 246, 248-250) were sequenced to confirm that the 13 nt deletion was corrected and subsequently transferred to pBIN_SN as described previously to generate five pBIN-e35S-

GTR1-2₍fl₎-cor-pA clones (J252-256). The corrected clones were agroinfiltrated into *N. benthamiana* plants and TPIA showed that none of the clones was infectious. At this point, the J245 clone was sequenced completely. Sequence analysis revealed a single nt change at position 4125 (Cytosine to Tymine) leading to a premature stop codon (TAG) in ORF 1. This results in a truncated polypeptide of 1346 aa in length. This mutation seemed significant as it could abolish replication of the virus.
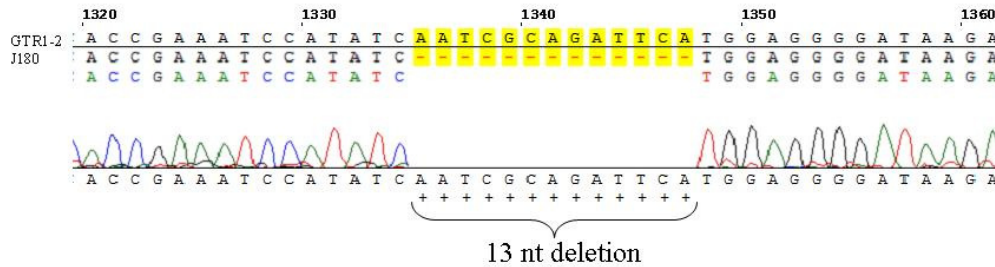

Figure 7. Sequence alignment of J180 clone with GTR1-2 sequence showing the 13 nt deletion at position 1335-1347.

4.4.2.5. Correction of the premature stop codon in clone J245

All pDRIVE-GTR1-2-internal clones were sequenced to identify one that did not contain the mutation. A correct clone (J387) was identified that could be used to replace the corresponding part in the J245 clone. J245 had to be transferred to pDRIVE to elimate a *Sac* I site that could interfere with cloning. The fragment containing the mutation was digested from J245 using *Hind* III and *Not* I and ligated into pDRIVE that was cut with the same enzymes, yielding pDRIVE-J245-tail. The correct fragment was digested from J387 and substituted into pDRIVE-J245-tail using *Hind* III and *Sac* I resulting in pDRIVE-J245-tail-cor. This clone was sequenced to confirm that the mutation was corrected. The corrected fragment was transferred back to J245 from pDRIVE-J245-tail-cor with *Hind* III, *Not* I and *Cla* I resulting in five pSKM-e35S-J245-PSC clones. *Cla* I was used to cut the backbone into smaller fragments for gel extraction purposes. The e35S-J245-PSC construct was transferred to pBIN_SN as described previously leading to three pBIN-e35S-GTR1-2₍fl*₎-pA clones (J396-398). These three clones were agroinfiltrated into *N. benthamiana* plants (2 plants per construct, repeated 3 times) to test infectivity. TPIA results revealed small clusters of infected mesophyll tissue in infiltrated areas for all three clones (figure 8). No symptoms were observed throughout the lifespan of the plant and GVA could not be detected in systemic leaves of infiltrated plants by RT-PCR. This showed that the clones were replicating locally, although, when compared to GVA118 or GR5 full-length clones, at a lower level, and that the clones could not move systemically throughout the plant. J245 sequence information was

used to translate all ORFs into proteins *in silico*. No insertions, deletions or significant amino acid substitutions were observed in the ORF 2-4 proteins. In ORF 1, however, 5 amino acid changes were observed that differed from other GVA variants, and the known GTR1-2 sequence at that specific position. These were 43: Aspartate (Asp/D)→ Glycine (Gly/G); 1264: Leucine (Leu/L)→ Phenyl-alanine (Phe/F); 1521: Cysteine (Cys/C) → Tyrosine (Tyr/Y); 1522: Serine (Ser/S)→ Proline (Pro/P); 1544: Cys→ Y. Three of these were in the RdRP domain that ranged from amino acids 1279 to 1603 in the GTR1-2 variant. Seeing that the replication efficiency of the J396, J397 and J398 clones were not optimal, these substitutions could provide a reason for this. However, before any assumptions can be made, these amino acid substitutions have to be compared to a clone that is infectious in plants. In ORF 5, only one significant amino acid change was observed. This was a change from a tyrosine (Tyr/Y) to cysteine (Cys/C) amino acid residue at position 13 (13:Y→C) (figure 10A).
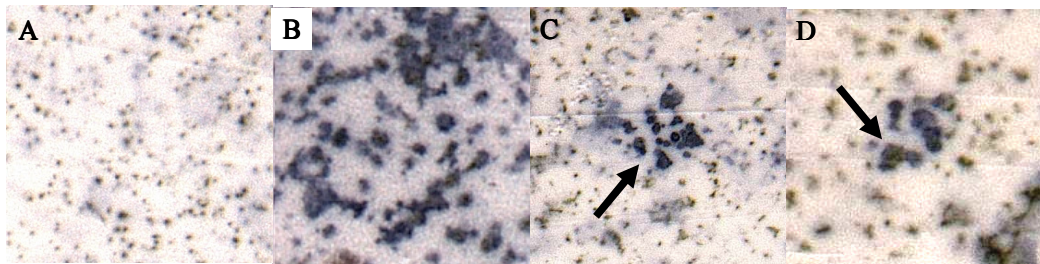
Figure 8. TPIA showing mesophyll cells of infiltrated *N. benthamiana* tissue. A) Mock-inoculated plant, B) 35S-GVA-GR5 inoculated plant, C,D) pBIN-e35S-GTR1-2fl*-pA (J396) inoculated plants showing small clusters of cells in which GVA is replicating.

As described previously, ORF 5 of GVA has been linked to symptomatology (Haviv et al., 2006) and silencing suppression (Chiba et al., 2006, Zhou et al., 2006). In order to test if this mutation could be the reason for the inefficacy of replication, an experiment was designed using previously described constructs 35S-GVA118ΔORF5 and 35S-GVA118ΔORF5-1-2 (Blignaut et al., 2009, figure 9). 35S- GVA118ΔORF5 is a construct from which the ORF 5 was removed and a short polylinker, containing *Pdi* I/*Ngo* MIV and *Mph* 1103I restriction sites, incorporated. The 35-GVA118ΔORF5-1-2 construct was generated by incorporation of ORF 5 of GVA variant GTR1-2. The ORF 5 containing the mutation at aa position 13 of the J396 construct was PCR amplified using primers GVA118-ORF5-allF and GVA118-ORF5-1-2R (table 1), cloned into pDRIVE (pDRIVE-J396-ORF5) and the sequence was confirmed by sequencing. The complete ORF 5 was digested from pDRIVE-J396-ORF5 with *Pdi* I and
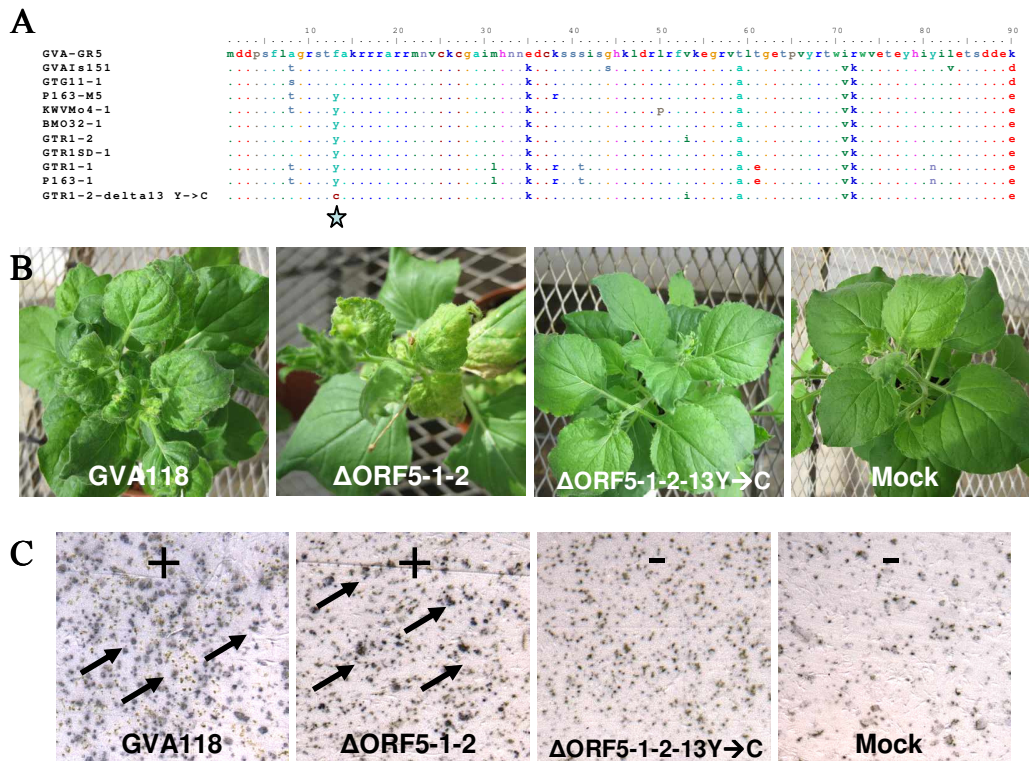
Mph 1103I and ligated into similarly digested 35S-GVA118-ΔORF5 (in a pBluescript backbone) leading to pBSM-35S-GVA118-ΔORF5-GTR1-2-13Y➔C. The part containing the substitution was sequenced to confirm that this construct differed only from the 35-GVA118ΔORF5-1-2 construct at aa position 13 in ORF 5. The 35-GVA118ΔORF5-1-2 cassette was digested from pBSM-35S-GVA118-ΔORF5-GTR1-2-13Y➔C with *Sna* BI and *Sal* I and transferred into a *Sna* BI and *Xho* I digested pBIN_SN binary vector. The constructs 35-GVA118ΔORF5-1-2, 35S-ΔORF5-GTR1-2-13Y➔C and 35S-GVA118-pA were agroinfiltrated into *N. benthamiana* plants and monitored for symptom development (table 3). At 6 dpi, TPIA tested positive for all plants infiltrated with 35-GVA118ΔORF5-1-2 and 35S-GVA118-pA, it was however negative for all plants infiltrated with 35S-GVA118ΔORF5-GTR1-2-13Y➔C (figure 10C). At 8 dpi all plants that tested positive in the TPIA showed symptoms while no symptoms were observed in plants infiltrated with 35S-GVA118ΔORF5-GTR1-2-13Y➔C (figure 10B and table 3). In fact, these plants stayed symptom-free throughout their lifespan. It seems that symptom development and replication was completely abolished or extremely reduced by the 13Y➔C mutation. This suggests that a significant amino acid change at residue 13, in the P10 protein of GVA, influences the efficiency of replication in *N. benthamiana*. RT-PCR detection for GVA in apical leaves to assess systemic movement was not performed, however. Several amino acids in the GVA P10 protein have been shown to influence symptomology, replication and silencing suppression of GVA in *N. benthamiana,* but none of those completely abolished replication of the virus (Munir Mawassi, pers. Comm.). Several studies have shown that mutations – even single amino acid changes – in a viral silencing suppressor abolish or strongly reduce RNA accumulation and silencing suppression leading to restriction of viral movement and decreased symptom severity in plants (Chu et al., 2000; Kasschau & Carrington, 2001; Qu & Morris, 2002; Liu et al., 2002; González-Jara et al., 2005; Klein et al., 2007; Carrasco et al., 2007; Mangwende et al., 2009; Hu et al., 2009). In the case of the 13Y➔C mutation, a hydrophobic, aromatic amino acid (Y) is changed to a small amino acid (C) with disulfide-bonding potential. This change could putatively cause a conformational change in the P10 protein, resulting in loss of function as disulfide bonds play an important role in folding and stability of proteins. In the near future, as final confirmation, this mutation will be corrected in the J396 clone to assess whether infectivity can be re-established. The three amino acid substitutions in the RdRP of the same clone have also to be investigated further.
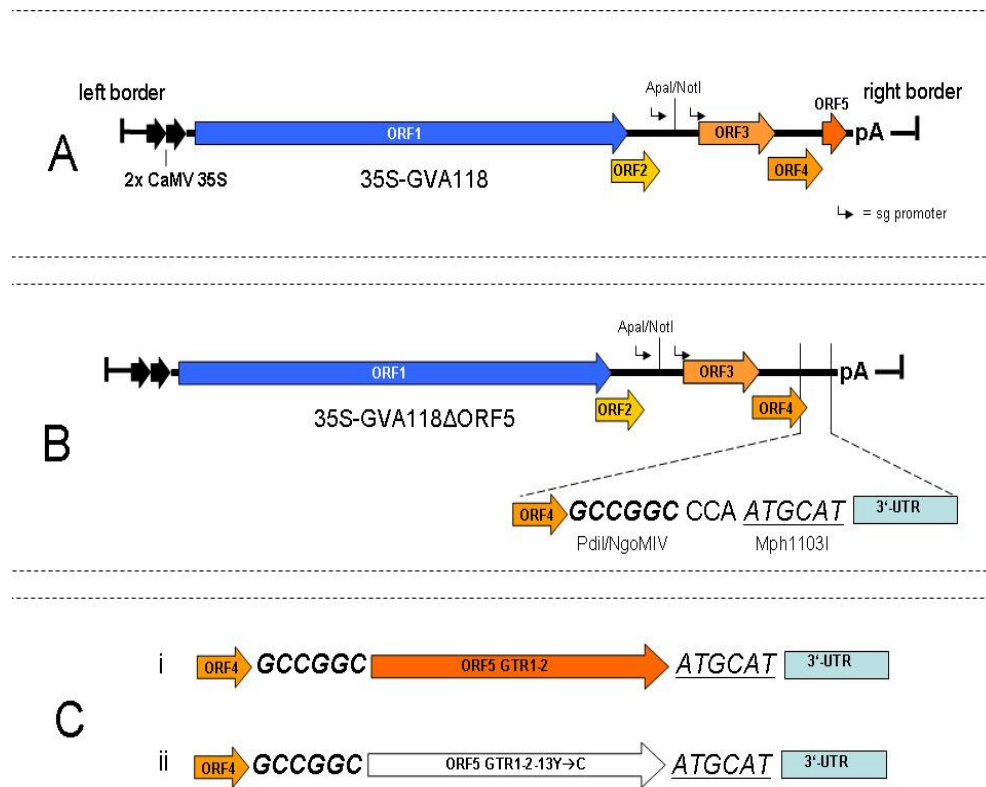
Figure 9. Graphic representation of clones used to test the GTR1-2-13Y→C mutation. A) 35S-GVA118, B) 35S-GVA118ΔORF5, ORF 5 of 35S-GVA118 was removed and a polylinker containing *Pdi* I/*Ngo* MIV and Mph1103I restriction sites introduced with overlap extension (Blignaut et al., 2009). Ci) 35S-GVA118ΔORF5-1-2, ORF 5 of the GVA GTR1-2 variant was cloned into construct B (Blignaut et al., 2009), Cii) 35S-GVA118ΔORF5-GTR1-2-13Y→C, ORF 5 of clone J396, containing single amino acid mutation, was cloned into construct B.

Table 3. Table showing specifics of GTR1-2-13Y→C mutation experiment in *N. benthamiana*.

Construct	Number of plants	TPIA	Symptoms
pBIN-35S-GVA118-ΔORF5-1-2	2	+	+
pBIN-35S-GVA118-ΔORF5-GTR1-2-13Y→C	2	-	-
pBIN-e35S-GVA118-pA	2	+	+
Mock	2	-	-

A

```
                      ....|....|  10    |    20   |    30   |    40   |    50   |    60   |    70   |    80   |    90   |
GVA-GR5             mddpsflagrstfakrrrarrmnvckcgaimhnnedcksssisghkldrlrfvkegrvtltgetpvyrtwirwvveteyhiyiletsddek
GVAIs151            .......t..........................k.........s...........a..........vk.........v......d
GTG11-1            .......s..........................k.................a...........vk...............d
P163-M5            .......t...y......................k..r..............a...........vk..............e
KWVMo4-1           .......t...y......................k.............P..............a...........vk..............e
BMO32-1            ...........y......................k.................a...........vk..............e
GTR1-2            ...........y......................k...............i.....a..........vk.............e
GTR1SD-1          ...........y......................k.................a...........vk..............e
GTR1-1            .......t...y................l..k..r..t.................a.e........vk........n........e
P163-1            .......t...y................l..k..r..t.................a.e........vk........n........e
GTR1-2-delta13 Y->C ...........c......................k...............i.....a..........vk..............e
                              ☆
```

B

C

Figure 10. A) Amino acid sequence alignment of ORF 5 of respective GVA variants showing the 13Y→C mutation (indicated by star) in J396, B) Symptoms observed on infiltrated *N. benthamiana* plants showing typical GVA symptoms on plants infiltrated with GVA118 and ΔORF5-1-2, but none on those mock-infiltrated or ΔORF5-GTR1-2-13Y→C-infiltrated, C) TPIA performed on infiltrated tissue of agroinoculated *N. benthamiana* plants showing that the single amino acid mutation 13Y→C abolishes replication of the virus. GVA118 and ΔORF5-1-2 show single cells where GVA-CP is detected, confirming replication of the constructs.

4.5. CONCLUSION

Shiraz disease remains to be a major problem in South African vineyards. To further studies into the aetiology of this disease, and the specific role that GVA contributes, infectious clones of associated and non-associated GVA variants are needed. In this chapter, cDNA clones of three South African GVA variants (GTR1-1, GTG11-1 and GTR1-2) were assembled cloned downstream of an enhanced CaMV 35S promoter and transferred into the binary vector pBIN_SN for agroinfiltration of *N. benthamiana*. During assembly of these clones, different CaMV 35S intermediate and binary vectors were tested. At first, after TPIA and RT-PCR assessment, none of the cDNA clones were able to replicate in infiltrated *N. benthamiana* plants to a detectable level. This could be due to a number of contributing factors. In the first assembly attempts only 1-3 clones per fragment were used during each step. This could improve the chances for incorporating a mutation in the final full-length clone. Furthermore,

even though proofreading enzymes were used in assembly, there is always a chance of PCR incorporation of point mutations during amplification that could influence infectivity of the final cDNA clone. It has been reported previously that fragments containing mutations are more stable in *E. coli* (Yamshchikov et al., 2001). Whether these mutations are from viral replication errors, PCR-incorporated mutations or spontaneous rearrangements in *E. coli*, it seems that these are selected during propagation in bacteria. The choice of vector and bacterial strain can influence infectivity of infectious cDNA clones (reviewed in Boyer and Haenni, 1994). Due to the lack of useful restriction enzymes for assembly of overlapping fragments in some regions of the GVA genomes, overlap extension PCR had to be used. This is not optimal, because this technique is based on PCR and in every round of amplification the chances for incorporation of mutations are increased.

When using a population cloning strategy, as was used in the second assembly attempt of GTR1-2, 10-12 clones were used during each assembly step. This improves the chances of obtaining a clone that is infectious. Even after making use of this strategy, no clones out of twelve were able to replicate to a detectable level after agro-infiltration in *N. benthamiana*. Full-length sequencing of one clone from this population identified two significant mutations, a 13 nt deletion and a premature stop codon in ORF 1. After correction of these mutations the resulting clone was able to replicate in *N. benthamiana* plants, but was not able to move systemically or induce symptoms. One nucleotide change from an Adenine (A) to a Guanine (G) at position 39 in ORF 5, resulting in an amino acid substitution from a Tyr/Y to a Cys/C at position 13, was identified that could give a possible explanation for the lack of systemic movement and symptoms. This was tested and results suggested that one amino acid change seems to have completely abolished replication in the ΔORF5-GTR1-2-13Y→C clone. This 13Y→C mutation will be investigated further in the future. Similar mutations in the GTR1-1 and GTG11-1 clones could also have rendered these non-infectious.

The Israeli variants, GR5 and GVA118 brought under control of a CaMV 35S promoter were able to replicate, move systemically an induce symptoms in *N. benthamiana* plants after agroinfiltration. The fact that cDNA clones of South African variants were assembled, but were not infectious (except for GTR1-2, containing 13Y→C mutation, that was able to replicate) prompted us to use the Israeli infectious clones for further studies. These clones were used as starting point for characterisation of expression and VIGS vectors in *N.*

benthamiana and *Vitis vinifera* (chapter 5) and elucidation of ORF 2 gene function (chapters 5 and 6).

Further experiments are needed for generation of South African GVA cDNA clones. The 13Y➔C mutation in the GTR1-2 clone will have to be corrected to confirm that this mutation is the cause of less efficient or abolished replication. The systemic movement of this clone was not assessed in this study. As for GTR1-1 and GTG11-1 variants, a population cloning strategy will have to be devised in assembly of cDNA clones even though limited success was found using this strategy. To circumvent possible incorporation of mutations it is also recommended to sequence as often as possible and to use restriction enzymes instead of PCR-based assembly techniques. A new trend has evolved in recent years where infectious clones are commercially synthesised *de novo*, although more expensive, to circumvent problems discussed (Saad Masri, Centre for Plant Health, Canada, pers. Comm.). As alternative, GR5 and GVA118 could be used as scaffold for the construction of GVA hybrids carrying different ORFs from South African variants. This could give more insight in the role that different genomic regions play in the aetiology of SD in the future.

4.6. REFERENCES

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Chapter 5: The characterisation of GVA vectors for transient expression and virus-induced gene silencing in *N. benthamiana* and *V. vinifera*

5.1. ABSTRACT

The development of tools for functional genomic studies in *Vitis vinifera* is an important step in grapevine genomics. *Grapevine virus A* is a candidate for use as expression and silencing vector in *Nicotiana benthamiana* (Nb) and *V. vinifera* (Vv). The function of the 20 kDa protein (P20) encoded by ORF 2 of GVA is still not known. Previous studies have shown that ORF 2 is not needed for replication or movement in *N. benthamiana*. The use of the full-length GVA cDNA clone (35S-GVA118; gene insertion vector) compared to an ORF 2 deletion mutant (35S-GVA-GR5-ΔORF2+sgMP; gene exchange vector) as expression and VIGS vector for both plant hosts was assessed. The GUS and PDS genes were used for expression and VIGS, respectively. In *N. benthamiana* both constructs showed comparable levels of GUS expression confirming that ORF 2 is not needed for replication and systemic movement in this host. It also showed that transgene expression from the subgenomic promoter of ORF 2 (sgORF2) is comparable to expression from the subgenomic promoter of the MP (sgMP) in *N. benthamiana*. Similar patterns of photobleaching were observed upon VIGS of NbPDS, for both vectors. In *V. vinifera* limited GUS expression levels and VIGS photobleaching symptoms were observed for the gene insertion vector, 35S-GVA118. No GUS expression was observed for the gene exchange vector 35S-GVA-GR5-ΔORF2+sgMP in this host. As for silencing, one plant, agroinfiltrated with 35S-GVA-GR5-ΔORF2-VvPDS+sgMP, developed photobleaching symptoms in 3 systemic leaves after 4 months. This study showed that GVA can be used as gene insertion and gene exchange vector for expression and VIGS *in N. benthamiana*, but in grapevine it's use is limited. It is also the first report that ORF 2 of GVA might not be needed for long distance movement in grapevine.

5.2. INTRODUCTION

Through the years, grapevine has been subjected to continuous selection of qualities that humanity have found appealing. Currently, a wealth of sequence information is generated by EST and genome sequencing projects performed on phylogenetically and agronomically important crops. The majority of these crops are not amenable to stable genetic transformation and are thus difficult to subject to functional genomics. Virus-induced gene silencing provides an attractive, powerful tool to enable functional analysis of genomes of these species (Becker & Lange, 2009). Recently, a high-quality sequence outline of the grapevine cultivar Pinot Noir has been published (Jaillon et al., 2007; Velasco et al., 2007). This leads to an increasing demand for functional genomic studies in this crop. The development of tools for this purpose

will lead to a better understanding of grapevine gene function and accelerate the breeding and selection of superior grapevines.

A number of plant viruses have been converted into expression and VIGS vectors for foreign protein expression (reviewed by Gleba et al., 2007) and silencing (reviewed by Senthil-kumar et al., 2008) of target genes in major crop plants. For grapevine, a possible candidate for this purpose is GVA. This virus naturally infects *Vitis vinifera* and the herbaceous host *Nicotiana benthamiana*. It was revealed that ORF 2 is dispensible for replication and movement in *N. benthamiana* (Galiakparov et al., 2003c). Two full-length T7-promoter driven infectious clones of GVA were reported earlier, namely GVA-GR5 and GVA118 (Haviv et al, 2006). The GR5 clone facilitated functional analysis of the GVA genome in *N. benthamiana* (Galiakparov et al., 2003c) and was engineered into an expression vector (GVA118) for herbaceous plants (Haviv et al, 2006). There was however some room for improvement on this vector. The GVA118 vector was under the control of a T7 promoter and was based on a gene insertion strategy. This vector hosts the full-length viral genome with the addition of a MCS and a duplicated subgenomic promoter of the MP for cloning and expression of the transgene. The loss of large transgenes was reported after the fourth plant-to-plant passage therefore the stability of this vector was problematic. By transferring GVA118 to CaMV 35S promoter control, it will be conducive to agroinfiltration. It has been reported that gene exchange vectors based on a full-length virus, where an endogenous gene has been substituted with a heterologous transgene, has advantages over gene insertion vectors (Nagyova & Subr, 2007). Viral genes involved in insect transmission that are not needed for replication or systemic movement of the virus, are useful targets for elimination. These vectors disable the horizontal transfer of the virus to other hosts, leading to increased control (Scholthof et al., 1996). Gene exchange vectors based on flexuous viruses, of which GVA is one, have the tendency to be packaged more efficiently than gene insertion vectors due to the smaller size. In the GVA genome, ORF 2 is a promising target for removal in the development of a gene exchange vector as it is not needed for replication and systemic infection in *N. benthamiana* (Galiakparov et al., 2003).

The aim of this study was to evaluate the use of GVA in either full-length (35S-GVA118; gene insertion vector) or ΔORF 2 (35S-GVA-GR5-ΔORF2+sgMP; gene exchange vector) state as a transient expression vector and a VIGS vector in *N. benthamiana* and *V. vinifera*. To construct the 35S-GVA-GR5-ΔORF2+sgMP gene exchange vector, a GVA cDNA clone

(35S-GVA-GR5; chapter 4) was modified by removing ORF 2 and substituting it with a polylinker containing unique restriction sites (35S-GVA-GR5ΔORF2). The resulting construct was subsequently engineered to include a subgenomic promoter of the MP (35S-GVA-GR5-ΔORF2+sgMP).

5.3. MATERIALS AND METHODS

5.3.1. Plant material

Cultivation of *N. benthamiana* plants were described in chapter 4.3.1. *V. vinifera* plantlets of the cultivars Sultana and Red Globe were micropropagated *in vitro* in Perlite containing liquid Murashige and Skoog (MS) medium. Plantlets were grown at 23ºC under artificial light, until the formation of roots at ~14-21 days. Plantlets with roots were subsequently used in vacuum agroinfiltration experiments.

5.3.2. Relevent standard molecular techniques

Relevent standard molecular techniques i.e. polymerase chain reaction, restriction enzyme digestion, cloning, sequencing etc. were performed as decribed in chapters 3 and 4.

5.3.3. DNA constructs

The T7-promoter driven GVA cDNA clones (T7-GVA-GR5 & T7-GVA118) were described in chapter 4 (Haviv et al., 2006). These clones were brought under control of an enhanced CaMV-35S promoter and the resulting 35S-casettes cloned into a modified pBIN19 vector (pBIN-e35S-GVA-GR5 & pBIN-e35S-GVA118, Figure 1; Chapter 4) for agroinfiltration experiments.

5.3.3.1. DNA contructs based on GVA118 (gene insertion vector)

The enhanced green fluorescent protein (EmGFP) containing 35S:EmGFP (Ghazala et al., 2008) and the intron containing GUS construct 35S:GUSi (Vaucheret et al., 1994) were described earlier. For transient expression experiments, the EmGFP gene and the GUS gene were PCR amplified from these constructs using primers containing *Not* I sites on flaps (EmGFP-F-*Not*/EmGFP-R-*Not* & *Not*I_GUS_s/*Not*I_GUS_as; table 1). These fragments were subsequently cloned into the *Not* I site of 35S-GVA118, resulting in 35S-GVA118-EmGFP (figure 1b) and 35S-GVA118-GUS [constructed by Marike Visser (Department of

Genetics, Stellenbosch University); figure 1c] respectively. For silencing, short fragments of the *N. benthamiana* phytoene desaturase (NbPDS) and *V. vinifera* phytoene desaturase (VvPDS) genes were amplified, from existing contructs, with primers containing *Not* I flaps (PDS-F-*Not*/PDS-R-*Not* & VvPDS-F-*Not*/VvPDS-R-*Not*; table 1) and cloned into the *Not* I site of 35S-GVA118. This resulted in the constructs 35S-GVA118-NbPDS (figure 1d) and 35S-GVA118-VvPDS (figure 1e). The allignment of the 363 bp VvPDS nucleotide sequence of the fragment used for silencing from different grapevine cultivars revealed a 99-100 % identity (Stephan et al., 2009) showing that this gene is highly conserved and that the same construct can be used for VIGS in different grapevine cultivars. The 35S-cassettes were transferred to pBIN_SN as described in section 4.3.5.1.
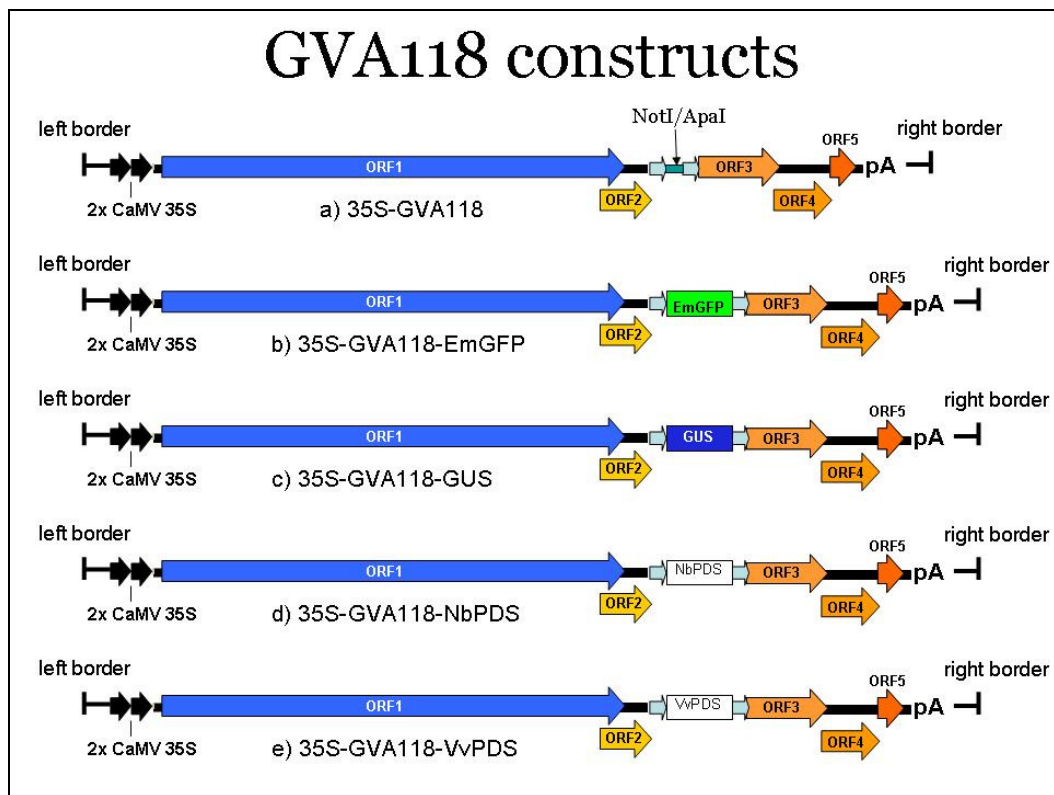
Figure 1. Graphic representation showing expression and silencing constructs based on the gene insertion vector GVA118. a) unmodified 35S-GVA118. All fragments were cloned into the *Not* I site of the MCS. b) 35S-GVA118-EmGFP containing the EmGFP gene. c) 35S-GVA118-GUS containing the GUS gene. d) 35S-GVA118-NbPDS containing a fragment of the NbPDS gene. e) 35S-GVA118-VvPDS containing a fragment of the VvPDS gene.

5.3.3.2. DNA constructs based on GVA-GR5 (gene exchange vector)

The e35S-GVA-GR5-pA cassette (chapter 4, figure 1A) was transferred to pBluescript II SKM to facillitate the use of *Aat* II and *Pst* I sites for cloning and generation of ΔORF 2 constructs. This was done by digestion of pSKM-e35S-GVA-GR5-pA with *Sna* BI/*Sal* I and ligation of resulting cassette into *Pst* I/*Sal* I digested pBluescript (native *Aat* II site eliminated in MCS during this process) resulting in pSKM-e35S-GVA-GR5-pA. The *Pst* I site was blunted with T4 DNA polymerase to generate a compatible end with *Sna* BI. Open reading frame 2 of the pSKM-e35S-GVA-GR5-pA construct was deleted by overlap extension PCR. This was accomplished as follows: Two overlapping PCR fragments were generated with primer pairs GVA-GR5-EF/GVA-GR5-IR and GVA-GR5-IF/GVA-GR5-ER. These overlapping fragments were joined in an overlap extension PCR using primers GVA-GR5-EF/GVA-GR5-ER (table 1). In this procedure, two unique restriction sites (*Sna* BI and *Bbv* CI) were incorporated to facilitate cloning of ORF 2 from GVA variants (chapter 6). The native ORF 2 start codon, at the 3'-terminal region of ORF 1, was silently mutated. The resulting fragment generated with overlap extension was digested with unique enzymes *Mlu* I/*Pst* I and ligated into similarly digested pGEM-T Easy to generate pGEM-1565. This construct was digested with *Aat* II and *Pst* I and the 1565 fragment ligated into similarly digested pSKM-e35S-GVA-GR5-pA to generate pSKM-e35S-GVA-GR5-ΔORF2-pA. The e35S-GVA-GR5-ΔORF2-pA cassette was digested out with *Not* I and *Sal* I and transferred to pBIN_SN, digested with *Not* I and *Xho* I, to generate pBIN-e35S-GVA-GR5-ΔORF2-pA (figure 2B).

In another construct, the sub-genomic promoter of the movement protein (sgMP) and a unique *Kpn* 2I restriction site were introduced into e35S-GVA-GR5-ΔORF2-pA via overlap extension PCR resulting in 35S-GR5-ΔORF2+sgMP (figure 2C). For expression and silencing experiments, the GUS gene, GFP gene, and short fragments of the NbPDS and VvPDS genes were incorporated into this construct using *Sna* BI and *Kpn* 2I sites, leading to 35S-GR5-ΔORF2-GUS+sgMP, GR5-ΔORF2-GFP+sgMP, 35S-GR5-ΔORF2-NbPDS+sgMP and 35S-GR5-ΔORF2-VvPDS+sgMP, respectively (figure 3). These 35S-cassettes were digested from the intermediate vector with *Not* I/*Sal* I and transferred to pBIN_SN digested with *Not* I/*Xho* I. For *trans* complementation experiments a pBIN61S-35S-GR5-ORF2 construct was made. ORF 2 of GVA variant GR5 was PCR amplified from 35S-GR5 using primers GR5-ORF2-F-*Sac*I and GR5-ORF2-R-*Xba*I. This PCR fragment was digested with *Sac* I/*Xba* I and cloned into pBIN61S that was digested with *Sac* I/*Xba* I.
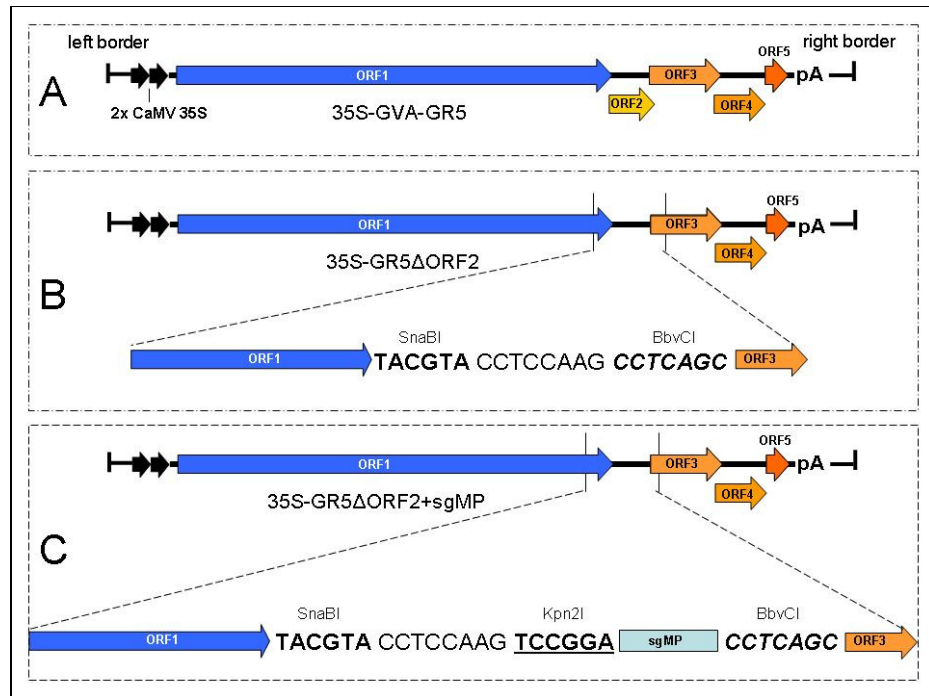
Figure 2. Graphic representation of modifications made to 35S-GVA-GR5 clone to facillitate ORF 2 substitution experiments (chapter 6) and to construct the gene exchange vector for transient expression and VIGS. A) Unmodified 35S-GVA-GR5, B) 35S-GVA-GR5-ΔORF2 constructed by deletion of ORF 2 and generation of *SnaBI-BbvCI* polylinker with overlap extension PCR. ORF 2 of South African GVA variants were cloned into this construct using *SnaBI* and *BbvCI*. C) The 35S-GVA-GR5-ΔORF2+sgMP gene exchange vector constructed with overlap extension PCR to include a unique *Kpn2I* restriction site and sgMP. GUS, NbPDS and VvPDS were cloned into this construct using *SnaBI* and *Kpn2I*.
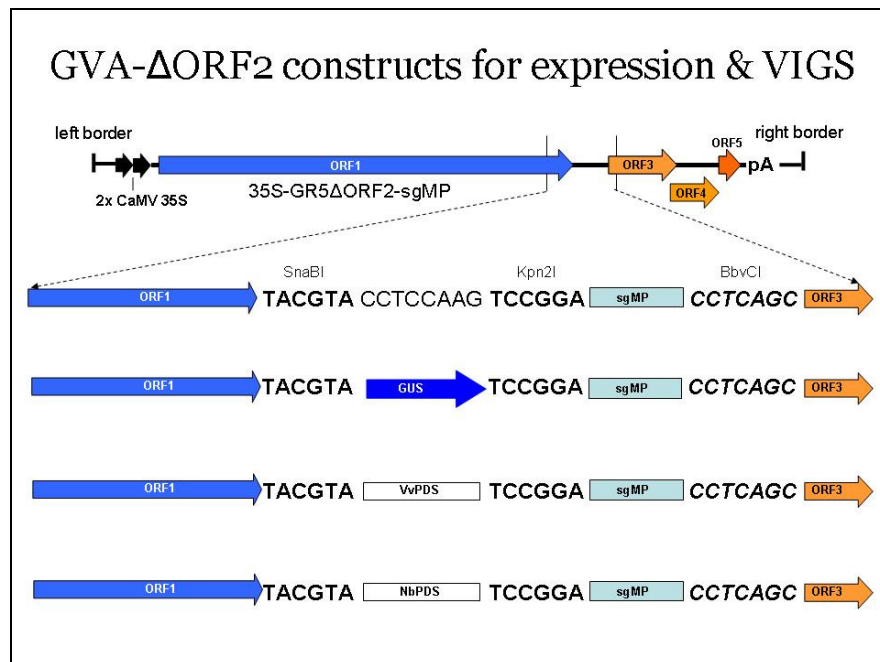
Figure 3. Graphic representation of the gene exchange vector 35S-GVA-GR5-ΔORF2+sgMP constructed with overlap extension PCR to include a unique *Kpn2I* restriction site and sgMP. GUS, NbPDS and VvPDS were cloned into this construct using *SnaBI* and *Kpn2I*.

5.3.4. Agroinfiltration

Agroinfiltration of *N. benthamiana* was performed as described previously in chapter 4.

N. benthamiana plants were infiltrated with an Agrobacterium-solution (strain C58CI + pCH32; Santos-Rosa *et al.*, 2008) containing 35S-GVA constructs using a 2 mL syringe (Voinnet *et al.*, 1998). Micropropagated *V. vinifera* plantlets were used for vacuum-agroinfiltration. Using a scalpel, 3-5 upper leaves of each plant were cut several times in the interveinal area. The leaf midrib and 2nd order veins, near the petiole onset, were also cut to allow direct infiltration into the phloem. Cut plantlets were submerged into a suspension containing recombinant *A. tumefaciens* (VvPDS: $OD_{600} = 0.1$; Other constructs: $OD_{600} = 0.5$). By using a BioRAD PDS1000 Gene Gun, the plantlets were exposed to a strong vacuum ($\sim$90 kPa) for 2 x 2 minutes, followed by a sterile water rinse step and subsequent transfer into a tub containing perlite and water. Plantlets were cultivated at 23ºC under artificial light conditions. *V. vinifera* plantlets were not co-infiltrated with 35S:BMYV-P0 containing the strong viral suppressor P0 of BMYV, (Stephan & Maiss, 2006) because this suppressor only functions in herbaceous plants. No suppressor that functions in grapevine was available for use in this study.

5.3.5. TPIA, GUS assay, and GFP detection

At 6-7 dpi Tissue-print Immuno-assay (TPIA; Franco-Lara et al., 1999) was performed on infiltrated *N. benthamiana* leaves to detect GVA-CP in mesophyll leaf tissue (see chapter 4). At 5-6 dpi, GUS assay was performed on detached infiltrated leaves (Jefferson, 1987). GUS staining solution was vacuum infiltrated using the BioRAD PDS1000 Gene Gun as described above. Pictures of whole leaves were taken with a Canon IXUS 90 IS camera using the macro function. Pictures of single cell clusters showing GUS expression were taken with a Zeiss HBO 100 Axioskop microsocope with an attached Nikon Digital Sight DS-SM camera. At 3-4 dpi, GFP detection was performed with the Zeiss HBO 100 Axioskop microsocope with an attached Nikon Digital Sight DS-SM camera using a GFP/dsRed filter combination.

5.3.6. Total RNA extraction, RT-PCR and sequencing to detect GVA constructs in apical leaves of infiltrated plants

In order to detect viral progeny of infiltrated GVA constructs in *N. benthamiana* and *V. vinifera* plants, 2 apical leaves per plant were harvested and 100 mg leaf material used in total RNA extraction (White et al., 2008). RNA was quantified using the NanoDrop 1000 spectrophotometer, and run on a 1 % Agarose/TAE gel to assess integrity. RNA was DNAse treated (1 h, 37ºC) and 400ng-1μg used in Two-step RT-PCR (This was performed as described in chapter 3). The primer pair GVA-GR5-4344F and GVA-GR5-ER (table 1) was used for constructs based on GVA-GR5 to generate RT-PCR fragments. To detect constructs based on GVA118, the primer pair GVA-GR5-5781-F and GVA-GR5-6188-R (table 1) was used. Fragments were seperated on a 1% Agarose/TAE gel, excised using a scalpel, and gel extracted using the Zymoclean gel DNA recovery kit™ (Zymo Research) according to the manufacturer's instructions. Extracted fragments were quantified with the NanoDrop 1000 spectrophotometer. Ten ng/μL dilutions were made and used as direct template for sequencing with primer GVA-GR5-ER or GVA-GR5-6188-R for GVA-GR5 and GVA118 respectively. Sequencing and sequencing analysis were performed as described in chapter 3.

5.3.7. Quantitative Reverse Transcription Real-Time PCR (qRT-PCR)

In this study qRT-PCR optimisation and analysis were performed by Melanie Grobbelaar (Department of Genetics, Stellenbosch University).

The Ubiquitin reference gene primers (ubi3-For and ubi3-Rev, table 1; Rotenberg et al., 2006) were used for generation of three independent standard curves. Primers designed to anneal at another region (than is present in the NbPDS fragment in construct 35S-GVA-GR5-ΔORF2-NbPDS+sgMP) in the NbPDS gene (NbPDS-s1 and NbPDS-as1, table 1) were used for generation of three independent standard curves for this gene. To generate each standard curve, a 4-fold dilution serial dilution of total RNA was made (568ng – 0.55ng) and amplified in triplicate using the RotorGene 6000™ real time thermal cycler. The individual standard curves was covered by a linear range of six orders of magnitude. For each reaction 1μl of each RNA dilution was added into 25μl of qRT-PCR reaction mix (1X Sensi mix One step, 1X SYBR® Green, 5U RNase inhibitor, 0.2μM forward primer, 0.2μM reverse primer) and amplified using the following cycling conditions: one hold at 42°C for 45min, one hold at 95°C for 10min, 40 cycles of 95°C for 20sec, annealing at 55°C for 20sec, elongation at 72°C

Table 1. Characteristics of primers used in this study.

Construct	Primers used	Sequence (5'-3')
35S-GR5-ΔORF2	GVA-GR5-EF	ATAGACGAGGAGGGGTATAGCGGGGAAGAGGCCAATC
	GVA-GR5-IR	GCTGAGGCTTGGAGGTACGTATCAACCAACTCTGTACAACTTTGCGATGTCGTTACTC
	GVA-GR5-IF	TACGTACCTCCAAGCCTCAGCATGTCGCACGGAGATTCT
	GVA-GR5-ER	ATTTCGGTCGTAGCCACTTTCGG
35S-GR5-ΔORF2+sgMP[b]	GVAsgMP_F[a]	ATACGTAGAGGGTCCGGATTGGTTACATTCCAACCG
	GVAsgMP_Rnew[a]	AGCTGAGGAGAACCTGCGTCGCCTTTG
35S-GR5-ΔORF2-GUS+sgMP	GUSiStuI_F[a]	AAGGCCTATGTTACGTCCTGTAGA
	GUSiKpn2I_R[a]	ATCCGGATCATTGTTTGCCTCCCT
35S-GR5-ΔORF2-GFP+sgMP	EmGFPORF2+5_F[a]	ATACGTAATGGTGAGCAAGGGCGA
	EmGFPORF2_R[a]	ATCCGGATTACTTGTACAGCTCGTCC
35S-GR5-ΔORF2-NbPDS+sgMP	NbPDS-F-SnaBI	ATTACGTAACGTCGACATATATGGACA
	NbPDS-R-Kpn2I	ATTCCGGATTCAGGTTAGAATCCCGAT
35S-GR5-ΔORF2-VvPDS+sgMP	VvPDS-F-SnaBI	ATTACGTATTTCAGAACTCCCAATGTAC
	VvPDS-RKpn2I	ATTCCGGATCTTGCTTCCAACAATAT
35S-GR5-ORF2	GR5-ORF2-F-SacI	ATGAGCTCATGACATCGCAAAGTTGT
	GR5-ORF2-R-XbaI	ATTCTAGATTAATTTCCTTGGGATCT
35S-GVA118-GUS[c]	NotI_GUS_s[d]	aagcggccgcATGTTACGTCCTGTA
	NotI_GUS_as[d]	aagcggccgcTCATTGTTTGCCTCC
35S-GVA118-EmGFP	EmGFP-F-Not	GCGGCCGCATGGTGAGCAAGGGCGAGGA
	EmGFP-R-Not	GCGGCCGCTTACTTGTACAGCTCGTCCATGCC
35S-GVA118-NbPDS	PDS-F(NotI)	GCGGCCGCACGTCGACATATATGGACA
	PDS-R(NotI)	GCGGCCGCTTCAGGTTAGAATCCCGAT
35S-GVA118-VvPDS	VvPDS-R(not)	GCGGCCGCTTTCAGAACTCCCAATGTAC
	VvPDS-F1(not)	AGCGGCCGCTTTCAGAACTCCCAATGTAC
Detection and sequencing of GVA-GR5 constructs	GVA-GR5-4344F	GCCCACTCCGTGCTTTGTAG
	GVA-GR5-ER	ATTTCGGTCGTAGCCACTTTCGG
Detection and sequencing of GVA118 constructs	GVA-GR5-5781-F	ATGTCCTCAGAAGATTCTCA
	GVA-GR5-6188-R	CATGAGGCTTCTTGAGATTC
Ubiquitin gene (qRT-PCR)	ubi3-For	GCCGACTACAACATCCAGAAGG
	ubi3-Rev	TGCAACACAGCGAGCTTAACC
PDS gene (qRT-PCR)	NbPDS_s1	ATGCCCCAAATCGGACTTGT
	NbPDS_as1	ACAAACCACCCAAACCTGCA

for 30sec, a hold of 72°C for 5min and a final melting step rising with 1°C each step from 72°C to 95°C. To determine whether NbPDS was silenced using the 35S-GVA-GR5-ΔORF2-NbPDS+sgMP construct, 4 plants were agroinfiltrated with this construct and compared to 2 plants infiltrated with 35S-GR5. Total RNA was extracted from plants at 24 dpi, 125ng of each sample ampified in duplicate and the results compared to the generated standard curves. The relative expression of each target gene was determined with the Relative expression software tool (REST©; Pfaffl et al., 2002).

5.4. RESULTS AND DISCUSSION

5.4.1. Biological characterisation of constructs based on GVA118 (gene insertion vector)

5.4.1.1 Transient expression and VIGS in *N. benthamiana*

Typical GVA symptoms were observed on *N. benthamiana* plants as early as 3-4 dpi after agroinfiltration of 35S-GVA118 (figure 4A). These symptoms included vein clearing, interveinal chlorosis, necrosis, stunting of plants and downward rolling of leaves. Mock inoculated plants stayed symptom-free. TPIA performed at 6-7 dpi was able to detect GVA-CP confirming that this clone is replicating (chapter 4). Co-infiltration (1:1) of 35S-GVA constructs with 35S:BMYV-P0 containing the strong viral suppressor P0 of BMYV (Pfeffer et al., 2002) enhanced replication and improved detection of GVA-CP through TPIA (chapter 4, figure 6). Intact GVA particles were also detected by electronmicroscopy (figure 4B). RT-PCR and sequencing performed on total RNA extracted from apical leaves showed systemic movement of the virus in all plants infiltrated, with the exception of the mock inoculated plants. The same was shown for 35S-GVA118-GUS, 35S-GVA118-EmGFP, 35S-GVA118-NbPDS and 35S-GVA118-VvPDS. Although, for these clones, the symptoms developed a few days later (7-11 dpi), TPIA performed at 6-7 dpi was sufficient to confirm replication of the virus by detection of GVA CP in infiltrated tissue. GUS assay performed on leaves infiltrated with 35S:GUSi and 35S-GVA118-GUS showed high levels of GUS expression in the infiltrated tissue confirming that 35S-GVA118 is an efficient transient expression vector for *N. benthamiana* (figure 5). Systemic GUS expression was observed at 17 dpi in systemic leaves of infiltrated plants. The stability of this vector was not assessed as this was already published earlier (Haviv et al., 2006). GFP expression was observed in infiltrated tissue of plants agro-infiltrated with 35S:EmGFP (Ghazala et al., 2008; data not shown), but no GFP expression could be observed for 35S-GVA118-EmGFP. EmGFP was PCR amplified from an existing functional EmGFP clone. Upon sequencing, no amino acid changes were observable

in the GFP ORF to suggest loss of function and consequently no further EmGFP expression experiments were pursued. Silencing of GFP was shown with the construct 35S-GVA118-EmGFP in GFP-transgenic *N. benthamiana* (16C) plants (Brigneti et al., 1998; Ruiz et al., 1998). The first silencing phenotypes were observed on leaves of infiltrated plants at 11 dpi. Silencing of transgenic GFP was initially confined to major veins of leaves and was visible by a reduction of GFP fluorescence, leading to an enhanced detection of red chlorophyll fluorescence under UV-light (figure 4C). The development of the silencing phenotype was monitored at regular intervals and at 40 dpi, most of the leaves exhibited silencing. Some leaves showed complete silencing of GFP and only the red fluorescence of chlorophyll could be detected. For the 35S-GVA118-NbPDS construct, photobleaching symptoms were observable 11-14 dpi and were mostly confined to veins and interveinal areas of leaves of *N. benthamiana* (figure 4D). After 24 dpi, most of the leaves showed photobleaching symptoms with some leaves having turned completely white. These findings correlated with the silencing phenotypes described by Muruganantham et al. (2009) for silencing of NbPDS with this construct. They confirmed silencing of NbPDS with semiquantitative PCR and showed a reduction in the endogeneous PDS mRNA transcript in the photobleached tissue. They also showed that the viral RNA accumulation levels were higher in silenced tissue than in non-silenced tissue and that the photobleached phenotype was not dependent on the specific subgenomic promoter (Muruganantham et al., 2009). The test whether the 35S-GVA118-VvPDS (constructed for silencing in *V. vinifera*) construct was infectious, it was infiltrated into *N. benthamiana*. Typical GVA symptoms were observed and the modified virus moved systemically, but did not result in any photobleaching. The lack of silencing was expected as a nucleotide sequence comparison revealed a 78.8% identity between the VvPDS and the NbPDS genes, but no identical 23-25mer stretches were present in the short VvPDS fragment incorporated into the GVA118 vector, when aligned to NbPDS. For silencing to occur there should be a 100% homologous region of at least 23 nucleotides present in the fragment used to induce silencing (Thomas et al., 2001).
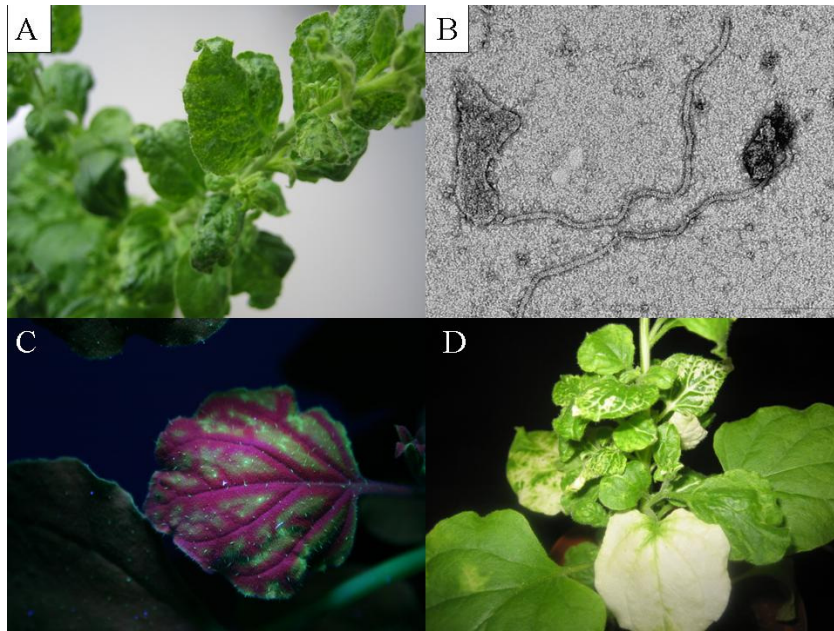
Figure 4. A) Symptoms observed on *N. benthamiana* plants agroinfiltrated with 35S-GVA118. B) Electronmicrograph showing GVA virus particles from *N. benthamiana* infiltrated with 35S-GVA-GR5 (chapter 4). C) GFP silencing phenotype on *N. benthamiana* (16c) plants agroinfiltrated with 35S-GVA118-EmGFP. D) Photobleaching observed in *N. benthamiana* plants agroinfiltrated with 35S-GVA118-NbPDS.
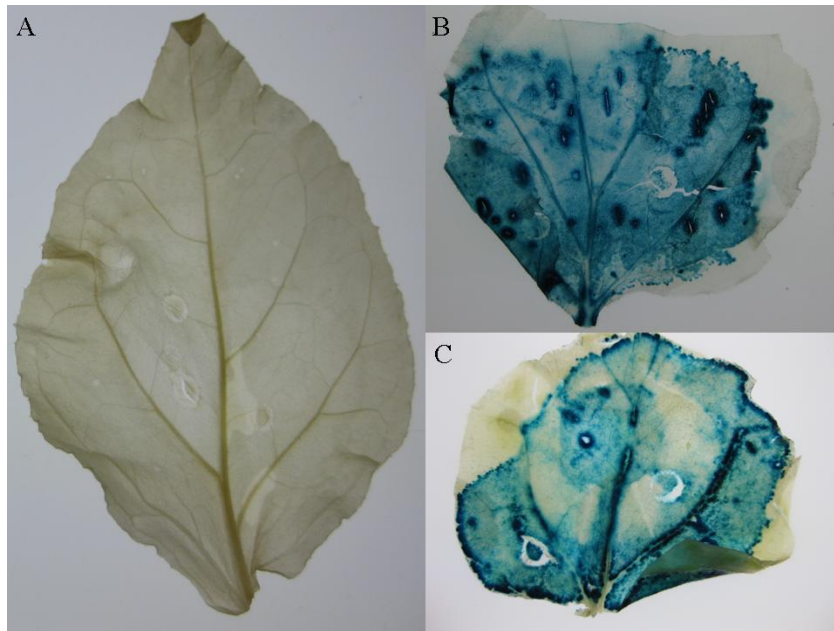

Figure 5. Results of GUS assay performed 6-7 dpi on infiltrated *N. benthamiana* leaves A) Photograph of a leaf agroinfiltrated with 35S-GVA-GR5 showing no GUS expression B) Photograph of a leaf agroinfiltrated with 35S-GUS showing GUS expression. C) Photograph of a leaf agroinfiltrated with 35S-GVA118-GUS showing GUS expression comparable to 35S-GUS.

Upon vacuum-agroinfiltration of 35S-GVA118 into *V. vinifera* cv Sultana, the detection of the virus by RT-PCR using generic GVA primers was problematic at first. Muruganantham et al., (2009) also reported difficulty in GVA118 detection by one-step RT-PCR in the cultivar Prime. Improved detection was obtained by using a GVA118-specific primer pair GVA-GR5-5781-F and GVA-GR5-6188-R (table 1). Infiltration of 35S-GVA118-GUS showed limited GUS expression in infiltrated leaves at 6-7 dpi. GUS expression was confined to major leaf veins and tissue adjacent to veins (figure 6). This expression pattern could be due to the phloem-limitation of GVA in that the virus can possibly only replicate in phloem and phloem-associated cells in the grapevine host. It is also possible that the GUS distribution pattern was caused by dispersion of this protein from cells, in which the virus is replicating, to adjacent cells after infiltration of the GUS colouring solution. Low GUS expression levels indicated that 35S-GVA118 is not optimal in the current state for use as high level transient expression vector in all cells of *V. vinifera*. This vector could however prove useful for specialised protocols where confined expression of target proteins are needed in the phloem tissue. A study by Chiba et al., (2006) has shown that co-infiltration of viral expression constructs with strong silencing suppressors can enhance replication and transgene expression by up to 10 000 fold after agroinfiltration of plants. Co-infiltration of suppressors that function in grapevine, such as the P24 of *Grapevine leafroll-associated virus-2* (GLRav-2) with 35S-GVA118-GUS could increase transgene expression in the same way. In Sultana plantlets infiltrated with the 35S-GVA118-VvPDS constructs, limited systemic silencing symptoms (only 2 leaves per plant) were observed in 20-50 % of plants, varying between different rounds of infiltration. The photobleaching symptoms were mainly confined to veins and spread only minimally into the interveinal areas (figure 7). This could be due to the replication of GVA mainly in phloem tissue. The development of silencing symptoms varied between different rounds of agroinfiltration. Silencing symptoms could be observed as early as 2 weeks post infiltration. GVA was detected in leaves showing silencing symptoms by RT-PCR (data not shown). Muruganantham et al., (2009) used an agrodrenching method to deliver the 35S-GVA118 vector (named pGVA378 in their study), carrying a 304 nt fragment of the VvPDS gene into *V. vinifera* cv Prime. They reported silencing symptoms at 14 to 20 dpi, in 40-60 % of plants infiltrated. Photobleaching symptoms were initially confined to leaf veins and in a few instances progressed into silencing of the complete leaf. They reported that the silencing symptoms were only observed on ~3 leaves per plant and suggested that this could be due to GVA-mediated silencing suppression of VIGS. In their report they showed

silencing of VvPDS with semiquantitative PCR. In the current study using *V. vinifera* cv Sultana plants, no leaves turned completely white due to silencing of VvPDS mediated by 35S-GVA118-VvPDS. Possible reasons for the difference in photobleaching symptoms could be related to the agro-delivery method, the grapevine cultivar used, or the length and position (within the gene) of the VvPDS fragment incorporated into the GVA VIGS vector. The Agrodrenching method (Ryu et al., 2004) where Agrobacterium is delivered directly to plant roots, might be a more effective method than vacuum agro-infiltration of leaves and could possibly lead to better silencing. Different grapevine cultivars might have a difference in cell or tissue physiology that subsequently could influence spread and replication of GVA, thereby influencing the efficiency of VIGS. A recent study using Apple latent spherical virus VIGS vectors to silence PDS in tobacco revealed that the length and position of the fragment used to induce silencing, is important and can result in a variation of silencing phenotypes (Igarashi et al., 2009). The difference in length and region of the VvPDS gene used in the current study, when compared to Muruganatham et al. (2009) could explain the variation seen in the photobleaching patterns in *V. vinifera*.
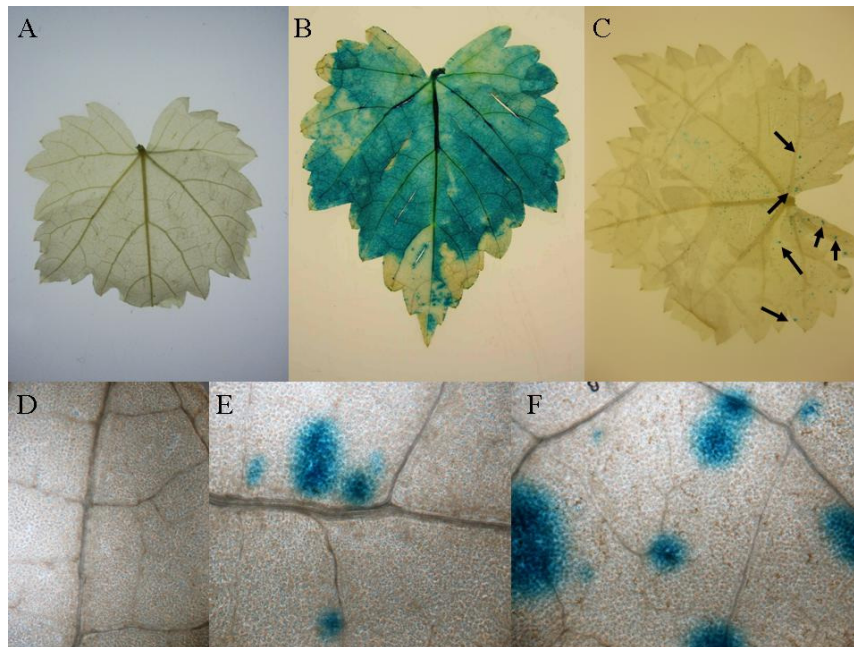
Figure 6. A) *V. vinifera* cv Sultana leaf infiltrated with GVA-GR5 showing no GUS expression. B) 35S-GUS positive control. C) Limited GUS expression (indicated by arrows) observed at 6 dpi in *V. vinifera* plant infiltrated with 35S-GVA118-GUS. D) Negative leaf shown at 100 x magnification. E & F) 35S-GVA118-GUS infiltrated leaf shown at 100 X magnification. GUS expression was confined to major leaf veins and cells adjacent to veins.
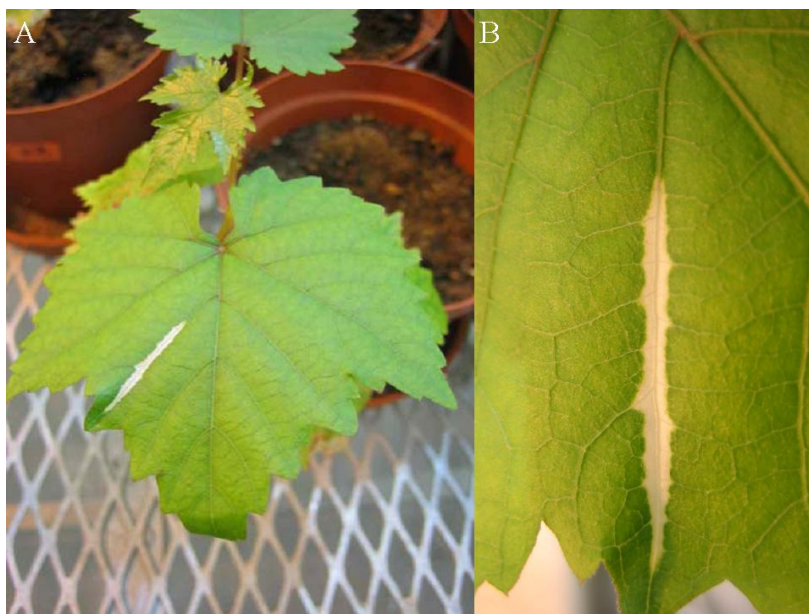
Figure 7. A) Photobleaching phenotype observed on a systemic *V. vinifera* cv. Sultana leaf. Silencing was confined to leaf veins and spread minimally into interveinal area. B) Close up of leaf shown in A (Pictures taken by D. Stephan).

5.4.2. Biological characterisation of constructs based on GVA-GR5-ΔORF2+sgMP (gene exchange vector)

### 5.4.2.1. Transient expression and VIGS in *N. benthamiana*

Upon agroinfiltration of 35S-GVA-GR5-ΔORF2+sgMP in *N. benthamiana* typical GVA symptoms developed 3-4 dpi. Typical GVA-GR5-induced symptoms which included vein clearing, interveinal chlorosis, necrosis, stunting of plants and downward rolling of leaves were observed. As was expected from literature (Galiakparov et al., 2003c), the modified virus resulting from this deletion construct was able to replicate, and move systemically throughout the plant without ORF 2. Introduction of the GUS gene into 35S-GVA-GR5-ΔORF2+sgMP leading to 35S-GVA-GR5-ΔORF2-GUS+sgMP did not affect any of the aforementioned parameters, but symptom development was delayed and appeared at 10-12 dpi when compared to 35S-GVA-GR5-ΔORF2+sgMP. This was not strange as studies have shown that modified viruses differed from their parental viruses at the tempo at which symptoms appeared in plants (van der Walt et al., 2008). The delayed appearance of symptoms have been correlated to the slower cell-to-cell movement of modified viruses within plants (Choi et al., 2005). High GUS expression levels (figure 8A) were observed 6-7 dpi in the infiltrated tissue which was comparable to the expression levels and pattern seen for 35S-GVA118-GUS. This shows that the subgenomic promoter of ORF 2 (sgORF2) drives

high level transgene expression in the gene exchange vector. Systemic GUS expression was observed at 16 dpi in apical leaves of infiltrated plants showing that the GUS gene is not knocked out in the viral progeny (figure 8B). Stability of the construct through several plant-to-plant passages was not investigated. In infiltrated leaves of *N. benthamiana*, GUS expression was observed in all infiltrated tissue, suggesting that GVA can replicate in non-phloem associated cells in this host. Systemic GUS expression was only confined to major leaf veins and clusters associated to the phloem tissue highlighting the long distance movement of GVA via the phloem. Delayed symptom development was also observed with the silencing constructs 35S-GVA-GR5-ΔORF2-NbPDS+sgMP and 35S-GVA-GR5-ΔORF2-VvPDS+sgMP. These constructs were able to replicate and move systemically throughout infiltrated plants. Photobleaching symptoms confined to leaf veins were observed 11-14 dpi for 35S-GVA-GR5-ΔORF2-NbPDS+sgMP. After 24 dpi, all plants showed these symptoms in most leaves (figure 9). The confinement of the photobleaching phenotype primarily to the phloem, could be due to the presence of the virus mainly in this tissue and the long-distance spread of the systemic silencing signal through the phloem system (Kalantidis et al., 2008). Total RNA was extracted and used in qRT-PCR to assess whether this photobleaching phenotype is due to a reduction in NbPDS mRNA. Three independent standard curves were generated for the Ubiquitin and NbPDS genes. The generated curves met the reliability criteria specified by a reaction efficiency of 1.0-0.8, a slope value (M) between -3.6 and -3.2 and a correlation coefficient (R^2 value) of 0.99 (Yuan et al., 2006). R^2 values for Ubiquitin and NbPDS were 0.99 and 0.98 respectively (figure 10). REST© analysis of the data suggested that there was ~50% reduction of NbPDS mRNA in 3 out of 4 plants showing the photobleaching phenotype when compared to NbPDS mRNA levels in plants infiltrated with 35S-GR5 (table 2, figure 11; Grobbelaar, 2009). As expected for 35S-GVA-GR5-ΔORF2-VvPDS+sgMP, no silencing symptoms developed in *N. benthamiana* due to the same reasons discussed in section 5.4.1.1.
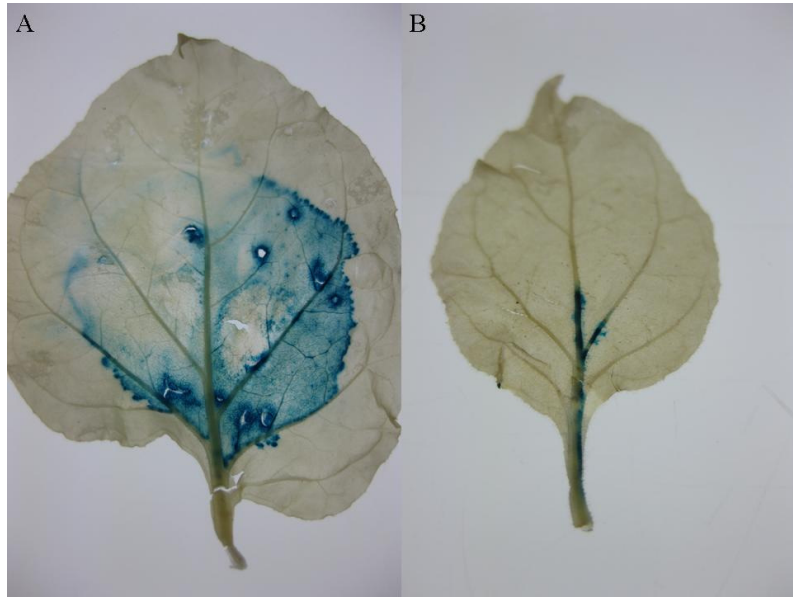
Figure 8. A) Photograph of a *N. benthamiana* leaf agroinfiltrated with 35S-GVA-GR5-ΔORF2-GUS+sgMP showing local GUS expression 7 dpi, B) GUS expression 16 dpi in a systemic infected leaf.
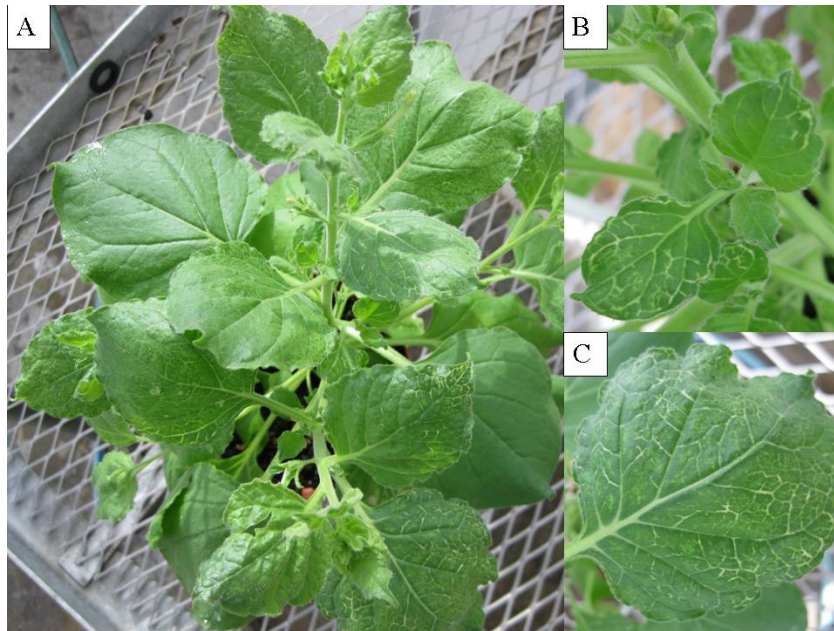

Figure 9. Photobleaching symptoms observed in *N. benthamiana* plant infiltrated with 35S-GR5-ΔORF2-NbPDS+sgMP. A) whole plant B) younger leafs C) older leaf. Silencing symptoms were mostly confined to leaf veins.
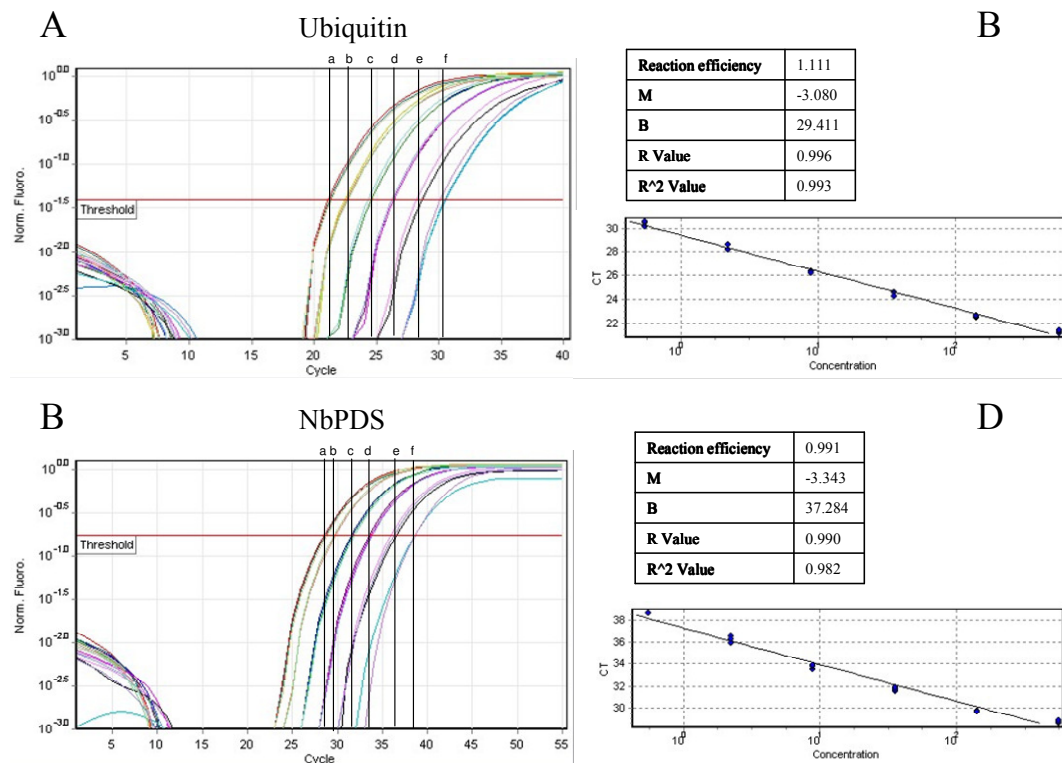
Figure 10: Standard curves of Ubiquitin and NbPDS for qRT-PCR amplification. (A and C) Amplification plot of the Ubiquitin and NbPDS primer sets showing a 4-fold triplicate dilution series containing total control RNA: a) 568.4ng: b) 142.1ng: c) 35.5ng: d) 8.88ng: e) 2.22ng: f) 0.55ng. R value: square root of correlation coefficient, R^2 Value: correlation coefficient, M: slope of each standard curve, B: intercept of the standard curve.   (B and D) A standard curve was generated where the Ct-values were plotted against the log in vitro RNA concentrations (ng/reaction) produced from triplicate dilution series (Grobbelaar, 2009).

Table 2: REST© value analysis compared with different NbPDS standard curves (Grobbelaar, 2009). GR5/1 and GR5/2 refers to plants infiltrated with 35S-GR5. These plants did not show any signs of photobleaching. NbPDS/1-4 refers to plants infiltrated with 35S-GVA-GR5-ΔORF2-NbPDS+sgMP. These plants showed photobleaching symptoms mostly confined to veins of the leaf. When comparing the average REST values of the GR5 with NbPDS, a reduction of NbPDS is observed in 3 out of the 4 plants, signifying the silencing of NbPDS.

| REST© values						
	GR5/1	GR5/2	NbPDS/1	NbPDS/2	NbPDS/3	NbPDS/4
PDS standard curve 1	0.821	0.724	0.362	0.398	0.635	0.365
PDS standard curve 2	0.785	0.599	0.361	0.42	0.603	0.342
PDS standard curve 3	0.892	0.763	0.37	0.392	0.662	0.356
Average	**0.833**	**0.695**	**0.364**	**0.403**	**0.633**	**0.354**

NbPDS expression levels
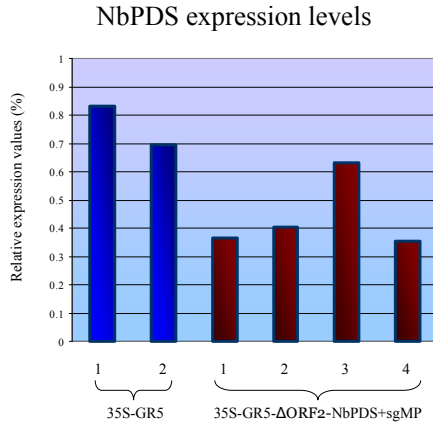
Figure 11: Bar chart showing the expression values of the NbPDS gene relative to the Ubiquitin reference gene (Grobbelaar, 2009). Blue bars represent 2 plants infiltrated with 35S-GR5 which did not show photobleaching symptoms. Brown bars represent 4 plants infiltrated with 35S-GVA-GR5-ΔORF2-NbPDS+sgMP which showed photobleaching symptoms mostly confined to veins of the leaf. When comparing the average REST values of the GR5 with NbPDS, a reduction of NbPDS is observed in 3 out of the 4 plants, signifying that NbPDS is silenced. The relative expression of NbPDS is indicated by a percentage of the Ubiquitin expression levels, which is standardised by the REST software to a 100% level in all plants tested.

### 5.4.2.2. Transient expression and VIGS in *V. vinifera*

No local GUS expression could be observed (6-9 dpi) in infiltrated leaves for 35S-GVA-GR5-ΔORF2-GUS+sgMP after vacuum-agroinfiltration of Sultana and Red Globe plantlets (figure 12B-D). This was unexpected as with the same construct high GUS expression levels was observed in *N. benthamiana*. High GUS expression levels were also observed in grapevine plantlets infiltrated with 35S:GUSi, showing that the infiltration procedure was efficient (figure 12A). Heterologous protein expression in the gene exchange vector is facillitated by the sgORF2, which was shown to be active in most tissues in *N. benthamiana* (section 5.4.1.1). In grapevine this promoter might only be functional in certain tissues or the ORF 2 protein product might only be expressed during a certain stage in the viral lifecycle. When driving GUS expression from the sgMP in the gene insertion vector (35S-GVA118), limited expression was observed only associated to the phloem in grapevine. The MP is needed during early stages of virus infection for movement mainly in the phloem, and the sgMP could possibly also only function in this tissue, giving another possible explanantion for the GUS distribution pattern. To assess whether the lack of GUS expression was due to the absence of the ORF 2 protein a pBIN61S-35S-GR5-ORF2 construct was co-infiltrated with 35S-GVA-GR5-ΔORF2-GUS+sgMP. This experiment did not show *trans*-complementation with the ORF 2 protein and no GUS expression was observed in the infiltrated tissue. This

showed that the lack of GUS expression was not due to the absence of the ORF 2 protein and preliminary results suggest that this protein does not function as a suppressor of silencing. The expression of the ORF 2 protein in grapevine from the 35S-GR5-ORF2 was not confirmed serologically, however. Co-infiltration of an unmodified 35S-GR5 clone with 35S-GVA-GR5-ΔORF2-GUS+sgMP also did not lead to any GUS expression. As no GUS expression was observed with 35S-GVA-GR5-ΔORF2-GUS+sgMP in grapevine, it seems the sgORF 2 might not be optimal for heterologous transient protein expression in this host. The lack of GUS expression could also be explained by the fact that *V. vinifera* plantlets were analysed for GUS expression at 6-9 dpi and not at later stages. Only one *V. vinifera* cv Sultana plantlet was agroinfiltrated with 35S-GVA-GR5-ΔORF2-VvPDS+sgMP, because taking into account our previous GUS expression experiments, no silencing was expected to occur without the presence of ORF 2. The plant was monitored for the development of silencing symptoms over a period of time. Only after 4 months 3 systemic leaves showed photobleaching symptoms (figure 13) similar to phenotypes observed for 35S-GVA118-VvPDS. Total RNA extraction from these leaves followed by RT-PCR confirmed the presence of 35S-GVA-GR5-ΔORF2-VvPDS+sgMP in this plant (data not shown). As this is only a single plant, no conclusions can be drawn until a repetition with more plants is performed. These silencing results, however show that the removal of ORF 2 in grapevine, does not prevent the virus from replicating and moving systemically throughout the plant.
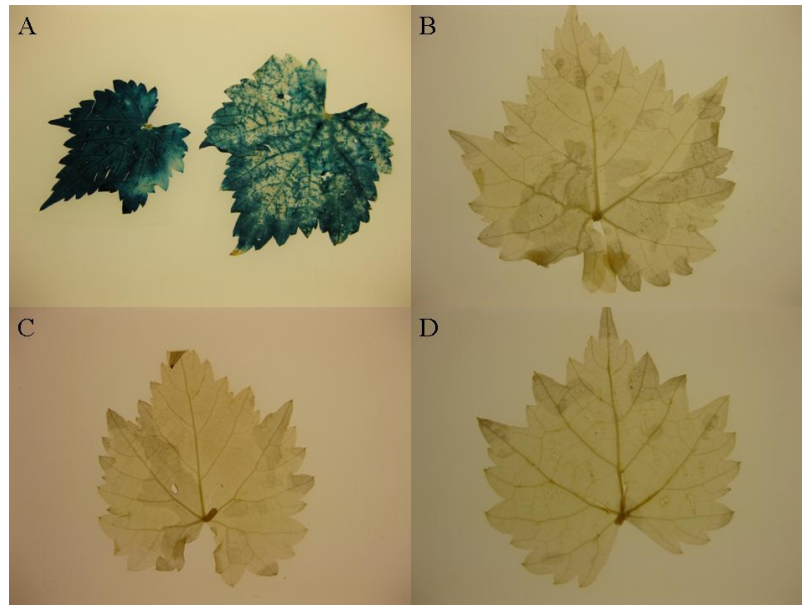
Figure 12. A) Photograph showing GUS assay performed 6 dpi on grapevine leaf infiltrated with 35S-GUS, B,C & D) Photographs showing GUS assay performed 6 dpi on grapevine leaves infiltrated with 35S-GVA-GR5-ΔORF2-GUS+sgMP.
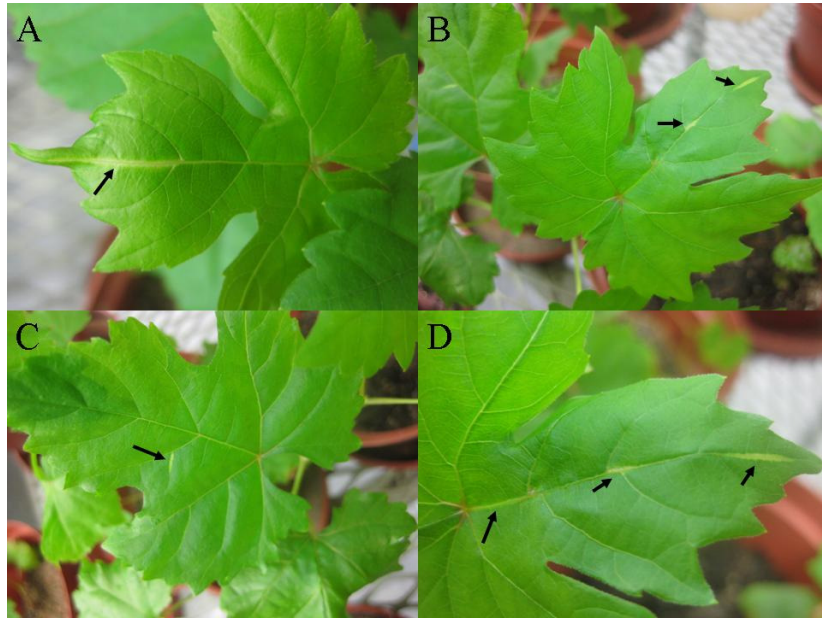
Figure 13. Photographs showing silencing symptoms observed in grapevine with the gene exchange vector 35S-GVA-GR5-ΔORF2-VvPDS+sgMP. A-C) Photobleaching phenotype (indicated by arrows) observed on systemic *V. vinifera* cv. Sultana leaves. Silencing was confined to leaf veins and spread minimally into interveinal areas. D) Close up of leaf shown in B.

5.5. CONCLUSIONS

Grapevine virus A seemed like an ideal candidate for utilisation as expression and VIGS vector in *N. benthamiana* and *V. vinifera* as it is well studied and can use both these plant species as host. Functional analysis of the GVA genome was performed earlier in *N. benthamiana* and putative functions could be assigned to all ORFs except for ORF 2. It was reported that ORF 2 was not essential in the herbaceous host and could be removed for the development of a new GVA VIGS and expression vector based on the gene exchange strategy. A gene insertion vector based on the full-length GVA genome (GVA118) was compared to the gene exchange vector of GVA that did not contain an ORF 2 (GVA-GR5-ΔORF2+sgMP) for expression and VIGS in *N. benthamiana* and *V. vinifera*. These vectors however also contained ORFs 3-5 of different GVA variants as was described in section 4.2. In *N. benthamiana*, both vectors were able to replicate and move systemically throughout the plant and high GUS expression levels were observed locally and systemically. Both vectors were comparable in their application to VIGS in *N. benthamiana*. Silencing symptoms however, were mostly confined to veins and areas adjacent to veins, which could be due to the phloem-association of the virus. The insertion of the PDS fragment, used in VIGS, at the two different positions in the GVA genome (when using the two different vectors) does not seem to change the resulting photobleaching phenotype. In *V. vinifera*, the use of GVA as

expression and VIGS vector is less-efficient. Limited GUS expression and silencing, only confined to major veins and adjacent tissues, were observed with the gene insertion vector (GVA118) and this could be due to the phloem limitation of the virus and the spatial activity of the sgMP. In contrast to *N. benthamiana*, no GUS expression could be observed when using the gene exchange vector GVA-GR5-ΔORF2+sgMP in grapevine. This could be due to limited activity of the sgORF2 in certain tissues in grapevine. It seemed at first that the ORF 2 protein could play a role in efficient initial replication of the virus in infiltrated tissue. Co-infiltration with 35S-ORF2 or 35S-GR5 did not result in any GUS expression in infiltrated tissue. These results suggested that it might not be the ORF 2 protein, but the ORF 2 nucleotide sequence that may have a regulatory function during GVA replication and initial infection. Results obtained from the VIGS and the detection of the virus by RT-PCR, in systemic leaves in grapevine indicates that ORF 2 is not needed for long distance movement in this host. Silencing symptoms, comparable with GVA118, were observed on three leaves in a single plant 4 months after agroinfiltration with 35S-GVA-GR5-ΔORF2-VvPDS+sgMP. Reasons for the delay in silencing symptoms could be due to an environmental factor, such as temperature as problems were encountered with conditions in the glasshouse during that time. It could also be that the removal of ORF 2 hampered the initial replication of the virus in infiltrated tissue, leading to delayed movement and systemic silencing symptoms. The lack of GUS expression and the delay in silencing symptoms obtained with the gene exchange vector in grapevine highlights the importance of functional analysis and study of a viral genome in the natural host. This study is the first to report that ORF 2 of GVA is not needed for long distance movement of the virus in the grapevine host.

Results from this study showed that GVA can be used as gene insertion or gene exchange vector for expression and VIGS in *N. benthamiana*. In grapevine the use of GVA appears to be more limited. GVA is a phloem-associated virus (Tanne et al., 1989) and expression of proteins or silencing of specific genes in this tissue could prove invaluable. Several pathogens spread through the plant via the phloem transport system. Expression of antimicrobial proteins in the phloem tissue that have an antibiotic effect on these pathogens can lead to an increased resistance in grapevine plants (Vidal et al., 2006). In addition, silencing of specific genes in the phloem tissue and phloem-associated cells, can lead to the elucidation of gene functional and discovery of new genes involved in i.e. metabolic processes of nutrient transport and systemic movement of viruses in plants. When high level somatic expression is needed in grapevine alternative viruses should be investigated. *Grapevine fanleaf virus*

(GFLV), genus *Nepovirus*, family *Secoviridae* is not phloem-limited and has a bipartite genome which in essence makes it an ideal candidate for conversion into a deconstructed viral vector. To date, the study of viruses in woody, perennial hosts is limited due to a lack of molecular and functional genomics tools. For grapevine it is no different, in the near future, most of the tools needed for study of viruses in this host will be developed. Recently, a few laboratories worldwide have taken on the challenge to develop tools for transient expression and functional genomics in grapevine. A vector for expression and VIGS based on the phloem restricted geminivirus, *Tomato yellow leaf curl virus* (TYLCV) was reported to function in every single plant (even grapevine) tested until present (Peretz et al., 2007), but this was met with some speculation at the recent 16th Meeting of the International Council for the Study of Virus and Virus-like diseases of the Grapevine, Dijon, France. At the same meeting the development of a VIGS vector based on *Grapevine rupestris stempitting-associated virus* (GRSPaV), genus *Foveavirus*, family *Flexiviridae* (Meng et al., 2009) and the efficient delivery of, and high-level transgene expression through a GLRaV-2 infectious cDNA clone in *V. vinifera* (Dolja et al., 2009) were reported. Once all these tools are firmly in place, the functional genomics of grapevine and the unravelling of symptomology and pathogenecity of GVA in *V. vinifera* can commence.

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Chapter 6: Towards the elucidation of *Grapevine virus A* ORF 2 gene function

6.1. ABSTRACT

Chimaeric viruses are often constructed and deployed to identify the determinants involved in pathogenicity of viruses to plants. The function of the 20 kDa protein (P20) encoded by ORF 2 of GVA is still not known. A recent study reported that, of all GVA genes, ORF 2 is the most diverse (Goszczynski et al., 2008). A South African GVA variant (P163-M5) was identified that contained a 119 nt duplication between ORF 1 and 2. This variant induced an exceptionally severe symptomology in *N. benthamiana*. The alignment of the P10 protein of this variant, to other GVA variants, showed no significant amino acid changes that could contribute to the severity of symptoms. To investigate the potential role of the 119 nt duplication and P20, in expression of symptoms in plants, ORF 2 of a 35S-GVA-GR5 cDNA clone was removed and subsequently substituted by the corresponding ORFs of South African GVA variants. Upon agro-infiltration into *N. benthamiana* leaves, all chimaeric GVA constructs were able to move systemically through the plant. At this stage no correlation could be found between severity of symptoms, the presence of the P163-M5 insert and the specific GVA ORF 2 present in the chimaeras indicating that other factors in the viral genome or the host plant might be involved.

6.2. INTRODUCTION

Functional characterisation of the GVA genome in *N. benthamiana* attributed gene functions to every ORF of the virus with the exception of ORF 2 (Galiakparov et al., 2003c). Mutations introduced into this gene did not have an effect on viral replication, viral movement and symptomology in this herbaceous host (Galiakparov et al., 2003c). The function of the ORF 2 protein product is still uncertain for all recognized vitiviruses.

Interestingly, in a recent study in the discription and characterisation of two isolates of a new vitivirus, GVE it was found that the ORF 2 protein had only 42,4 % amino acid identity between these isolates even though the other proteins had an identity of more than 75 % to each other (Nakuane et al., 2008). This suggested that this gene is not under selective pressure in the case of GVE. For GVA, a study performed on the molecular divergence of South African GVA variants showed that members of group III (GTR1-1 and P163-1) boasted a significantly high level of nucleotide (59.2-59.5%) and protein (53.7–54.0%) sequence divergence in comparison to the Italian GVAIs151 variant (Goszczynski et al., 2008). The low level of nucleotide identity suggests that these sequences might not have originated from the same virus (Adams et al., 2004). Eventhough the amino acid identity was very low it showed a similarity of 70% to the corresponding gene of GVA Is151 signifying that in the case of GVA, contradictory to GVE, this gene is under selective pressure. The same study also revealed that the ORF 2 gene sequence of the GTR1-2 group II GVA variant was

distinctly divergent from other members of this group (Goszczynski et al., 2008). It is well-known that the respective South African GVA variants from the three groups, bring about different symptoms in *N. benthamiana* (Goszczynski & Jooste, 2003b). A variant, P163-M5, that induced exceptionally severe symptoms in *N. benthamiana* was recognized and was found to contain an imperfect duplication (insertion) of 119 nt between ORF 1 and 2 (Goszczynski et al., 2008) (see chapter 3.4.3). The P10 protein of GVA, encoded by ORF 5, has been correlated to pathogenicity and silencing suppression in *N. benthamiana* (Zhou et al., 2006; Chiba et al., 2006). Upon alignment of the ORF 5 protein sequence of the P163-M5 variant to those of other variants, no significant amino acid substitutions were observable that suggested a contribution of P10 to severity of symptoms. This prompted the investigation into the possible role of the 119 nt duplication and ORF 2 in symptom expression in *N. benthamiana* and *V. vinifera*. The possible role of ORF 2 in insect vector transmission has been speculated (Galiakparov et al., 2003c). This speculation however is only based on functional analysis studies of the GVA genome in *N. benthamiana* and has not been tested with transmission experiments thus far. In order to obtain the complete picture, functional analysis of the GVA genome should be performed in the natural viral host, *V. vinifera*. Up to now, no information is available about the GVA genomic determinants of pathogenicity in grapevine. In order to identify and comprehend the determinants involved in pathogenicity of viruses to plants, different techniques are often employed. These include mutation studies where specific genomic elements are changed, deleted or exchanged among different isolates of viruses (resulting in chimaeras or hybrids) towards the elucidation of genes involved in insect transmission or host specificity for example (van der Walt et al., 2008).

The aim of this study was to investigate the possible gene function of ORF 2 of GVA by using a GVA cDNA clone (T7-GVA-GR5, Haviv et al., 2006). This clone was converted to be under control of a CaMV 35S promoter (35S-GVA-GR5, chapter 4) and modified by removing ORF 2 (35S-GVA-GR5ΔORF2, chapter 5). The corresponding ORFs of South African GVA variants GTR1-1, GTR1-2, GTG11-1 and P163-M5, the latter one with and without 119 nt insert, were substituted into this construct, respectively. Subsequently, the biological characterisation of the clones was performed by agroinfiltration of *N. benthamiana* and *V. vinifera*.

6.3. MATERIALS AND METHODS

6.3.1. Plant material

Propagation of plants were described in chapters 4 and 5.

6.3.2. DNA constructs

The pGEM-1565 construct was described in chapter 5.3.3.2. ORF 2 of GVA variants [GTR1-1, GTR1-2, GTG11-1 and P163-M5 (with and without duplication)] were obtained via RT-PCR on dsRNA extracted from GVA-infected *N. benthamiana* (Goszczynski & Jooste, 2003c). This was discussed in chapter 3. Primers used (table 1), included *Sna* BI and *Bbv* CI restriction sites on flaps with the exception of primers used for the insert-P163-M5 construct that contained a *Stu* I site instead of *Sna* BI. The resulting PCR fragments were cloned into pGEM-1565 using *Sna* BI (or *Stu* I)/*Bbv* CI restriction sites resulting in pGEM-1565-GTR1-1-ORF2, pGEM-1565-GTR1-2-ORF2, pGEM-1565-GTG11-1-ORF2, pGEM-1565-P163-M5-ORF2, pGEM-1565-ins-P163-M5-ORF2. All these constructs were digested with *Aat* II and *Pst* I and fragments carrying ORF 2 modifications were ligated into similarly digested pSKM-e35S-GVA-GR5-pA to generate pSKM-e35S-GVA-GR5-ΔORF2-pA, pSKM-e35S-GVA-GR5-ΔORF2-GTR1-1-pA, pSKM-e35S-GVA-GR5-ΔORF2-GTR1-2-pA, pSKM-e35S-GVA-GR5-ΔORF2-GTG11-1-pA, pSKM-e35S-GVA-GR5-ΔORF2-P163-M5-pA, pSKM-e35S-GVA-GR5-ΔORF2-ins-P163-M5-pA. All these 35S-constructs (figure 1) were digested out with *Not* I/*Sal* I and transferred to *Not* I/*Xho* I digested pBIN_SN to generate pBIN-e35S-GVA-GR5-ΔORF2-pA, pBIN-e35S-GVA-GR5-ΔORF2-GTR1-1-pA, pBIN-e35S-GVA-GR5-ΔORF2-GTR1-2-pA, pBIN-e35S-GVA-GR5-ΔORF2-GTG11-1-pA, pBIN-e35S-GVA-GR5-ΔORF2-P163-M5-pA, pBIN-e35S-GVA-GR5-ΔORF2-ins-P163-M5-pA.
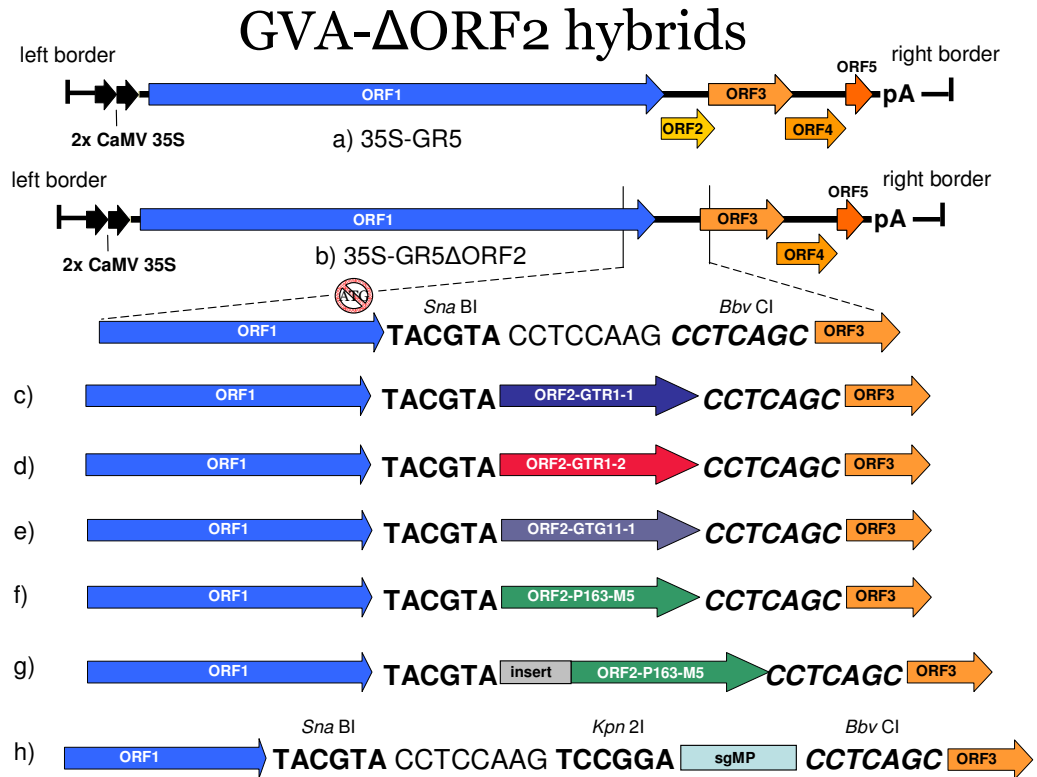
GVA-ΔORF2 hybrids

Figure 1. Schematic representation of GVA ORF 2 hybrids. a) 35S-GVA-GR5; b) 35S-GVA-GR5-ΔORF2 constructed by deletion of ORF 2 and generation of *Sna* BI-*Bbv* CI polylinker with overlap extension PCR. ORF 2 of South African GVA variants were cloned into this construct using *Sna* BI and *Bbv* CI; c) 35S-GR5-ΔORF2-GTR1-1; d) 35S-GR5-ΔORF2-GTR1-2; e) 35S-GR5-ΔORF2-GTG11-1; f) 35S-GR5-ΔORF2-P163-M5; g) 35S-GR5-ΔORF2-ins-P163-M5; h) 35S-GR5-ΔORF2+sgMP.

Table 1. Primers used in this study.

Construct	Primers used	Sequence (5'-3')
35S-GR5-ΔORF2-GTR1-1	GTR1-1-ORF2-F-SnaBI	ATTACGTAATGATGTCGGGCGAGAGT
	GTR1-1-ORF2-R-BbvCI	ATGCTGAGGTCATGAAGAGCGTGTGAT
35S-GR5-ΔORF2-GTR1-2	GTR1-2-ORF2-F-SnaBI	ATTACGTAATGTCGCAGAATTGTACA
	GTR1-2-ORF2-F-BbvCI	ATGCTGAGGTTAATTTCCTGATGACCT
35S-GR5-ΔORF2-GTG11-1	GTG11-1-ORF2-F-SnaBI	ATTACGTAATGACATCGCAAAATTGTACA
	GTG11-1-ORF2-F-BbvCI	ATGCTGAGGTTAATGTCTCGCGGTTCG
35S-GR5-ΔORF2-P163-M5	P163-M5-1-ORF2-F-SnaBI	ATTACGTAATGTCGCAGGATTGTACA
	P163-M5-1-ORF2-F-BbvCI	ATGCTGAGGTTAATTTCCTGTAGATCT
35S-GR5-ΔORF2-ins-P163-M5	P163-M5-1-insORF2-F-Stu	ATAGGCCTGGTTAGTTGAACTCTTAGG
	P163-M5-1-ORF2-F-BbvCI	ATGCTGAGGTTAATTTCCTGTAGATCT

6.3.3. Agro-infiltration of ΔORF2 constructs into *N. benthamiana*

The contructs 35S-GVA-GR5-ΔORF2 (described in chapter 5), 35S-GVA-GR5-ΔORF2+sgMP (descibed in chapter 5.3.3.2), 35S-GVA-GR5-ΔORF2-GTR1-1, 35S-GVA-GR5-ΔORF2-GTR1-2, 35S-GVA-GR5-ΔORF2-GTG11-1, 35S-GVA-GR5-ΔORF2-P163-M5 & 35S-GVA-GR5-ΔORF2-ins-P163-M5 were Agroinfiltrated into *N. benthamiana* plants and assessed for replication, systemic movement and the development of symptoms. The construct 35S-GVA-GR5 (described in chapter 4.3.5.2) was used as positive control. Overnight transgenic *Agrobacterium* cultures were diluted in resuspension buffer to an OD_{600} = 0,6. For each of the constructs, four plants were infiltrated and only two plants were mock-inoculated with resuspension buffer for use as negative control. One millilitre of Agrobacterium solution was infiltrated into two leaves per plant.

6.3.4. TPIA and symptom develoment

TPIA was performed (described in chapter 4.3.10) at 6 dpi on infiltrated tissue and petiole prints were made at 13 dpi. The rate of symptom development was monitored up till 28 dpi.

6.3.5. Total RNA extraction, RT-PCR and sequencing to detect GVA constructs in apical leaves of infiltrated plants

In order to detect viral progeny of infiltrated GVA constructs in *N. benthamiana* plants, 2 apical leaves per plant were harvested and 100 mg leaf material used in total RNA extraction (described in chapter 4; White et al., 2008). RNA was quantified using the NanoDrop 1000 spectrophotometer, and run on a 1 % Agarose/TAE gel to assess integrity. RNA was DNAse treated (1 h, 37ºC) and 400ng-1μg used in Two-step RT-PCR (described in chapter 3). The primer pair GVA-GR5-4344F and GVA-GR5-ER (chapter 5, table 1) was used to generate RT-PCR fragments. Fragments were seperated on a 1% Agarose/TAE gel, excised using a scalpel, and gel extracted using the Zymoclean gel DNA recovery kit™ (Zymo Research) according to the manufacturer's instructions. Extracted fragments were quantified with the NanoDrop® ND-1000 spectrophotometer. Ten ng/μL dilutions were made and used as direct template for sequencing with primer GVA-GR5-ER. Sequencing and sequencing analysis was performed as described in chapter 3.

6.3.6. Agro-infiltration of ΔORF2 constructs into *V. vinifera*

Agro-infiltration of *V. vinifera* was described in chapter 5.3.5.

### 6.4. RESULTS AND DISCUSSION

6.4.1. Characterisation of ΔORF2 constructs in *N. benthamiana*

The constructs 35S-GVA-GR5-ΔORF2 (described in chapter 5.3.3.2), 35S-GVA-GR5-ΔORF2+sgMP (desribed in chapter 5.3.3.2), 35S-GVA-GR5-ΔORF2-GTR1-1, 35S-GVA-GR5-ΔORF2-GTR1-2, 35S-GVA-GR5-ΔORF2-GTG11-1, 35S-GVA-GR5-ΔORF2-P163-M5 & 35S-GVA-GR5-ΔORF2-ins-P163-M5 and 35S-GVA-GR5 (described in chapter 4.3.5.2) were agroinfiltrated into *N. benthamiana* plants and assessed for local replication by TPIA, systemic movement by RT-PCR and the rate of development of symptoms. The native subgenomic promoter of the MP is contained in ORF 2. Therefore the 35S-GVA-GR5-ΔORF2 construct, where ORF 2 was deleted does not contain a subgenomic promoter for expression of the MP. If the ORF 3 encoded MP is not expressed, systemic movement of the virus throughout the plant would not be expected (Galiakparov et al., 2003c). TPIA performed at 6 dpi on infiltrated tissue and petiole prints performed at 13 dpi (figure 2), confirmed that all constructs were replicating locally and moving systemically throughout the plants. No CP could be detected in the mock inoculated plants by TPIA. The rate of symptom development and severity was monitored up to 28 dpi and results are summarized in tables 2 and 3. For most constructs, the first symptoms appeared at 4-6 dpi, which included downward curling and mottling of apical leaves. Symptom development was delayed for 35S-GVA-GR5-ΔORF2 and 35S-GVA-GR5-ΔORF2-GTR1-2 and the first symptoms on apical leaves appeared at 11 dpi and 13 dpi, respectively. It seems, for the 35S-GVA-GR5-ΔORF2, that expression of the MP is facilitated by the subgenomic promoter of ORF 2 that is present in ORF 1. However, expression of MP was not confirmed serologically. At 18 dpi, for all constructs infiltrated, all plants showed symptoms (figure 3) and at this point total RNA was extracted from apical leaves. Sequencing of RT-PCR fragments generated from total RNA confirmed that all constructs were able to move throughout the plant and that the modifications were present in the viral progeny. Symptoms ranged from very mild chlorosis in the case of 35S-GVA-GR5-ΔORF2-GTR1-2 to severe stunting of plants, deformation of leaves, chlorosis and necrosis in the case of 35S-GVA-GR5 (table 3).
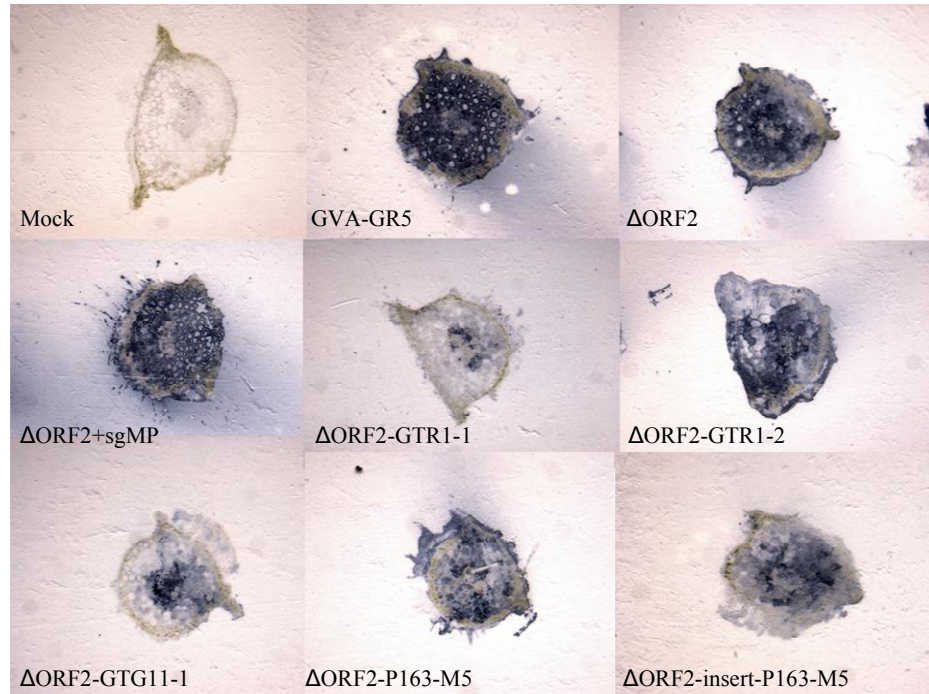
Figure 2. TPIA performed on petioles from infiltrated plants at 13 dpi showing replication of the virus in all constructs except the mock inoculated plants.
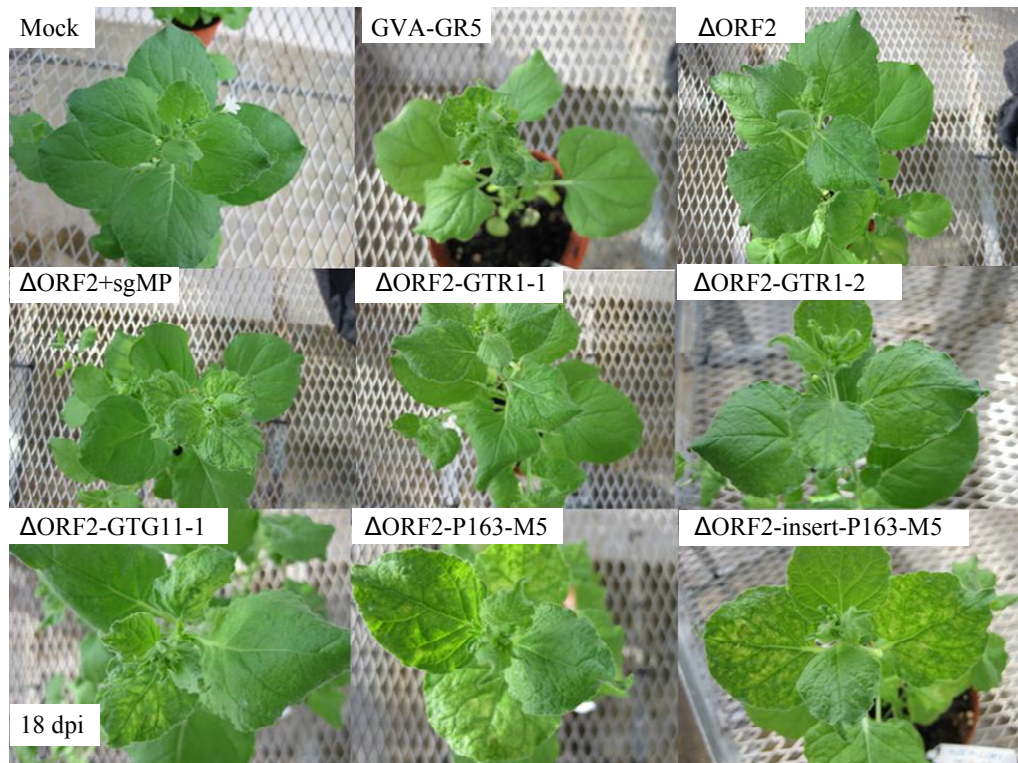
Figure 3. Photographs of agroinfiltrated *N. benthamiana* plants showing different symptoms induced by the specific ΔORF2 constructs at 18 dpi.

Table 2. Table showing symptom development and detection results for ΔORF2 experiments. IT = ifiltrated tissue, P = petiole, Seq = sequencing

| Construct	# plants								TPIA		RT-PCR
		Symptom development							IT	P	Seq
		6 dpi	7 dpi	11 dpi	13 dpi	15 dpi	18 dpi	28 dpi			
ΔORF2	4	0/4	0/4	2/4	3/4	4/4	4/4	4/4	+	+	+
ΔORF2+sgMP	4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	+	+	+
GTR1-1	4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	+	+	+
GTR1-2	3	0/3	0/3	0/3	1/3	1/3	3/3	3/3	+	+	+
GTG11-1	4	2/4	2/4	4/4	4/4	4/4	4/4	4/4	+	+	+
P163-M5	4	3/4	3/4	3/4	4/4	4/4	4/4	4/4	+	+	+
Ins-P163-M5	4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	+	+	+
GR5	4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	+	+	+
MOCK	2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	-	-	-

No correlation could be found between different ΔORF2 chimaeras, symptom severity and the molecular group that specific GVA variants were earlier devided into (Goszczynski et al., 2008). This was illustrated by the fact that 35S-GVA-GR5-ΔORF2-GTR1-2 induced the mildest symptoms in this study, whereas the GVA GTR1-2 variant is present in molecular group II and associated with severe symptoms. Furthermore, the 35S-GVA-GR5-ΔORF2-GTR1-1, hosting ORF 2 from the mild variant GTR1-1 (group III), induced medium to severe symptoms in this study. As yet, no correlation could be found between the presence of the 119 nt P163-M5 insert and the severity of symptoms. It seems that the presence of the insert resulted in milder symptoms in this experiment. Figure 4 shows symptoms observed on leaves of plants infiltrated with different GVA chimaeric constructs at 28 dpi. In an attempt to explain the obtained results, modifications made to the GVA constructs were assessed in terms of duplication of sequences and evolutionary relationship of different variants. The stability of hybrid constructs could be compromised in variants that are closely related to the GR5 variant due to some duplication of sequences in the modified areas leading to possible recombination. Problems leading to viral vector instability due to homologous recombination have been reported by other groups (Dawson et al., 1989; Haviv et al., 2006). Instability of chimaeric viruses did not seem to be a problem in this experiment, however as RT-PCR and sequencing performed on systemic leaves revealed that modifications were passed on to the viral progeny. The cloning strategy used, generated hybrid constructs where two regions in the GVA genome are duplicated (figure 5). These are not complete duplications as there are some variation between different GVA variants. Naturally, ORF 2 of GVA overlaps with ORF 1 and ORF 3 (MP). In the constructed ORF 2 hybrids, the first 32 nt of ORF 2 and the first 59 nt of the MP are duplicated. During the cloning strategy, the ORF 2 ATG start codon in ORF 1 of the GVA-GR5 variant used as acceptor was silently mutated to prevent translation from this start codon. Alignment of the ORF 2 3' terminal 32 nt region is shown in figure 5B and phylogenetic analysis of this region showed that this region of GTR1-2 and

P163-M5 was more distant from the other variants (not shown). Alignment of the 59 nt region is shown in figure 5C. This region varied in length of 53 nt (GTR1-1), 59 nt (GTR1-2, P163-M5) or 68 nt (GTG11-1) depending on the specific variant from which the ORF 2 originated. Phylogenetic analysis once again showed that for this region GTR1-2 and P163-M5 was more distant from the other variants (not shown). Upon assessment of duplicated genomic areas it was found that an elongated MP resulted in the modified constructs due to the cloning strategy used (figure 5D). The native start codon of the MP is present in ORF 2 (this was not silenced as in the case for ATG of ORF 2) and during construction of different GVA ORF 2 hybrids, a duplication of the MP start codon arrose. This led to an elongation ranging from 20 – 25 extra amino acids at the N-terminus of the MP, depending on the respective variant from which ORF 2 originated. Several studies have shown that GFP fusions at the N- or C-terminus of the MP did not affect the ability of viruses to move intercellularly in plants (Boyko et al., 2000; Sanchez-Navarro & Bol, 2001; Canto & Pulakaitis, 2005). If a MP fusion of a ~240 aa GFP protein can be tolerated it seems that in the case of the GVA chimaeras that an elongation of 20 - 25 amino acids should not influence systemic movement of the chimaeras throughout the plant. The MP of Begomoviruses has been proven to play a role in the effectiveness of infection, the tempo at which symptoms develop and the severity of symptoms (van der Walt et al., 2008). For several viruses, the characterisation of the MP through mutational analysis have implicated both the N- and C-termini to have an effect on the systemic development of symptoms due to cell-to-cell movement that occur at different tempos (Boyko et al., 2000; Sanchez-Navarro & Bol, 2001; Takeda et al., 2004; Choi et al., 2005). In seems in the current study that eventhough the chimaeric GVA viruses were able to move systemically throughout the plants, the elongation to the MP could probably influence the rate of cell-to-cell movement leading to a differentiation in symptoms observed. The range of symptoms observed for the different chimaeras can thus be due to 2 contributing factors: 1) the presence of the specific ORF 2, and/or 2) the elongation of the MP.

Table 3. Table showing symptomology of ΔORF2 plants.

Construct	Dpi First Symptoms appeared	Severity of symptoms	Type of symptoms	Dpi all showing symptoms
ΔORF2	11	1	**Very mild:** leaf curling in apical leaves, mild to medium chlorosis in other leaves	15
ΔORF2+sgMP	6	4.5	**Severe:** Comparable to GR5, but plants are only slightly stunted	6
GTR1-1	6	3.5	**Medium:** Mild downward curling of leaves, medium chlorosis	6
GTR1-2	13	0.5	**Very mild:** Very mild chlorosis	18
GTG11-1	6	4	**Severe:** Slight deformation of leaves, vein clearing, chlorosis and necrosis	11
P163-M5	6	3	**Medium:** Mild deformation and downward rolling of leaves, big patches showing chlorosis proceeding into necrosis	13
Ins-P163-M5	6	2.5	**Mild-Medium:** No leaf curling, large chlorotic patches proceeding into necrosis	7
GR5	6	5	**Severe:** Stunting of plants, deformation of leaves, chlorosis and necrosis	6
MOCK	-	-	-	-
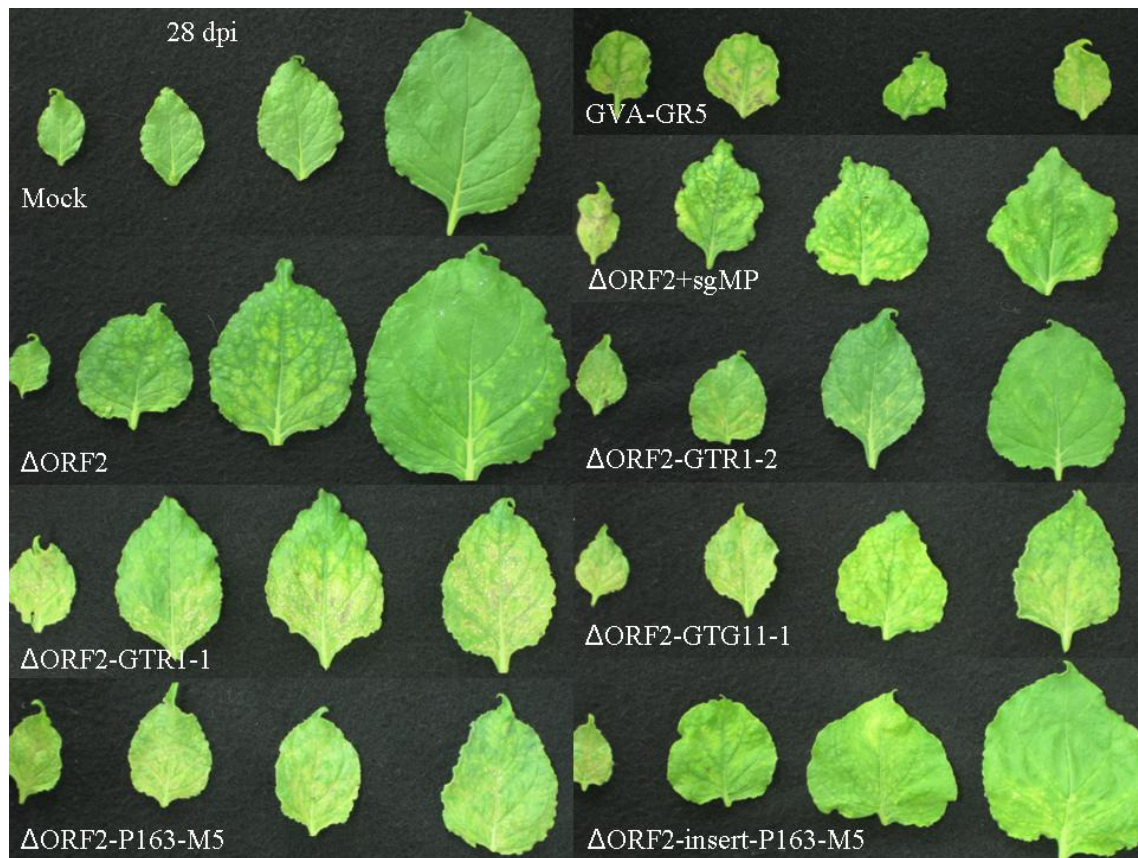
Figure 4. Photographs of leaves harvested from agroinfiltrated *N. benthamiana* plants showing different symptoms induced by the specific ΔORF2 constructs at 28 dpi.

Figure 5. Schematic representation of duplications in ΔORF2 constructs and elongation of MP resulting from the cloning strategy used. A) In the generated hybrid constructs, two regions in the GVA genome are duplicated: the first 32 nt (shown in green) of ORF 2 and the first 59 nt of the MP (shown in purple). The native start codon (ATG) of ORF 2 was silently mutated to prevent translation. B) Alignment of 32 nt duplicated region showing homology between GVA variants used in hybrid construction. C) Alignment of 59 nt (53, 59 or 68 bp depending on variant) duplicated region showing homology between GVA variants used in hybrid construction. D) The native start codon of the MP is present in ORF 2 (this was not silenced as in the case for ATG of ORF 2) and during construction of different GVA ORF 2 hybrids, a duplication of the MP start codon arrose leading to an elongation at the N-terminus. This allignment is shown in the blue box.

6.4.2. Characterisation of ΔORF2 constructs in *V. vinifera*

Only 35S-GVA-GR5, 35S-GVA-ΔORF2 and 35S-GVA-GR5-ΔORF2+sgMP, which were not affected by the elongated MP were vacuum agroinfiltrated into *V. vinifera* cv Sultana plantlets. Results of GVA detection in the new leaves was not available at the time of submission of this thesis.

6.5. CONCLUSION

For all known vitiviruses, the function of the ORF 2 protein product is still not known. In order to investigate the possible function of this protein molecular tools are needed to

facilitate mutational analysis and characterisation in *N. benthamiana* and *V. vinifera*. In this study, a GVA cDNA clone (35S-GVA-GR5) was modified by deletion of ORF 2 (35S-GVA-GR5-ΔORF2). This clone was used to substitute ORF 2 of different South African GVA variants, resulting in different chimaeras, followed by biological characterisation in *N. benthamiana*. All constructs tested were able to replicate, move systemically and induce symptoms in *N. benthamiana*. The fact that the 35S-GVA-GR5-ΔORF2 construct, that did not contain the sub-genomic promoter of the MP, was able to move systemically was unexpected. Symptoms induced by this construct were very mild when compared to normal 35S-GVA-GR5 and the 35S-GVA-GR5-ΔORF2+sgMP construct, containing the sub-genomic promoter of the MP. It has been reported earlier that of all GVA proteins, the MP is expressed at highest levels (Haviv *et al.*, 2006), suggesting that the sub-genomic promoter driving the expression is the most active. It seems in the case of 35S-GVA-GR5-ΔORF2, that the very mild symptoms observed could be due to inefficient movement of the virus due to lower levels of MP. It could be that low level expression of the MP was driven by the subgenomic promoter of ORF 2, although this was not tested. In *N. benthamiana*, no correlation could be found between severity of symptoms, the presence of the P163-M5 insert and the specific GVA ORF 2 contained in the chimaeras. This experiment however showed the versatility of GVA in the way it can tolerate duplications, foreign nucleotides and even extensions to the MP in its small, compact genome. Conversely in chapter 4 it was shown, that a single amino acid change, in ORF 5 of the GTR1-2 variant reduced replication of GVA to below a detectable level (described in chapter 4).

6.6. REFERENCES

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Chapter 7: Conclusion and future prospects

The development of molecular tools to facilitate studies on grapevine is essential for the improvement and protection of this crop. *Grapevine virus A* seemed like an ideal candidate for use as expression and silencing vector in *Nicotiana benthamiana* and *Vitis vinifera*. In this study the use of GVA as transient expression and VIGS vector in grapevine was assessed. At first, three South African GVA variants (GTR1-1, GTR1-2 and GTG11-1) were completely sequenced and compared to 7 other GVA variants in a phylogenetic analysis (chapter 3). Previous variability studies were only based on short genomic regions of GVA (Goszczynski & Jooste, 2002, 2003b, 2003c; Goszczynski, 2007). It was found that the heterogenic population structure of the virus and the division into three groups, with the mild variants in group III being the most divergent, correlated to findings from earlier studies. The generated sequences contributed to a publication in collaboration with D.E. Goszczynski (Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa): "Molecular divergence of GVA variants associated with Shiraz disease in South Africa", 2008, Virus Research 138: 105-110. In this paper two interesting Group II GVA variants were described namely GTR1-2 and P163-M5. The Group II variants were found to be closely linked to the expression of SD. GTR1-2 was isolated from a grapevine plant that never showed SD symptoms (Goszczynski 2007). A variant (P163-M5) that resulted in exceedingly severe symptoms in *N. benthamiana* that is used as SD positive control by the grapevine industry, was found to contain a 119 nt insert within the native ORF2. The role of this insert and other genomic pathogenicity factors of GVA, in expression of disease in both plants, remains to be determined (chapter 6). Comparative analysis performed on group II GVA variants (Goszczynski et al., 2008) suggest that pathogenicity determinants in the GVA genome are more complex in *V. vinifera* than in *N. benthamiana*. This highlights the importance for the development of *in vivo* tools to answer these questions.

Infectious clones of RNA viruses infecting grapevine are among these tools that need to be developed. In order to disentangle the contribution of different GVA variants to the aetiology of SD, infectious clones of these variants are needed. In chapter 4, the construction of full-length cDNA clones of three South African GVA variants (GTR1-1, GTR1-2 and GTG11-1), were discussed. After several cloning strategies were attempted, none of these clones (pBIN-e35S-Asc-GTR1-1-pA, pGREEN-e35S-Stu-GTR1-2 and pBIN-e35S-Asc-GTG11-1-pA) were infectious upon agroinfiltration in *N. benthamiana*. A population cloning strategy was

used in a second attempt to assemble variant GTR1-2 into an infectious cDNA clone. After correction of a 13 nt deletion and a premature stop codon in ORF 1, a full-length cDNA clone (pBIN-e35S-GTR1-2$_{fl*}$-pA) showed small clusters of infected cells in infiltrated mesophyll tissue of *N. benthamiana* plants after TPIA detection of GVA CP. This indicated that this clone was replicating locally, but as the plants stayed symptom-free, it seemed not to be able to move systemically. A single amino acid mutation at position 13 [Tyrosine (Tyr/Y)→Cysteine (Cys/C)] in the ORF 5-encoded P10 of this clone was found and preliminary experiments suggested that this single amino acid mutation reduced replication of the virus to a lower than detectable level. Furthermore, two infectious clones of Israeli variants of GVA (T7-GVA-GR5 and T7-GVA118, obtained from M. Mawassi) were brought under control of a CaMV 35S promoter (35S-GVA-GR5 and 35S-GVA118). Both clones were infectious, able to replicate, move systemically and induce typical GVA symptoms after agroinfiltration in *N. benthamiana*. Seeing as no cDNA clones of South African GVA variants were able to replicate in *N. benthamiana*, the Israeli clones served as backbone for further experiments in characterisation of transient expression and VIGS vectors in *N. benthamiana* and *V. vinifera* (chapter 5). In future it could be worthwhile exploring and correcting the mutations in the J396 GTR1-2 clone in order to obtain an infectious clone of a South African GVA variant. The use of ssRNA, instead of dsRNA, as starting material in infectious clone construction could be explored as this has been shown to be more successful (M. Mawassi, Agricultural Research Organization, Israel, pers. Comm.). The assembly of the P163-M5 variant into an infectious clone could serve as an invaluable tool for the study of the GVA genomic determinants of SD.

In chapter 5 the use of GVA as gene insertion vector (35S-GVA118) and gene exchange vector (35S-GVA-GR5-ΔORF2+sgMP) in *N. benthamiana* and *V. vinifera* was compared. The gene insertion vector, 35S-GVA118 (described in chapter 4) was based on the full-length GVA genome. The gene exchange vector, 35S-GVA-GR5-ΔORF2+sgMP, was constructed in this study by elimination of ORF 2 and insertion of a sgMP and unique restriction sites to facilitate transgene insertion. In *N. benthamiana* both vectors showed similar GUS expression levels and photobleaching symptoms upon virus-induced NbPDS silencing. In *V. vinifera* limited GUS expression levels and VIGS photobleaching symptoms were observed for the gene insertion vector, 35S-GVA118. No GUS expression was observed for the gene exchange vector 35S-GVA-GR5-ΔORF2+sgMP in this host. As for silencing, one plant, agroinfiltrated with 35S-GVA-GR5-ΔORF2-VvPDS+sgMP, developed photobleaching symptoms in 3

systemic leaves after 4 months. This study showed that GVA can be used as gene insertion and gene exchange vector for expression and VIGS *in N. benthamiana*, but in grapevine it's use is limited. It is also the first report that ORF 2 of GVA is not needed for long distance movement in grapevine.

The function of the 20 kDa protein (P20) encoded by ORF 2 of GVA is still not known. In this study it was found that, of all GVA genes, ORF 2 is the most diverse (Goszczynski et al., 2008). A South African GVA variant (P163-M5) was identified that contained a 119 nt duplication between ORF 1 and 2. This variant induced an exceptionally severe symptomology in *N. benthamiana*. The alignment of the P10 protein of this variant, to other GVA variants, showed no significant amino acid changes that could contribute to the severity of symptoms. To investigate the potential role of the 119 nt duplication and P20, in expression of symptoms in plants, ORF 2 of a 35S-GVA-GR5 cDNA clone was removed and subsequently substituted by the corresponding ORFs of South African GVA variants (chapter 6). Upon agro-infiltration into *N. benthamiana* leaves, all chimaeric GVA constructs were able to move systemically through the plant. At this stage no correlation could be found between severity of symptoms, the presence of the P163-M5 insert and the specific GVA ORF 2 present in the chimaeras indicating that other factors in the viral genome or the host plant might be involved. In future elucidation of ORF 2 gene function, experiments should be mainly performed in the natural grapevine host of GVA. Studying the cellular localization of the ORF 2 protein product (fused to GFP) in grapevine protoplasts could give hints to where and when in the GVA lifecycle this protein is expressed and needed. Mutational analysis studies on ORF 2 artificially made mutants, in grapevine/protoplasts, could prove valuable in the characterisation of this gene in the future.

Results presented in this thesis showed that GVA is useful as gene insertion or gene exchange vector for expression and VIGS in *N. benthamiana*. In grapevine the use of GVA appears to be more restricted seeing as GVA is a phloem-associated virus. However, the expression of proteins or silencing of specific genes in this tissue could prove invaluable as many pathogens use the phloem system for long-distance spread. Expression of antimicrobial proteins in the phloem tissue that have an antibiotic effect on these pathogens can lead to an increased resistance in grapevine plants (Vidal et al., 2006). In addition, silencing of specific genes in the phloem tissue and phloem-associated cells, can lead to the elucidation of gene functional and discovery of new genes involved in i.e. metabolic processes of nutrient transport and

systemic movement of viruses in plants. When high level somatic expression is needed in grapevine alternative viruses should be investigated.

To date, the study of viruses in woody, perennial hosts is limited due to a lack of molecular and functional genomics tools. For grapevine it is no different, in the near future, most of the tools needed for study of viruses in this host will be developed. Recently, a few laboratories worldwide have taken on the challenge to develop tools for transient expression and functional genomics in grapevine (Peretz et al., 2007; Meng et al., 2009; Dolja et al., 2009). This study contributed to the available pool of molecular tools for grapevine and once these are securely in place, the functional genomics of grapevine and the unravelling of symptomology and pathogenecity of GVA in *V. vinifera* can begin. Of specific interest will be the contribution of the P163-M5 119 nt insert and the role of ORF 2. This study has also opened in interesting avenue to pursue in the future characterisation of ORF 2 in *V. vinifera*. The established ORF 2 chimaeras can also prove very useful in mealybug transmission experiments, because it has been speculated that this gene is involved in insect transmission (Galiakparov et al., 2003c).

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Appendix A: Phylogenetic trees

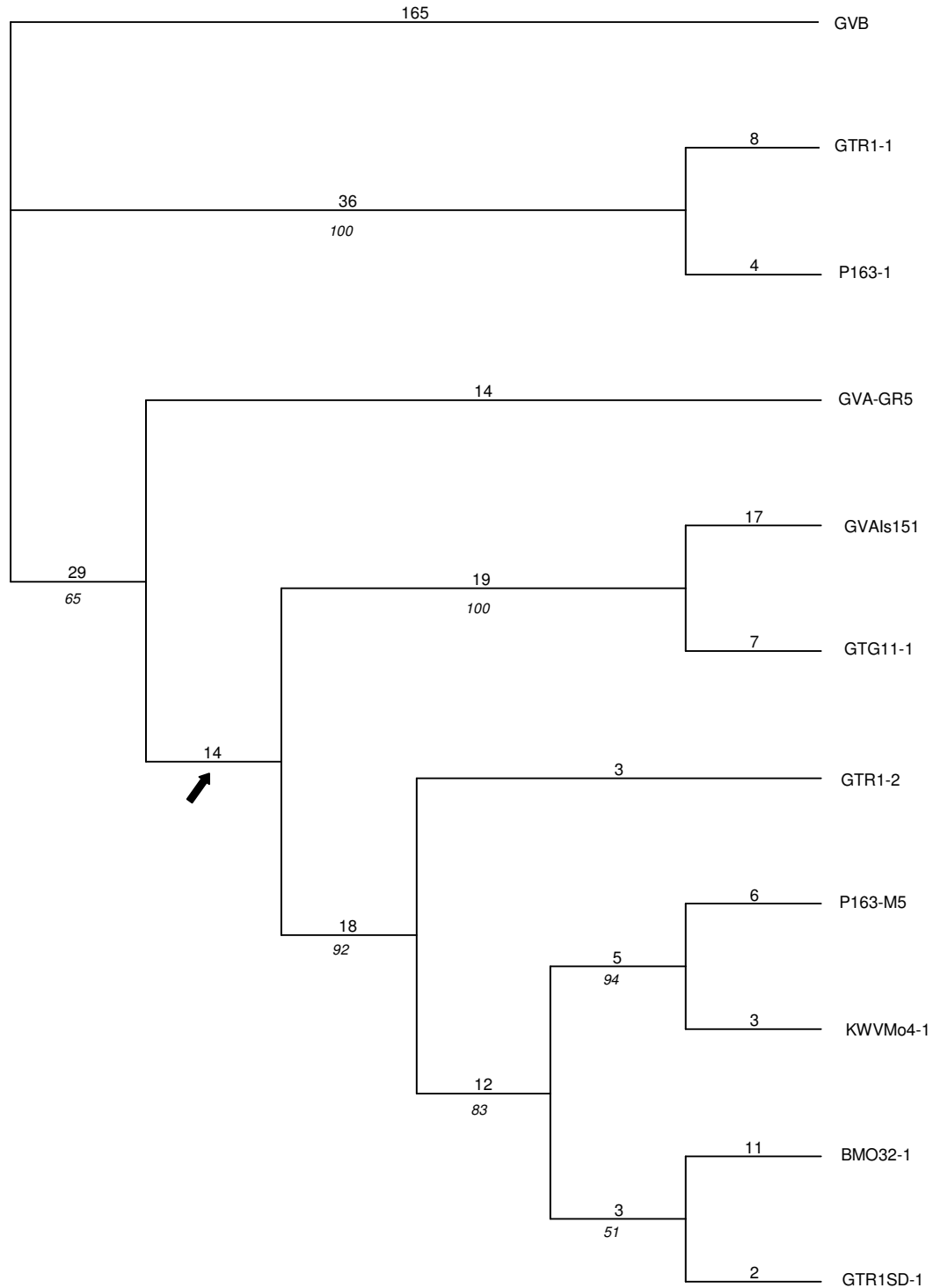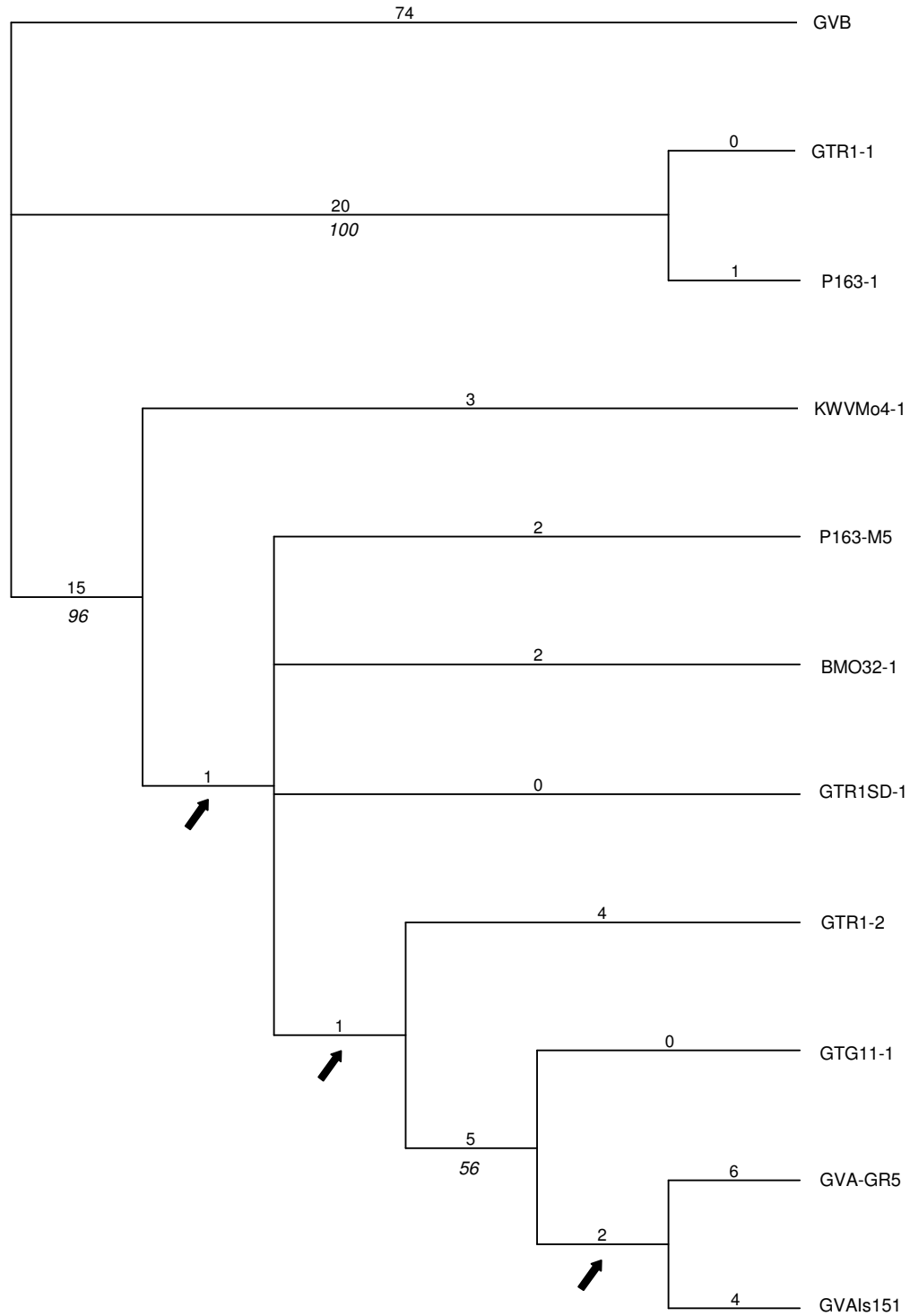*In this appendix, the phylogenetic trees described in chapter 3 are included.*
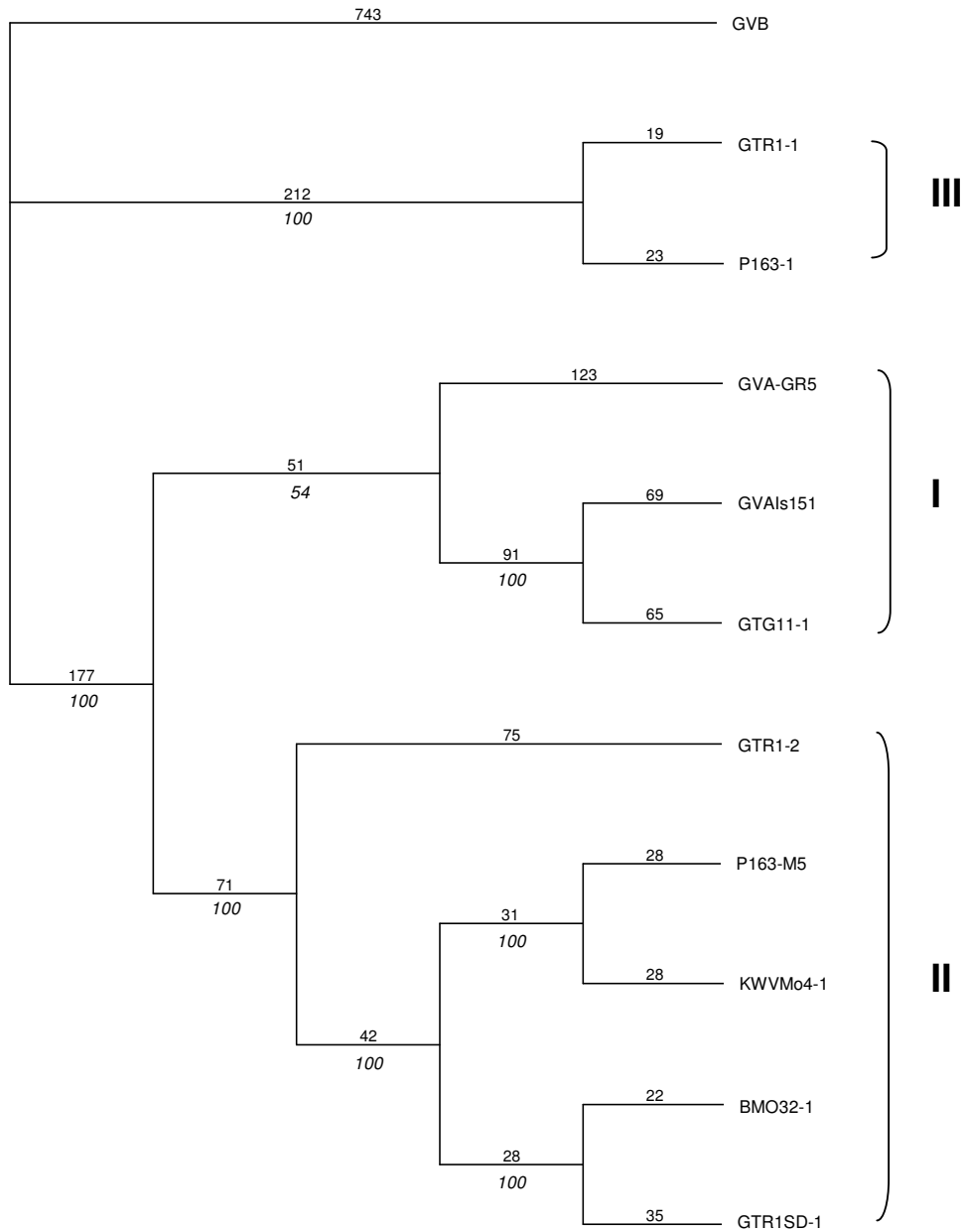
Figure 1. Phylogenetic analysis showing the shortest tree drawn from the heuristic search performed on the GVA ORF 1 (aa) data matrix. Values above branches indicate branch lengths and values (*italics)* beneath branches show bootstrap values. GVA molecular groups (I, II & III) are shown on the right hand side of the tree.
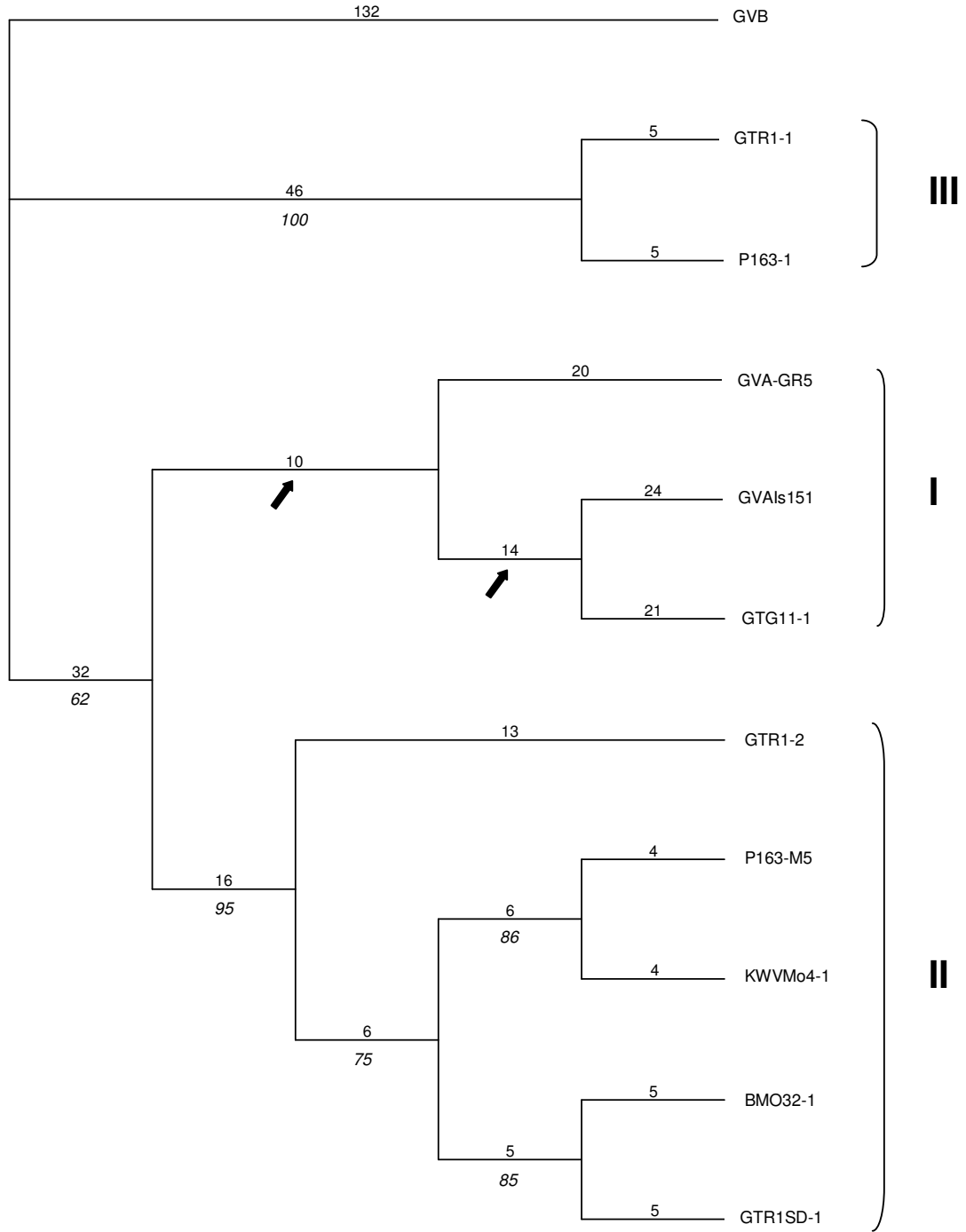
Figure 2. Phylogenetic analysis showing the shortest tree drawn from the heuristic search performed on the GVA ORF 2 data matrix. Values above branches indicate branch lengths and values (*italics)* beneath branches show bootstrap values. GVA molecular groups (I, II & III) are shown on right hand side of tree. Nodes that collapse in the strict consensus tree are indicated by arrows.
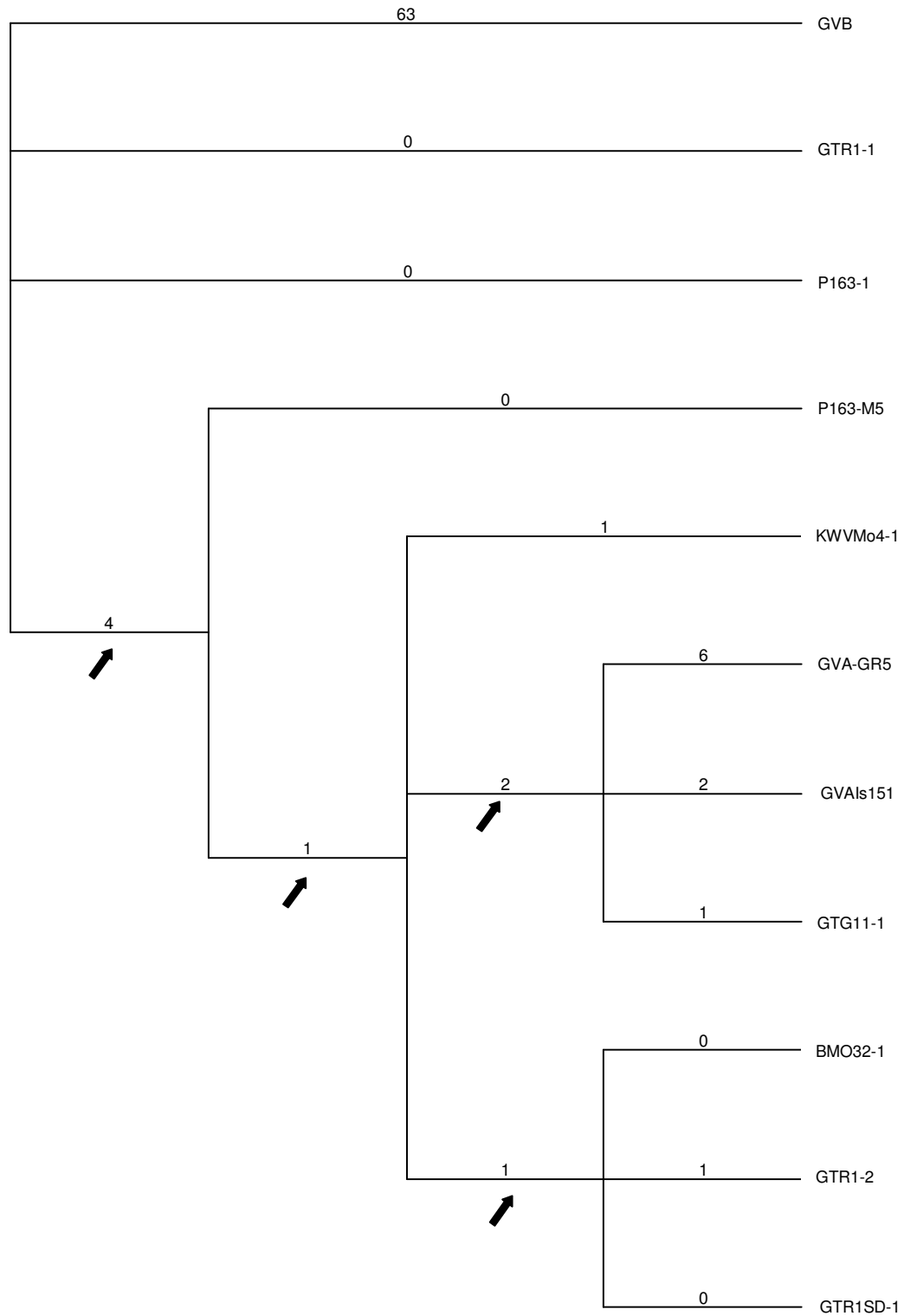
Figure 3. Phylogenetic analysis showing the shortest tree drawn from the heuristic search performed on the GVA MP data matrix. Values above branches indicate branch lengths and values (*italics)* beneath branches show bootstrap values. GVA molecular groups (I, II & III) are shown on right hand side of tree. Nodes that collapse in the strict consensus tree are indicated by arrows.

Figure 4. Phylogenetic analysis showing the shortest tree drawn from the heuristic search performed on the GVA CP data matrix. Values above branches indicate branch lengths and values (*italics)* beneath branches show bootstrap values. GVA molecular groups (I, II & III) are shown on right hand side of tree. Nodes that collapse in the strict consensus tree are indicated by arrows.

Figure 5. Phylogenetic analysis showing the shortest tree drawn from the heuristic search performed on the GVA P10 data matrix. Values above branches indicate branch lengths and values (*italics)* beneath branches show bootstrap values. Nodes that collapse in the strict consensus tree are indicated by arrows.

Appendix B: GTR1-2 population cloning strategy

In this appendix, a diagrammatic representation of the GTR1-2 population cloning strategy (described in chapter 4, sections 4.4.2.3, 4.4.2.4 and 4.4.2.5) and the correction strategies of the 13 nt deletion and the premature stop codon, is included. Please refer to these sections for a detailed description of the steps followed.

Figures 1-4 show the steps followed to construct pBIN-e35S-GTR1-2$_{fl}$-pA.

Figure 5 shows the steps followed in correction of the 13 nt deletion in ORF 1 of pBIN-e35S-GTR1-2$_{fl}$-pA.

Figure 6 shows the steps followed in correction of the premature stop codon in ORF 1 to result in pBIN-e35S-GTR1-2$_{fl*}$-pA (J396-398).
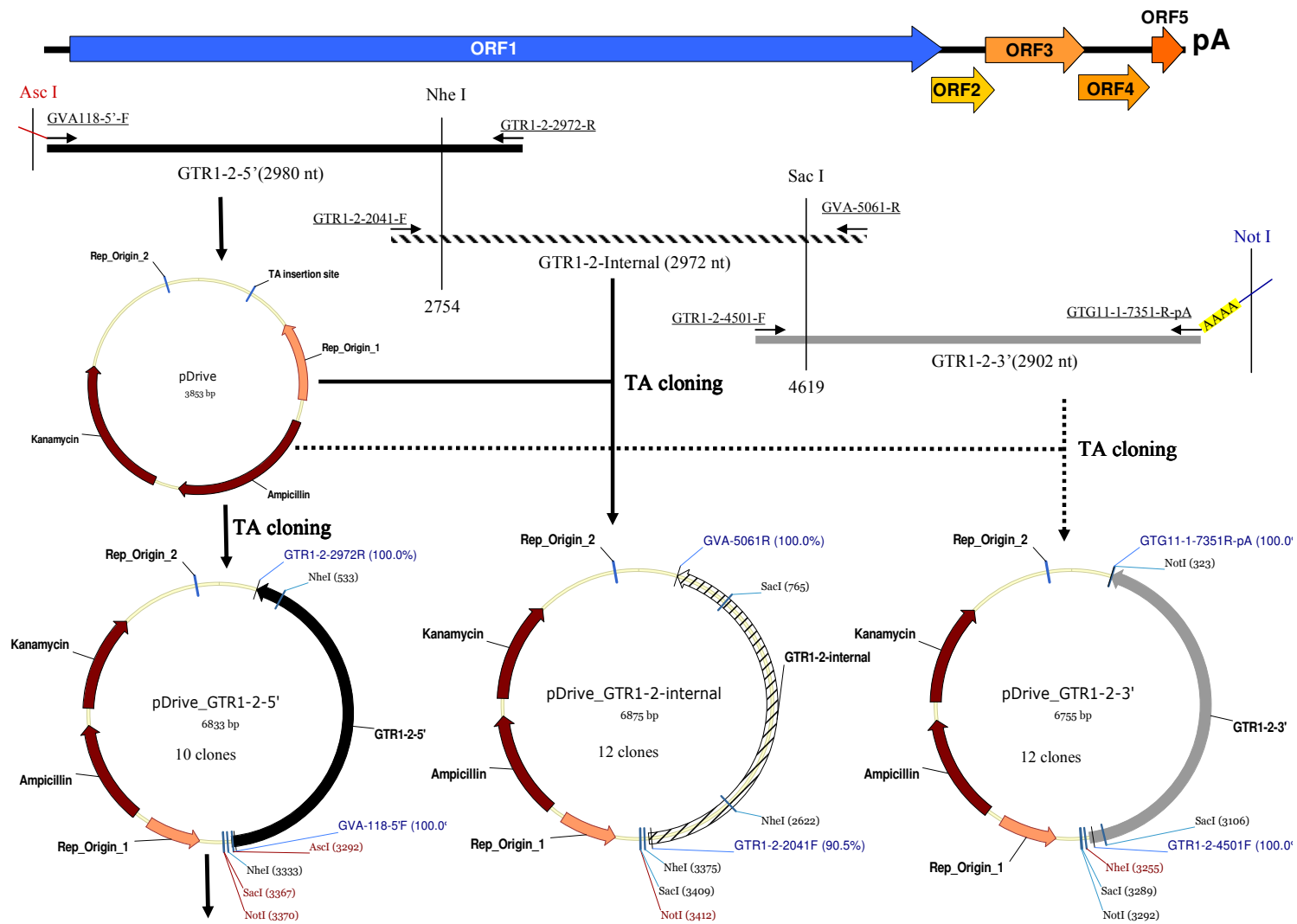
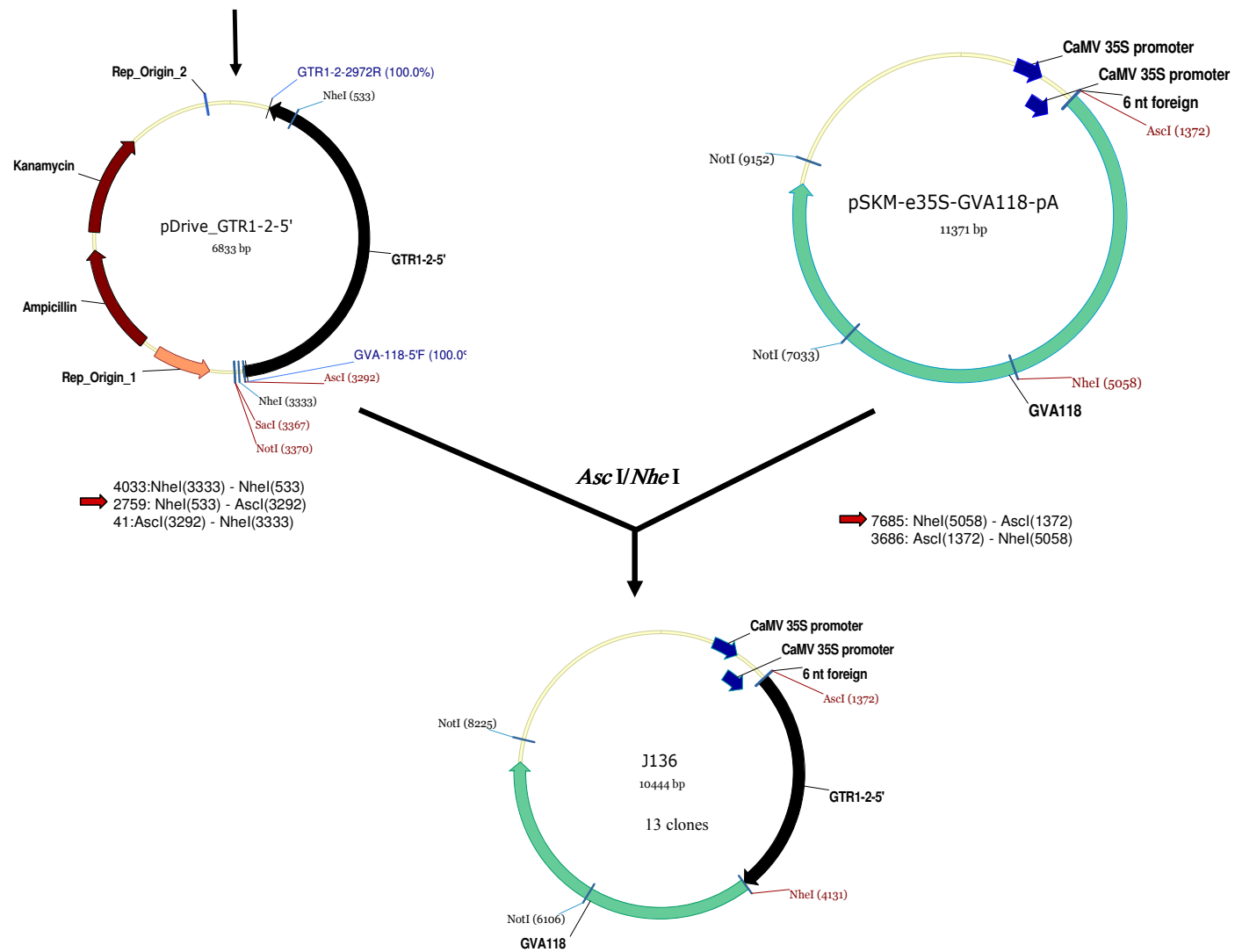**Figure 1. Steps followed to construct pBIN-e35S-GTR1-2fl-pA**

pDrive_GTR1-2-5'
6833 bp

GTR1-2-2972R (100.0%)
NheI (533)
Rep_Origin_2
Kanamycin
Ampicillin
Rep_Origin_1
GTR1-2-5'
GVA-118-5'F (100.0%
AscI (3292)
NheI (3333)
SacI (3367)
NotI (3370)

4033:NheI(3333) - NheI(533)
2759: NheI(533) - AscI(3292)
41:AscI(3292) - NheI(3333)

pSKM-e35S-GVA118-pA
11371 bp

CaMV 35S promoter
CaMV 35S promoter
6 nt foreign
AscI (1372)
NotI (9152)
NotI (7033)
NheI (5058)
GVA118

7685: NheI(5058) - AscI(1372)
3686: AscI(1372) - NheI(5058)

Asc I/*Nhe* I

J136
10444 bp
13 clones

CaMV 35S promoter
CaMV 35S promoter
6 nt foreign
AscI (1372)
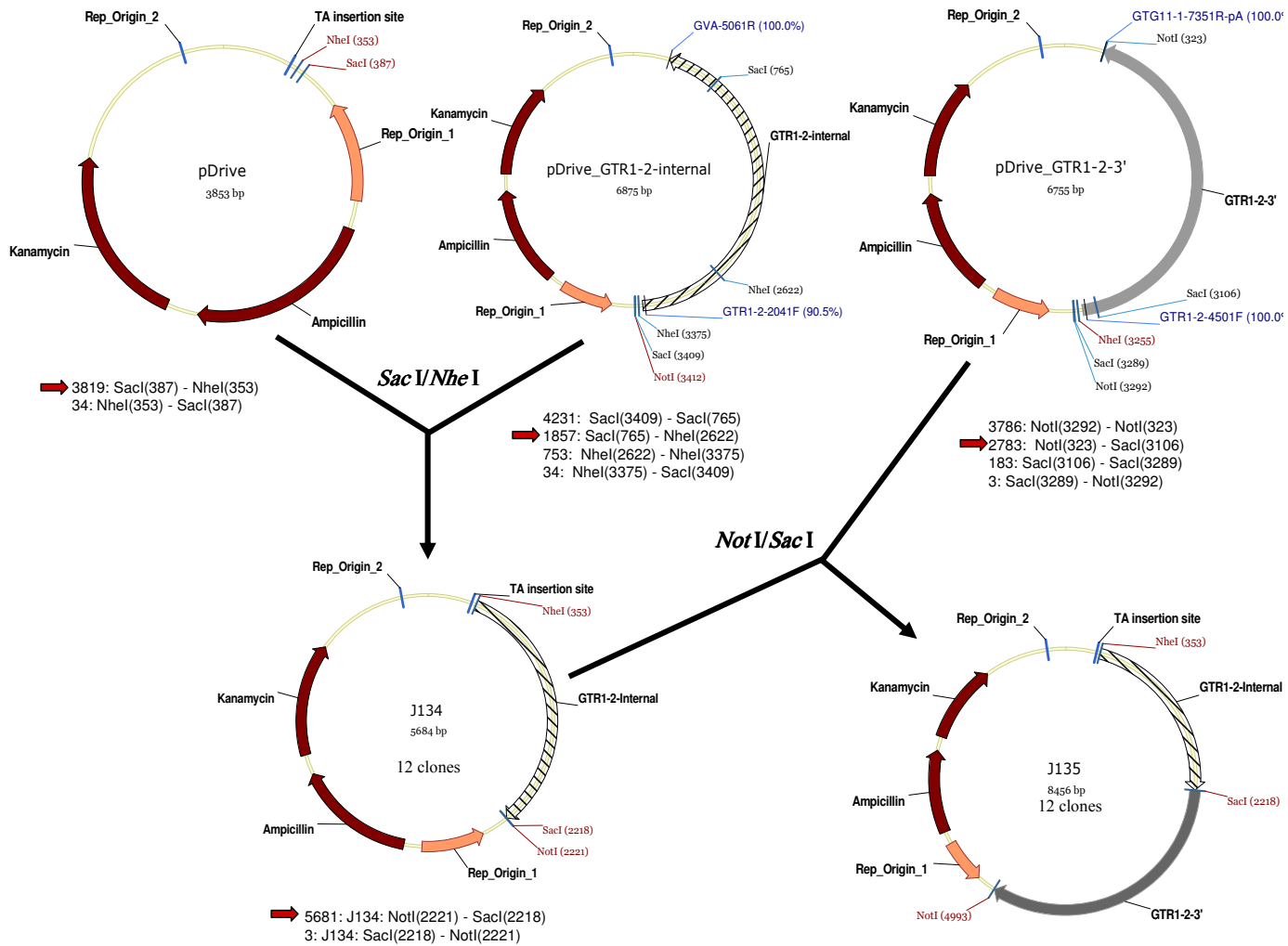NotI (8225)
GTR1-2-5'
NheI (4131)
NotI (6106)
GVA118

Figure 2. Steps followed to construct pBIN-e35S-GTR1-2fl-pA continued…

135

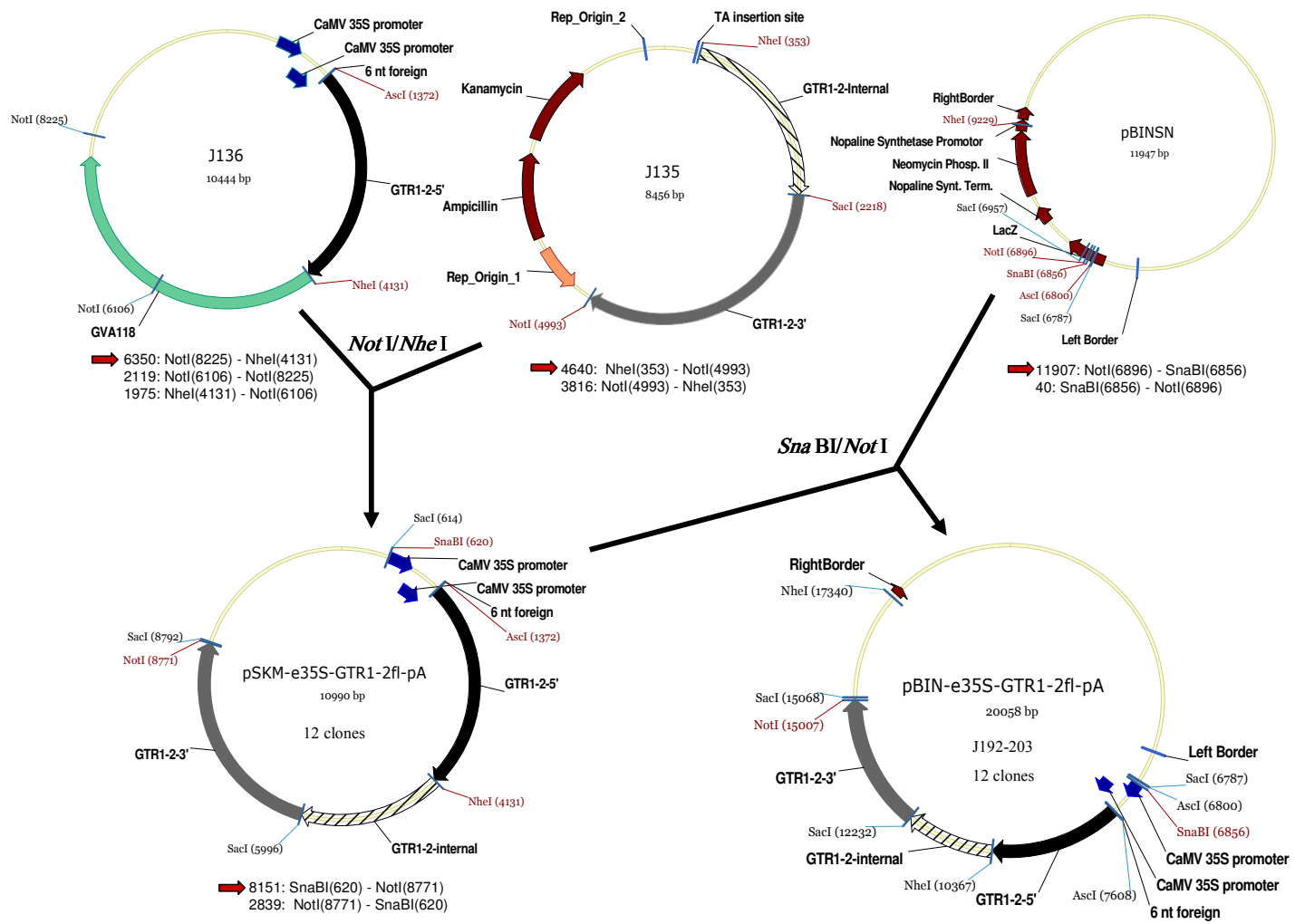**Figure 3. Steps followed to construct pBIN-e35S-GTR1-2fl-pA continued…**

136

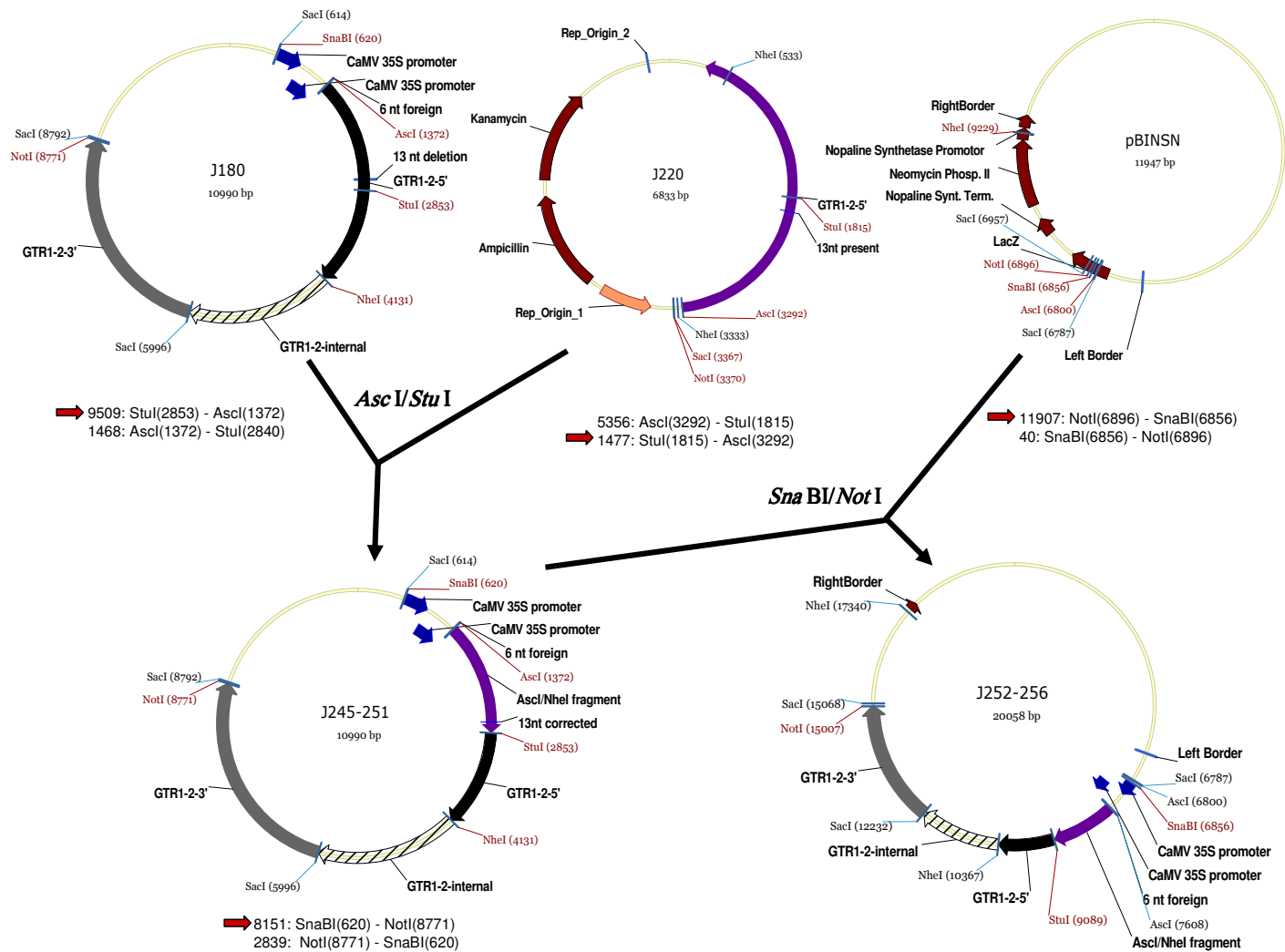**Figure 4**. **Steps followed to construct pBIN-e35S-GTR1-2fl-pA continued.**

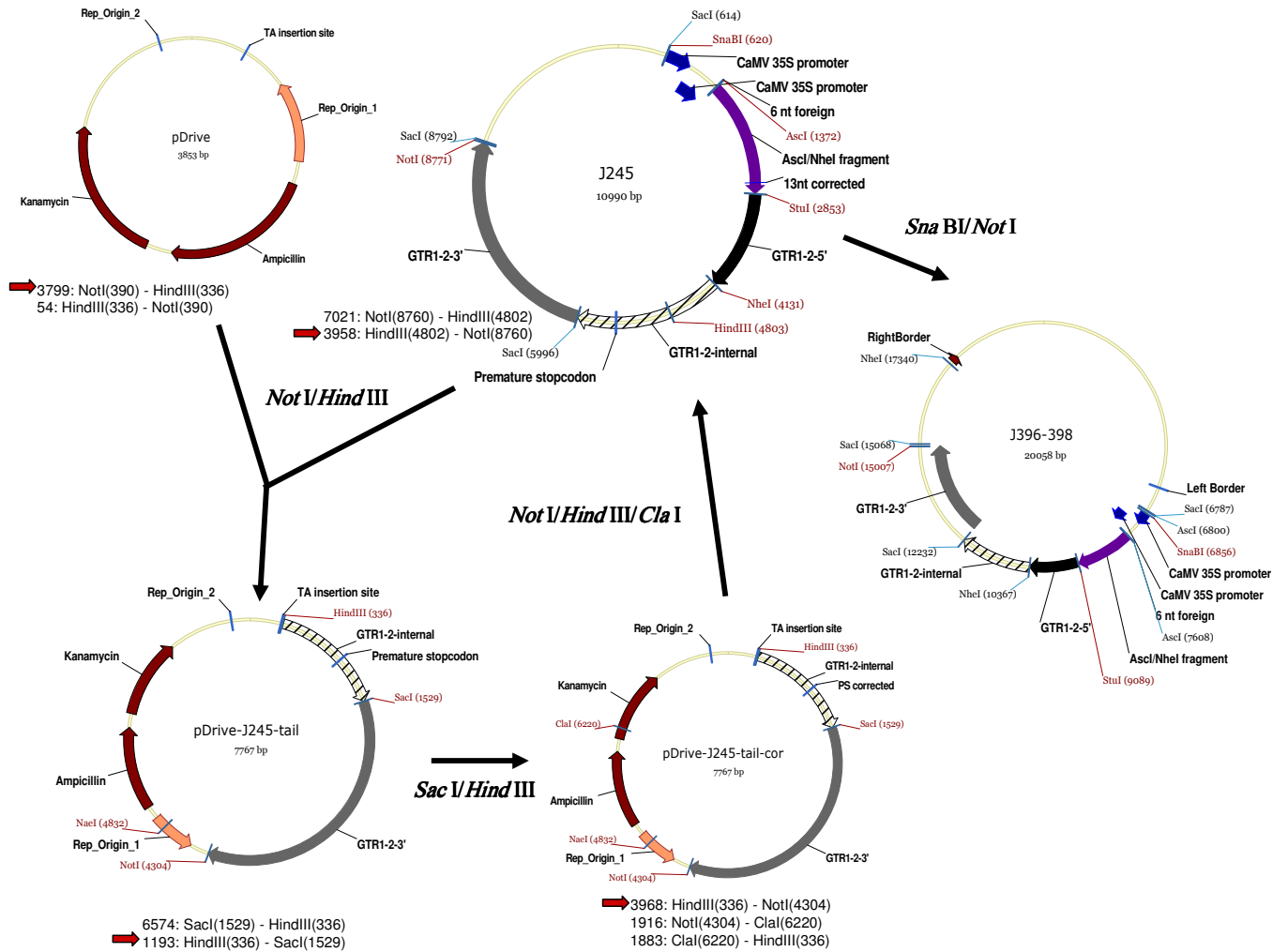**Figure 5. Steps followed in correction of the 13 nt deletion in ORF 1 of pBIN-e35S-GTR1-2fl-pA.**

Figure 6. Steps followed in correction of the premature stop codon in ORF 1 to result in pBIN-e35S-GTR1-2fl*-pA (J396-398).