The molecular and biological characterisation of ORF5 of three South African variants of Grapevine Vitivirus A

by

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Declaration

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Abstract

Grapevine Vitivirus A (GVA), genus *Vitivirus*, family *Flexiviridae* is a well characterised single-stranded RNA virus that has been implicated in the grapevine diseases, Kober stem grooving and Shiraz disease. The virus infects both its host, *Vitis vinifera* and the experimental model plant, *Nicotiana* spp.. Biological studies performed on the virus in its herbaceous host, *Nicotiana benthamiana*, revealed that many divergent variants of the virus exists in South Africa and can induce different symptoms in the model plant. Further molecular analysis divided the variants into three molecular groups based on molecular heterogeneity and nucleotide identity. The establishment of an infectious full-length cDNA clone of GVA contributed towards the elucidation of gene functions for 4 of the 5 open reading frames (ORF's), and indicated ORF5 as the pathogenicity determinant within the genome. Further studies also showed that ORF5 encodes for a nucleic acid binding protein that exhibits suppression activity of a plants' natural virus silencing mechanism. Many proteins that have previously been identified as the pathogenicity determinant within a viral genome have been found to encode for suppression activity. Although suppression activity has been elucidated within the ORF5 of the Italian cDNA clone of GVA, IS 151, no such study has yet been performed on the divergent South African variants of GVA. Three variants, GTR1-1, GTR1-2 and GTG11-1, which represent each of the molecular groups (Group III, II and I), were selected for this study. The aim of this study was to visually elucidate suppression activity of RNA transgene silencing by the ORF5's of GTR1-1, GTR1-2 and GTG11-1 in a transient expression assays in transgenic *N. benthamiana* (line 16c). Pathogenicity studies for these variants were also performed. The ORF5 of the infectious full-length clone, GVA118, which can also serve as an expression vector, was deleted and provided with restriction enzyme sites into which the respective ORF5s and the marker genes, GFP and GUS could be cloned directionally. Infectivity, symptom development and systemic movement were compared between the different full length clones after co-infiltration in *N. benthamiana*. Preliminary results obtained in this study failed to visually indicate any suppression activity encoded by the ORF5 of GTR1-1, GTR1-2 and GTG11-1. The deletion of ORF5 within GVA118 was successful and rendered the infectious full length clone asymptomatic. Directional cloning of the ORF5 of GTR1-1 into the unique restriction enzymes provided previously, resulted in much milder symptoms than those observe for GTR1-2 and GTG11-1. No GFP and GUS accumulation could be detected. This study has established an infectious full-length cDNA clone, pBINSN-e35S-GVA118ΔORF5-1-1-pA, that can possibly induce much milder symptoms in the herbaceous host, *N. benthamiana*. This construct can be further characterised as a possible expression vector of foreign proteins in herbaceous hosts and grapevine.

Opsomming

Grapevine Vitivirus A (GVA), genus *Vitivirus*, familie *Flexiviridae* is 'n goed-gekaraktiseerde enkelstring RNS virus wat ge-assosieer word met wingerdsiektes soos 'Kober stem grooving' en Shiraz siekte. Die virus kan infeksie in beide die gasheer, *Vitis vinifera* en die eksperimentele model gasheer, *Nicotiana* veroorsaak. Biologiese studies van die virus in sy eksperimentele gasheer, *Nicotiana benthamiana*, het gewys dat baie diverse variante van die virus bestaan in Suid Afrika, wat ook verskillende simptome in die modelplant veroorsaak. Verdere molekulêre analise, gebaseer op diversiteit en nukleotied identiteit, het die variante in drie molekulêre groepe geplaas. Die konstruksie van 'n infektiewe vol-lengte kDNS kloon van GVA het ook bygedra tot die identifikasie van geen funksies vir 4 van die 5 oop leesrame (OLR), en het aangedui dat OLR5 die bepalende faktor in die virus se vermoë om simptome te veroorsaak is. Verdere studies het aangedui dat OLR5 kodeer vir 'n nukleïensuur-bindende protein wat 'n plant se natuurlike vermoë om virale infeksie te inhibeer, kan onderdruk. Baie proteïene wat die bepalende faktor in 'n virus se vermoë is om simptome te veroorsaak beskik ook oor die vermoë om 'n plant se inhibisie meganisme te onderdruk. Die onderdrukkingsvermoë van GVA is in die Italiaanse kDNS kloon van die virus, Is151, ontdek, maar is nog glad nie in die Suid Afrikaanse variante geïdentifiseer nie. Die drie Suid Afrikaanse variante, GTR1-1, GTR1-2 and GTG11-1, wat die drie molekulêre groepe verteenwoordig, is gekies vir die studie. Die doel van die studie was om die onderdrukkingsvermoë van die onderskeie variante se OLR5'e op 'n plant se inhiberingsmeganisme te bepaal en te ondersoek in 'n tydelike uitdrukkings studie op transgeniese *N. benthamiana* (lyn 16c). Die vermoë van diverse variante om verskillende simptome te veroorsaak is ook ondersoek. Die OLR5 van die infektiewe vol-lengte kDNS kloon , GVA118, wat as 'n uitdrukkingsvektor kan funksioneer, is verwyder en vervang met unieke restriksie-ensiemsetels. Die onderskeie OLR5'e van die diverse variante en die merker gene, GFP en GUS kon in 'n spesifieke oriëntasie met behulp van die unieke restriksie-ensiemsetels in die kDNS kloon, GVA118, gekloneer word. Die vermoë van die verskillende klone om infeksie in *N. benthamiana* plante te veroorsaak, sistemies te beweeg en verskeie simptome te induseer is ondersoek en vergelyk tussen die onderskeie klone. Hierdie studie kon nie daarin slaag om onderdrukkingsaktiwiteit te identifiseer vir die OLR5 van GTR1-1, GTR1-2 and GTG11-1 nie. Die verwydering van OLR5 in GVA118 het die kloon asimptomaties gelaat. Die vervanging van die OLR5 met die OLR5 van GTR1-1 het meer gematigde simptome tot gevolg gehad as wat waargeneem is vir die vervanging met die OLR5 van GTR1-2 en GTG11-1. Geen GFP fluoresensie of GUS versameling in die sitosol van die plant se selle is waargeneem nie. Tydens hierdie studie is 'n vol-lengte kDNS kloon, pBINSN-e35S-GVA118ΔORF5-1-1-pA daar gestel wat meer gematigde simptome in die modelgasheer, *N. benthamiana* veroorsaak. Hierdie konstruk kan verder gekaraktiseer word as 'n moontlike uitdrukkingsvektor van vreemde proteïene in model gashere en wingerd.

Abbreviations

A	Adenine
Ago	ARGONAUTE
Amp	Ampicilin
ATP	Adenosine Triphosphate
bp	Base pairs
β-ME	β- Mercaptoethanol
BMYV	Beet Mild Yellowing Virus
BSA	Bovine Serum Albumin
C	Cytosine
CaMV	Cauliflower Mosaic Virus
CMV	Cucumber Mosaic Virus
CHS	Chalcone synthase gene
cDNA	Complementary deoxyribonucleic acid
CP	Coat Protein
DCL	Dicer-like
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleoside triphosphate (s)
DTT	Dithiothreitol
dH_2O	Distilled water
dpi	days post inoculation
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylenediamine Tetra-acetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EtBr	Ethidium bromide
GFP	Green Fluorescent Protein
GUS	β-glucuronidase
GVA	Grapevine virus A

GVB	Grapevine virus B
GVC	Grapevine virus C
GVD	Grapevine virus D
HEPES	*N*-(2-hydroxyethyl)piperazine-*N'*-(ethanesulfonic acid)
HC-Pro	Helper Component proteinase
HLV	Hercaleum Latent Virus
hpRNA	hairpin RNA
ICTV	International Committee on Taxonomy of Viruses
Kn	Kanamycin
LB	Luria Bertoni broth
MES	10 mM 2-[Morpholino]ethanesulfonic acid
mRNA	messenger RNA
miRNA	microRNA
MP	Movement Protein
nts	Nucleotides
OD_{600}	Adsorption value at 600 nm
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PPV	Plum Pox virus
PTGS	Post-transcriptional gene silencing
RdRp	RNA-dependent RNA polymerase
Rif	Rifampicin
RISC	RNA-induced silencing complex
RITS	RNA-induced transcriptional silencing complex
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute

SEM	Scanning electron microscopy
siRNA	small interfering RNA
ssRNA	single-stranded RNA
TAE	Tris, acetic acid and EDTA
Tet	Tetracycline
Tris	tris(hydroxymethyl)aminomethane
TPIA	Tissue print immuno-assay
μg	microgram
UTR	Untranslated region
UV	Ultraviolet
VIGS	Virus induced gene silencing
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Zn	Zinc

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Contents

1 Introduction **1**

2 Literature Review **4**
- 2.1 Background . . . 4
- 2.2 Genomic and biological properties of GVA . . . 7
  - 2.2.1 Taxonomy and genomic properties . . . 7
  - 2.2.2 Biological properties . . . 9
  - 2.2.3 Molecular diversity . . . 10
- 2.3 Suppression of gene silencing in plants by viral suppressors . . . 11
  - 2.3.1 RNA silencing . . . 11
  - 2.3.2 Post-transcriptional gene silencing (PTGS) . . . 14
  - 2.3.3 Suppression of PTGS . . . 16
  - 2.3.4 GVA-ORF5 as suppressor . . . 17
- 2.4 Viral full-length cDNA clones and their uses . . . 19

3 Materials and methods **21**
- 3.1 General materials and methods used during this study . . . 21
  - 3.1.1 Primer design . . . 21
  - 3.1.2 PCR amplification . . . 22
  - 3.1.3 Agarose gel electrophoresis . . . 23
  - 3.1.4 PCR product gel purification . . . 23
  - 3.1.5 Cloning of PCR and restriction enzyme digest products . 23
  - 3.1.6 Cloning of purified PCR products into the pDRIVE cloning vector . . . 24
  - 3.1.7 Transformation and selection of recombinant colonies by PCR . . . 24
  - 3.1.8 Plasmid extraction and purification . . . 25
  - 3.1.9 Restriction enzyme digestion reactions . . . 25
  - 3.1.10 Sequencing . . . 26
  - 3.1.11 Freezer cultures . . . 26
- 3.2 Suppressor activity assay in transgenic *N. benthamiana* (16c) plants . . . 26
  - 3.2.1 pT7T318U vector constructs . . . 26
  - 3.2.2 Construction of the binary vector clones . . . 27
  - 3.2.3 Plasmids used as controls during the suppressor activity assay . . . 27

3.2.4 Electroporation of *Agrobacterium* cells . . . 28
3.2.5 Growth conditions for the transgenic *N. benthamiana* (16c) plants . . . 29
3.2.6 *Agrobacterium*-infiltration of plants . . . 29
3.2.7 Visualization of GFP fluorescence in transgenic *Nicotiana benthamiana* (16c) plants . . . 31
3.3 Deletion and replacement of ORF5 in GVA118 . . . 31
3.3.1 Overlap extension PCR . . . 31
3.3.2 Cloning of the overlap extension PCR product . . . 34
3.3.3 Construction of a partial GVA118-pA construct with PCRΔORF5 . . . 34
3.3.4 Deletion and replacement of ORF5 in pSKM-e35S-GVA118-pA with GVA118ΔORF5 . . . 35
3.3.5 Re-introduction of ORF5 from three variants, GFP and GUS into pSKM-e35S-GVA118ΔORF5-pA . . . 35
3.3.6 Construction and infiltration of binary vectors with the complete viral cassettes for GVA118ΔORF5, -1-1, -1-2, -11-1, -GFP and -GUS . . . 38
3.3.7 Tissue prints . . . 39
3.3.8 Recording of symptom development . . . 39
3.3.9 Rapid direct-one-tube RT-PCR . . . 39

4 Results 41
4.1 Suppression assay using GVA ORF5 in transgenic *N. benthamiana* 41
4.1.1 Cloning of ORF5 of GTR-1-2 and GTG11-1 into pBIN61S 41
4.1.2 Co-infiltration assay in transgenic *N. benthamiana* (line 16c) . . . 45
4.2 Deletion and replacement of ORF5 in the expression vector, GVA118 . . . 49
4.2.1 The deletion of ORF5 by overlap extension PCR . . . 49
4.2.2 Construction of partial GVA118-pA construct with PCRΔORF5 . . . 51
4.2.3 Deletion and replacement of ORF5 in pSKM-e35S-GVA118-pA with GVA118ΔORF5 . . . 52
4.2.4 Cloning of ORF5, GFP and GUS into pSKM-e35s-GVA118-ΔORF5-pA . . . 53
4.2.5 Construction and infiltration of pBINSN-GVA118ΔORF5-1-1, -1-2, -11-1, GUS and GFP . . . 56
4.2.6 Viral replication and symptom development in *N. benthamiana* . . . 58

5 Discussion 66
5.1 Suppression activity of ORF5 of GTR1-1, GTR1-2 and GTG11-1 66
5.2 Deletion and replacement of ORF5 in the expression vector, GVA118 . . . 69
5.3 Future work . . . 73

6 Conclusion 75

Bibliography 77

7 Vector Maps 87

8 General Reagents and Media 95

9 Antibiotic selection for different plasmids, *E. coli* and *Agrobacterium* strains 97

List of Figures

2.1 A diagrammatic representation of the GVA genome 5
2.2 Phylogenetic analysis and neighbour-joining tree indicating the three molecular groups observed for GVA 11
2.3 A simplified model of RNA silencing showing the importance of dsRNA 13
2.4 Amino acid alignment of ORF5 18
2.5 Schematic diagram of the expression vector, GVA118 20

3.1 Overlap extension PCR used to create a chimeric GVA118 fragment 32
3.2 The ΔORF5 site of pSKM-e35S-GVA118ΔORF5-pA 36
3.3 The ΔORF5-restriction enzyme sites with the respective inserts 37

4.1 A chromatogram indicating the incorporation of unique restriction enzyme sites at the 5'- and 3'-terminal of ORF5 42
4.2 The digestion of the pDRIVE-ORF5 constructs to obtain the respective ORF5s 43
4.3 The respective ORF5's were obtained from the intermediary vector, pT7T318U by restriction enzyme digestion 44
4.4 PCR results for the constructs, pBIN61S-ORF5-1-1, -1-2 and 11-1 after successful cloning 45
4.5 GFP fluorescence 3dpi in transgenic *N. benthamiana* (line 16c) co-infiltrated with GFP and the strong suppressors, HC-Pro and P0 46
4.6 Screening of ORF5 of GTR1-1, GTR1-2 and GTG11-1 for suppression activity in transgenic *N. benthamiana* 47
4.7 Fluorescence microscopy of transgenic leaves co-infiltrated with GFP and the strong suppressors, HC-Pro and P0 48
4.8 Fluorescence microscopy of leaves co-infiltrated with pBIN61S-ORF5-1-2:pBIN61S-GFP and pBIN61S-ORF5-11-1:pBIN61S-GFP 49
4.9 Overlap extension PCR products 50
4.10 Sequence chromatogram showing the successful incorporation of the overlap extension PCR products that replaces ORF5. 51
4.11 The cloning of PCRΔORF5 into pLitmus38-118 52
4.12 The construction of pSKM-e35s-GVA118ΔORF5 53
4.13 PCR amplification of ORF5 of GTR1-1, GTR1-2 and GTG11-1 for cloning into pSKM-e35S-GVA118ΔORF5-pA 54

4.14 Sequence chromatogram of the ORF5 of GTR1-2 with the restriction enzyme sites, PdiI and Mph11031 54
4.15 Digestion of ORF5, GFP and GUS for cloning into pSKM-35s-GVA118ΔORF5 55
4.16 Digestion of the constructs, pSKM-e35S-GVA118ΔORF5, -1-1, -1-2, -11-1, -GFP and -GUS for cloning into pBINSN 57
4.17 A example of an EcoRI restriction enzyme digestion to confirm the cloning of the full-length e35S-GVA118ΔORF5-pA construct into pBINSN 58
4.18 TPIA results indicating replication for the construct, pBINSN-e35S-GVA118ΔORF5-1-2-pA 59
4.19 Microscopy of GFP fluorescence and GUS colouring reaction in leaf tissue co-infiltrated with the strong suppressor, P0 and the constructs, pBINSN-GVA118ΔORF5-GFP and -GUS 60
4.20 Transmission electron microscopy of GR-5 and pBINSN-e35S-GVA118Δ-ORF5-1-2-pA 61
4.21 Symptom development in *N. benthamiana* after co-infiltration with the suppressor, P0 and the constructs, pBINSN-GVA118Δ-ORF5, -1-1, 1-2, -11-1, -GFP and -GUS at 14 dpi 62
4.22 Symptom development in plants infiltrated with pBINSN-e35S-GVA118ΔORF5-pA constructs at 20 dpi 63
4.23 The asymptomatic *N. benthamiana* plants at 30 dpi 64
4.24 Symptom development in *N. benthamiana* plants at 30 dpi 65

7.1 pT7T318U cloning vector 87
7.2 pBIN61S-talk 88
7.3 pBIN_SN 89
7.4 pSKM-e35S-GVA118-pA 90
7.5 pLitmus38-118 91
7.6 pLitmus38-118deltaORF5 92
7.7 pSKM-e35S-GVA118ΔORF5-pA 93
7.8 pSKM-e35S-GVA118deltaORF5-pA 94

List of Tables

3.1 Primers to obtain ORF5 of three SA GVA variants with unique restriction enzyme sites 22
3.2 Primer sequences for the amplification of ORF5 of GTR1-1, GTR1-2 and GTG11-1, for ligation into pSKM-e35S-GVA118-ΔORF5-pA 22
3.3 Summary of positive and negative controls included in the final 16c suppression activity assay 30
3.4 Overlap extension PCR primers to create a chimeric GVA118 without ORF5 33

4.1 A summary of the final pBINSN-e35S-GVA118ΔORF5-pA constructs 57

Chapter 1

Introduction

Grapevine Vitivirus A (GVA) was initially detected in Italian vineyards almost thirty years ago and is commonly found in grapevines worldwide today (Conti *et al.*, 1980). Although the virus's role in grapevine diseases is still largely unknown, studies have shown close association with Kober stem grooving disease, Shiraz disease of grapevines in South Africa and Syrah decline, notably in France, California and Australia (Garau *et al.*, 1994; Goszczynski, 2007).

GVA is a phloem-limited virus and can be transmitted biologically between grapevines by several species of pseudococcid and coccid mealybugs, and mechanically to herbaceous host plants such as *Nicotiana benthamiana* (Rosciglione *et al.*, 1983). The virus has been fully sequenced (Minafra *et al.*, 1994, 1997) and characterised extensively through molecular and biological techniques. The positive sense single-stranded (ss) RNA genome of GVA is organised into five open reading frames (ORF) and functions have been identified for 4 of the 5 ORF's (Galiakparov *et al.*, 2003c). Although the protein product of ORF2 shows some homology to a similar protein product in Grapevine Vitivirus B (GVB) (Minafra *et al.*, 1997), the function of the polypeptide encoded by the ORF is still unknown (Galiakparov *et al.*, 2003c).

The ORF5 of GVA encodes for a nucleic-acid binding protein (p10) that is also the pathogenicity determinant within the GVA genome (Galiakparov *et al.*, 2003b). Conserved regions that are associated with suppression activity within known and putative silencing suppressors have been identified within the amino acid sequence of the ORF (Chiba *et al.*, 2006). Suppression activity, by means of siRNA sequestering, was observed during transient expression assays for the ORF5 of the infectious cDNA clone, Is151 (Zhou *et al.*, 2006) . These kind of assays have not yet been performed for any of the divergent South African variants of GVA.

The isolation of several South African strains of GVA from mechanically inoculated *N. benthamiana* made the identification of divergent variants of the virus possible (Goszczynski and Jooste, 2003a). The virus is heterogeneous and identified variants of the virus are grouped into three molecular groups (I, II and III) based on nucleotide identity (Goszczynski and Jooste, 2003b). The divergent GVA isolates were found to induce symptoms of different severity in *N. benthamiana* plants which can be associated with the three molecular

groups (Goszczynski *et al.*, 2008). The isolates in molecular group III were found to induce mild symptoms such as mild vein clearing, whilst group I an II isolates were found to cause more severe symptoms (Goszczynski and Jooste, 2003b). Pathogenicity determinants within viral genome sequences are often associated with silencing suppression activity (Voinnet *et al.*, 1999), that make divergent variants of GVA which differ in pathogenicity, an interesting target for suppression activity identification.

RNA silencing is a general term that describes a RNA-based immune reaction phenomenon observed in animals, plants, fungi and protists (Baulcombe, 2004). In plants this RNA-based immunity, referred to as post-transcriptional gene silencing (PTGS), results in the degradation of replicating ssRNA viruses by small RNA strands, and so protects the plant genome from invading viruses (Baulcombe, 2004). Through co-evolution virus genomes have evolved the ability to encode silencing suppression proteins which interfere with the silencing mechanism of plants, and allow for the expression of the virus within the plants' genome (Waterhouse *et al.*, 2001).

In order to understand and elucidate pathogen-host interactions such as pathogenicity and suppression it is necessary to study viral genomes at a molecular level. A powerful tool for such studies are full-length infectious clones of the viral genome in question. A full-length infectious clone is a copy (cDNA) of a viral genome that is obtained with a reverse transcription reaction. Such clones have been constructed previously for several viruses including GVA. Infectious full-length cDNA clones can be produced with either *in vitro* transcription of cDNA that is driven by a T7 or SP6 phage promoter, or *in vivo* transcription that is driven by a Cauliflower Mosaic Virus (CaMV) 35S promoter. Many infectious full-length cDNA clones have been constructed for GVA, but none for the South African variants of GVA. An infectious, full-length cDNA clone (GVA118) has been constructed previously by Haviv *et al.* (2006a) and is comprised of the ORF1 and 2 of the infectious full-length clone, GR-5 (Galiakparov *et al.*, 2003a) and the ORF3, 4 and 5 of the South African variant, GTR1-3 (Goszczynski and Jooste, 2003a). This full-length clone has also been modified into an expression vector (Haviv *et al.*, 2006a).

Viral vectors offer an alternative method for transgenic modification of plants, and can be utilised to study expression or silencing of genes in plants or screening of unknown sequences for possible functions. Transient expression vectors are based on full-length cDNA clones that have been modified to express foreign gene inserts in plants. An application for such technology includes the cheaper and safer production of diagnostic or therapeutic proteins and/or enzymes in plants when compared to other eukaryotic systems (Lico *et al.*, 2008). A GVA-based transient expression vector could contribute towards the investigation of some grapevine diseases, and be used as a vector for the production of resistance genes in grapevine (Haviv *et al.*, 2006a).

Although GVA has been identified as suitable target for modification into a viral vector because of its host range that includes grapevine and *Nicotiana* spp., the virus is phloem-limited, which restricts foreign protein expression to the vascular tissue of the plant. The virus is also associated with diseases

such as Kober stem grooving and Shiraz disease in grapevine which can be transmitted easily by mealybugs. The expression vector, GVA118 has not yet been characterised within grapevine, but induces severe symptoms that could mask protein expression in herbaceous hosts such as *N. benthamiana* and is not as effective as other viral-based vectors (Haviv *et al.*, 2006a).

To further understand symptom severity encoded by different variants of the same virus and possibly identify a correlation between suppression activity and pathogen severity, three South African variants of GVA, representing each of the three molecular groups, were identified for further biological and molecular characterisation in this study.

Project proposal

The ORF5 of GVA encodes for a nucleic-acid binding protein (p10) that exhibits silencing suppression activity and is also the pathogenicity determinant within the GVA genome.

This project proposes to elucidate the suppression activity encoded by the ORF5's of three South African variants (GTR1-1, GTR1-2 and GTG11-1) of GVA, representing each of the three molecular groups (I, II and III), in a co-infiltration experiment with the green fluorescent protein-gene (GFP) in transgenic *N. benthamiana* plants (line 16c). The suppression activity assay will entail the cloning of ORF5 of three South African variants (GTR1-1, GTR1-2 and GTG11-1) into a TA-cloning vector for sequencing purposes, after which the ORF5s will be cloned into a binary plant vector for the transient expression assay using the plant pathogen, *Agrobacterium tumefaciens*. The plants will be evaluated visually for GFP fluorescence with ultra violet (UV) light.

The ORF5 of GTR1-1, GTR1-2 and GTG11-1 will be evaluated as pathogenicity determinants within the infectious full-length cDNA clone, GVA118. The ORF5 of GVA118 will be deleted and replaced with unique restriction enzyme sites by overlap extension PCR. The unique restriction enzyme sites will allow for the integration of the respective ORF5 sequences and marker genes such as GFP and GUS by site directed cloning. The completed clones will be sub-cloned respectively into a plant binary vector and tested for infectivity in *N. benthamiana* and *V. vinifera* plants. If the clones prove to be infectious within *N. benthamiana*, differences in symptom severity and systemic movement between the constructs will be evaluated. The clones with the marker genes, GFP and GUS will also be evaluated for GFP fluorescence and GUS accumulation with the appropriate assays.

The aim of this study is thus to elucidate suppression activity for the ORF5 of the South African variants of GVA, GTR1-1, GTR1-2 and GTG11-1, which represents the three molecular groups. The divergent variants of GVA also differ in pathogenicity, and these differences will be studied with the use of the full-length infectious cDNA clone, GVA118 in *N. benthamiana* and *V. vinifera* plants.

Chapter 2

Literature Review

2.1 Background

Grapevine stem-pitting disease was initially described in Italy, followed by reports of the disease in other European countries, as well as Israel and South Africa. It was also in Italy where GVA was first reported and described as a closterovirus associated with stem-pitting diseased vines (Conti *et al.*, 1980). The virus was provisionally referred to as Grapevine stem-pitting (GSP)-associated virus (GSP-AV) and found to consist of phloem-limited, flexuous filamentous particles that were approximately 800 nm in size (Conti *et al.*, 1980). It was determined that the genome of the virus consisted of single-stranded RNA with base composition, ribonucleic A sensitivity and melting behavior (Boccardo and D'Aquilio, 1981). By 1983, the virus was named Grapevine virus A (GVA), and was found to be transmitted by mealybugs (*Pseudococcidae*) between grapevines (*Vitis vinifera*), but not from grapevine to a herbaceous host such as *Nicotiana benthamiana* (Rosciglione *et al.*, 1983). It was found that GVA could be transmitted mechanically to *N. benthamiana* and *N. clevelandii* by sap inoculation, and was also the first grapevine virus transmitted in such a manner (Conti *et al.*, 1980).

In the early 1990's GVA was the only grapevine closterovirus that was extensively characterised and investigated in terms of biological, serological and epidemiological properties, and a reliable ELISA protocol utilising monoclonal antibodies could be established for the detection of the virus (Boscia *et al.*, 1992). An association between GVA and Rupestris stem pitting, a disease of the rugose wood complex, was suggested in 1990 (Gugerli *et al.*, 1991) and association with Kober stem grooving of grapevines was suggested in 1993 by Chevalier *et al.* (1993). GVA was also suggested to be associated with Shiraz disease of grapevine in South Africa by Burger and Spreeth (1993).

The nucleotide sequence of the 3' terminal region of GVA (a total of 1883 bp) was reported by Minafra *et al.* (1994), and three putative open reading frames were identified. Although GVA has been characterised extensively, molecular properties of the virus were still largely unknown. The 5' terminal region, encompassing 5 466 bp, was reported three years later (Minafra *et al.*, 1997), and based on the sequence data, GVA was tentatively grouped in the

Trichovirus genus. Two more putative ORF's were identified in the single strand RNA genome that was approximated to consist of 7 800 nucleotides. The availability of the complete sequence of GVA (isolate Is151) allowed for the tentative assignment of gene functions to the 5 ORF's based on sequence homology to known genes. ORF1 contains conserved regions of replication-related proteins of positive-strand RNA viruses, and although ORF2 showed 44% homology to a similar ORF in GVB, no function could be assigned (Minafra *et al.*, 1997). The putative movement protein was detected with serological methods and confirmed to be expressed by ORF3 (Rubinson *et al.*, 1997). ORF4 was identified as the coat protein cistron and substantial sequence homology was shared with other *Tricho*-and *Capilloviruses*(Minafra *et al.*, 1994).
The organization of the GVA genome into its 5 ORF's is shown in Figure 2.1.

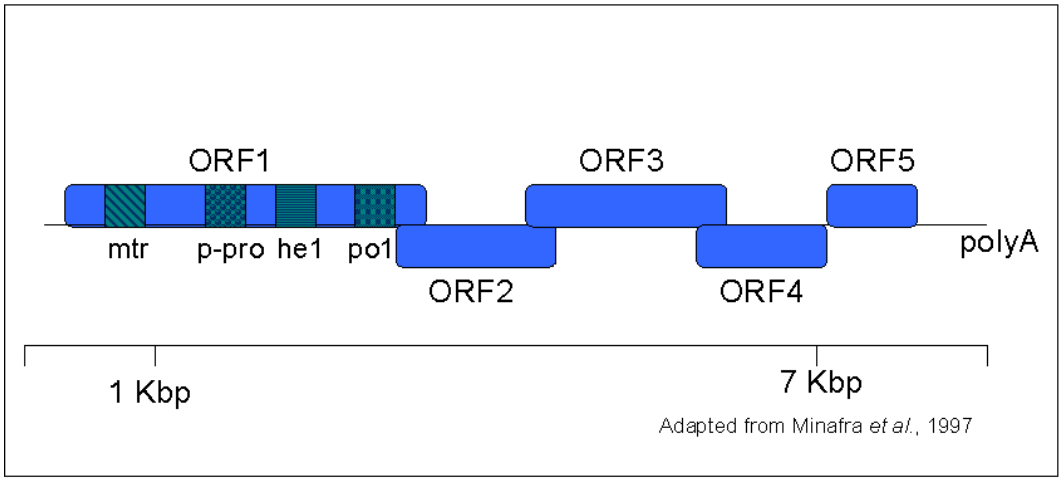

Figure 2.1: A schematic representation of the genome of Grapevine virus A suggested by Minafra *et al.* (1997), after the complete genome of GVA was fully sequenced. Amino acid sequence alignment revealed homology between ORF1 and replication-related proteins. The four regions indicated in ORF1 corresponds to the putative methyltransferase domain of the 'Sindbis-like' super group of positive-strand RNA viruses, NTP-binding motifs of helicases and the GDD signature motif of RdRp (Minafra *et al.*, 1997). ORF2 did not show any significant homology to any known proteins (Minafra *et al.*, 1997). ORF3 and 4 have previously been found to show strong homology to the movement and coat protein of tricho- and capilloviruses, respectively, and have since been shown to encode for these proteins (Minafra *et al.*, 1994; Galiakparov *et al.*, 2003c).

The complete sequence of GVA made more complete phylogenetic studies of the virus possible. GVA was previously placed in the genus *Trichovirus* together with two definitive species, Apple chlorotic leafspot virus (ACLSV) and Potato virus T (PVT), and three tentative species, Grapevine virus B (GVB), Grapevine virus D and Heracleum latent virus (HLV), which have been sequenced completely with the exception of the two latter viruses (Monette and James, 1991; Minafra *et al.*, 1997; German *et al.*, 1990). Phylogenetic analysis of the sequences showed molecular and biological differences that suggested a re-evaluation of the taxonomy of GVA, GVB and GVD (Martelli *et al.*, 1997).

The new genus, *Vitivirus*, was established and approved by the ICTV. The four viruses, GVA (type species), GVB, GVD and HLV were included in the genus, and Grapevine virus C (GVC) was proposed as a tentative species (Martelli *et al.*, 1997). Mint-virus 2 has also recently been identified as a member of *Vitivirus* (Tzanetakis *et al.*, 2007). The genus *Vitivirus* of which GVA is the type species have been included in the new plant virus family, *Flexiviridae*, that has been described by Adams *et al.* (2004).

The first full-length cDNA clone for GVA (isolate PA3), and also the first for a member of the *Vitivirus* genus, was constructed by Galiakparov *et al.* (1999). Infectious RNA transcripts were transcribed *in vitro* from the cDNA clone with T7 polymerase and inoculated mechanically in *N. benthamiana* and *N. clevelandii*. It was proposed that the cDNA clone could contribute to the genetic analysis of the RNA genome, enable studies on gene function and expression, the replication cycle, as well as pathogenicity determinants of GVA (Galiakparov *et al.*, 1999). Another GVA full-length cDNA clone was constructed for the Italian isolate, Is151 by Saldarelli *et al.* (2000), under the control of the T7 bacteriophage promoter. The assembly of a GVA cDNA clone under control of a CaMV-35S promoter was attempted, but was not successful (Saldarelli *et al.*, 2000). Functional analysis of the GVA genome was performed in 2002 by Galiakparov *et al.* (2003c), through systematic mutation of each of the ORF's in the previously constructed infectious clone (Galiakparov *et al.*, 1999). Functions could be assigned to four of the 5 ORF's, but the function of the polypeptide encoded by ORF2 is still unknown.

Different strains of plant viruses are commonly found, and although previous studies have indicated the presence of different variants, it was only found in 2002 that GVA has extensive molecular heterogeneity (Goszczynski and Jooste, 2002). Molecular and biological studies of various isolates retrieved from mechanically inoculated *N. benthamiana* made the identification of divergent variants of GVA possible (Goszczynski and Jooste, 2003a). Eight isolates of GVA were transmitted mechanically to *N. benthamiana* from various grapevines and were found to induce four kinds of symptoms in the herbaceous host, the first being mild vein clearing, the second was vein clearing and interveinal chlorosis, third was vein clearing, interveinal chlorosis and strong curling of the upper leaves and the last was extensive necrosis (Goszczynski and Jooste, 2003a).

Sequencing of the isolates revealed that the variants of GVA clusters in three molecular groups (I, II and III) based on nucleotide identity. The isolates in molecular group III were found to induce mild symptoms whilst group I and II isolates were found to induce more severe symptoms in *N. benthamiana* (Goszczynski and Jooste, 2003a). Mixed infections by divergent variants of GVA also seems to be common in South African grapevines and a variant specific RT-PCR technique was developed for the identification of these variants (Goszczynski and Jooste, 2003b).

The genome of GVA was engineered into an expression vector in 2006 (Haviv *et al.*, 2006a) to express proteins in a herbaceous host such as *N. benthamiana*. The vector, GVA118, was assembled with duplicated movement protein

(MP) controller elements (CE) from the GVA strain, GTR1-3 (Goszczynski and Jooste, 2003b), and the ORF3 (MP), ORF4 (CP) and ORF5 of this strain. The ORF1 and ORF2 was obtained from the infectious GVA clone, GR-5 (Galiakparov *et al.*, 2003a), and the vector was constructed to contain restriction enzyme sites between two MP-subgenomic promoters (MP-CE) where foreign genes can be inserted for transient expression (Haviv *et al.*, 2006a). The genomic organisation of the of the vector, GVA118, is represented schematically in figure 2.5.

The ORF5 of GVA was identified as a RNA-silencing suppressor in 2006 (Zhou *et al.*, 2006) with transient expression experiments in transgenic *N. benthamiana* expressing GFP. The p10 protein encoded by ORF5 suppress post transcriptional gene silencing of the host plant by the sequestering of siRNAs (Zhou *et al.*, 2006). Possible weak suppression activity of GVA p10, which was shown to belong to a family of proteins containing Zn-ribbons, was also reported by Chiba *et al.* (2006).

Other applications for GVA, with the exception of expression vectors already mentioned, also include pathogen-derived resistance. The identification of the coat protein (CP) of GVA opened up the possibility of the use of this protein to confer resistance to *N. benthamiana* and grapevine through genetic transformation. The coat protein of GVA was transformed with *A. tumefaciens* into *N. benthamiana* and a number of these transformed plants were resistant to GVA (Radian-Sade *et al.*, 2000). The insertion and expression of an antisense RNA construct of the movement protein (MP) of GVA in grapevine (*Vitis rupestris*) also showed to be a promising tool for attaining pathogen-derived resistance in grapevine (Martinelli *et al.*, 2002).

Although GVA has been characterised extensively through biological and molecular techniques, its involvement in grapevine diseases such as Kober stem grooving, Shiraz disease and Rugose-wood disease complex is still largely unknown. The characterisation of the virus revealed a broad host range which makes it a suitable candidate for alternative plant transformation strategies, and the virus has been modified into an infectious expression vector. Suppression of post-transcriptional gene silencing in plants has not yet been characterised in South African variants of GVA. Severe symptoms induced by viral expression vectors can mask the expression of foreign proteins. The determination of pathogenicity and suppression activity within specific variants of GVA genome is necessary if the virus is to be developed into an effective expression vector.

2.2 Genomic and biological properties of GVA

2.2.1 Taxonomy and genomic properties

Grapevine Vitivirus A is the type species of the genus, *Vitivirus* (Martelli *et al.*, 1997) and belongs to the plant virus family, *Flexiviridae* (Adams *et al.*, 2004). The viral genome has been sequenced fully and is capped at the 5' terminus and polyadenylated at the 3' terminus, with untranslated regions

(UTR) at both the 5' and 3' regions of 86 and 68 nt respectively (Minafra *et al.*, 1997).The virions are helical, flexuous (hence the name of the virus family) filaments that are 800 nm in length, and contain a single-stranded positive-sense RNA genome of 7 349 nucleotides (nt) with five ORF's of which the first four are overlapping (Adams *et al.*, 2004).

The functions of the ORF's were proposed by systematic mutation of the ORF's of an infectious cDNA clone of GVA and biological analysis of the protein products encoded by these ORF's (Galiakparov *et al.*, 2003c). Open reading frame 1 encodes a 194 kDA polypeptide with conserved regions corresponding to the 'Sindbis-like' supergroup of replication-related proteins in single-stranded RNA viruses (Galiakparov *et al.*, 2003c). These conserved motifs correspond to the methyl-tranferase domain, helicase motif and the RNA-dependent RNA-polymerase domain (Minafra *et al.*, 1997). Systemic mutation of ORF1 of GVA abolished RNA replication, and confirmed that the polypeptide encoded by ORF1 is responsible for RNA replication of GVA.

Open reading frame 2 of GVA encodes for a 19.8 kDa polypeptide, which has no significant homology to any other known proteins. A decrease of the MP is noted in the presence of a mutated form of ORF2 when compared to the wild type, but no specific function has yet been attributed to ORF2 (Galiakparov *et al.*, 2003c).

Open reading frame 3 of GVA encodes for a 31 kDa protein that was identified through homology as the putative movement protein (Minafra *et al.*, 1994; Rubinson *et al.*, 1997). The protein shows a conserved G/D motif that corresponds to the movement protein '30K superfamily' (Minafra *et al.*, 1994) and functional analysis through mutation of this ORF resulted in the loss of the virus's ability to move from cell to cell (Galiakparov *et al.*, 2003c). This confirmed that ORF3 encodes the MP of GVA.

Open reading frame 4 was experimentally identified as the coat protein (CP) cistron of GVA and also contains the conserved region associated with salt bridge structures found in the CP sequences of filamentous viruses (Minafra *et al.*, 1994). Mutation of the CP results in a lack of viral cell-to-cell movement, and indicates that the CP is required for this type of movement (Galiakparov *et al.*, 2003c).

Open reading frame 5 encodes for a nucleic-acid binding protein (p10) that is indicated as a pathogenicity determinant (Galiakparov *et al.*, 2003b). The ORF5 polypeptide is 10 kDa and can impair viral movement when mutated (Galiakparov *et al.*, 2003c). The protein acts as a RNA-silencing suppressor (Zhou *et al.*, 2006) which binds nucleic acid non-specifically that requires a stretch of basic amino acids known as the "zinc' finger domain (Galiakparov *et al.*, 2003b; Minafra *et al.*, 1997; Chiba *et al.*, 2006).

The virus is transcribed through direct translation of the 5' proximal ORF1 and subgenomic (sg) RNA production for the translation of the ORF's downstream, and replicates within the cytoplasm of a plant cell (Martelli *et al.*, 2008). GVA produce two classes of sgRNA's possibly through three controller elements adjacent to the 5' terminal of the 3' ORF's, excluding ORF5(Galiakparov *et al.*, 2003a). Translation of GVA occurs through the production of 3'-terminal

sgRNAs, with the exception of ORF5 which is possibly expressed by a bi- or polycistronic mRNA (Galiakparov *et al.*, 2003a).

2.2.2 Biological properties

The virus is phloem-limited and naturally infects *V. vinifera* as well as a narrow range of herbaceous hosts, which include *N. clevelandii* and *N. benthamiana*, by sap inoculation (Conti *et al.*, 1980). The virus is transmitted between grapevine, and from grapevine to *N. clevelandii* and *N. benthamiana* by mealybug vectors. These mealybug vectors have been identified as *Pseudococcus longispinus*, *Pseudococcus affini,*, *Planococcus ficus*, *Planococcus citri* (Rosciglione *et al.*, 1983; Engelbrecht and Kasdorf, 1990; Garau *et al.*, 1995) and *Heliococcus bohemicus* (Zorloni *et al.*, 2006). Soft scale mealybugs (Coccidae) have also been identified as vectors for the transmission of GVA and include *Neopulvinaria innumerabilis* (Fortusini *et al.*, 1997) and *Parthenolecanium corni* (Hommay *et al.*, 2008).

The virus is indicated in the aetiology of Kober stem grooving, one of the four syndromes associated with the rugose wood complex (Chevalier *et al.*, 1993; Garau *et al.*, 1994; Credi, 1997), Shiraz disease in South Africa (Burger and Spreeth, 1993; Goszczynski, 2007), and Syrah decline in France, California and Australia.

Rugose wood complex is a severe group of disorders which mainly result in the pitting and grooving of scion and/or rootstock material and occurs worldwide (Credi, 1997; Martelli *et al.*, 2008). This disease is etiologically complex and of economic importance because of reduced growth and yield caused by the disease (Credi, 1997; Savino *et al.*, 1985). GVA has been associated with Kober stem grooving, one of the causative agents in rugose wood complex (Credi, 1997; Garau *et al.*, 1994; Chevalier *et al.*, 1995). Syrah decline and Syrah disorder, found in France and California respectively, is believed to be caused by GVA, although this association has not yet been confirmed experimentally.

Shiraz disease affects Shiraz, Merlot, Gamay, Malbec and Viognier grapevine cultivars in South Africa and gives rise to plants that never mature fully (Goszczynski, 2007; Goszczynski *et al.*, 2008). The disease causes non-lignification of xylem and enlargement of phloem tissue, reduced fruit yield and finally results in death 3-5 years after infection (Goszczynski *et al.*, 2008; Goussard and Bakker, 2006). The GVA variants in molecular group II, with the exception of GTR1-2, have consistently been associated with Shiraz disease, whilst variants in group III have been associated with grapevines susceptible to Shiraz disease that do not show any symptoms of the disease (Goszczynski, 2007; Goszczynski *et al.*, 2008). The difference in symptomology of Shiraz disease caused by the different molecular groups are due to extensive molecular heterogeneity between GVA variants and low nucleotide similarity between parts of the genome (Goszczynski and Jooste, 2002; Goszczynski *et al.*, 2008).

2.2.3 Molecular diversity

Extensive molecular heterogeneity of GVA was shown with single-strand conformation polymorphism (SSCP) (Goszczynski and Jooste, 2002) which led to the identification of divergent South African variants of GVA (Goszczynski and Jooste, 2003a) that could be grouped according to nucleotide similarity into three molecular groups (I, II and III) (Goszczynski and Jooste, 2003a). Similar grouping of GVA was observed in Italy, based on the variability of the CP sequence (Murolo *et al.*, 2008).

Nucleotide sequence identity of 91-99.8% is shared between GVA variants within a group, while nucleotide sequence identity of 78-89.3% is shared between groups (Goszczynski and Jooste, 2003a). Molecular group I and II is associated with severe symptoms in both grapevine (Shiraz disease) and the herbaceous host plant, *N. benthamiana*, and group III, which shares 78.0-79.6% of nt sequence identity with group I and II, is associated with the disease in grapevine but does not induce symptoms, which correspond to mild symptoms in *N. benthamiana* plants (Goszczynski and Jooste, 2003a; Goszczynski, 2007; Goszczynski *et al.*, 2008).

Many molecular variants of GVA have been identified in South Africa (Goszczynski and Jooste, 2003a) of which three are of importance to this study. The isolates used in this study, GTR1-1, GTR1-2 and GTG11-1 represent the three molecular groups (Group III, II and I, respectively) and are associated with Shiraz disease susceptible plants that do not show symptoms of the disease.

Phylogenetic analysis was performed with MEGA version 4 (Tamura *et al.*, 2007) to illustrate the placement of GTR1-1, GTR1-2 and GTG11-1 within the respective molecular groups with other South African variants and the reference isolate of GVA, Is151 (Minafra *et al.*, 1997) as well as the complete sequence of GVB (Saldarelli *et al.*, 1996) as outgroup. A phylogenetic tree (figure 2.2) of the full-length sequences of the respective variants of GVA, was constructed with the neighbour-joining method (Saitou and Nei, 1987) and includes bootstrap analysis (Felsenstein, 1985). A similar tree has been constructed previously by Goszczynski *et al.* (2008). The full-length genome sequences used for phylogenetic analysis were obtained from the GenBank/EMBL database with the following accession numbers: GTR1-1 (DQ787959), GTR1-2 (DQ855086), GTG11-1 (DQ855084), P163-1 (DQ855088), KWVMo4-1 (DQ855083), P163-M5 (DQ855082), GTR1-SD1 (DQ855081), BMO32-1 (DQ855087), Is151 (X75433) and GVB (X75448).

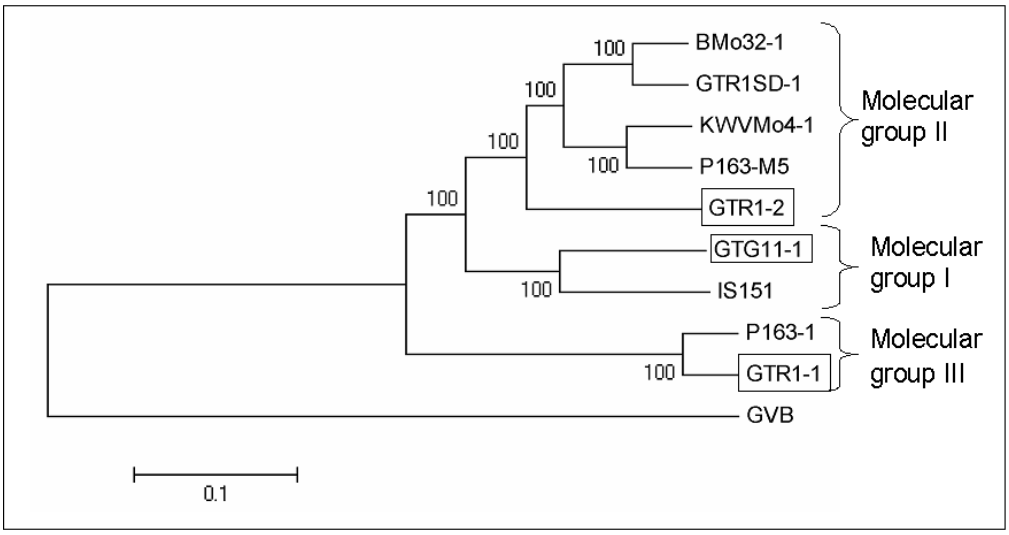

Figure 2.2: A neighbour-joining phylogenetic tree showing the evolutionary relationships of the full-length sequences of previously identified South African variants of GVA depicted within the three molecular groups, I, II and III. The variants, GTR1-1, GTR1-2 and GTG11-1, are shown in blocks. The evolutionary history was inferred with the neighbour-joining method (Felsenstein, 1985) and bootstrap analysis (1000 replicates) which indicate the percentage of replicate trees in which the variants cluster, are shown next to the branches of the tree (Felsenstein, 1985). Evolutionary distances were computed with the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and branch lengths are indicated in the same units. Phylogenetic analysis was performed with MEGA 4.0 (Tamura *et al.*, 2007).

Open reading frame 5 of GVA has been indicated as a pathogenicity determinant (Galiakparov *et al.*, 2003b,c; Zhou *et al.*, 2006) and differences observed in the severity of symptoms, or lack of symptoms could be due to the molecular heterogeneity between the variants (Haviv *et al.*, 2006b). It has been suggested that the determinants of pathogenicity in grapevine differ from those in experimental host plants such as *N. benthamiana* and might involve other ORF's in the GVA genome (Goszczynski *et al.*, 2008).

The 8th amino acid residue, which is highly diverse within ORF5, can have an affect on the different symptoms induced by the divergent variants of GVA in *N. benthamiana* plants (Haviv *et al.*, 2006b).

2.3 Suppression of gene silencing in plants by viral suppressors

2.3.1 RNA silencing

The phenomenon of gene silencing was first observed and described in 1928 by S. A Wingard (Wingard, 1928). The paper described tobacco plants which acquired immunity in the upper leaves to tobacco ring spot after initial infection of the lower leaves with the virus. Although this 'resistance' to secondary infection of the virus in the upper leaves could not be explained at the time, it

can be explained today by diverse RNA silencing pathways present in plants (Baulcombe, 2004).

RNA silencing is a broad term that describes a RNA based eukaryotic defense system, and is also known as gene silencing or RNA interference (RNAi) (Vance and Vaucheret, 2001; Roth *et al.*, 2004). This defense system relies on small non-protein coding RNA molecules that regulate gene expression in protists, fungi, plants and animals (Vaucheret, 2006). Silencing is described as a sequence-specific RNA degradation system that results in gene inactivation (Lakatos *et al.*, 2006) through the action of transcription inhibition (transcriptional gene silencing [TSG]) or RNA degradation (post-transcriptional gene silencing [PTGS]) (Vaucheret, 2006) that is mediated by double-stranded RNA (dsRNA) (Fire *et al.*, 1998).

A general silencing pathway is triggered by the cleavage of dsRNA with a dsRNA-specific endonuclease (RNaseIII-like enzyme referred to as Dicer) into RNA duplexes called small interfering RNA's (siRNA's) or microRNA (miRNA) that are 21-25 nt in length (Bernstein *et al.*, 2001; Hamilton and Baulcombe, 1999; Lakatos *et al.*, 2006). These small RNA's guide a RNA-induced silencing complex (RISC), which is a multi-subunit ribonuclease, to degrade sequence-specific RNA that is homologous to the dsRNA that initially triggered the pathway (Elbashir *et al.*, 2001; Nykanen *et al.*, 2001; Hamilton *et al.*, 2002). The RISC complex contains an Argonaute (Ago) protein which has been identified in plants, animals and fungi, and is the 'slicer' ribonuclease in the RISC complex which cuts the ssRNA complementary to siRNAs or 'dice' dsRNA into siRNA fragments (Tomari and Zamore, 2005). This process results in ssRNA (also mRNA) or dsRNA degradation which in turn causes gene inactivation in plants and animals which protects the genomes against transposons and foreign DNA or RNA (Mello and Conte Jr., 2004).

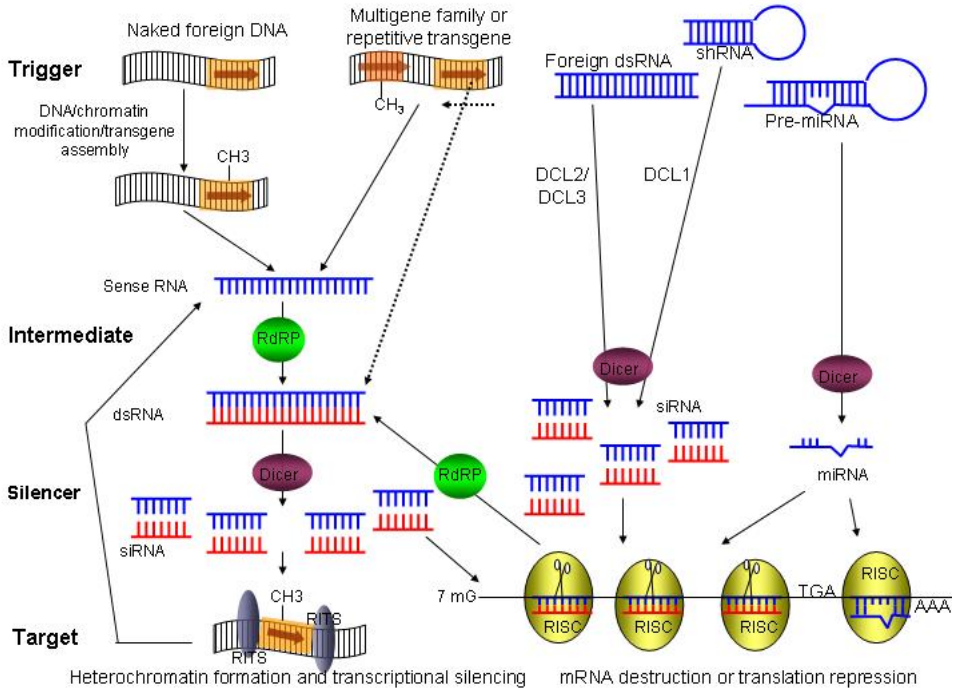

Figure 2.3: A simplified model adapted from Mello and Conte Jr. (2004) showing the importance of dsRNA in interacting RNA silencing pathways found in animals, plants, fungi and protists. Double stranded RNA can either serve as an intermediate, when mRNA's are transcribed into dsRNA by RNA-dependent RNA polymerase (RdRp), or as the trigger, where foreign dsRNA is introduced as a replicating RNA virus (Waterhouse *et al.*, 2001). In plants the dsRNA is then cleaved by four Dicer-like (DCL) proteins, of which DCL1 processes miRNA precursors and DCL2 and 3 is required for the production of siRNA's from plant viruses and rasiRNA's (Meister and Tuschl, 2004).The small RNA's (siRNA) then guide silencing complexes (RISC) to their targets, where they are implicated in the destruction of mRNA's and translational repression, whilst RITS complexes are indicated in chromatin silencing (Mello and Conte Jr., 2004).

RNA silencing was first recognized as an antiviral defense mechanism in plants (Meister and Tuschl, 2004; Vance and Vaucheret, 2001; Ratcliff *et al.*, 1997) and was discovered through transgenic experiments which resulted in the silencing of introduced genes in transgenic plants, and homologous endogenous genes when a second copy of the gene was introduced (hence the term transgene) (Hamilton and Baulcombe, 1999; Vance and Vaucheret, 2001). At least three pathways have been identified in plants that have diversified to protect the plant genome against transposons and viral infection as well as regulate gene expression (Baulcombe, 2004).

The first of these pathways is the silencing of endogenous messenger RNA (mRNA) by miRNA's (which are endogenous small RNA's produced by genes distinct from the genes they regulate) that binds specifically to mRNA and so repress gene expression by RNA cleavage or disruption of protein translation (Baulcombe, 2004; Vaucheret, 2006). A proposed mechanism for mRNA silencing suggests that cleavage of mRNA occurs when Dicer, loaded with dsRNA,

separate the dsRNA into ssRNA that hybridizes to homologous ssRNA, which is then cleaved in the middle of the 21 nt guide-recognized sequence (Waterhouse *et al.*, 2001). It is also suggested that the mechanism has sense-specificity which is enabled by the separation of the dsRNA (dimer) into two ssRNA fragments that directs hybridization in the sense or anti-sense direction, and accordingly allows for the cleavage of sense and anti-sense RNA strands (Waterhouse *et al.*, 2001). Silencing of mRNA by miRNA-mediated regulation thus results in RNA degradation or disruption of protein translation, and has been referred to as heterosilencing (Bartel, 2004).

The second pathway is associated with DNA methylation and suppression of gene transcription in plants, which at chromatin level can protect the plant genome against damage caused by transposons (Waterhouse *et al.*, 2001; Lippman and Martienssen, 2004). RNA-directed DNA methylation requires the cleavage of dsRNA into small RNAs that guide the RITS (RNA-induced transcriptional silencing) complex which modifies DNA sequences complimentary to the siRNA or miRNA (Matzke *et al.*, 2001; Baulcombe, 2004; Mello and Conte Jr., 2004). Regions that are usually not transcribed, such as promoters can be subject to transcriptional silencing and methylation (Matzke *et al.*, 2001), where increased methylation corresponds to transcriptional silencing, and decreased methylation corresponds to increased transcription (Hirochika *et al.*, 2000). Transposable elements are also targets of dsRNA-induced silencing Waterhouse *et al.* (2001) and the involvement of methylation in transposon silencing has been shown through various studies (Brutnel and Delaporta, 1994; Lui *et al.*, 1994). These studies have found that suppressed transposable elements can be reactivated in PTGS and RNAi defective mutants (Elbashir *et al.*, 2001), which provides evidence for a dsRNA-induced silencing mechanism that relies on the methylation of transposon DNA and degradation of transposase mRNA Waterhouse *et al.* (2001).

The third diverse pathway identified in plants is cytoplasmic siRNA silencing (Hamilton and Baulcombe, 1999) which can result in either transgene-induced PTGS or virus-induced PTGS, and is of importance in this study.

2.3.2 Post-transcriptional gene silencing (PTGS)

Post-transcriptional gene silencing, also referred to as autosilencing, has been described as an RNA-mediated immune response in plants where virus-derived siRNAs degrade the RNAs from which they derive (*cis*-acting siRNAs) (Vaucheret, 2006; Bartel, 2004). In this pathway, dsRNA can be a replication intermediate or a secondary-structure feature of viral ssRNA (Baulcombe, 2004) that triggers the silencing pathway. The triggered silencing pathway reduces the steady-state levels of mRNA in the cytoplasm of the plant cells by the binding of the siRNAs derived from the initial trigger to the viral mRNA (Voinnet *et al.*, 1999; Waterhouse *et al.*, 2001). This sequence-specific degradation of mRNA in cytoplasm has been termed transgene-RNA-directed RNA degradation and is caused by the same mechanism that can result in co-suppression (De Carvalho *et al.*, 1995), where co-suppression is described as a form of

PTGS that causes the silencing of two or more homologous loci (Baulcombe and English, 1996). Co-suppression describes the inhibition of gene expression and subsequent silencing of an endogenous gene after a homologous gene has been introduced, and was first described for the chalcone synthase (CHS) gene in *Petunia* where the insertion of an extra copy of the gene behind a strong promoter resulted in the loss of colour, contrary to an expected darker hue of purple (Van der Krol *et al.*, 1990; Napoli *et al.*, 1990; Jorgensen *et al.*, 1996).

Similar results were observed when plants were transformed to contain genes derived from the cDNA of plant viruses (transgenes) coupled with a CaMV promoter (35S) and resulted in resistance when the plants were challenged with the same or similar viruses (Baulcombe and English, 1996). The resistance that resulted in the suppression of transgene mRNA accumulation was RNA-mediated, and has been described as pathogen-derived resistance (PDR) (Ratcliff *et al.*, 1997; Vance and Vaucheret, 2001). Plant viruses can thus be the targets of transgene RNA silencing but has also been found to induce gene silencing (Vance and Vaucheret, 2001). Virus-induced gene silencing (VIGS) targets transgenes or endogenous genes (Ruiz *et al.*, 1998) and exploits the plant's ability to degrade sequence-specific RNA. A virus is modified to act as a vector and carry an insert homologous to the host gene which results in specific mRNA degradation of the host gene, and has been applied as a useful tool to indicate gene function in plants (Lu *et al.*, 2003).

Virus resistance, mediated by PTGS, has also been observed in non-transgenic plants (Covey *et al.*, 1997). The general RNA silencing pathway triggered by replicating viruses in transgenic and non-transgenic plants are shown in figure 2.3. The dsRNA is transcribed by the ssRNA-virus's own RdRp during replication or RdRp native to the plant (Dalmay *et al.*, 2000) (with the exception of retroviruses) in the cytoplasm of the plant cell (Waterhouse *et al.*, 2001). Long dsRNA's from plant viruses are processed into 21-23 bp dsRNA intermediates by Dicer, and are then incorporated in an ATP-mediated reaction with a RNA helicase (putative RNA helicase, Armitage) and Argonaute protein into a RISC-complex (Meister and Tuschl, 2004). The siRNA fragments within the RISC-complex can be targeted by the ssRNA from which they derive and also result in a feedback mechanism (Baulcombe, 2004) that is self-regulating. An increase in the levels of ssRNA (replicating virus) will result in an increase of siRNA, which would in turn result in a decrease of ssRNA (Baulcombe, 2004).

Diversification of different PTGS pathways mediated by siRNAs in plants are indicated by the existence of 10 Ago proteins, 4 DCL proteins and as many as 6 RdR-proteins in the model plant, *Arabidopsis*, (Yu *et al.*, 2003) which can explain varying responses to infection by different viruses (Vaucheret, 2006).

Post transcriptional gene silencing can be divided into three stages : initiation, maintenance and systemic movement of the silencing signal throughout the plant, which is important for antiviral defense (Baulcombe, 2004; Waterhouse *et al.*, 2001). It is suggested that the silencing signal can move in parallel to the virus, where, when the virus enters a cell, replicates and triggers a silencing response, the silencing signal (which is a RNA [siRNA, ssRNA or dsRNA] or a RNA component, (Baulcombe, 2004)) can move with the virus to the

next cell. Movement of the silencing signal is enabled by the plasmodesmatal channels that are a continuation of the endoplasmic reticulum and include the phloem cells of the vascular system (Haywood *et al.*, 2002; Vionnet and Baulcombe, 1997; Vance and Vaucheret, 2001). A study on the systemic silencing signal revealed that a virus (PVX, *Potyvirus*) can encode for a protein that prevents the spread of the silencing signal (Voinnet *et al.*, 2000). Viruses have evolved various strategies to overcome PTGS, of which the primary defense is proteins that suppress silencing activity in plants (Brigneti *et al.*, 1998; Voinnet *et al.*, 2000).

2.3.3 Suppression of PTGS

Suppressor proteins are encoded in the genomes of most RNA and DNA viruses, although the molecular basis of silencing suppression differs between most of these viruses (Lakatos *et al.*, 2006), and is still largely unknown (Silhavy *et al.*, 2002). A common characteristic shared by viral suppressor proteins is that many of the proteins were initially identified as pathogenicity determinants within their respective viral genomes (Voinnet *et al.*, 1999; Qu and Morris, 2005). A number of viral suppressors are also implicated in long distance movement of the virus (Qu and Morris, 2005). Divergent suppressors can target different steps in the PTGS pathway, and the molecular action of some of these suppressors have been described (Silhavy *et al.*, 2002; Voinnet *et al.*, 2000; Ye *et al.*, 2003; Voinnet *et al.*, 1999; Pfeffer *et al.*, 2002). Silencing suppressors are generally identified in silencing suppression assays that utilise a reporter transgene (Li and Ding, 2001). The three general approaches used to elucidate silencing suppression are transient expression assays which are based on *Agrobacterium* co-infiltration, reversal of silencing assays and stable expression assays (Roth *et al.*, 2004). The viral suppressors HC-Pro from potato virus Y (PVY)(Brigneti *et al.*, 1998), P1 of rice yellow mottle virus (RYMV), AC2 from Alfalfa Mosaic virus (AMV) (Voinnet *et al.*, 1999), 2b from Cucumber Mosaic virus (CMV) (Ding *et al.*, 1994), and p25 from Potato virus X (PVX) (Voinnet *et al.*, 2000) are some of the viruses identified with the reversal of silencing assays. This type of assay utilise a transgenic *N. benthamiana* (line 16c) plant that expresses GFP, which is systemically silenced by infiltration with *Agrobacterium* expressing GFP (Brigneti *et al.*, 1998). Once the plant is completely silenced, it is infiltrated with the virus containing a possible suppressor gene and evaluated for re-activated GFP fluorescence (Roth *et al.*, 2004) which will indicate that the virus suppresses gene silencing induced by the presence of an homologous transgene (Voinnet *et al.*, 1999). Other suppressors identified in this manner is described by Voinnet *et al.* (1999). The suppressor, P0 of BWYV was identified in a transient expression assay, which provides a rapid and easy method to identify viral suppressors, and is currently the most used technique Voinnet *et al.* (2000). This method makes use of two *Agrobacterium* cultures to be infiltrated in a transgenic *N. benthamiana* (line 16c) plant: the first contains the reporter gene, GFP, which induces silencing, and the second culture expresses the candidate suppressor gene (Roth *et al.*,

2004). These 2 cultures are co-infiltrated into the same leaf of the transgenic plant and evaluated over time. If the candidate gene is an active suppressor, GFP fluorescence will be sustained over a longer period of time, and if not, gene silencing of GFP will be observed 3-5 dpi (Roth *et al.*, 2004). The suppressor protein, p10 of GVA was also identified in such a transient expression assay (Zhou *et al.*, 2006).

This study utilised the suppressor, Helper component-proteinase (HC-Pro, potyviruses) and P0 of Beet Mild Yellows Virus (BMYV, poleroviruses), and their general suppression mechanisms will be discussed. The suppressor, HC-Pro (Brigneti *et al.*, 1998) from potyviruses is a very effective silencing suppressor that targets transgene and virus-induced gene silencing by binding siRNAs to form duplexes. This prevents the formation of a RISC-complex which results in miRNA and siRNA inactivation and prevents or reduces the downstream destruction of viral mRNA (Silhavy and Burgyán, 2004; Lakatos *et al.*, 2006). It is known that HC-Pro can suppress VIGS and transgene-induced gene silencing (Brigneti *et al.*, 1998), and also suppress the systemic silencing signal (Mallory *et al.*, 2002; Pfeffer *et al.*, 2002). The molecular mechanism by which HC-Pro mediates suppression of RNA silencing is the binding of 21-nt siRNA duplexes that contain 3' 2-nt overhangs, which is a common strategy employed by various suppressor proteins (Lakatos *et al.*, 2006). The suppressor protein is also a pathogenicity determinant within potyviruses, is known to bind RNA (HC-Pro from PVY), and has shown involvement in the long distance movement of the virus (Silhavy and Burgyán, 2004).

The suppressor, P0 of BMYV is also a strong suppressor of RNA silencing in plants but differs significantly from HC-Pro (Pfeffer *et al.*, 2002). The suppressor, P0 can suppress PTGS-activity for a longer period after inoculation in co-infiltration experiments (up to 25 dpi) than HC-Pro but cannot suppress the systemic silencing signal (Pfeffer *et al.*, 2002). The novel molecular mechanism that mediates suppression activity of P0 targets the PAZ (piwi-argonaute-zwille) domain, which binds siRNA within a RISC complex, and the adjacent upstream sequence in AGO1, which results in the degradation of this protein (Pazhouhandeh *et al.*, 2006; Baumberger *et al.*, 2007). The Argonaute proteins forms a part of the RISC complex which binds small RNAs, and AGO1 is implicated in the cytoplasmic RNA and miRNA silencing pathways (Baulcombe, 2004). The systemic silencing signal is not blocked by P0, because the signal does not contain an Ago1 protein, and it is suggested that this can also contribute to the restriction of viral movement to the phloem of the plant (Baumberger *et al.*, 2007).

2.3.4 GVA-ORF5 as suppressor

Grapevine virus A encodes for a nucleic-acid binding protein, p10, which binds nucleic acids non-specifically, but requires a stretch of basic amino acids to do so (Galiakparov *et al.*, 2003b). It was suggested by Chiba *et al.* (2006) that GVA p10 possesses weak silencing suppressor activity and belongs to the only suppressor-protein family (comprised of p10-like proteins) that spans three

viral genera and two families with a putative host ancestor (Chiba *et al.*, 2006). Alignment of the nucleotide and amino acid sequence of ORF5 of GTR1-1, GTR1-2 and GTG11-1 was performed with BioEdit version 7.0.0 (Hall, 1999) and is shown in figure 2.4 where the conserved basic, arginine rich motif is indicated in a block. This region is involved in nucleic acid binding activity of p10 encoded by the ORF5 of GVA (Galiakparov *et al.*, 2003b) and is, together with the zinc-finger motif (C-X-C-X_4-H-X_4-C, where X can be any amino acid)(Chiba *et al.*, 2006) highly conserved in all the aligned variants of GVA (figure 2.4). These conserved amino acid sequence motifs have also been found in known and putative silencing suppressors encoded by filamentous viruses (Chiba *et al.*, 2006).

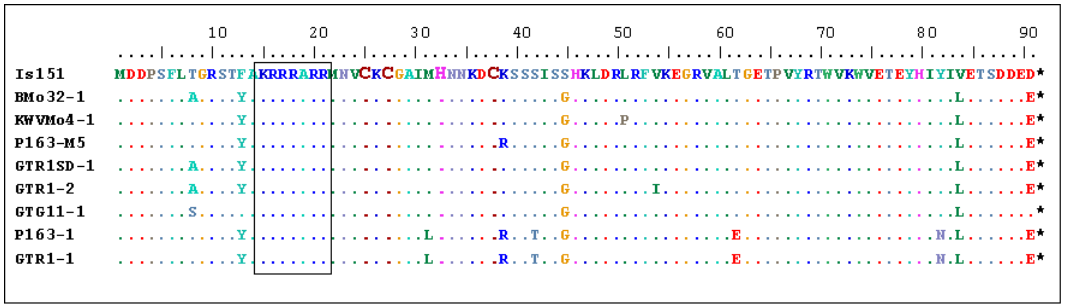

Figure 2.4: Amino acid alignment of ORF5 of GTR1-1. GTR1-2 and GTG11-1 as well as other South African variants used for the phylogenetic analysis. The ORF5 sequence of the Italian isolate, Is 151, has been included as the reference sequence. The amino acid alignment indicates molecular heterogeneity between the different variants but also shows the highly conserved basic, arginine rich motif (KRRRARR) required for nucleic-binding activity (Galiakparov *et al.*, 2003b). This is followed by a typical zinc-finger motif (C-X-C-X_4-H-X_4-C, where X can be any amino acid, shown in enlarged letters) which is conserved in known and putative silencing suppressors encoded by various diverse viruses (Chiba *et al.*, 2006).

RNA-silencing suppressor activity of GVA-p10 was identified by Zhou *et al.* (2006) in co-infiltration experiment with the ORF5 of the infectious clone, Is151 (Minafra *et al.*, 1997). The p10 protein was screened for suppression activity in a green fluorescent protein (GFP)-based transient expression assay, where the ORF5 was co-infiltrated with GFP in transgenic *N. benthamiana* plants that expresses GFP constutively (Zhou *et al.*, 2006). This assay resulted in a decrease of GFP siRNA levels in co-infiltrated tissue and the study suggested that p10 binds small RNA molecules involved in RNA silencing (Zhou *et al.*, 2006) which corresponds with previous results that showed that p10 binds long ssRNAs and dsRNAs due to the presence of the basic, arginine-rich domain at the amino terminus of the protein (Galiakparov *et al.*, 2003b). The suppressor activity of GVA-p10 is most probably due to the ability of the protein to sequester siRNA's involved in local and systemic silencing of the virus in plants (Zhou *et al.*, 2006).

2.4 Viral full-length cDNA clones and their uses

Infectious full-length cDNA clones of RNA viruses have provided a useful tool for research on virus-host interactions and viral gene functions (Nagyová and Subr, 2007). The availability of an infectious cDNA clone of GVA has enabled the study of the genome of the virus (Galiakparov *et al.*, 2003c), and has led to the identification of a suppressor protein (Zhou *et al.*, 2006). Infectious cDNA clones of the divergent variants of GVA will eventually also contribute towards the determination of the role of the virus in diseases such as Kober stem grooving and Shiraz disease (Saldarelli *et al.*, 2000).

Another application of infectious cDNA clones are their ability to be manipulated into virus-based expression vectors that are used for the rapid, high-level transgene expression of foreign proteins in plants (Lico *et al.*, 2008). Plant viruses are applied for the expression of these foreign proteins in plants because of the ease with which they can be manipulated, site-specific recombination ability, enhanced replication and high infectivity (Lico *et al.*, 2008; Giddings, 2001). The genome of GVA has been engineered into an expression vector for proteins in herbaceous hosts (Haviv *et al.*, 2006a).

The ability of GVA to infect both *V. vinifera* and herbaceous plants such as *N. benthamiana* (Galiakparov *et al.*, 1999) enables the characterisation of such an expression vector before application of the vector in *Vitis (Haviv* et al., *2006a).* The vector, GVA118, is capable of expressing heterologous proteins in *N. benthamiana* under control of an internal GVA promoter that produces sgRNA (Haviv *et al.*, 2006a; Galiakparov *et al.*, 2003a). The sequence upstream of the MP (ORF3) encodes for the *cis*-acting controller elements (CE) and regulates expression of the MP by production of sgRNAs (Galiakparov *et al.*, 2003a). The MP-CE site was therefore chosen because of the high expression observed for the MP (Galiakparov *et al.*, 2003c; Rubinson *et al.*, 1997). The vector was constructed to contain the MP-CE of the infectious clone, GR5 (Galiakparov *et al.*, 2003a) within the 3' region of ORF2 and the distantly related MP-CE of the GVA clone, GTR1-3 (Goszczynski and Jooste, 2003b) directly upstream of ORF3 (MP) (Haviv *et al.*, 2006a). The related MP-CE sequences were separated with a sequence containing the restriction enzyme sites, *NotI*, *SpeI*, *SplI*, *HpaI* and *ApaI*, to serve as a multiple cloning site where foreign genes can be inserted for transient expression (Haviv *et al.*, 2006a). The use of two different strains for the construction of the full-length clone proved to be more stable than the use of one strain (Haviv *et al.*, 2006a). The genomic organisation of GVA118 is represented in figure 2.5.

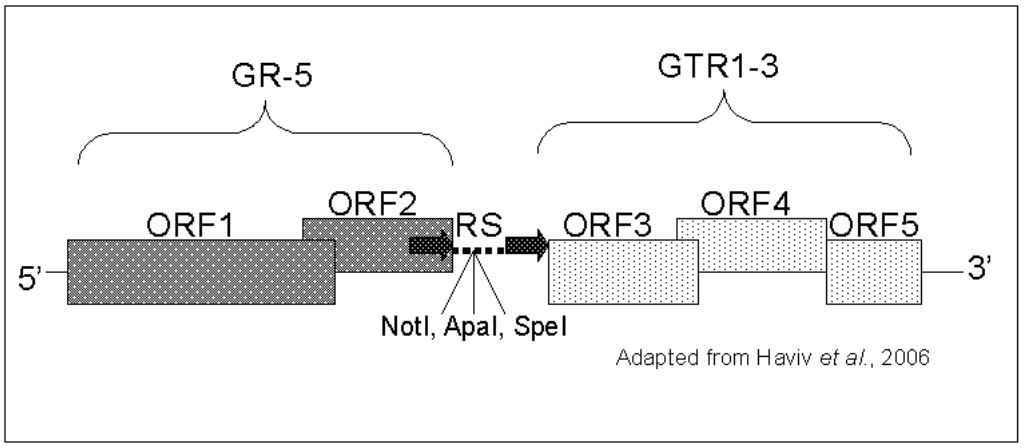

Figure 2.5: A schematic diagram of the genomic organization of the expression vector, GVA118 (Haviv *et al.*, 2006a). The ORF1 and 2 are indicated in grey and the two subgenomic promoters, MP-CE, are indicated with arrows. These two promoters are separated by a dotted line, which represents the restriction enzyme sites that were inserted during construction of the vector. The lighter boxes represent ORF3, 4 and 5 with the 3' UTR directly downstream of ORF5. ORF1 and 2 derive from the infectious clone, GR-5 (Galiakparov *et al.*, 2003a) and ORF3, 4 and 5 from the clone, GTR1-3 (Goszczynski and Jooste, 2003b).

The use of GVA118 as an infectious full-length clone in this study might contribute towards a better understanding of the ORF5 from divergent variants of GVA and ideally contribute towards a GVA-based expression vector that is less virulent than the current vector.

Chapter 3

Materials and methods

This study was designed to determine the suppression activity of ORF5 of GTR1-1, GTR1-2 and GTG11-1 in a transient expression assay in transgenic *Nicotiana benthamiana* (line 16c) plants expressing GFP. The second part of the study consisted of the deletion and replacement of ORF5 in the infectious full-length cDNA clone, GVA118. The ORF5 of this construct was removed and replaced with the ORF5's of GTR1-1, GTR1-2 and GTG11-1 respectively, as well as the marker genes GFP and GUS. The materials and methods is divided into three parts: first, general materials and methods used for both parts of the study, second, the suppression assay of ORF5, and thirdly, the deletion and replacement of ORF5 in GVA118.

3.1 General materials and methods used during this study

The ORF5's of three South African GVA variants (GTR1-1, GTR1-2 and GTG11-1) were obtained by PCR for cloning into the cloning vector, pDRIVE (QIAGEN), for both the suppression assay and the replacement of ORF5 in GVA118. Full-length cDNA clones of the three South African GVA variants have been constructed previously in our laboratory (Du Preez, 2005), and were used as templates to obtain the ORF5 of each variant.

3.1.1 Primer design

PCR primers were designed for the suppression assay from the known sequences of the full-length clones of GTR1-1, GTR1-2 and GTG11-1 (DQ787959, DQ855086 and DQ855084, respectively) found in the GenBank database (NCBI). Unique restriction enzyme flaps (5'- *SacI* and 3'-*BamHI*) were added to the primers for cloning purposes. The nucleotide identity of the GVA ORF5 5'-end allowed for the design of an universal forward primer for all three of the variants. Reverse primers, specific to each variant, were designed because of the nucleotide heterogeneity of the ORF5 3'-end. The primers were designed with a GC content of 50 -55% and an annealing temperature of 55 - 62°C. The annealing temperature was calculated based on the melting temperature

of the primer as supplied by the manufacturer. The primers were synthesized by Inqaba Biotech (Pretoria, South Africa), and are summarised in Table 3.1.

Table 3.1: Primers to obtain ORF5 of three SA GVA variants with unique restriction enzyme sites

Primer name	Sequence	Orientation
GVAO1-1ORF5as	*GGATCC*TCATTCCTCATCATCTGAGG	Reverse
GVAO11-1ORF5as	*GGATCC*TCAATCCTCATCGTCTGAGG	Reverse
GVAO1-2ORF5as	*GGATCC*TCATTCCTCATCGTCTGAGG	Reverse
GVAallORF5s	*GAGCTC*ATGGATGACCCATCGTTTC	Forward

Restriction enzymes sites are indicated in italic font within the primer sequence

For the insertion of ORF5 into GVA118, primers were designed to contain the restriction enzyme sites, *PdiI* on the 5'-end and *Mph11031* on the 3'-end. The forward and reverse primers were designed based on nucleotide identity between the variants, as mentioned previously. The primers were designed to be 24-26 nucleotides in length, with a GC-content of 50-55%, and are summarised in Table 3.2. The primers were synthesized by Integrated DNA Technologies (IDT, Iowa, USA), except GUSiNgoMIV_F and GUSiNsiI_R, which were synthesized by Inqaba Biotech (Pretoria, South Africa).

Table 3.2: Primer sequences for the amplification of ORF5 of GTR1-1, GTR1-2 and GTG11-1, for ligation into pSKM-e35S-GVA118-ΔORF5-pA

Name	Sequence	Orientation
GV118_ORF5_allF	A*GCCGGC*ATGGATGACCCATCGTTTC	Forward
GV118-ORF5_1-1R	A*ATGCAT*TTATTCCTCATCATCTGAGG	Reverse
GV118-ORF5_1-2R	A*ATGCAT*TTATTCCTCATCGTCTGAGG	Reverse
GV118-ORF5_11-1R	A*ATGCAT*TTAATCCTCATCGTCTGAGG	Reverse
GFP-ORF2+5_F	A*TACGTA*ATGGTGAGCAAGGGCGA	Forward
GFP-ORF5_R	A*ATGCAT*TTACTTGTACAGCTCGTCCA	Reverse
GUSiNgoMIV_F	AA*GCCGGC*ATGTTACGTCCTGTAGAA	Forward
GUSiNsiI_R	A*ATGCAT*TCATTGTTTGCCTCCCTG	Reverse

Restriction enzyme sites are indicated in italic font within the primer sequence

3.1.2 PCR amplification

PCR amplification reactions were performed to obtain the ORF5 of each GVA variant for cloning purposes, and were also used for bacterial colony screening.

General PCR reactions were performed with Kapa Biosystems (Cape Town, South Africa) reagents according to the manufacturer's protocol and reaction conditions. Approximately 1 ng of plasmid DNA was used for amplification with the NH_4buffer containing $MgCl_2$ (Buffer A), and enriched with a further 1.5 mM $MgCl_2$. A negative control was included for each PCR reaction. One time (1X) loading buffer (20% (w/v) sucrose, 1mM cresol red) was also added to the PCR reaction to facilitate later loading of the reaction into the wells of

a 1% (w/v) TAE/agarose gel. The primers in all PCR reactions were present in a final concentration of 0.4 mM, except where stated differently. A standard reaction cycle, with one cycle at 94°C for 30 seconds, followed by 30 cycles at 94°C for 30 seconds, variable annealing temperatures depending on the primer set for 30 seconds, and elongation at 72°C for 30 seconds. Final elongation of the PCR products were done in a single cycle at 72°C for 5 minutes, followed by cooling at 4°C for an undetermined length of time. All PCR reactions were performed with an AB 2720 Thermal Cycler (Applied Biosystems).

3.1.3 Agarose gel electrophoresis

All reaction products (PCR and digest) were visualised with agarose gel electrophoresis. Agarose gel electrophoresis was performed as described by Sambrook *et al.*, 2001.

DNA fragments were separated on an 1 % (w/v) agarose (D1-LE Whitehead Scientific) gel in 1X TAE buffer prepared from a 50X stock solution of TAE buffer (2M Tris, 5.71% (w/v) glacial acetic acid, 0.05M EDTA at pH 8). Ethidium bromide (0.5 $\mu g/$ ml) was added to the 1% (w/v) TAE/agarose gel prior to electrophoresis.

DNA in agarose gels were visualised with UV (ultra violet) trans-illumination. A DNA ladder (Generuler^{TM}1 kb DNA Ladder, Fermentas) was also loaded on each agarose gel.

3.1.4 PCR product gel purification

All PCR gel fragments were purified with a Zymoclean Gel DNA recovery Kit^{TM}(Zymo Research Corp.), as set out by the manufacturer's protocol.

The PCR DNA gel fragments were excised from the 1X TAE/agarose gel with sterile scalpel blades and transferred to a 1.5 ml microfuge tube, in which the fragments were weighed. Solvent buffer (ADB buffer) was added based on the weight of the fragment in micrograms, and the agarose gel fragments were incubated at 42°C for 15 minutes to dissolve. The manufacturer's instructions were followed and the DNA was eluted in a total volume of 10 μl sterile water. The purified DNA was stored at -20°C until use.

3.1.5 Cloning of PCR and restriction enzyme digest products

All ligations were performed with T4 DNA ligase (Fermentas) in 1X T4 DNA ligase buffer (Fermentas), except where specified differently for the QIAGEN® PCR Cloning Kit.

A volume of insert was added to half a volume of vector (2:1) in a sterile 1.5 ml microfuge tube. A volume of T4 DNA ligase (Fermentas) equal to 5 Weiss units were added to the reaction, as well as 1X T4 DNA Ligase buffer (10 X Ligation buffer : 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5

mM ATP, pH 7.8 at 25°C) (Fermentas). The reaction volume was filled up to 20 μl with sterile, deionized water, and incubated overnight at 4°C.

3.1.6 Cloning of purified PCR products into the pDRIVE cloning vector

All ORF5 product fragments that were generated by PCR amplification, were cloned initially into the pDRIVE Cloning Vector (QIAGEN) with a QIAGEN® PCR Cloning kit for sequencing purposes. Ligation and cloning were done according to the manufacturer's protocol described in the QIAGEN®PCR Cloning handbook (April 2001). A volume of 4 μl of purified PCR product was added to the ligation mixture for a final reaction volume of 10 μl. The ligation-reaction mixture was incubated overnight at 4°C.

3.1.7 Transformation and selection of recombinant colonies by PCR

Preparation of chemically competent DH5α, JM109 and NM522 cells

Chemically competent *Escherichia coli* cells were prepared as described by Hanahan (1985).

The *E. coli* cells (DH5α, JM109 or NM522) (Chapter 9) were plated out on a LB/agar (bacteriological, Biolab, Merck) plate (without selection) (Chapter 8) and grown overnight at 37°C. A single colony was selected and inoculated in 5 ml LB (Biolab, Merck) media (Chapter 8) without selection, which was grown overnight at 37°C, whilst shaking at 225 rpm. The overnight culture was added to 500 ml LB media (1:100 dilution) and incubated at 37°C until an optimal density (OD_{600} 0.5-0.6) was reached. The cells were centrifuged at 4°C for 10 minutes at 2 300 x g. The supernatant was discarded and the pellet was resuspended in 100 ml $MgCl_2$ by pipetting. The resuspended cells were incubated on ice for 30 minutes before centrifugation at 4°C for 10 minutes at 2 300 x g. The supernatant was discarded and the pellet was resuspended in 10 ml 100 mM $CaCl_2$with 15% sterile glycerol. Aliquots of 100 μl were placed in pre-cooled sterile microfuge tubes and quick frozen in liquid nitrogen. The cells were stored at -80°C.

E. coli transformation and bacterial colony screening by PCR

All plasmids were transformed into chemically competent *E. coli* cells, following the protocol as set out in Sambrook *et al.*, 2001. All plasmids were transformed into either DH5α-, JM109- or NM522 chemically competent cells.

Overnight ligation reactions were added to 100 μl chemically competent cells in 1.5 ml microfuge tubes, and lightly mixed by gentle flicking. The transformations were incubated on ice for 20 minutes, followed by a heat shock at 42°C for 45 seconds. The cells were immediately returned to ice for 2 minutes, after which 900 μl SOB medium (Chapter 8) was added to each of the transformations. The transformation-reactions were incubated at 37°C for

1 hour and 30 minutes. After incubation, the transformation-reactions were plated out on agar/LB plates with antibiotic selection specific to the plasmid's antibiotic resistance gene. The agar/LB plates were incubated overnight (16 hours) at 37°C.

Plasmids containing the LacZ gene (pDRIVE), were screened based on blue/white selection induced by 20 mg/ml 5-bromo-4-chloro-3-indocyl-β-D-galactoside (X-Gal) (Promega) and 0.1 mM IPTG (isopropyl-beta-D-thiogalacto-pyranoside). Other plasmids (Chapter 9) not containing the lacZ gene, or containing a disrupted LacZ gene, were only screened based on the antibiotic selection. White colonies on X-Gal/antibiotic selection plate were assumed to contain pDRIVE cloning vector with insert. These colonies were further screened with PCR to determine whether the insert (ORF5 of GTR1-1, GTR1-2 and GTG11-1) is indeed present in the plasmid.

The PCR reactions were performed with Kapa Biosystems (Cape Town, South Africa) reagents according to the manufacturer's protocol and reaction cycle. The PCR reaction and reaction cycle was performed as described in section 3.1.2. The primers used for the colony screen PCR of the pDRIVE-ORF5 clones for GTR1-1, GTR1-2 and GTG11-1 are summarised in Table 3.1 and Table 3.2. The primer sets for screening of ORF5 for the suppression assay were used at annealing temperatures of 60°C and the primer sets for the GVA118 ORF5s at an annealing temperature of 58°C. The colonies were picked with sterile toothpicks and gently mixed with the PCR reagent mix in a sterile PCR reaction tube. A negative control was included for each PCR reaction.

3.1.8 Plasmid extraction and purification

All plasmids were extracted with a GeneJET^{TM}Plasmid Miniprep Kit (Fermentas), following the manufacturer's protocol.

Single bacterial colonies that have been screened positively for the specific plasmid and insert after cloning, were picked with a sterile toothpick and inoculated in 5 ml LB media with the appropriate selection antibiotics. The culture was grown to saturation at 37°C whilst shaking at 225 rpm overnight

A total volume of 2 ml saturated culture was harvested by centrifugation at 12 000 x g in a 2 ml microfuge tube for 5 min at room temperature. The plasmid was eluted in 30-50 μl elution buffer supplied with the kit, or sterile water where stated. The eluted plasmids were stored at -20°C until use.

3.1.9 Restriction enzyme digestion reactions

Restriction enzyme digestion reactions were performed to create constructs and confirm their integrity during this study. This technique was also utilised to confirm the presence of inserts in vectors.

Digestion reactions were performed at 37°C and fragments obtained with these reactions were visualized on 1% (w/v) 1X TAE/agarose gels (section 3.1.3). Separated fragments were compared to expected fragment sizes created

virtually on plasmid maps (VectorNTI, Invitrogen) to confirm the fragments.

3.1.10 Sequencing

All clones that were constructed during this study were sequenced at the Central DNA Sequencing Facility (University of Stellenbosch).

3.1.11 Freezer cultures

Freezer cultures were made of all saturated bacterial cultures that contained confirmed plasmids and positive constructs after cloning. A volume of 500 μl of saturated culture was added to a volume of 500 μl of sterile 80 % glycerol in a 1.5 ml microfuge tube. Freezer cultures were stored at -80°C.

3.2 Suppressor activity assay in transgenic *N. benthamiana* (16c) plants

Suppressor activity of GVA-ORF5 of each variant was determined in a suppressor activity assay in transgenic *N. benthamiana* expressing GFP. The ORF5-constructs were cloned into the binary plant vector, pBIN61S, under 35S control, and were co-infiltrated with pBIN61S-GFP. This was done to determine whether the ORF5 of the three South African variants have suppression activity and to which degree this suppression activity influences silencing in transgenic *N. benthamiana* expressing GFP.

3.2.1 pT7T318U vector constructs

The ORF5-fragments were cloned directionally into the intermediate vector, pT7T318U (Figure 7.1) with the restriction enzymes, BamHI and SacI. After successful cloning, the inserts were digested and cloned into the binary vector, pBIN61S. Direct cloning of the respective ORF5's from pDRIVE into pBIN61S, resulted in mixed species of plasmid due to the same antibiotic (kanamycin) selection. The plasmid, pT7T318U has ampicillin selection, and the intermediate cloning step into this vector could eliminate any contaminating pDRIVE-ORF5 clones after cloning into the binary vector.

The GTR1-1-ORF5, GTR1-2-ORF5 and GTG11-1-ORF5 in pDRIVE were sequenced, and found to be homologous to the corresponding sequences in the GenBank database (DQ787959, DQ855086 and DQ855084, respectively), with the exception of ORF5 of GTR1-1, which contained two substitutions. These substitutions did not result in a frame shift or change of amino acid sequence after translation, and were used in subsequent experiments. The ORF5 fragments were digested from the pDRIVE cloning vector with the restriction enzymes, *BamHI* (Fermentas) and *SacI* (Fermentas) in a double digestion reaction with 1X Buffer BamHI (10 mM Tris-HCl pH8.0, 5 mM $MgCl_2$, 100 mM KCl, 0.02% Triton X-100, 1 mM 2-mercapthoethanol, 0.1 mg/ml BSA). The

reaction was performed in a reaction volume of 20 μl and incubated at 37°C for two hours. The vector, pT7T318U, was subjected to the same restriction enzyme digest conditions as the pDRIVE-ORF5 constructs.

The digestion-reaction products were separated and visualized by agarose gel electrophoresis and were eluted out of the 1% (w/v) TAE/agarose gel. The respective gel-eluted restriction-enzyme ORF5 fragments were ligated into the eluted vector, pT7T318U. The ligation-reaction were transformed into competent *E. coli* cells (NM522).

These constructs will henceforth be referred to as pT7T318U-ORF5-1-1, pT7T318U-ORF5-1-2 and pT7T318U-ORF5-11-1. Colonies were grown overnight at 37°C on ampicillin selection plates (LB/agar) and screened with colony screen PCR with the primers sets described in Table 3.1, to determine whether the ORF5s of all three variants were successfully ligated into pT7T318U. All positive colonies were inoculated in 5 ml LB media with 100 μg/ml ampicillin selection and grown to saturation (37°C, 225 rpm). The plasmids, pT7T318U-ORF5-1-1, pT7T318U-ORF5-1-2 and pT7T318U-ORF5-11-1 were subjected to a double digest reaction with *BamHI* and *SacI* to confirm that the ORF5-fragment of each variant was present.

3.2.2 Construction of the binary vector clones

All pT7T318U constructs containing an ORF5 (pT7T318U-ORF5-1-1, pT7T318U-ORF5-1-2 and pT7T318U-ORF5-11-1), as well as the empty binary vector, pBIN61S (Figure 7.2), were subjected to a double digest reaction with *BamHI* (Fermentas) and *SacI* (Fermentas). The digested fragments were separated with gel electrophoresis and purified from the agarose gel.

Ligation reactions were performed to ligate the gel eluted ORF5 fragment of each variant, into the linearized pBIN61S vector. The reactions were incubated overnight at 4°C.

The ligated pBIN61S-ORF5-1-1, -1-2 and -11-1 constructs were transformed into NM522 chemically competent *E. coli* cells and plated on 50 μg/ml kanamycin selection plates. The selection plates were incubated at 37°C overnight and selected colonies were screened by colony PCR reactions. The same primer sets described in Table 3.1 were used for the colony screen by PCR of the pBIN61S-ORF5 constructs. A single positive colony of each construct was inoculated in 5 ml LB medium with 50 μg/ml kanamycin and grown to saturation at 37°C whilst shaking at 225 rpm.

Plasmid extraction was performed on each saturated culture for each of the three respective pBIN61S-ORF5 constructs. The plasmids were eluted in a final volume of 50 μl of sterile, deionized water and stored at -20°C.

3.2.3 Plasmids used as controls during the suppressor activity assay

The binary plant expression vector, pBIN61S (Silhavy *et al.*, 2002) is a derivative of the binary vector, pBIN19 (Bevan, 1984), which contains an enhanced

CaMV 35S promoter and poly(A) terminator cassette. The plasmid, pBIN61S-GFP, was constructed by Haseloff *et al.* (1997), and used in all co-infiltration experiments during this study. The plasmid, pBIN61S-HC-Pro (from Plum Pox Virus), was constructed by Varrelmann *et al.* (2007), and used as one of three control plasmids for suppression activity in this study. All pBIN61S-derived plasmids were kindly supplied by Dr. M Varrelmann, Department of Crop Science, Section Plant Virology, University of Göttingen, Grisebach-straSSe 6, D-37077 Göttingen, Germany. The second control was pBIN61S-BMYV-Po and was constructed by Stephan and Maiss (2006). The plasmid was kindly supplied by Dr. D Stephan, Department of Genetics, University of Stellenbosch. The published plasmids, constructed by Zhou *et al.* (2006), which contains the ORF5 of the GVA clone, Is151 in the binary vector, pBI121, was kindly provided by Prof. P Saldarelli, Centro di Studio del CNR sui Virus e le Virosi delle Coltore Mediterranee and Dipartmento di protezione delle Pianti, Università degli Studi, Bari, Italy. All plasmids and their antibiotic selection are described in Chapter 9.

3.2.4 Electroporation of *Agrobacterium* cells

Preparation of electrocompetent *Agrobacterium* cells

The *Agrobacterium tumefaciens* strain, C58C1 (pGV2260), was used for all agro-infiltrations relevant to the suppressor assay. The strain, C58CI with the helper plasmid, pCH32, was used for the infiltration of the modified GVA118 constructs. The C58C1 *A. tumefaciens* strain was streaked out on agar/LB plates containing 50 μg/ml Rifampicin (Rif) and incubated for 48 hours at 28°C. Ten colonies were picked with sterile toothpicks and transferred to 20 ml of sterile liquid LB media. The media was incubated overnight at 28°C whilst shaking at 225 rpm. A volume of 10 ml of the overnight culture was transferred into 500 ml LB media (at room temperature) which contained 50 μg/ml Rifampicin. The culture was incubated at 28°C and shaken at 225 rpm until an OD_{600} of 0.5 - 0.8 was reached. The culture was cooled on ice for 20 minutes after which it was transferred to two pre-cooled centrifuge tubes and centrifuged at 4°C for 15 minutes at 4000 x g. The supernatant was discarded and the pellet was re-suspended in a 100 ml 1 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*'-(ethanesulfonic acid)] (pH 7.0), which was kept on ice. The re-suspended solution was centrifuged again at 4°C for 15 minutes at 4000 x g, and the supernatant discarded. The pellet was again re-suspended in 100 ml 1 mM HEPES (pH 7.0). The solution was then centrifuged at 4°C for 15 minutes at 4000 x g. The supernatant was discarded and the pellet was re-suspended in 10 ml sterile 10 % glycerol, which was kept on ice, and centrifuged again at 4°C for 15 minutes at 4000 x g. The supernatant was discarded and the pellet was finally suspended in 1 ml sterile 10 % glycerol. Aliquots of 40μl in sterile 1.5 ml microfuge tubes were shock frozen in liquid nitrogen and stored at -80°C.

Electroporation of electrocompetent *Agrobacterium* cells

Electroporation of all electrocompetent cells were performed on a Bio-Rad Gene Pulser®II and Bio-Rad Pulse Controller Plus. All cuvettes and media used for electroporation were pre-cooled on ice and kept on ice during electroporation.

Previous aliquots of electrocompetent cells were thawed on ice and 2-3 μl of plasmid DNA was mixed with the cells by gentle pipetting. Each of the three pBIN61S-ORF5 constructs were mixed with the electrocompetent cells. The cells containing plasmid DNA were incubated on ice for 1 minute, and transferred into pre-cooled cuvettes (0.1 cm) ensuring that no bubbles were present. The cuvette contacts were dried before insertion into the electroporater. The power supply settings were 1500V, 200 Ohm and 25 μF, and the electroporator was set to a capacitance of 50 μF, a resistance of 150 Ohm and voltage at 2.5 kV. After the pulse, the cuvette was quickly removed and the bacterial cells mixed with 1 ml ice cold sterile SOC medium, after which the suspension was transferred to a new, sterile 1.5 ml microfuge tube. The microfuge tubes containing the bacterial SOC suspension were incubated at 28°C shaking at 225 rpm for 3-4 hours, and plated out after incubation on LB/agar plates with 50 μg/ml Rifampicin and 50 μg/ml Kanamycin selection. The plates were incubated for 48 hours at 28°C.

3.2.5 Growth conditions for the transgenic *N. benthamiana* (16c) plants

The suppressor activity assay utilised the transgenic *N. benthamiana* line (16c) which is homozygous for green fluorescent protein (GFP), and show constitutive expression of the stable GFP transgene. These plants have been described previously by Brigneti *et al.*, 1998; Ruiz *et al.*, 1998.

The 16c plants were cultivated from seed (kindly supplied by Dr. D Baulcombe, the John Innes Centre, Sainsbury Laboratory, Colney, Norwich, UK), and germinated in a growth room under controlled conditions. Temperatures of 22°C - 28°C were maintained in a relative humidity of 70 % with a 16 hour to 8 hour light/dark cycle. Plants were grown on soil in a 4:1:1 potting soil: sand: vermiculite mixture.

3.2.6 *Agrobacterium*-infiltration of plants

Recombinant *A. tumefaciens* C58C1 cell suspensions, harbouring the constructs, pBIN61S-ORF5-1-1, pBIN61S-ORF5-1-2 and pBIN61S-ORF5-11-1, were prepared as described previously by Voinnet *et al.* (1998). A single colony (for each of the three constructs and the controls described in Section 3.2.3) was inoculated in 5 ml LB media with 50 μg/ml Kanamycin and 50 μg/ml Rifampicin, and incubated overnight at 28°C whilst shaking at 225 rpm. A total volume of 2 ml of overnight culture was re-inoculated into 20 ml LB media, containing 50 μg/ml Kanamycin , 10 mM 2-[Morpholino]ethanesulfonic acid (MES) and 20 μM acetosyringone, and incubated overnight at 28°C and 225

rpm. The saturated bacterial culture was centrifuged for 5 minutes at 4000 x g and suspended in re-suspension solution (10 mM $MgCl_2$, 10 mM MES, 100 μM acetosyringone) to an OD_{600} of 1. The re-suspended solutions were rested at room temperature for three hours before infiltrations were performed. Co-infiltration experiments were performed with 0.4 volumes of *Agrobacterium* culture containing 35S-GFP mixed with 0.6 volumes of each *Agrobacterium* culture containing the 35S-ORF5-constructs as well as the controls, immediately before infiltration (Zhou *et al.*, 2006).

The pBIN61S-constructs (controls and ORF5 constructs) were mixed in a ratio of 0.6 volumes to 0.4 volumes (final OD_{600} of 1) with an *Agrobacterium* culture containing the binary vector, pBIN61S, as negative control. A control of 0.6 volumes of *Agrobacterium* culture containing pBIN61S and 0.4 volumes of *Agrobacterium* culture containing 35S-GFP was also included. Positive controls with the suppressors, PPV-HC-Pro and BMYV-Po, under 35S control in pBIN61S, were included in the co-infiltration experiments, and were mixed as follows: 0.4 volumes of *Agrobacterium* culture containing 35S-GFP with 0.6 volumes of each *Agrobacterium* culture containing the constructs, pBIN61S-HC-Pro and pBIN61S-BMYV-Po. All controls (positive and negative) are summarised in Table 3.3.

Table 3.3: Summary of positive and negative controls included in the final 16c suppression activity assay

Co-infiltration constructs	Volume ratio	OD_{600}
Negative controls		
pBIN61S-HC-Pro : pBIN61S-empty	0.6:0.4	1
pBIN61S-BMYV-Po : pBIN61S-empty	0.6:0.4	1
pBIN61S-GFP : pBIN61S-empty	0.6:0.4	1
pBIN61S-ORF5-1-1 : pBIN61S-empty	0.6:0.4	1
pBIN61S-ORF5-1-2 : pBIN61S-empty	0.6:0.4	1
pBIN61S-ORF5-11-1 : pBIN61S-empty	0.6:0.4	1
Positive controls		
pBIN61S-HC-Pro : pBIN61S-GFP	0.6:0.4	1
pBIN61S-BMYV-Po : pBIN61S-GFP	0.6:0.4	1
Constructs being tested		
pBIN61S-ORF5-1-1 : pBIN61S-GFP	0.6:0.4	1
pBIN61S-ORF5-1-2 : pBIN61S-GFP	0.6:0.4	1
pBIN61S-ORF5-11-1 : pBIN61S-GFP	0.6:0.4	1

All infiltrated 16c plants were monitored over a period of 3-25 days post inoculation under UV illumination for suppression activity in the infiltrated leaves and local as well as systemic silencing throughout the plant.

3.2.7 Visualization of GFP fluorescence in transgenic *Nicotiana benthamiana* (16c) plants

GFP fluorescence in the transgenic *N. benthamiana* plants were observed with a hand held 100W long wavelength (320 nm) ultraviolet (UV) lamp (SB-100F Series model, Spectroline). The plants were photographed with a digital camera (Fuji Finepix S5600). GFP fluorescence was also subjected to GFP fluorescence microscopy with the quantitive imaging system, IVIS®100 Series (Xenogen).

3.3 Deletion and replacement of ORF5 in GVA118

The ORF5 of GVA has been identified as a pathogenicity determinant in the GVA genome. The effect of ORF5 on pathogenicity and infectivity can be determined by the deletion of ORF5 in a known infectious full-length clone of GVA (GVA118). The three South African variants, GTR1-1, GTR1-2 and GTG11-1, are known to show different degrees of symptom severity in *N. benthamiana* plants (Goszczynski and Jooste, 2003b), and by replacing the original ORF5 in GVA118, the effect of the individual ORF5's on the pathogenicity of the full-length clone could be determined.

An infectious full-length cDNA clone of Grapevine Vitivirus A, under control of a T7-promoter (GVA118, Haviv *et al.*, 2006a) was kindly provided by Dr. M Mawassi (Robert H. Smith Institute of Plant Science and Genetics in Agriculture, The Hebrew University of Jerusalem, Israel). In order to use GVA118 in agroinoculation experiments, the viral sequence including the p(A) was brought under the control of an enhanced CaMV-35S promoter and introduced in the plant binary vector pBINSN (Figure 7.3), a derivative of pBIN19 (Bevan, 1984), by Mr. J du Preez (Department of Genetics, University of Stellenbosch, South Africa). This clone is referred to as pBIN-e35S-GVA118-pA. A subclone in pBluescript II SKM, containing the complete viral cassette and regulatory elements (pSKM-e35S-GVA118-pA) was also constructed by Mr. J du Preez (Figure 7.4). The subclone, pSKM-e35S-GVA118-pA was digested with *NheI* (Fermentas) and *SalI* (Fermentas), which cuts within ORF1(5') and after the poly-A tail (3'), respectively. The partial GVA118-pA fragment was ligated into the vector, pLitmus38 and is referred to as pLitmus38-118 (Figure 7.5). This clone was constructed by Dr. D Stephan (Department of Genetics, University of Stellenbosch, South Africa).

3.3.1 Overlap extension PCR

An adaptation of the method described by Heckman and Pease (2007) was used to perform overlap extension PCR. The vector pLitmus38-118 (figure 7.5) was used as template for the initial PCR reaction.

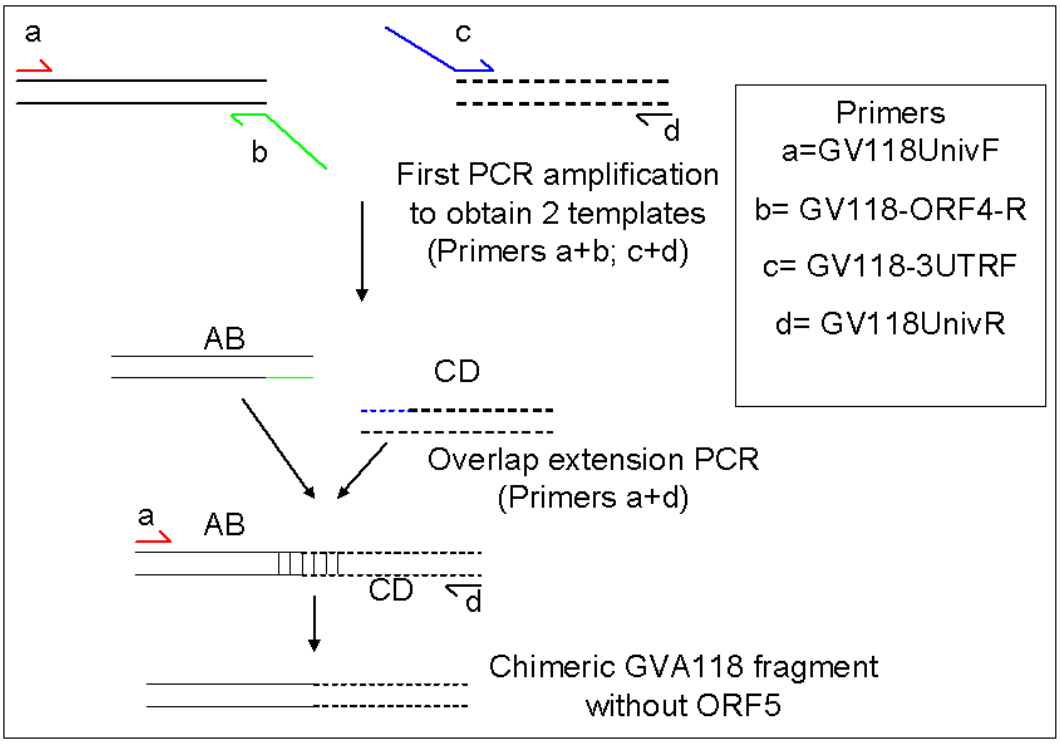

Figure 3.1: Overlap extension PCR was used to delete the ORF5 in GVA118 and replace it with unique restriction enzyme sites incorporated into the overlap extension PCR primers, b and d. These sites were used to introduce the three ORF5 fragments, from each of the three South African variants of GVA, into GVA118. This figure was adapted from Heckman and Pease (2007).

Primer design

Four primers were designed for the initial amplification reactions to obtain the template fragments for the overlap extension PCR reaction. A universal forward primer (GV118-UnivF), flanking the NcoI restriction enzyme site within ORF3 of GVA118 was designed with a universal reverse primer (GV118-UnivR), corresponding to the 3'-UTR directly upstream from the poly(A) tail, which includes a SalI restriction enzyme site. These primers were designed to be 22-25 nucleotides (nts) in length with a GC content of 55 - 60%.

The primers that contained the overlapping fragments which needed to be incorporated for the second amplification, were designed to be 39-45 nts in length, with a GC content of 50-57%. The 3'-UTR forward primer (GV118-3UTR-F) was designed to correspond to the first 9 nts of the 3' UTR, with a 27 nts flap which includes a *PdiI* restriction site, followed by three random nucleotides, and a *Mph11031* restriction site (Fermentas). The flap containing the restriction sites are followed by 9 nts corresponding to the last 9 nts of ORF4 of GVA118.

The ORF4-reverse primer (GV118-ORF4-R) was designed to correspond to the last 18 nts of ORF4, followed by the flap containing the *PdiI* and *Mph11031* restriction enzyme sites, and the first 6 nts of 3'UTR. The restriction enzyme,

PdiI, is a blunt cutter, but has an isoschizomer, *NgoMIV*, which results in overhanging single-stranded tails (sticky ends).

All the primer sequences are summarised in Table 3.4.The primers were synthesized by Integrated DNA Technologies (IDT, Iowa, USA).

Table 3.4: Overlap extension PCR primers to create a chimeric GVA118 without ORF5

Name	Sequence	Orientation
GV118-UnivF	GGCTCTGATGCCCCATGGTAAGAAC	Forward
GV118-UnivR	AGGCCTTGACTAGAGGGTCGAC	Reverse
GV118-3UTR-F	GAGTTATA*GGCCGG*CCCAATGCATTCGTCTATCCTAGCTAAGGAG	Forward
GV118-ORF4-R	AGACGA*ATGCAT*TGGGCCGGCCTATAACTCGACAGCCTG	Reverse
P17	ACTCACAGTTAGCAACTA	Forward

Restriction enzyme sites are shown in italic font within the primer sequence

Amplification to obtain templates for the overlap extension PCR

Two PCR reactions were performed initially to obtain the templates for the overlap extension PCR.

The PCR reaction to obtain the fragment including ORF3 and ORF4 of GVA118 (PCR product AB, Figure 3.1), with the added flap, was performed with the primer set, GV118-UnivF and GV118-ORF4-R (Table 3.4). The PCR reaction was performed with the proofreading polymerase enzyme, *ExTaq*™(TaKaRa, Japan) reagents, according to the manufacturer's protocol and reaction cycle. Approximately 1 ng of DNA was used, and one time (1X) loading buffer (20% (w/v) sucrose, 1mM cresol red) was added prior to the PCR reaction.

The second PCR reaction was performed to obtain the 3'UTR, poly-A tail and added flap (PCR product CD, Figure 3.1), with the primer set GV118-3UTR-F and GV118-UnivR (Table 3.4). The reaction was performed with Kapa Biosystems (Cape Town, South Africa), because of the small product size. Reagents were added in accordance to the manufacturer's instructions and reaction cycle. One time (1X) loading buffer (20% (w/v) sucrose, 1mM cresol red) was added prior to the PCR reaction. A negative control with water as template was included for both PCR reactions.

The two PCR products obtained with the respective PCR reactions were used as templates for the overlap extension PCR reaction.

Overlap extension PCR reaction

The overlap extension PCR reaction was performed with *ExTaq*™(TaKaRa, Japan) reagents according to the manufacturer's protocol and reaction cycle. The annealing temperature in the overlap extension PCR, of the primer set, GV118-UnivF and GV118-UnivR (Table 3.4) was adjusted to 55°C. The primers were present in concentrations of 1.0 μM each. The general reaction mix for the overlap extension PCR reaction contained 1.25 units *ExTaq*™polymerase enzyme, 1X *ExTaq*™Buffer (Mg^{2+} free), 100 mM $MgCl_2$

and 0.8 mM dNTP Mixture. Both previously described PCR reaction products were used as templates in the single overlap extension reaction, and were present in quantities of 70 ng each. The volume of the PCR reaction was 50 μl. A negative control with water as template was also included. One time (1X) loading buffer (20% (w/v) sucrose, 1mM cresol red) was added prior to the PCR reaction. The PCR cycling conditions were as follows: 94°C for 30 seconds, 30 cycles at 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and a final elongation step of 72°C for 5 minutes. The PCR machine was cooled to 4°C indefinitely. These conditions were suggested by the manufacturer.

3.3.2 Cloning of the overlap extension PCR product

The overlap extension PCR product (henceforth referred to as PCRΔORF5) was purified after electrophoresis and cloned into the pDRIVE Cloning Vector that was sequenced with the T7-forward primer [(QIAGEN®PCR Cloning handbook (April 2001)], and the P17-forward primer (Table 3.4).

3.3.3 Construction of a partial GVA118-pA construct with PCRΔORF5

The original ORF5 found in GVA118 was replaced with the PCRΔORF5 fragment in the clone, pLitmus38-118. This was done with restriction enzyme digestion, and resulted in the clone, pLitmus38-118ΔORF5 (figure 7.6), which lacks ORF5.

The plasmid, pDRIVE-PCRΔORF5 was digested with the restriction enzymes *NcoI* (Fermentas) and *SalI* (Fermentas) (which were included in the GV118-UnivF and GV118-UnivR primer sites) in a double digest reaction. The reaction was performed in a reaction volume of 20 μl with 2X Buffer Tango^{TM}(33 mM Tris-acetate pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA) at 37°C for two hours. The restriction enzyme digest reaction was performed to generate the overlap extension PCR product, PCRdeltaORF5-fragment (which does not contain the ORF5) and replace the original GVA118-fragment which contains the ORF5 in pLitmus38-118.

The original pLitmus38-118 was digested with *NcoI* (Fermentas) and *SalI* (Fermentas) to obtain the pLitmus38-118 backbone and discard the original GVA fragment containing the ORF5.

The digest fragments of both restriction enzyme digestion reactions were separated and visualized on a 1% (w/v) TAE/agarose gel and the appropriate fragment was gel eluted.

The insert, PCRΔORF5 was ligated into the pLitmus38-118 backbone, and the plasmid, henceforth referred to as pLitmus38-118ΔORF5, was digested and screened with *EcoRI* (Fermentas) and *PauI* (Fermentas) in a double digestion reaction to determine whether the replacement of the GVA118 fragment with the PCRΔORF5-fragment was successful.

3.3.4 Deletion and replacement of ORF5 in pSKM-e35S-GVA118-pA with GVA118ΔORF5

The aim of this experiment was to replace ORF5 in pSKM-e35S-GVA118-pA with the partial GVA118ΔORF5 construct. The result of this is the infectious, full-length cDNA clone, GVA118, in pSKM-e35S-GVA118ΔORF5-pA (figure 7.7), lacking the ORF5.

The clone, pLitmus38-118ΔORF5, was digested with the restriction enzymes, *NheI* (Fermentas) and *SalI* (Fermentas). The restriction enzyme buffers were not compatible in a double digest reaction, and two separate restriction enzyme digestion reactions were performed. The plasmid was first linearised with the enzyme, *NheI* (Fermentas), in a reaction volume of 20 μl and 1X Tango^{TM}(33 mM Tris-acetate pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA). The digestion reaction was incubated at 37°C for two hours. The enzyme and buffer were removed with SureClean (Bioline), and the DNA was resuspended in 10 μl sterile water. SureClean (Bioline) is a column-free clean up kit, used to remove restriction enzymes, and purify as well as concentrate DNA. The linearised plasmid was cleaned up in this way to ensure that the initial concentration of the plasmid remains approximately the same.

The resuspended, linearised vector served as template for the restriction enzyme digestion reaction with *SalI* (Fermentas). The restriction enzyme reaction was performed in a volume of 20 μl in the presence of 1X Buffer O (50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$,100 mM NaCl, 0.1 mg/ml BSA), and incubated for two hours at 37°C.

The clone, pSKM-e35S-GVA118-pA, was also digested with *NheI* (Fermentas) and *SalI* (Fermentas) as described for the subclone, pLitmus38-118deltaORF5, in the previous paragraph. This was done to create a backbone in which the original full-length clone, GVA118 containing ORF5, can be replaced with the GVA118ΔORF5 construct.

The fragments were separated on a 1% TAE/agarose gel as described in section 3.1.3 and gel purified. The fragments were cloned, transformed and the plasmid was extracted.

The final plasmid, pSKM-e35S-GVA118ΔORF5-pA (Figure 7.8), was sequenced with the P17-forward primer (Table 3.4) to confirm that the fragment present in the plasmid, pSKM-e35S-GVA118deltaORF5-pA (figure 3.2), indeed contained the restriction enzyme sites, *Mph11031* and *PdiI* instead of ORF5.

3.3.5 Re-introduction of ORF5 from three variants, GFP and GUS into pSKM-e35S-GVA118ΔORF5-pA

The ORF5's of GTR1-1, GTR1-2 and GTG11-1 were amplified with primers (section 3.1.1) containing *Mph11031* and *PdiI* sites. With these restriction enzyme sites directional cloning into pSKM-e35S-GVA118ΔORF5-pA was possible, allowing for the re-introduction of ORF5 into the clone. The primer set used to amplify GFP contains the restriction enzyme sites, *SnaBI* and *Mph11031*, whilst the primer set used to amplify GUS contains the restriction

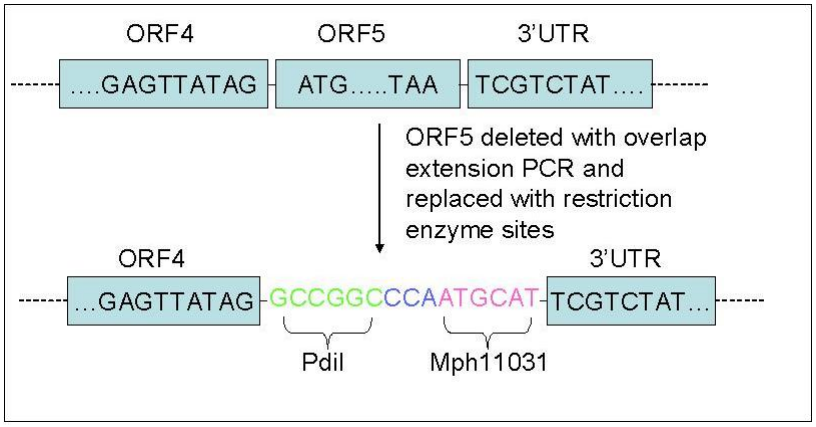

Figure 3.2: The ΔORF5 site within the construct, pSKM-e35S-GVA118ΔORF5. The ORF5 was replaced with the two restriction enzyme sites, *Mph11031* and *PdiI/NgoMIV*, into which ORF5 from GTR1-1, GTR1-2 and GTG11-1 and the genes, GFP and GUS can be re-introduced.

enzyme sites, *Pdi* (*NgoMIV*) and *Mph11031*. All primers are summarised in Table 3.2.

PCR amplification and cloning of ORF5 of GTR1-1, GTR1-2, GTG11-1, GFP and GUS

PCR amplification was performed using the primer sets used for the PCR amplification of ORF5 of each variant, GFP and GUS as summarised in Table 3.2, with an annealing temperature of 58°C for each PCR reaction. The gel-purified PCR products (except GUS) were cloned into pDRIVE, transformed and colony screened with PCR. The amplified product for GUS was cloned into the pGEM®T-Easy Vector (Promega) according to manufacturer's protocol.

Plasmid purification was done after successful cloning and the final pGEM-GUS, respective pDRIVE-ORF5 and -GFP constructs were sequenced .

Introduction of ORF5, GFP and GUS into pSKM-e35S-GVA118ΔORF5

The GUS-fragment and the ORF5 of each of the variants (GTR1-1, GTR1-2 and GTG11-1), with the enzyme flaps (*PdiI/NgoMIV* and *Mph11031*), could be cloned directionally into pSKM-e35S-GVA118ΔORF5 at the enzyme sites, *PdiI/NgoMIV* and *Mph11031* (Figure 3.2). The GFP-fragment was also cloned directionally into pSKM-e35S-GVA118ΔORF5 with the exception of GFP being digested with *SnaBI* for blunt ligation compatible with the *PdiI* site. The ORF5 of GTR1-2 is homologous to the ORF5 originally found in the infectious, full-length clone, GVA118, and could thus serve as control for the modified plasmid, pSKM-e35S-GVA118ΔORF5-pA. The insertion of ORF5 of GTR1-2 into pSKM-e35S-GVA118ΔORF5-pA, would complete the genome of the full-length clone, with the introduced restriction enzyme sites, *PdiI* and *Mph11031*. The effect of these foreign nucleotides could be determined when

compared to the symptomology and infectivity of the pSKM-e35S-GVA118-pA construct in *N. benthamiana* plants.

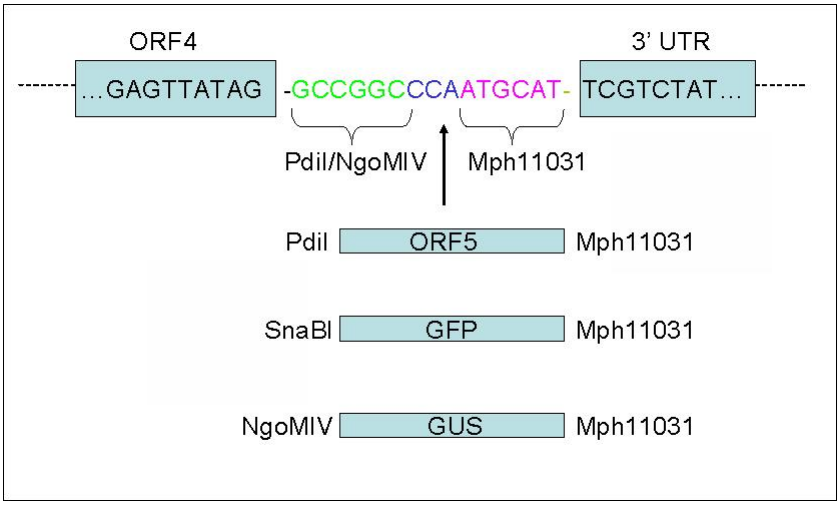

Figure 3.3: This figure is based on Figure 3.1 and shows the different inserts with their specific flanking primer sites for ligation into pSKM-e35S-GVA118ΔORF5 which is partially shown at the top with the restriction enzyme sites, *PdiI* and *Mph1103*. The ORF5-fragment represents ORF5 of GTR-1-1, GTR-1-2 and GTG-11-1. The fragments, GFP and GUS were also cloned into pSKM-e35S-GVA118ΔORF5, respectively with the flanking restriction enzyme sites indicated next to the fragments. It is important to note that *PdiI* is a blunt cutter which is compatible with *SnaBI*. The restriction enzyme, *NgoMIV*, is an isoschizomer of *PdiI*, and is thus also compatible.

The pDRIVE-constructs containing GFP and the ORF5 of each variant as well as the plasmid, pSKM-e35S-GVA118ΔORF5, were digested with *PdiI* (Fermentas) and *Mph11031* (Fermentas).

The GUS-fragment was obtained in a sequential digest from pGEM-GUS. The constructs, pGEM-GUS and pSKM-e35S-GVA118ΔORF5 were first subjected to respective digestion reactions with the enzyme, *NgoMIV* (NEB) in 1X NEBuffer 4 (50 mM K-acetate, 20 mM Tris-acetate, 10 mM Mg-acetate, 1 mM DTT, pH 7.9 at 25° C) in a total reaction volume of 20 μl. The reactions were incubated at 37°C for 2 hours, after which both digestion reactions were cleaned with Sureclean (Bioline) and resuspended in 10 μl sterile water. The purified, linearised plasmids served as template for the digestion reactions with *Mph11031*.

The pDRIVE-ORF5 fragments, GFP and GUS were purified and cloned into the linearised pSKM-e35S-GVA118ΔORF5-pA, transformed and screened by PCR. Positive colonies were inoculated, plasmids were extracted, and digested with *EcoRI* as control for the colony screen PCR reactions. All pSKM-e35S-GVA118ΔORF5-pA plasmids, containing an ORF5 of each variant, GFP and GUS respectively, were sequenced.

3.3.6 Construction and infiltration of binary vectors with the complete viral cassettes for GVA118ΔORF5, -1-1, -1-2, -11-1, -GFP and -GUS

The plasmid, pSKM-e35S-GVA118ΔORF5-pA and the pSKM-e35S-GVA118ΔORF5-constructs containing the ORF5, GFP and GUS (section 3.3.5) were digested and ligated into the binary plant vector, pBINSN (Figure 7.3), a derivative of pBIN19 (Bevan, 1984) for plant transformation. The binary vector, pBINSN, was kindly supplied by Prof. Edgar Maiss, Institute of Plant Diseases and Plant Protection, Hanover University, Germany.

All the pSKM-e35S-GVA118ΔORF5-constructs except -GUS were subjected to sequential digests with the restriction enzymes, *SnaBI* (Fermentas) and *SalI* (Fermentas). The construct, pSKM-e35S-GVA118ΔORF5-GUS, was digested initially with *SmaI* (Fermentas) in 1X Buffer Tango™(33 mM Tris-acetate pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA) in a reaction volume of 20 μl for 2 hours at 30°C, after which *SalI* (Fermentas) was added to the reaction, and the buffer concentration was adjusted to 2X Buffer Tango™in a total reaction volume of 40 μl. The digested fragments were separated with gel electrophoresis, and the appropriate fragments were purified and cloned into the digested pBINSN vector.

Similarly, the binary plant vector was digested sequentially with *SnaBI* (Fermentas) and *XhoI* (Fermentas). Although the digested vector was not subjected to gel electrophoresis, the digestion reaction was cleaned with the Zymoclean Gel DNA recovery Kit™(Zymo Research Corp.).

The cloned constructs were transformed (section 3.1.7) and plasmids were extracted and screened with *EcoRI* (Fermentas) digestion.

Electroporation of the pBINSN-e35S-GVA118ΔORF5-constructs

The constructs, pBINSN-e35S-GVA118ΔORF5, -1-1, -1-2, -11-1, -GFP and -GUS (figure 7.8) were electroporated (section 3.2.4) into electrocompetent C58CI *Agrobacterium* cells containing the helper plasmid, pCH32. The full length infectious clone, GR5 and GVA118 (Haviv *et al.*, 2006a) were also included as controls for the infectivity of the pBINSN-e35S-GVA118ΔORF5 constructs.

Electroporation was performed (section 3.2.4) and the cell suspensions were plated out on LB/agar plates with 5 μg/ml tetracycline and 50 μg/ml kanamycin selection. The plates were incubated for 48 hours at 28°C.

Growth conditions for *N. benthamiana* plants

Infectivity of the pBINSN-e35S-GVA118ΔORF5-constructs were tested in co-infiltration experiments in *N. benthamiana* plants. The plants were cultivated in tissue culture and kindly provided by the Institute of Plant Biotechnology (IPB), University of Stellenbosch, under conditions described before (section 3.2.5). Glasshouse facilities were also kindly provided and made available by the Institute of Plant Biotechnology (IPB), University of Stellenbosch.

Agro-infiltration of *N. benthamiana* plants

Recombinant *A. tumefaciens* C58C1-pCH32 cell suspensions, containing the pBINSN-constructs, were prepared as described previously (section 3.2.6) by Voinnet *et al.* (1998). The strong viral suppressor, pBINSN-BMYV-P0 was co-infiltrated with the pBINSN-e35S-GVA118ΔORF5-constructs and GVA118 in *N. benthamiana* plants (Chiba *et al.*, 2006).

3.3.7 Tissue prints

Tissue-print immuno-assay (Franco-Lara *et al.*, 1999) with modifications were performed to detect the CP of GVA in *N. benthamiana* plants 5-7 days post inoculation (dpi).

A hybond PVDF membrane was wetted in methanol, and immediately rinsed in water. The membrane was equilibrated in 1X PBS buffer for 15 minutes and dried on filter paper before blotting the leaf mesophyll tissue. A section of the epidermis of previously infiltrated leaves were removed with tweezers and the exposed mesophyll tissue was pressed firmly on the Hybond PVDF membrane for 2 seconds. The membrane was incubated in 4.5% milk powder dissolved in 1X PBS (Blocking solution) for 1 hour whilst gently shaking. This was followed by a washing step in PBS-T that was repeated 3X for 5 min per wash. The PBS-T was discarded and the membrane was incubated in the primary antibody solution (1 GVA-CP-antisera: 400 PBS-TPO) for 2 hours whilst gently shaking. The antibody solution was collected and the membrane was subjected to 3 washing steps of 5 min each with PBS-T. This was followed by incubation of the membrane in the secondary goat-antirabbit-antibody (GAR-AP) in PBS-T (1:10 000), gently shaking for at least an hour. The conjugate solution was collected and stored at 4°C, and the membrane was washed 3X in PBS-T, for 5 min per wash. This was followed by incubation in an AP-NBT-BCIP colour reaction solution (Reference Chapter 8 for solution compositions). The membrane was incubated for a maximum of 45 min, and rinsed in water, before drying overnight at room temperature.

3.3.8 Recording of symptom development

Symptom development was evaluated over a period of 30 dpi at a room temperature of 24°C, and was photographed with a digital camera (Fuji Finepix S5600).

3.3.9 Rapid direct-one-tube RT-PCR

Rapid direct-one-tube RT-PCR is a modified protocol adapted from the method described by La Notte *et al.* (1997).

Two to three *N. benthamiana* leaf disks were ground in 200 μl grinding buffer (pH 9.6, Na_2CO, $NaHCO_3$, 2 % PVP-40, 0.2% BSA, 0.05% Tween 20, 1% Sodium metabisulphide) with a tissue lyser and stored at -80°C until required. Four μl of the ground leave tissue was pipetted into a 1.5 ml microfuge tube

with 25 μl sterile 1X GES buffer (0.1 M Glycine, 50 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and denatured in a water bath at 95°C for 10 min , followed by rapid cooling on ice for 5 min. A volume of 2 μl was used in 23 μl RT-PCR mix for a total volume of 25μl.

The one-tube RT-PCR mix final reaction concentrations were: 1X NH_4 buffer, 1.5 mM $MgCl_2$, 0.625 μM forward primer, 0.625 μM reverse primer, 0.2 μM dNTP's, 5 mM DTT, 1 U *AMV-RT* (Fermentas), 1 U Kapa *Taq*-polymerase, 10% Cresol and sterile water for a total reaction volume of 25 μl. The PCR cycling conditions were: 1 cycle of 48°C for 30 min, 35 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 1 min, and a final cycle of 72°C for 7 min.

Chapter 4

Results

4.1 Suppression assay using GVA ORF5 in transgenic *N. benthamiana*

Suppression activity has been described previously for ORF5 of the Italian infectious clone, Is151 of GVA (Zhou *et al.*, 2006). A transient expression assay was used to determine suppression activity of the ORF5's of GTR1-2 and GTG11-1. The ORF5 of the full-length clone, GTR1-1 was subjected to the same cloning experiments and analysis as the ORF5's of GTR1-2 and GTG11-1. The results obtained for the pBIN61S-construct believed to contain the ORF5 of GTR1-1 will be shown up to where it was found that the ORF5 was that of GTR1-2. The results obtained for this construct will be omitted from the discussion.The construct have been corrected to contain the ORF5 of GTR1-1 but has not yet been subjected to a co-infiltration assay in transgenic *N. benthamiana*.

4.1.1 Cloning of ORF5 of GTR-1-2 and GTG11-1 into pBIN61S

The ORF5's of the three South African GVA variants, GTR1-1, GTR1-2 and GTG11-1 were amplified from the fully sequenced full-length clones of GTR1-1, GTR1-2 and GTG11-1 (Du Preez, 2005), and were cloned into the vector, pDRIVE. The pDRIVE-ORF5 constructs were sequenced to verify their homology with the original full-length clones and sequences on GenBank database, NCBI (Q787959, DQ855086 and DQ855084, respectively). Two substitutions were noted within the ORF5 of GVA1-1, but sequence alignment and translation revealed that the mutations did not result in a frame shift or change of the amino acid sequence and the clone was used for subsequent experiments.

The primers (Table 3.1), used for the amplification from the full-length clones, were designed to include the restriction enzyme sites, *SacI* and *BamHI*, which flank the respective ORF5's at the 5'- and 3'-termini. These restriction enzyme do not cut within the sequence of ORF5 and are available in the multiple cloning sites of the vector, pT7T318U and pBIN61S, but not in the

pDRIVE cloning vector. The sites were chosen with careful consideration to downstream cloning steps into the intermediary and binary vector. The integration of the restriction enzyme sites are indicated in figure 4.1, flanking the 5'- and 3'-terminal of ORF5 of GTR1-1. Sequencing results showing the correct integration of the restriction enzyme sites were obtained for ORF5 of GTR1-2 and GTG11-1.

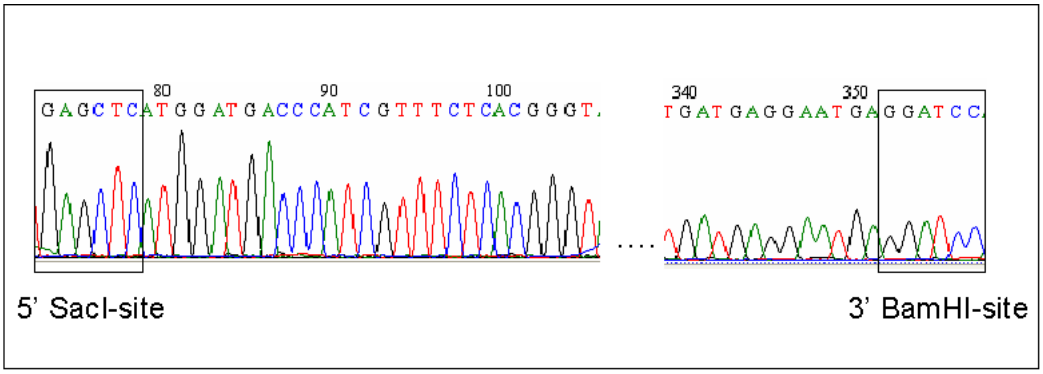

Figure 4.1: A chromatogram of the 5'- and 3'-terminals of ORF5 of GTR1-1 with the restriction enzyme sites, *SacI* and *BamHI* indicated in blocks. The ORF5 of GTR1-2 and GTG11-1 was also sequenced to confirm the integration of the RE sites.

The plasmids, pDRIVE-ORF5-1-1 -1-2 and -11-1 were digested with the restriction enzymes, *BamHI* and *SacI* (Fermentas) and the respective ORF5's were ligated into the vector, pT7T318U. The vector, pT7T318U, was chosen as an intermediary vector, because of its antibiotic resistance. Both the vectors, pBIN61S and pDRIVE contain kanamycin resistance genes which resulted in mixed species of plasmid after cloning of the ORF5 fragments from pDRIVE into the binary vector. This was overcome by the intermediate cloning of ORF5 of GTR1-1, GTR1-2 and GTG11-1 into pT7T318U, which has ampicillin resistance.

High plasmid concentrations were used for the digestion reaction of the pDRIVE-constructs, and it was found that the digestion reaction of the vector, pT7T318U, should not be run on a gel prior to ligation, as purification from agarose gels results in lower concentration and poor cloning results. The vector was therefore cleaned with Sureclean (Bioline) after digestion and prior to ligation of the ORF5 fragments, which were excised out of the agarose gel and cleaned with the Zymoclean Gel purification kit (section 3.1.4) , respectively. Results for the digestion of ORF5 from pDRIVE for ligation into pT7T318U are shown in figure 4.2.

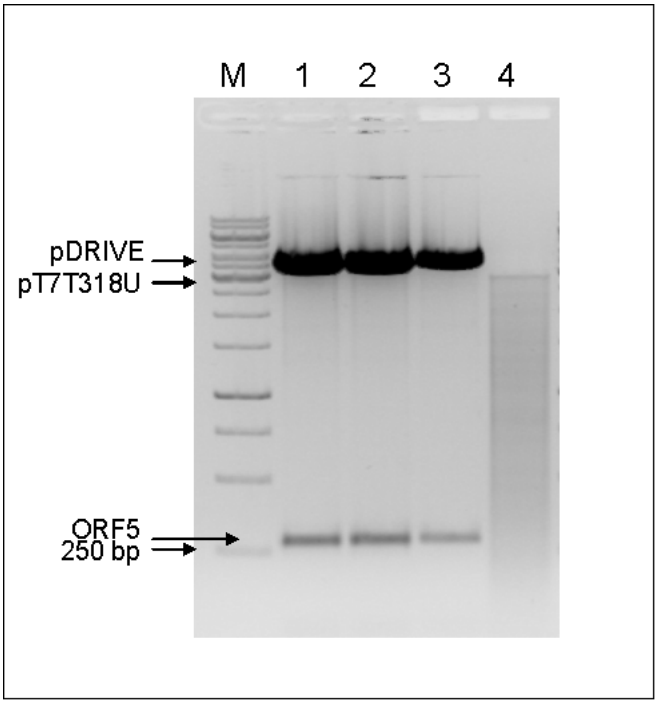

Figure 4.2: The digestion of ORF5 of GTR1-1, GTR1-2 and GTG11-1 from the cloning vector, pDRIVE, for ligation into the vector, pT7T318U, at the restriction enzyme sites, BamHI and SacI. Lane M: 1Kb marker; Lane 1: pDRIVE-ORF5-1-1 after digestion with *BamHI* and *SacI*; Lane 2: pDRIVE-ORF5-1-2 after digestion with *BamHI* and *SacI*; Lane 3: pDRIVE-ORF5-11-1 after digestion with *BamHI* and *SacI*; Lane 4: Linearised pT7T318U after digestion with BamHI and SacI. The different vectors and ORF5 are indicated with arrows.

After successful cloning of ORF5 of GTR1-1, GTR1-2 and GTG11-1 into pT7T318U, colonies were screened with PCR, and plasmid was extracted from positive colonies. These constructs were referred to as pT7T318U-ORF5-1-1, -1-2 and -11-1 and were subjected to digestion with *BamHI* and *SacI* to obtain ORF5 of each construct for ligation into the binary vector, pBIN61S. The binary vector, pBIN61S, was subjected to digestion with *BamHI* and *SacI* but was not visualised on an agarose gel after digestion. The digest reaction was cleaned with Sureclean (Bioline) to ensure a high concentration of vector for successful cloning of the excised ORF5 fragments from the agarose gel. The results for the digestion reactions of ORF5 from the pT7T318U-constructs are shown in figure 4.3.

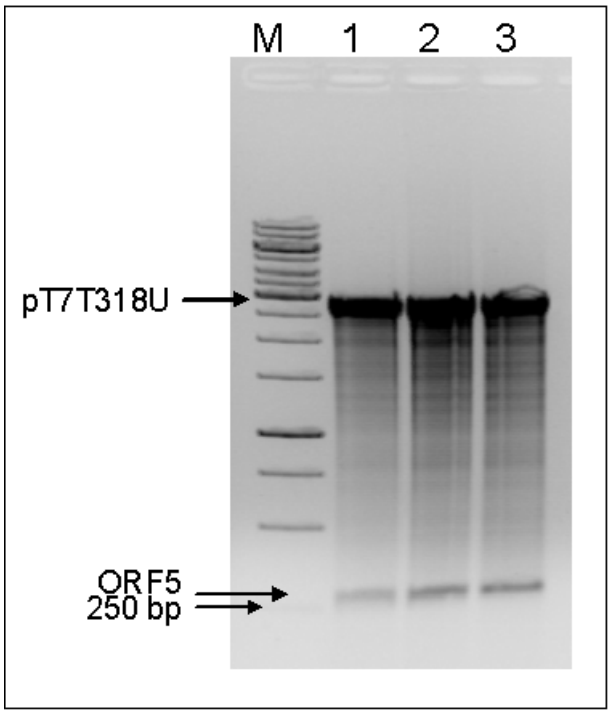

Figure 4.3: Digestion of ORF5 from the intermediary vector, pT7T318U, at the restriction enzyme sites, *BamHI* and *SacI*, for cloning into the binary vector, pBIN61S. Lane M: 1 Kb marker; Lane 1: pT7T318U-ORF5-1-1 after digestion with *BamHI* and *SacI*; Lane 2: pT7T318U-ORF5-1-2 after digestion with *BamHI* and *SacI*; Lane 3: pT7T318U-ORF5-11-1 after digestion with *BamHI* and *SacI*. The vector and ORF5 is indicated with arrows.

Few colonies were observed after the cloning of pBIN61S-ORF5-1-1, -1-2 and -11-1 and the colonies were grown for plasmid extraction, rather than screened with PCR because of the size of the colonies. Once the respective plasmids were obtained, they were screened with PCR to determine whether the cloning was successful. The PCR reactions were performed with the respective primer sets for the amplification of ORF5 described in Table 3.1, before electroporation into *A. tumefaciens* C58C1 cells. The PCR results are shown in figure 4.4.

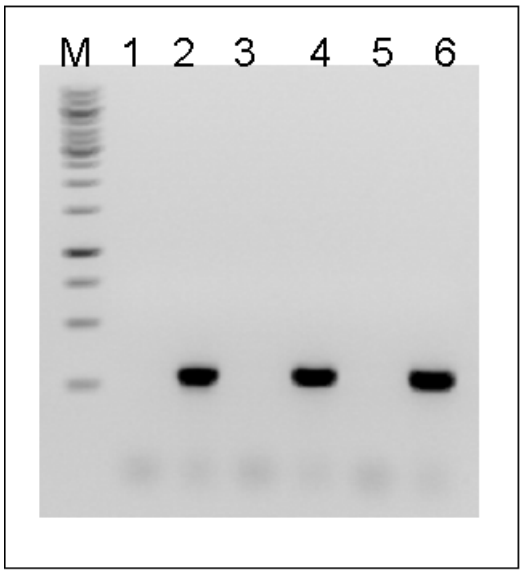

Figure 4.4: The constructs, pBIN61S-ORF5-1-1, -1-2 and -11-1 were screened with their respective primer sets for the amplification of ORF5. Lane M: 1 Kb marker; Lane 1: Negative water control for ORF5 from pBIN61S-ORF5-1-1; Lane 2: Amplified PCR product of ORF5 from pBIN61S-ORF5-1-1; Lane 3: Negative water control for ORF5 from pBIN61S-ORF5-1-2; Lane 4: Amplified PCR product of ORF5 from pBIN61S-ORF5-1-2; Lane 5: Negative water control for ORF5 from pBIN61S-ORF5-11-1; Lane 6: Amplified PCR product of ORF5 from pBIN61S-ORF5-11-1.

4.1.2 Co-infiltration assay in transgenic *N. benthamiana* (line 16c)

The co-infiltration experiments were repeated twice in three plants per construct. Three to four leaves were infiltrated per plant with the constructs that are summerized in Table 3.3. The plants were evaluated over a period of 10 days for GFP fluorescence. The expected outcome was sustained GFP fluorescence in plants co-infiltrated with GFP and the suppressors, HC-Pro, P0, as well as the ORF5's of GTR1-1, GTR1-2 and GTG11-1. This was compared to plants infiltrated with only the GFP gene, which should result in the systemic silencing of GFP expression in transgenic *N. benthamiana* (line 16c) Brigneti *et al.* (1998).

Plants that were infiltrated with the GFP-gene and the suppressors, HC-Pro and P0 in transgenic 16c *N. benthamiana* showed suppression of PTGS of GFP for up to 25 dpi (results not shown). These plants served as positive controls for the suppression assay, whilst the plants infiltrated with the GFP gene and the empty pBIN61S-vector served as negative control. At 3 dpi bright fluorescence of GFP was observed in plants co-infiltrated with the constructs, pBIN61S-HC-pro:pBIN61S-GFP and pBIN61S-BMYV-P0:pBIN61S-GFP as shown in figure 4.5. These results indicated that the infiltration was successful and that GFP was being expressed.

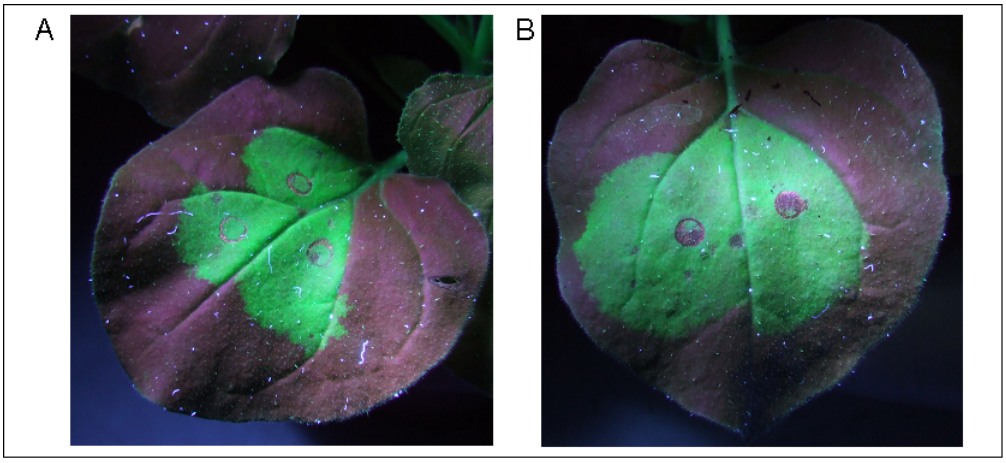

Figure 4.5: Co-infiltrated patches showing GFP fluorescence 3 dpi in transgenic *N. benthamiana* line (16c) that expresses a stable GFP transgene constitutively. The introduction of an extra copy of GFP should result in the systemic silencing of GFP in the plant, but when co-infiltrated with a strong suppressor such as HC-Pro or P0, silencing of the transgene can be suppressed for up to 25 dpi, and results in strong fluorescence of the GFP gene in infiltrated patches. **A)** Bright fluorescence due to GFP expression in co-infiltrated patches with pBIN61S-HC-Pro:pBIN61S-GFP; **B)** GFP fluorescence in co-infiltrated patches with pBIN61S-BMYV-P0:pBIN61S-GFP.

This could be compared to the fluorescence of GFP in plants co-infiltrated with pBIN61S-ORF5-1-1:pBIN61S-GFP, pBIN61S-ORF5-1-2:pBIN61S-GFP and pBIN61S-ORF5-11-1:pBIN61S-GFP. Some of the leaves were divided into two vertical parts on the adaxial side and infiltrated on one side with the pBIN61S-ORF5-constructs:pBIN61S-GFP and with only pBIN61S-GFP:pBIN61S-empty on the other side. This was done to eliminate the possibility of differences that can occur between different plants and leaves in the suppression assay. Results for these co-infiltration experiments 3 dpi are shown in figure 4.6 . The adjacent co-infiltrated patches can indicate suppression activity for ORF5 if GFP fluorescence can be sustained for a longer period of time than seen in the patch infiltrated with only the GFP gene, which will show the progression of PTGS without the interference of a suppressor. In figure 4.6 fluorescence of GFP, which is expected up to 4 dpi without the presence of a suppressor, is visible but cannot not be distinguished between the co-infiltrated patches with and without ORF5.

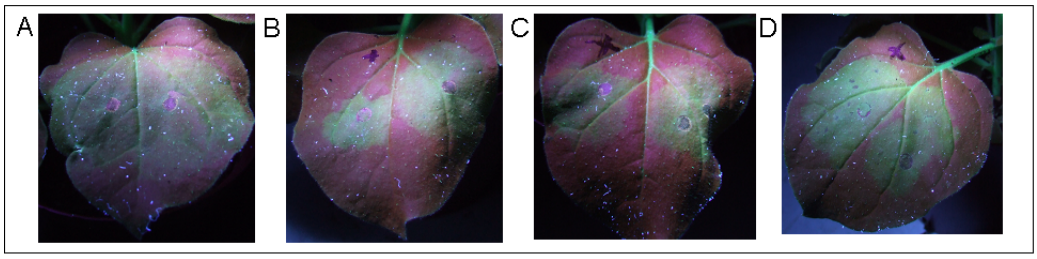

Figure 4.6: Screening of ORF5 of GTR1-1, GTR1-2 and GTG11-1 for suppression activity in co-infiltration experiments with an extra copy of the GFP gene in transgenic *N. benthamiana* (line 16c) which expresses stable GFP transgene. This was compared to adjacent patches that was co-infiltrated with pBIN61S-GFP:pBIN61S-empty, which served as a control for the progression of PTGS without the interference of a suppressor. **A)** Leaf infiltrated with pBIN61S-GFP:pBIN61S-empty. **B)** One side (left) is infiltrated with pBIN61S-ORF5-1-1:pBIN61S-GFP and is indicated with a + sign. The other side (right) is infiltrated with pBIN61S-GFP:pBIN61S-empty. **C)**One side (left) is infiltrated with pBIN61S-ORF5-1-2:pBIN61S-GFP and is indicated with a + sign. The other side (right) is infiltrated with pBIN61S-GFP:pBIN61S-empty. **D)**One side (left) is infiltrated with pBIN61S-ORF5-11-1:pBIN61S-GFP and is indicated with a + sign. The other side (right) is infiltrated with pBIN61S-GFP:pBIN61S-empty.

No GFP fluorescence could be observed by 7 dpi in the control plants that were infiltrated with pBIN61S-GFP:pBIN61S-empty as well as the plants that were co-infiltrated with pBIN61S-ORF5-constructs and pBIN61S-GFP. Sequence analysis of the C58CI:pBIN61S-constructs with which the plants were infiltrated revealed that the ORF5 of GTR1-1 was not present in the construct, pBIN61S-ORF5-1-1, but rather contained the sequence for ORF5 of GTR1-2. All results that were obtained for pBIN61S-ORF5-1-1 could thus not be taken into further consideration, but were used as controls for results obtained for pBIN61S-ORF5-1-2. No other anomalies were detected in the sequence analysis of pBIN61S-ORF5-1-2 and pBIN61S-ORF5-11-1 that could influence suppression activity.

The leaves of the plants infiltrated with the different constructs were subjected to GFP fluorescence microscopy with the quantitive imaging system, IVIS®100 Series (Xenogen) at 10 dpi to confirm the lack of suppression activity by the ORF5 of GTR1-2 and GTG11-1. GFP fluorescence based on photon count with a GFP filter (515 nm - 540 nm) is measured and presented on a scale of red to blue, where red indicates very little fluorescence (grey is none) and blue very high fluorescence. The results are presented in figure 4.7 for the leaves co-infiltrated with the control constructs, pBIN61S-HC-Pro:pBIN61S-GFP, pBIN61S-BMYV-P0:pBIN61S-GFP and pBIN61S-GFP:pBIN61S-empty. Non-infiltrated leaves of *N. benthamiana* and transgenic *N. benthamiana* (line 16c) were also included.

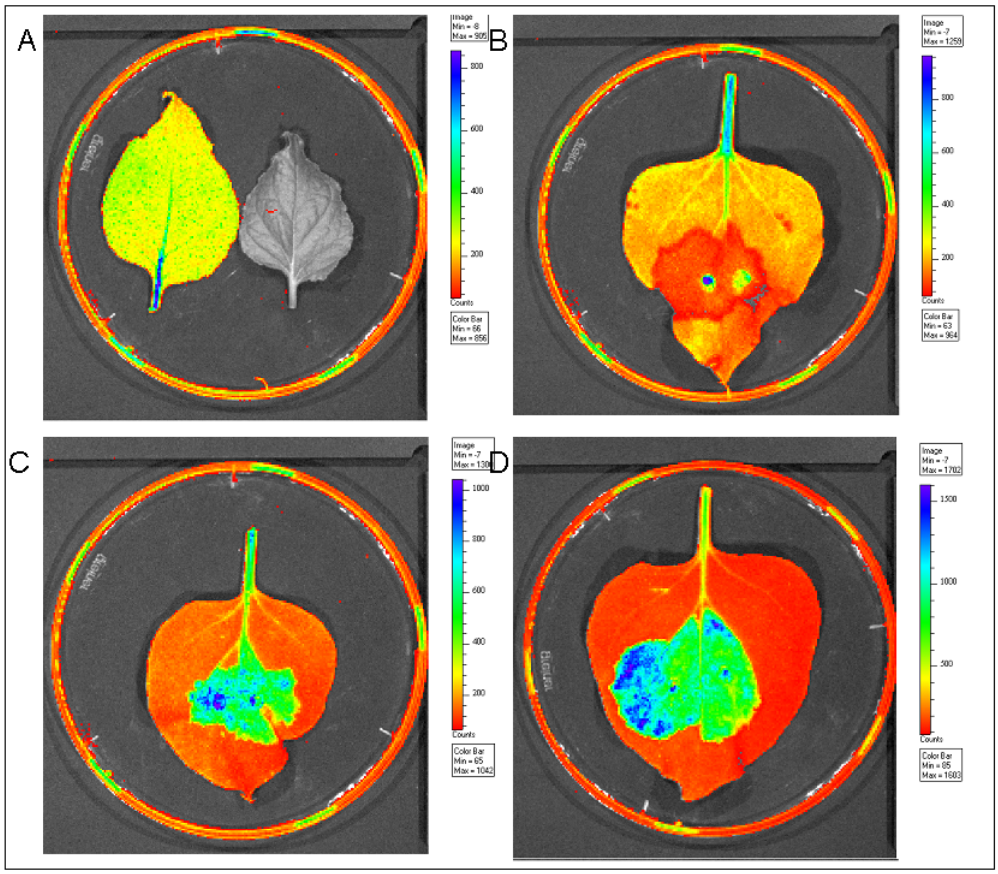

Figure 4.7: Leaves of plants infiltrated with the strong suppressors HC-Pro and P0 in comparison to non-infiltrated leaves and a leaf infiltrated with an extra copy of the GFP gene. **A)** The leaf on the left hand side shows constitutive expression of the GFP gene in transgenic *N. benthamiana* (line 16c) and the leaf on the right is *N. benthamiana* which shows no fluorescence at all (chlorophyll fluorescence cannot be measured with a GFP filter). **B)** A transgenic *N. benthamiana* (line 16c) leaf infiltrated with an extra copy of the GFP gene. A very strong decrease (low photon count) can be observed in the patch infiltrated with the GFP gene, which indicates PTGS. **C)** This leaf was infiltrated with pBIN61S-HC-Pro:pBIN61S-GFP and the presence of blue indicates a very high photon count and thus high GFP fluorescence. **D)** This leaf was infiltrated with pBIN61S-BMYV-P0:pBIN61S-GFP and the presence of blue indicates a very high photon count and thus high GFP fluorescence.

The results for the co-infiltrated leaves with the constructs, pBIN61S-ORF5-1-2:pBIN61S-GFP and pBIN61S-ORF5-11-1:pBIN61S-GFP are shown in figure 4.8. No suppression activity is observed for either of the constructs, and a complete lack of fluorescence is observed as a ring of cells that border the infiltrated patches which indicates that no suppression activity of the cell-to-cell spread of the silencing signal is present (Himber *et al.*, 2003; Zhou *et al.*, 2006).

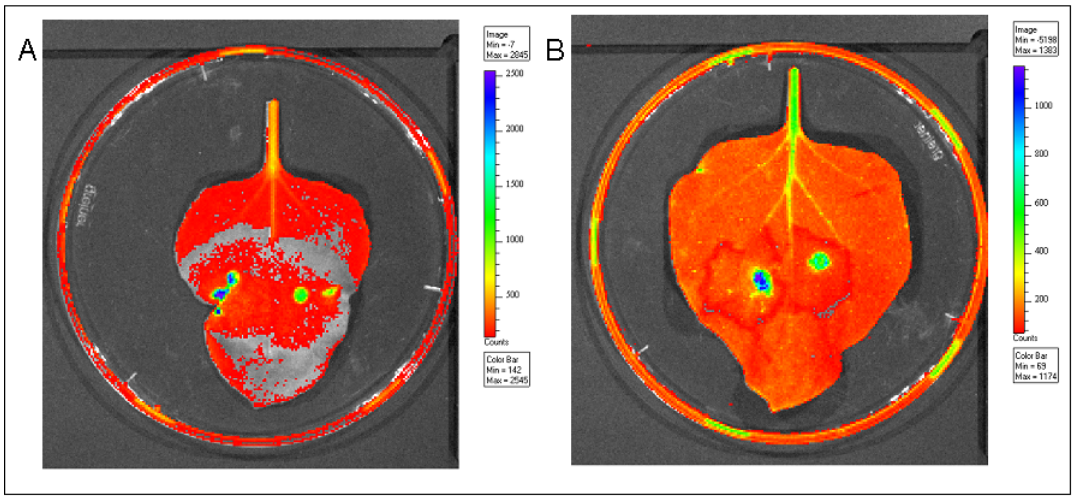

Figure 4.8: This figure shows leaves that were co-infiltrated with pBIN61S-ORF5-1-2:pBIN61S-GFP and pBIN61S-ORF-11-1:pBIN61S-GFP and are visualised with fluorescence microscopy. Brightly fluorescing spots within the infiltrated patches are necrotic spots caused by the infiltration process. **A)** A leaf infiltrated with pBIN61S-ORF5-1-2 that shows very little fluorescence (low photon count) and a non-fluorescence border around the infiltrated patch. **B)** A leaf infiltrated with pBIN61S-ORF5-11-1 that shows very little fluorescence (low photon count) and the start of a non-fluorescence border around the infiltrated patch.

The construct pBIN61S-ORF5-1-1 was corrected to contain the complete sequence of ORF5 of GTR1-1 and has been confirmed to contain the correct sequence within the binary vector, pBIN61S by sequencing. This construct has not yet been tested for suppression activity in a co-infiltration experiment with GFP in transgenic *N. benthamiana* (line 16c).

4.2 Deletion and replacement of ORF5 in the expression vector, GVA118

The ORF5 of the expression vector, GVA118 (Haviv *et al.*, 2006a) was deleted and replaced with two unique restriction enzyme sites into which the ORF5 of GTR1-1, GTR1-2 and GTG11-1 could be cloned. A GFP and GUS gene was also cloned into GVA118 in the place of ORF5. Plants were evaluated for viral replication and symptom development over a period of 30 days.

4.2.1 The deletion of ORF5 by overlap extension PCR

An adaptation of the method described by (Heckman and Pease, 2007) was used to delete the ORF5 from GVA118 and replace it with two unique restriction enzyme sites. The two overlapping fragments were generated in two separate PCR reactions with the construct, pLitmus38-118 as template. The overlapping fragment was designed to include the restriction enzyme sites, *PdiI*

and *Mph11031*, which are not present within the sequence of GVA118, and are unique within the modified GVA118ΔORF5 construct. The primer pairs for the individual PCR reactions, in which the two overlapping fragments were generated, are summarised in Table 3.4. The PCR reactions resulted in a PCR fragment of 1075 bp, which includes the 3'-terminal of ORF3, the complete ORF4 and the overlap fragment with the two RE sites, and a fragment of 143 bp that includes the overlap extension fragment with the two RE sites, 3'-UTR and the poly-A tail, followed by a SalI site. These fragments were joined by overlap extension PCR with the primer set, GV118-UnivF and GV118-UnivR (Table 3.4). The two overlapping PCR fragments and the final overlap extension PCR product constructed from the overlapping fragments are shown in figure 4.9. Although the sizes of the fragments have not been confirmed experimentally except for comparison to the 1 Kb marker, the fragments correspond to the theoretical sizes and are thus accepted to be the sizes indicated in figure (figure 4.9).

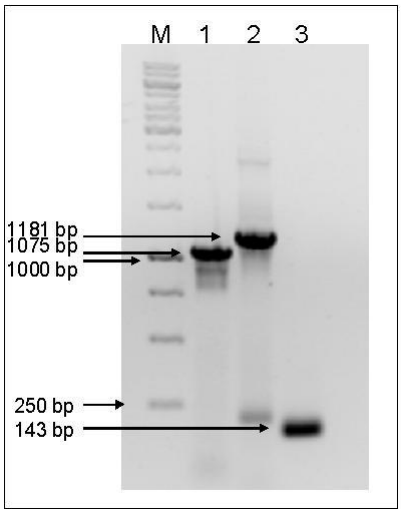

Figure 4.9: The overlap extension PCR results, showing the two fragments used to create the final overlap extension PCR product. Lane M: 1 Kb marker; Lane 1: 1075 bp fragment; Lane 2: Overlap extension PCR product that is the joined product of the 1075 bp fragment and 143 bp fragment; Lane 3: 143 bp fragment. Negative controls (water) were included for all PCR reactions but are not shown on this agarose gel.

The overlap extension PCR product was cloned into the cloning vector, pDRIVE and sequenced to confirm the correct nucleotide sequence. A partial sequencing chromatogram showing correct integration of the restriction enzyme sites in the overlap extension PCR product is shown in figure 4.10.

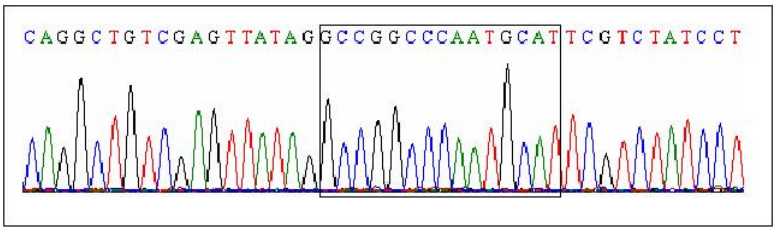

Figure 4.10: Sequence chromatogram showing the successful incorporation of the overlap extension PCR fragments in the place of ORF5. The restriction enzyme sites, *PdiI* (GCCGGC) and *Mph11031* (ATGCAT), separated by the nucleotides, CCA, are indicated in the block. The stop codon, TAG, for ORF4 is directly upstream of the RE sites, and the 3'UTR follows directly downstream.

The overlap extension PCR fragment (henceforth referred to as PCRΔORF5) was digested from pDRIVE and cloned into the pLitmus38-118 construct (section 3.3), to replace the original fragment that contains ORF5.

4.2.2 Construction of partial GVA118-pA construct with PCRΔORF5

The construct, pLitmus38-118 was subjected to digestion with the restriction enzymes, *NcoI* which cuts within ORF3 of GVA118 and *SalI*, directly after the polyA tail. Both of these enzymes are unique with the sequence of pLitmus38-118. The overlap extension PCR fragment, PCRΔORF5 was ligated into the construct, pLitmus38-118, at the above mentioned restriction enzyme sites to replace the original fragment which contains ORF5. The results are shown in figure 4.11.

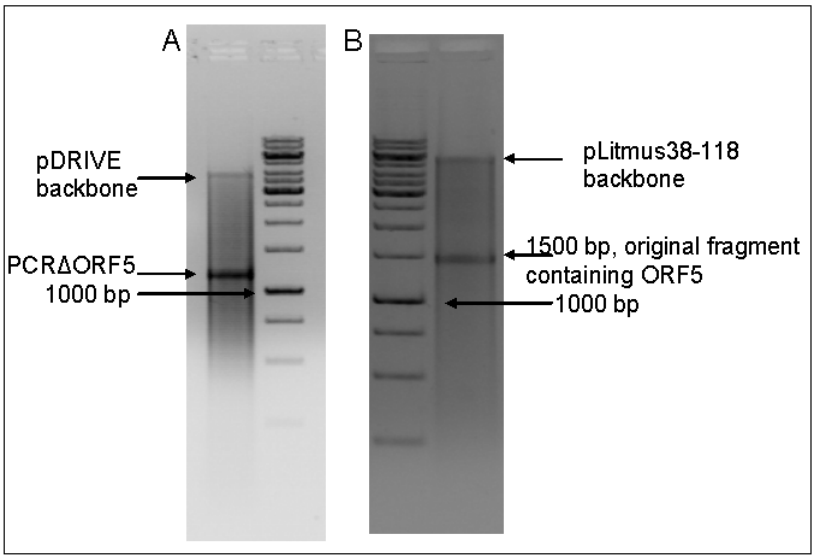

Figure 4.11: Restriction enzyme digestion reactions were performed to obtain the PCRΔORF5 fragment for ligation into the pLitmus38-118 backbone. **A)** The pDRIVE-PCRΔORF5 construct was digested with *NcoI* and *SalI* and the digest reaction was separated on an agarose gel. The smaller PCRΔORF5 fragment is visible just above the 1000 bp fragment of the 1 Kb marker. **B)** pLitmus38-118 was digested with *NcoI* and *SalI*. The upper band, indicated as the backbone was eluted and used in the consequent cloning with the PCRΔORF5 fragment.

The construct was cloned and will henceforth be referred to as pLitmus38-118ΔORF5. This vector (Figure 7.6) contains a partial construct of GVA118 but lacks ORF5, and was used to replace the original fragment in GVA118.

4.2.3 Deletion and replacement of ORF5 in pSKM-e35S-GVA118-pA with GVA118ΔORF5

The construct, pLitmus38-118ΔORF5 was digested to obtain the partial GVA118ΔORF5 from the pLitmus38-backbone. The partial fragment, GVA118ΔORF5 includes a part of ORF1, ORF2, ORF3, 4, 3'UTR and the poly-A tail, and was used to replace the homologous sequence in the original full-length GVA118 clone within a pSKM-backbone. This was done by the digestion with the restriction enzymes, *NheI* and *SalI*, both of which are unique restriction enzyme sites within the constructs, pSKM-e35S-GVA118-pA and pLitmus38-118ΔORF5. The results (shown in figure 4.12) show the successful digestion of both constructs.

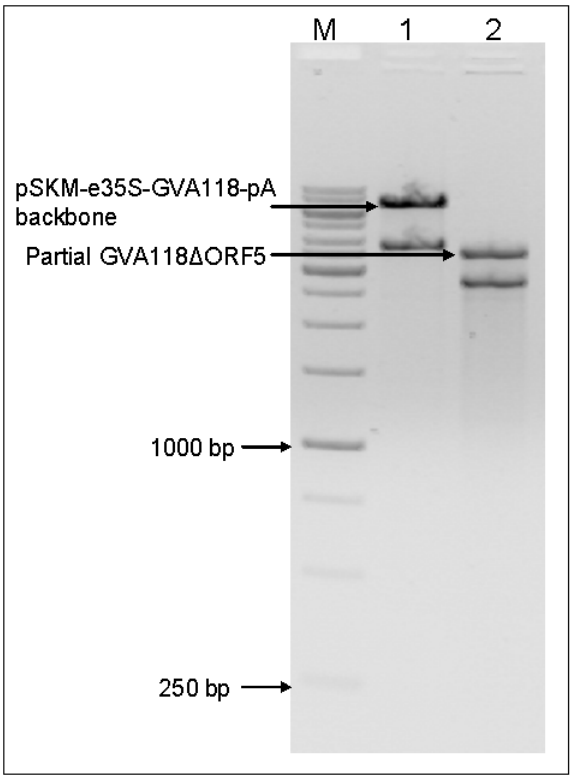

Figure 4.12: Digestion reactions of pSKM-e35S-GVA118-pA and pLitmus38-GVA118ΔORF5 to re-assemble the full-length GVA118 clone without ORF5. Lane M: 1Kb marker; Lane 1: pSKM-e35S-GVA118-pA digested with *NheI* and *SalI*; Lane 2: pLitmus38-GVA118ΔORF5 digested with *NheI* and *SalI*.

The bands indicated in figure 4.12 were eluted from the agarose gel, and cloned. The construct was screened with digestion to ensure successful re-assembly of the partial GVA118 ΔORF5 fragment into the pSKM-e35s-GVA118-pA backbone. This construct, henceforth referred to as pSKM-e35S-GVA118ΔORF5-pA was also partially sequenced.

4.2.4 Cloning of ORF5, GFP and GUS into pSKM-e35s-GVA118-ΔORF5-pA

The vector, pSKM-e35s-GVA118ΔORF5 was used for the subsequent cloning of the ORF5s, GFP and GUS-genes, before being cloned into the binary vector, pBINSN. The ORF5's of GTR1-1, GTR1-2 and GTG11-1 were amplified from the previously amplified ORF5 in pDRIVE (section 4.1.2) with the primer sets described in table 3.2. The results for the PCR reactions are shown in figure 4.13. The primers were designed to include the restriction enzyme sites, *PdiI* and *Mph11031*, which do not cut within the sequence of ORF5 and have previously been included in the construct, pSKM-e35s-GVA118ΔORF5-pA, to flank to ORF5 at the 5' and 3 '-termini.

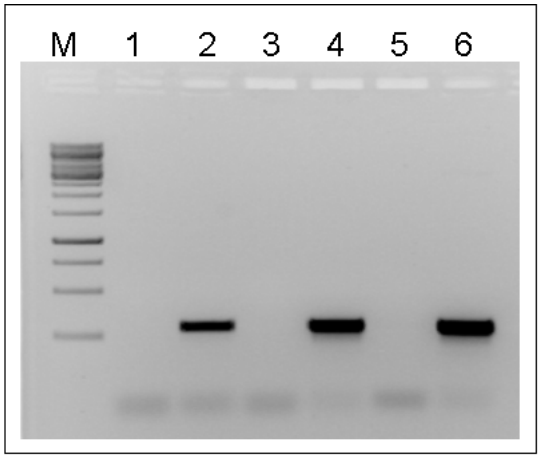

Figure 4.13: The PCR reaction products for the ORF5 of GTR1-1, GTR1-2 and GTG11-1. Lane M: 1 Kb marker; Lane 1: Negative control (water) for ORF5 of GTR1-1; Lane 2: ORF5 PCR product of GTR1-1; Lane 3: Negative control (water) for ORF5 of GTR1-2; Lane 4: ORF5 PCR product of GTR1-2; Lane 5: Negative control (water) for ORF5 of GTG11-1; Lane 6: ORF5 PCR product of GTG11-1.

The amplified ORF5 products for GTR1-1, GTR1-2 and GTG11-1 were cloned into the cloning vector, pDRIVE and sequenced to ensure the correct incorporation of the restriction enzyme sites. Sequence analysis was also performed to ensure that no point mutations were incorporated during the amplification reaction with *Taq*-polymerase. The sequencing chromatogram, showing the incorporation of the restriction enzyme sites, is shown for ORF5 of GTR1-2 in figure 4.14. The correct incorporation of the restriction enzyme sites were confirmed with the sequencing results were obtained for ORF5 of GTR1-1 and GTG11-1.

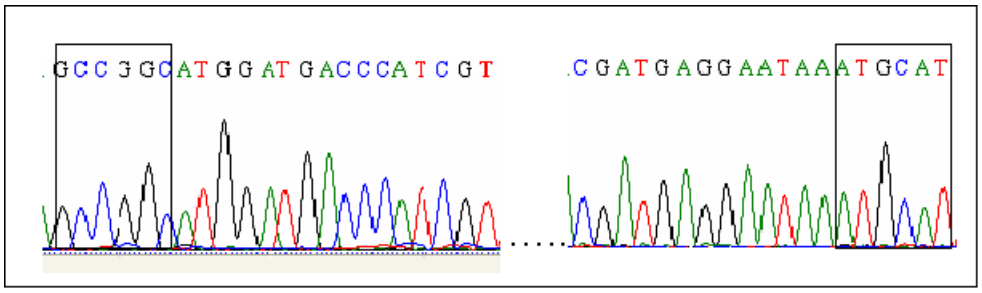

Figure 4.14: A sequence chromatogram showing the incorporation of the restriction enzyme site, *PdiI*, directly upstream of the ORF start codon, ATG, and the restriction enzyme site, *Mph11031*, directly downstream of the stop codon, TAA. The restriction enzyme sites are indicated in boxes. This sequence chromatogram was generated for the ORF5 of GTR1-2 in the cloning vector, pDRIVE.

The GFP- (735 bp) and GUS-gene (2012 bp) were amplified with primers also designed to include the flanking restriction enzyme sites (*PdiI* and *Mph11031*) and are described in table 3.2.The amplified GFP gene was cloned into pDRIVE,

and GUS was cloned into the cloning vector, pGEM. The constructs, pGEM-GUS and pDRIVE-GFP were screened respectively with digestion reactions and PCR to confirm the ligation of the gene into the cloning vector. Both constructs were subjected to sequence analysis to confirm that no point mutations were incorporated during the amplification reaction.

Open reading frame 5 of GTR1-1, GTR1-2 and GTG11-1, as well as the marker genes GFP and GUS were subjected to digestion to obtain the respective fragments from the cloning vectors and were cloned into pSKM-e35s-GVA118ΔORF5-pA. The results for the restriction enzyme digestion reactions are shown in figure 4.15.

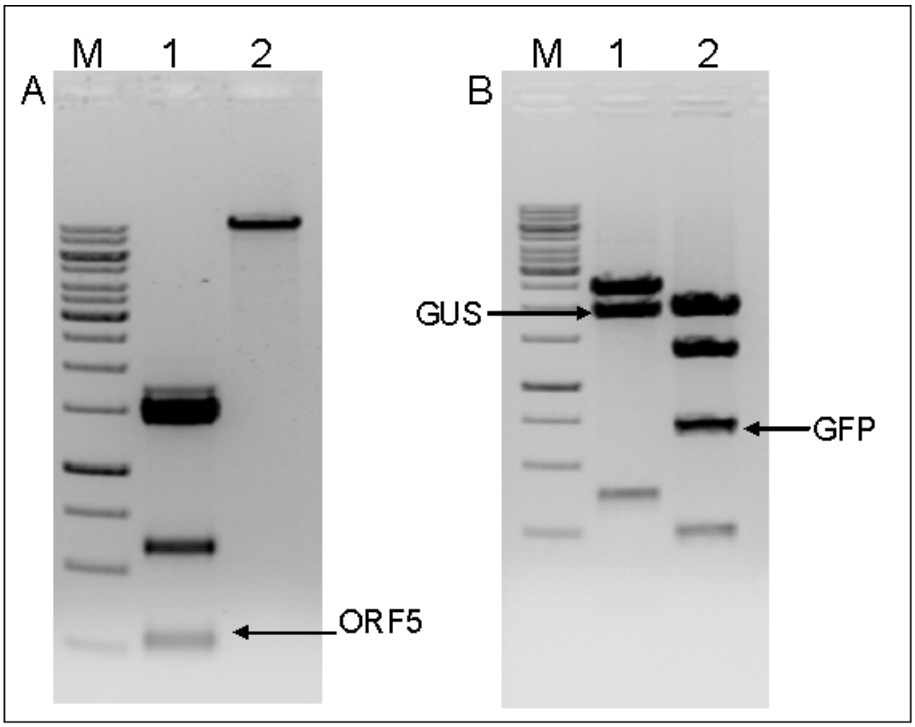

Figure 4.15: The digestion reactions to obtain ORF5, GFP and GUS from their respective cloning vectors with the restriction enzymes, *PdiI* and *Mph11031*, with the exception of GFP. **A)** An example of the digestion of ORF5 from pDRIVE. The restriction enzymes, *PdiI* and *Mph11031* cuts at three positions within the pDRIVE backbone. Lane M: 1 Kb marker; Lane 1: *PdiI* and *MPh11031* digestion of pDRIVE-ORF5-1-2. The ORF5 fragment is indicated with an arrow just above 250 bp. Lane 2: The vector, pSKM-e35S-GVA118ΔORF5 linearised with *PdiI* and *Mph11031*. **B)** Lane M: 1 Kb marker; Lane 2: pGEM-GUS. The GUS gene is indicated with an arrow. Lane 3: pDRIVE-GFP. THE GFP gene is shown just below 750 bp.

The GFP-gene was cloned into pSKM-e35s-GVA118ΔORF5 with the restriction enzyme sites, *SnaBI* and *Mph11031*. *SnaBI* is a blunt-end cutter and compatible with *PdiI*. The sticky-end cutter, *NgoMIV* (isoschizomer of *PdiI*) and *Mph11031* were used for the cloning of the GUS-gene into pSKM-e35S-GVA118ΔORF5. Once the constructs were confirmed to contain the inserts, ORF5 from GTR1-1, GTR1-2 and GTG11-1 as well as the GUS and GFP

genes by restriction enzyme analysis and sequencing, the full-length cDNA clones of GVA118ΔORF5 with the respective inserts were digested out of the pSKM-backbone, and cloned into the binary vector, pBINSN (Du Preez *et al.*, 2008).

4.2.5 Construction and infiltration of pBINSN-GVA118ΔORF5-1-1, -1-2, -11-1, GUS and GFP

The respective constructs, pSKM-e35S-GVA118ΔORF5-1-1, -1-2, -11-1, and -GFP, were digested with *SnaBI* and *SalI* for ligation into the binary plant vector, pBINSN, that was linearised with the restriction enzymes, *SnaBI* and *XhoI* (compatible with *SalI*) for directional cloning. The construct, pSKM-e35S-GVA118ΔORF5-GUS was digested with the restriction enzyme, *SmaI* (also a blunt-end cutter and compatible with *SnaBI*) and *SalI* and cloned into pBINSN, that was digested with *SnaBI* and *XhoI*, as described in section 3.3.6.

The digestion results for pSKM-e35S-GVA118ΔORF5-1-1, -1-2, and the empty vector, pSKM-e35S-GVA118ΔORF5 are shown in figure 4.16A, and the restriction enzyme digestion results for pSKM-e35S-GVA118ΔORF5-11-1, GFP and GUS are shown in figure 4.16B. The respective full-length GVA clones are indicated with arrows and include the e35S promoter from the pSKM-vector. Size differences in the GVA118ΔORF5-constructs are due to the size differences in the inserts cloned into the restriction enzyme sites, *PdiI* and *Mph11031*. The approximate length of GVA118ΔORF5 is 8 000 bp, and the genes, GFP and GUS, are respectively 750 bp and 2000 bp in size. ORF5 is approximately 280 bp in length when the flanking restriction enzyme sites are included.

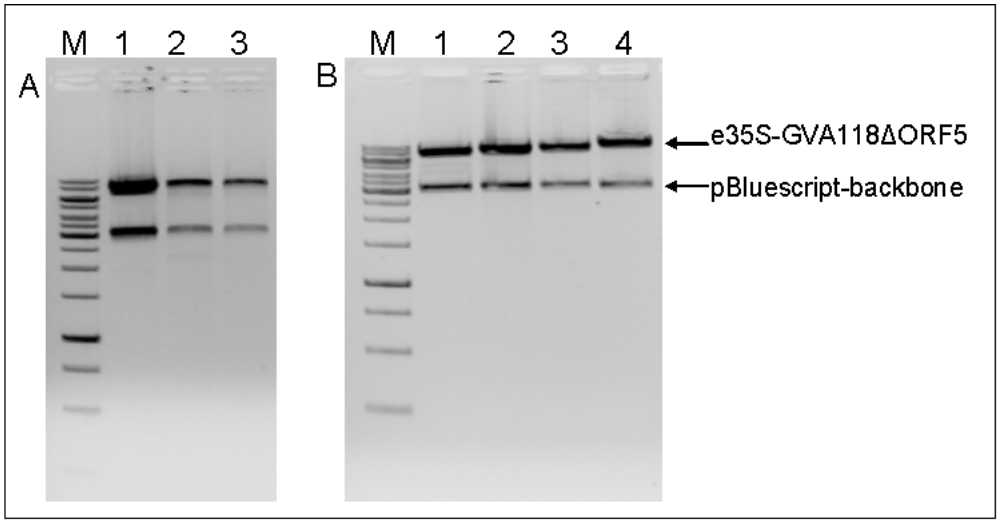

Figure 4.16: The digestion of the empty vector, pSKM-e35S-GVA118ΔORF5 and the constructs, pSKM-e35S-GVA118ΔORF5-1-1, -1-2, -11-1, GFP and GUS for ligation into pBINSN. **A)** Lane M: 1Kb marker; Lane 1: pSKM-e35S-GVA118ΔORF5; Lane 2: pSKM-e35S-GVA118ΔORF5-1-1; Lane 3: pSKM-e35S-GVA118ΔORF5-1-2. **B)** Lane M: 1 kb marker; Lane 1: pSKM-e35S-GVA118ΔORF5; Lane 2: pSKM-e35S-GVA118ΔORF5-11-1; Lane 3: pSKM-e35S-GVA118ΔORF5-GFP; Lane 4: pSKM-e35S-GVA118ΔORF5-GUS.

The full-length GVA clones with the ORF5 exchanges, and the introduced genes, GFP and GUS, were eluted from the respective agarose gels and cloned directionally into the binary vector, that was subjected to digestion but not agarose gel electrophoresis. Successful cloning was confirmed with restriction enzyme digestion. The pBINSN-GVA118ΔORF5-constructs are summarized in Table 4.1.

Table 4.1: A summary of the final pBINSN-e35S-GVA118ΔORF5-pA constructs

Construct name	Size	*A. tumefaciens*
pBINSN-e35S-GVA118ΔORF5-pA	19 856 bp	C58CI:pCH32
pBINSN-e35S-GVA118ΔORF5-1-1-pA	20 123 bp	C58CI:pCH32
pBINSN-e35S-GVA118ΔORF5-1-2-pA	20 123 bp	C58CI:pCH32
pBINSN-e35S-GVA118ΔORF5-11-1-pA	20 123 bp	C58CI:pCH32
pBINSN-e35S-GVA118ΔORF5-GFP-pA	20 605 bp	C58CI:pCH32
pBINSN-e35S-GVA118ΔORF5-GUS-pA	21 868 bp	C58CI:pCH32
Controls		
pBINSN-GR-5	20 000 bp	C58CI:pCH32
pBINSN-e35S-GVA118-pA	20 123 bp	C58CI:pCH32

An example of an *EcoRI* restriction enzyme digestion to confirm the successful cloning of the full-length GVA clones into pBINSN is shown in figure 4.17. The same restriction enzyme digestion reaction was used to confirm the successful electroporation of the pBINSN-e35S-GVA118ΔORF5-constructs

in the electrocompetent C58CI *Agrobacterium* cells with the helper plasmid, pCH32.

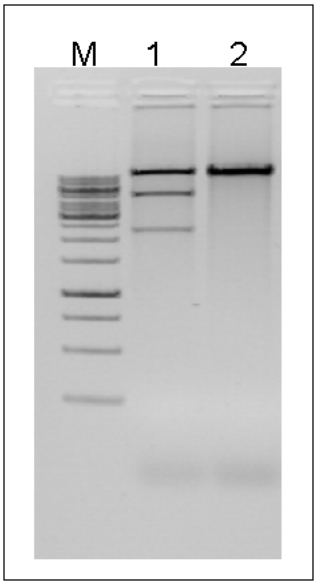

Figure 4.17: An example of an *EcoRI* restriction enzyme digestion to confirm the successful cloning of the full-length GVA118ΔORF5 constructs into the binary vector, pBINSN. The binary vector linearises in the presence of *EcoRI* (single cutter) but cuts twice within the full-length GVA118ΔORF5 constructs. Lane M: 1 Kb marker; Lane 1: pBINSN-GVA118ΔORF5; Lane 2: pBINSN-GVA118ΔORF5-11-1 that is negative for the full-length GVA118ΔORF5-11-1 construct and only shows linearised pBINSN.

The constructs, described in Table 4.1, were co-infiltrated with the strong viral suppressor, P0 in *N. benthamiana* and evaluated for viral replication and symptom development.

4.2.6 Viral replication and symptom development in *N. benthamiana*

Viral replication was determined with a tissue print immuno-assay (TPIA) 5 dpi that detects the coat protein of GVA. The epidermal cells of the abaxail side of the leaf was removed and the exposed mesophyll tissue was used for the TPIA with GVA coat protein antibodies. Plants were infiltrated in two respective infiltration experiments. The first experiment utilised GR-5 as the positive control, and the second experiment, GVA118 as positive control in *N. benthamiana*, whilst *N. benthamiana* that has not been infiltrated, was used as the negative control. The TPIAs were performed for each of the infiltration experiments and revealed that the construct, pBINSN-GVA118ΔORF5-1-2, which also serves as a control, was replicating within *N. benthamiana*. Although pBINSN-GVA118ΔORF5-11-1 seemed to be replicating, it has not been confirmed more than once. These results are shown in figure 4.18 and the leaf tissue positive for pBINSN-35S-GVA118ΔORF5-1-2-pA is indicated with arrows for both the first and second infiltration experiments.

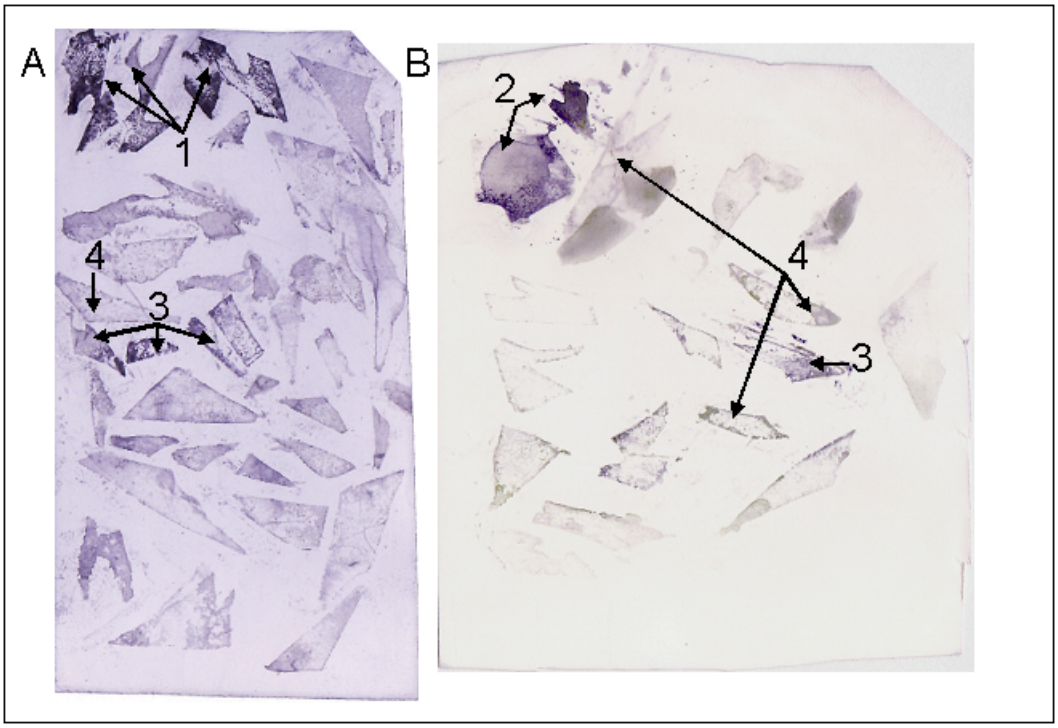

Figure 4.18: TPIA results for the mesophyll tissue of *N. benthamiana* after agroinfection with the all pBINSN-GVA118ΔORF5-constructs per membrane and pBINSN-GR-5/pBINSN-GVA118 as positive control. Both the positive controls, GR-5 (A1) and GVA118 (B2) are in the upper left hand corner of the respective membranes. **A)** The TPIA for the first infiltration experiment with GR-5(1) as the positive control. The leaf prints for pBINSN-e35S-GVA118ΔORF5-1-2-pA (3) is indicated with arrows, with a negative control (4) right above and below the prints. **B)** The TPIA membrane result, that was performed with modifications to reduce the background observed in **A**. The positive control, GVA118 (2) is in the upper left hand corner and the positive print for pBINSN-e35S-GVA118ΔORF5-1-2-pA (3) is shown with an arrow. A negative leaf print (4) is right above the indicated print.

Microscopy of cells infiltrated with the construct, pBINSN-GVA118ΔORF5-GFP did not reveal any GFP fluorescence at 5 or 7 dpi, and no GUS accumulation in the cytosol of the infiltrated plant cells was observed at 5 dpi. This corresponds with the lack of replication observed with TPAI at 5 dpi for these constructs. The microscopy results are shown in figure 4.19.

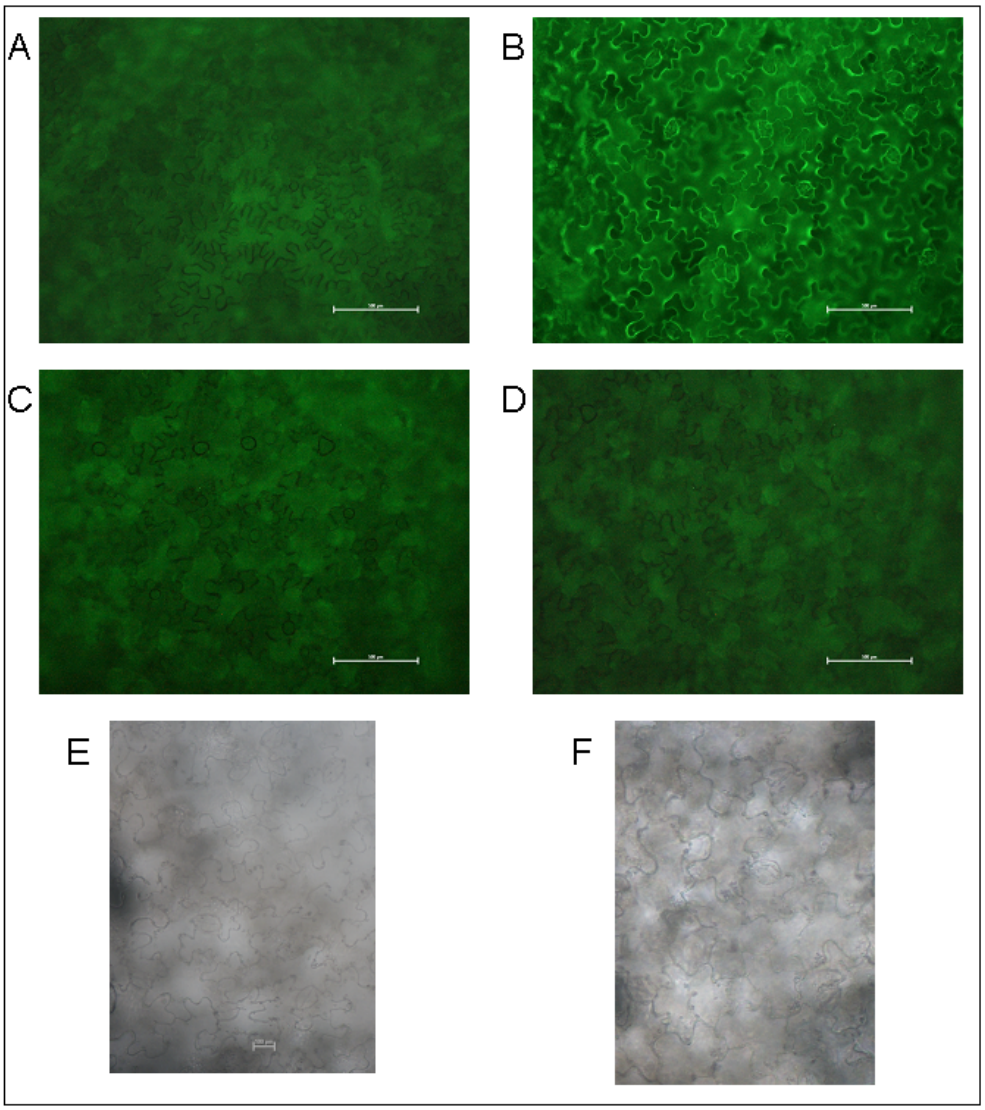

Figure 4.19: Microscopy of GFP fluorescence at 5 and 7 dpi in comparison to transgenic *N. benthamiana* (line 16c) expressing GFP and *N. benthamiana*. The GUS colouring reaction, which should show blue colouring within the cytosol of the plant cells, in the presence of the expressed GUS gene, is also shown. **A)** Non-infiltrated *N. benthamiana* leaf; **B)** Transgenic *N. benthamiana* (line 16c) expressing GFP; **C)** Leaf mesophyll tissue co-infiltrated with pBINSN-GVA118ΔORF5-GFP: pBINSN-P0 5 dpi; **D)** Leaf mesophyll tissue co-infiltrated with pBINSN-GVA118ΔORF5-GFP: pBINSN-P0 7 dpi; **E)** Transgenic *N. benthamiana* (line 16c) expressing GFP under general microscopy without a GFP filter; **F)** Leaf mesophyll tissue co-infiltrated with pBINSN-GVA118ΔORF5-GUS: pBINSN-P0 5 dpi.

Transmission electron microscopy (TEM) was performed on leaf samples infiltrated during the first experiment and corresponded to initial TPIA results which indicated replication for GR-5 and the construct, pBINSN-35S-GVA118Δ ORF5-1-2-pA at 5 dpi. These results are shown in figure 4.20.

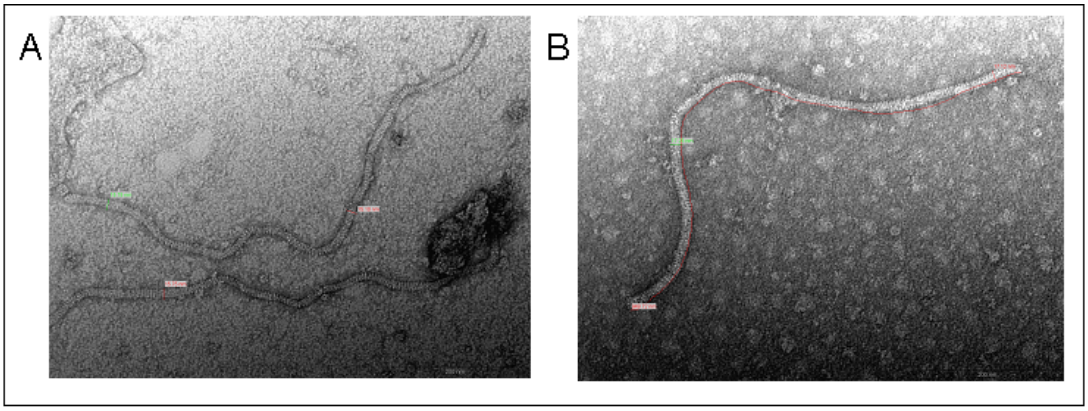

Figure 4.20: TEM of the positive control, GR-5 used during the first infiltration experiments of the modified GVA118 constructs and pBINSN-e35S-GVA118ΔORF5-1-2-pA. **A)** GR-5; **B)** pBINSN-e35S-GVA118ΔORF5-1-2-pA

In contrast to the lack of replication that was observed with TPIA at 5 dpi during the first and second infiltration experiments for the constructs, with the exception of GR-5-, GVA118 and pBINSN-e35S-GVA118ΔORF5-1-2-pA, symptom development was observed in all the plants infiltrated with the constructs, pBINSN-GVA118ΔORF5-1-1, pBINSN-GVA118ΔORF5-1-2 and pBINSN-GVA118ΔORF5-11-1 and the control, pBINSN-GVA118 between 10-14 dpi during the second infiltration experiment. The constructs, pBINSN-GVA118ΔORF5, pBINSN-GVA118ΔORF5-GFP and pBINSN-GVA118ΔORF5-GUS which lacks the ORF5 did not show any symptom development at all. Symptom development was evaluated over a period of 30 dpi and are shown respectively at 14 dpi (figure 4.21) and 20 dpi (figure 4.22) and 30 dpi (figure 4.24)

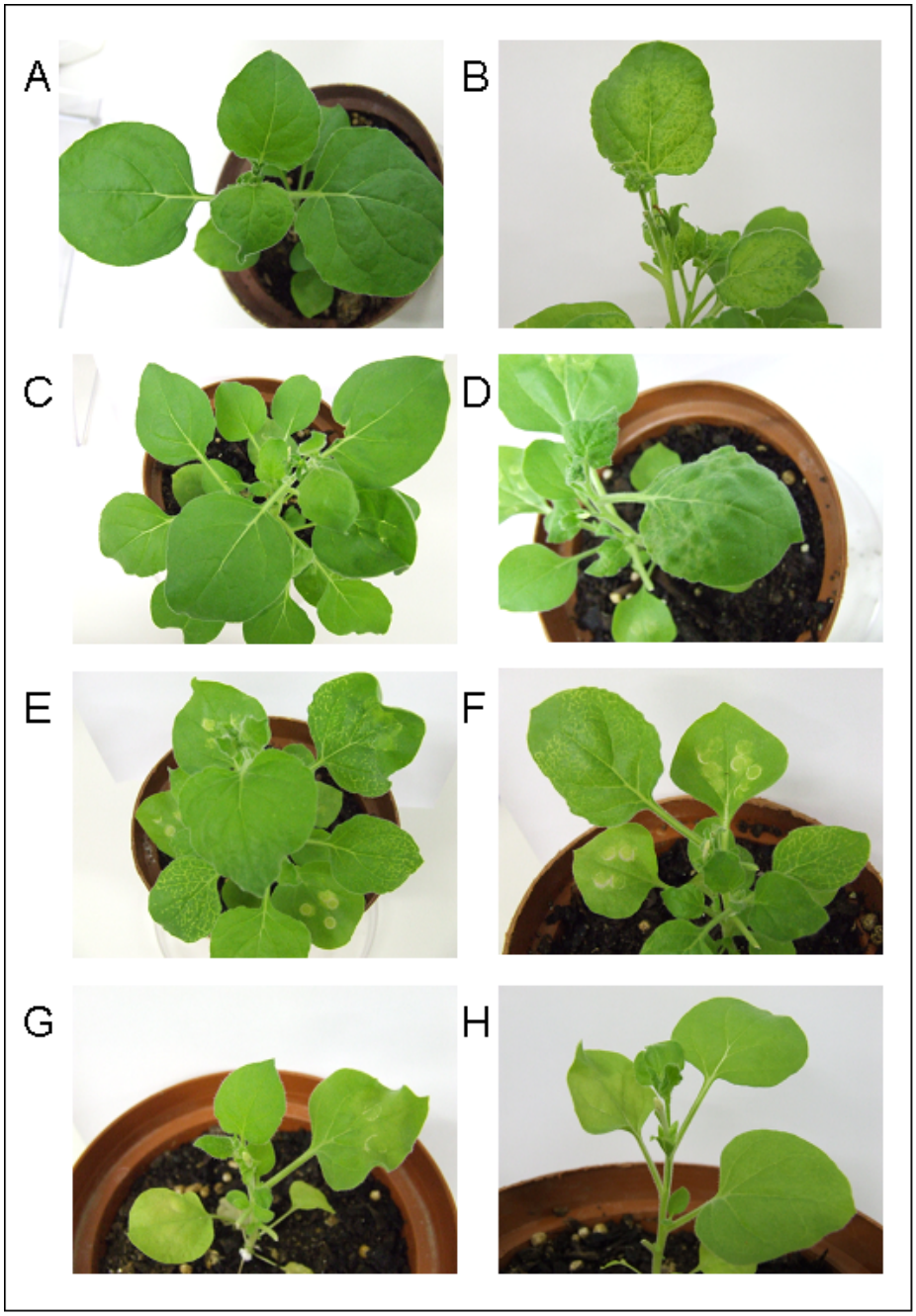

Figure 4.21: Symptom development in *N. benthamiana* after co-infiltration with the suppressor, P0 and the constructs, pBINSN-GVA118ΔORF5, -1-1, 1-2, -11-1, -GFP and -GUS at 14 dpi. A) *N. benthamiana* as negative control; B) *N. benthamiana* co-infiltrated with pBINSN-GVA118 and P0 as positive control; C) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5 and P0; D) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-1-1 and P0; E) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-1-2 and P0; F) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-11-1 and P0; G) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-GFP and P0; H) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-GUS and P0.

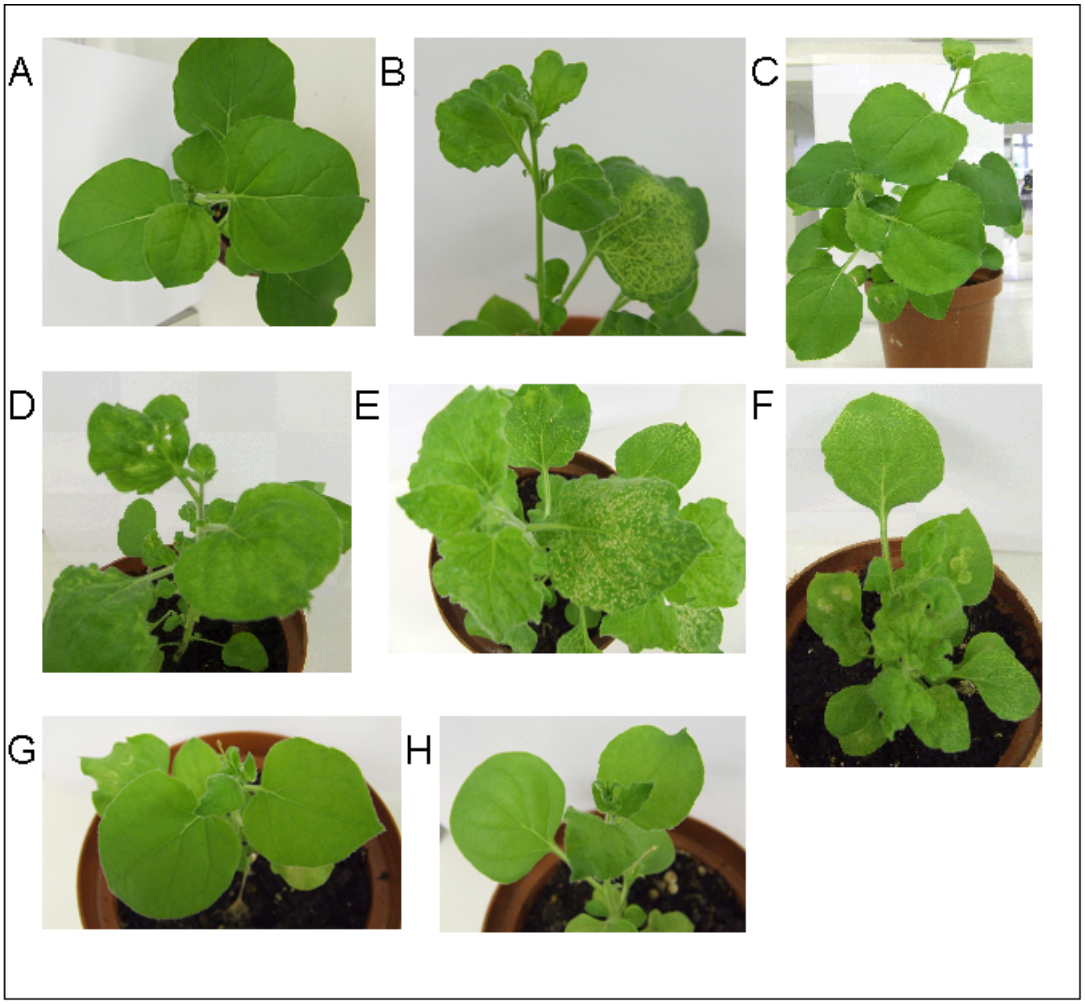

Figure 4.22: Symptom development in *N. benthamiana* after co-infiltration with the suppressor, P0 and the constructs, pBINSN-GVA118ΔORF5, -1-1, 1-2, -11-1, -GFP and -GUS at 20 dpi. A) *N. benthamiana* as negative control; B) *N. benthamiana* co-infiltrated with pBINSN-GVA118 and P0 as positive control; C) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5 and P0; D) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-1-1 and P0; E) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-1-2 and P0; F) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-11-1 and P0; G) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-GFP and P0; H) *N. benthamiana* co-infiltrated with pBINSN-GVA118ΔORF5-GUS and P0.

Symptoms could still be seen clearly at 30 dpi. The constructs, pBINSN-GVA118ΔORF5, pBINSN-GVA118ΔORF5-GFP and pBINSN-GVA118ΔORF5-GUS, still did not show any symptom development (figure 4.23), and the plants infiltrated with pBINSN-GVA118ΔORF5-1-1-pA, pBINSN-GVA118ΔORF5-1-2-pA and pBINSN-GVA118ΔORF5-11-1-pA and the control, pBINSN-e35S-GVA118-pA showed similar symptoms to those observed at 20 dpi (figure 4.24).

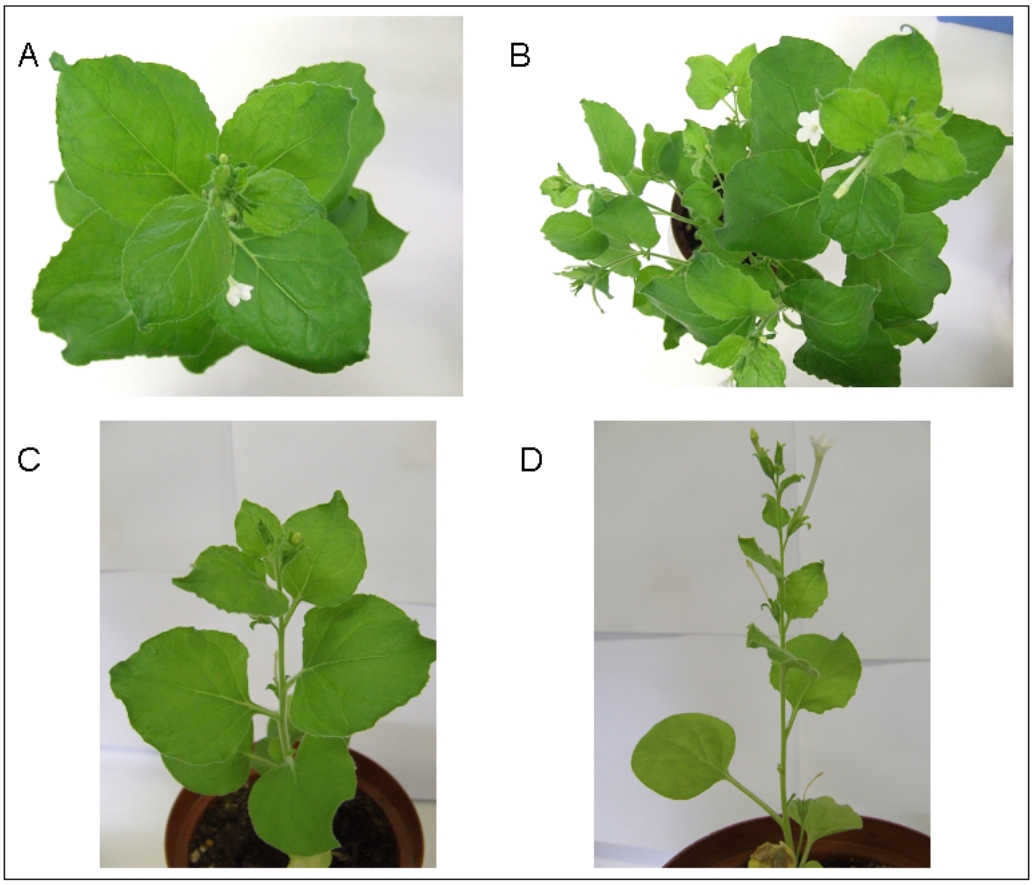

Figure 4.23: The asymptomatic N. benthamiana plants infiltrated with the constructs, pBINSN-GVA118ΔORF5, pBINSN-GVA118ΔORF5-GFP and pBINSN-GVA118ΔORF5-GUS and the N. benthamiana plant (negative control) that was not infiltrated at all at 30 dpi. **A)** N. benthamiana; **B)** N. benthamiana infiltrated with pBINSN-GVA118ΔORF5-pA; **C)** N. benthamiana infiltrated with pBINSN-GVA118ΔORF5-GFP-pA; **D)** N. benthamiana infiltrated with pBINSN-GVA118ΔORF5-GUS-pA.

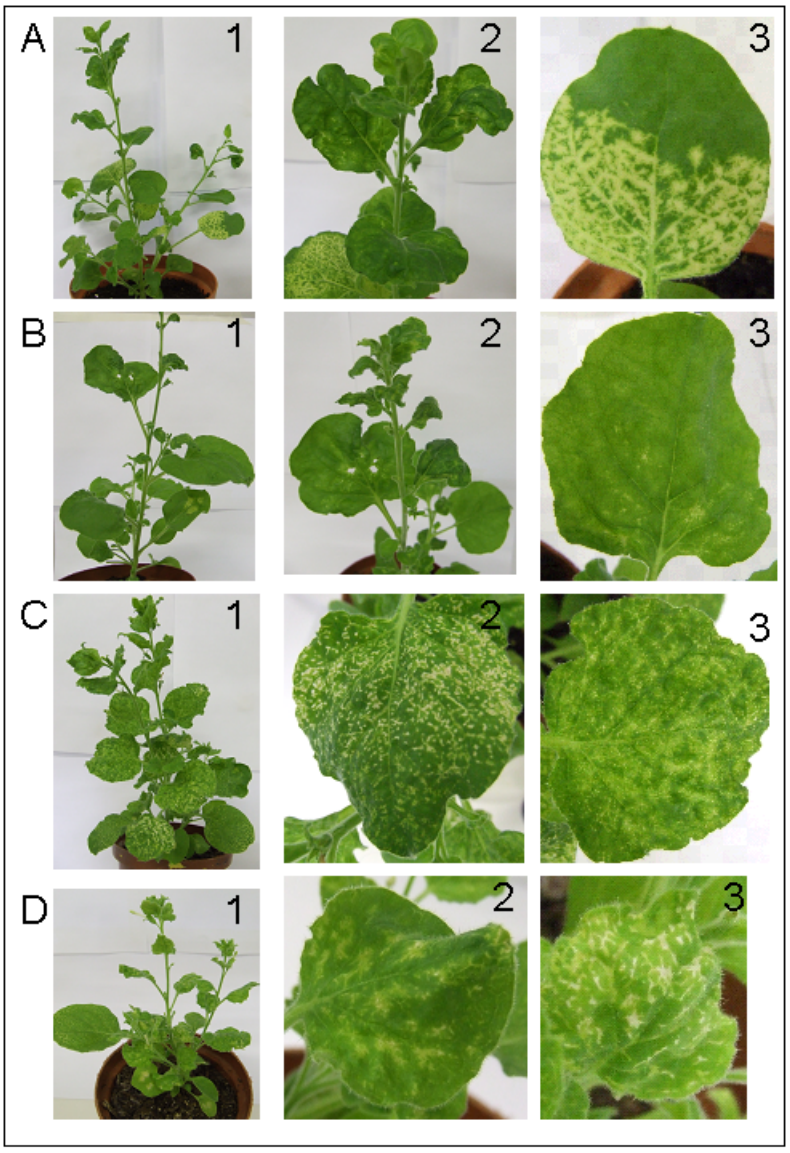

Figure 4.24: Symptom development in the respective N. benthamiana plants at 30 dpi. **A)** The positive control plant infiltrated with GVA118. Figures 1, 2 and 3 shows the whole plant, and typical symptomatic leaves from the plant; **B)** The plant infiltrated with pBINSN-GVA118ΔORF5-1-1-pA, and typical symptomatic leaves from the plant; **C)** The plant infiltrated with pBINSN-GVA118ΔORF5-1-2-pA and typical symptoms that is observed through out the plant; **D)** The plant infiltrated with pBINSN-GVA118ΔORF5-11-1-pA and typical symptoms that is observed through out the plant.

Rapid direct-one-tube RT-PCRs were performed to detect the various infectious full-length clones in the infiltrated *N. benthamiana* plants. This technique failed to give consistent results in visually infected upper leaves of the plants at 14 and 30 dpi (results not shown).

Chapter 5

Discussion

5.1 Suppression activity of ORF5 of GTR1-1, GTR1-2 and GTG11-1

The ability of the nucleic acid binding protein (p10), encoded by ORF5 of GVA, to suppress post transcriptional silencing activity in transgenic *N. benthamiana* (line16c) has been characterised previously (Zhou *et al.*, 2006) and supported the suggestion by Chiba *et al.* (2006) that p10 could act as a weak suppressor. Co-infiltration experiments with GFP performed by Zhou *et al.* (2006) suggested that the p10 of GVA suppresses transgene RNA silencing in transgenic *N. benthamiana* (line 16c) by the sequestering of siRNAs.

Open reading frame 5 of GVA contains a highly conserved arginine region and zinc-finger motif (Figure 2.4) which is associated with silencing suppressor activity (Chiba *et al.*, 2006) and affects pathogenesis (Galiakparov *et al.*, 2003b). The same conserved regions are observed in the ORF5 of the three South African variants of GVA (GTR1-1, GTR1-2 and GTG11-1) which suggests that these ORF5s should exhibit similar suppression activity observed for the ORF5 of Is151. Suppression activity has also been associated with pathogenicity determinants within viral genomes (Voinnet *et al.*, 1999) and supports p10 as a suppressor protein since this protein has been shown to be the pathogenicity determinant with GVA (Galiakparov *et al.*, 2003b; Zhou *et al.*, 2006; Goszczynski *et al.*, 2008)

This study resulted in the construction of three binary vectors with the ORF5 of the South African variants of GVA, GTR1-1, GTR1-2 and GTG11-1. The ORF5 of each of the above mentioned variants were amplified from the respective full-length cDNA clones, each of which represents a molecular group (Goszczynski and Jooste, 2003b) and were cloned directionally, downstream of an enhanced 35S promoter, into the T-DNA (transfer DNA) region of the binary vector, pBIN61S. The respective ORF5's of the three full-length clones were cloned into the intermediary vector pT7T318U, since direct cloning from pDRIVE into pBIN61S resulted in mixed populations of vectors after cloning, which were thought to be caused by the same antibiotic selection and the small electrophoretic size of the ORF5 fragment. Although the ORF5 fragments were eluted directly from the agarose gel after digestion, it is believed that linearised

or supercoiled pDRIVE-vector could still have been present within the elute and would select on kanamycin after cloning into pBIN61S. This problem was overcome by the cloning of the respective ORF5 fragments into the vector, pT7T318U which only selects on ampicillin. During infiltration of the binary constructs, the T-DNA region of the plasmid transfers into the plant nuclear DNA (Vionnet and Baulcombe, 1997), which should result in the expression of p10 of GVA.

The construct that was believed to contain the ORF5 of GTR1-1 was found, after infiltration, to contain the ORF5 of GTR1-2. The results obtained for the construct believed to be pBIN61S-ORF5-1-1 are thus omitted from any further discussion. Although the construct have also been corrected, it has not yet been tested in a co-infiltration experiment.

The expected outcome of the co-infiltration experiments with a GFP gene and the ORF5 of GTR1-2 and GTG11-1 in transgenic *N. benthamiana* plants were visually sustained fluorescence of GFP due to the suppression of silencing, in comparison to a decrease in GFP fluorescence 3-5 dpi due to active transgene silencing by the plant. No sustained fluorescence of GFP was observed in plants that were co-infiltrated with a 35S-GFP cassette and the ORF5 of GTR1-2 and -11-1 (figure 4.6 and 4.8) in comparison to plants infiltrated with the 35S-GFP cassette, which resulted in transgene RNA silencing. This suggests that the presence of ORF5 of GTR1-2 and GTG11-1 did not result in any suppression activity within the co-infiltrated leaf tissue, and that silencing of GFP progressed as it would have without the presence of a suppressor. Suppression activity of GFP in transgenic *N. benthamiana* (line 16c) was observed for the well characterised suppressors, HC-Pro (Brigneti *et al.*, 1998) of PPV and P0 (Pfeffer *et al.*, 2002) of BMYV. Plants infiltrated with these suppressors showed sustained suppression of PTGS and consequent GFP fluorescence for up to 25 days, respectively. The suppressors, HC-Pro an P0 also served as the positive controls for the co-infiltration experiments conducted during this study, which confirmed that the agro-inoculation of the constructs were successful, and resulted in the replication of the GFP gene and the suppressors, P0 and HC-Pro within the plant genome.

A common phenomenon observed in leaf patches infiltrated with a transgene is a characteristic ring of silenced cells surrounding the infiltrated patch. This is due to the cell-to-cell spread of the mobile silencing signal from the site of infiltration (Himber *et al.*, 2003). This was observed in leaf patches co-infiltrated with pBIN61S-GFP and pBIN61S-ORF5-1-2, and -11-1, respectively, which indicates that the systemic silencing signal is activated (Vionnet and Baulcombe, 1997) and supports the lack of suppression activity observed for the ORF5's (figure 4.8). The cell-to-cell spread of the silencing signal was suppressed by P0 and HC-Pro as no silenced cell ring was observed around the patches of leave tissue agro-infiltrated with pBIN61S-BMYV-P0:pBIN61S-GFP or pBIN61S-PPV-HC-Pro:pBIN61S-GFP constructs (figure 4.7).

Delayed systemic silencing of GFP due to the presence of ORF5 as suppressor was reported by Zhou *et al.* (2006) in transgenic *N. benthamiana* (line 16c) and corresponded well to the delayed systemic silencing observed in plants co-

infiltrated with a 35S-GFP cassette and the strong suppressor, HC-Pro during the study. Although systemic silencing was also observed during this project in the upper leaves of some of the transgenic *N. benthamiana* (line 16c) plants infiltrated with the respective constructs (results not shown), the cell-to-cell spread of the silencing signal is responsible for silencing and occured much earlier tan noted in the study performed by Zhou *et al.* (2006). The phenomenon of the systemic spread of the PTGS-signal, induced by a second copy of a transgene (GFP) in a plant, is well described in literature (Vionnet and Baulcombe, 1997; Baulcombe, 2004).

Sequencing of the vectors revealed that no point mutations or foreign nucleotides were incorporated during the construction of the vectors, although it did reveal that the construct, pBIN61S-ORF5-1-1 contained the ORF5 of GTR1-2. The construct has been corrected and now contains the ORF5 of GTR1-1.

The ORF5's of the three variants have been cloned directionally into the binary vector, under direct control of the enhanced 35S promoter, and should thus produce a sense transcript of the nucleic-acid binding gene within the plant genome. The age of the plant can play a role in the expression of the p10 protein as well as the co-infiltration ratio during agro-inoculation (Personal communication, Prof. P Saldarelli). Temperature also plays a crucial role in plant-virus interactions and high temperatures are frequently associated with attenuated symptoms (also known as 'heat-masking') and low virus content, whilst low temperatures are associated with the spread of viruses and symptom development (Hull, 2002; Gerik *et al.*, 1990). Increased RNA silencing activity, limited viral movement and reduced protein expression have been reported in *N. benthamiana* plants between 25°C and 27°C (Szittya *et al.*, 2003). Temperature was not optimally regulated during this study and could have resulted in the masking of weak suppression activity due to temperature fluctuations in sub-optimal conditions.

This study has failed to demonstrate visual suppression activity for the ORF5's of GTR1-1, GTR1-2 and GTG11-1, but has established all the ORF5 fragments in the pBIN19-derived (Bevan, 1984) binary vector, pBIN61S within the *A. tumefaciens* strain, C58C1. The constructs with which suppression activity have been shown previously (Zhou *et al.*, 2006) have also been obtained (established within the *A. tumefaciens* strain, C58C1) and can serve as controls towards the determination of suppression activity in the South African variants of GVA in a temperature regulated environment. It has also been suggested that GVA can act as a strong silencing suppressor but requires the complete genome of the virus as other components of the genome might be required by ORF5 (personal communication, Dr. M Mawassi).

5.2 Deletion and replacement of ORF5 in the expression vector, GVA118

The infectious full-length cDNA clone, GVA118 was constructed by Haviv *et al.* (2006a) and consists of the ORF1 and 2 of GR-5 and the ORF3, 4 and 5 of the full-length GVA clone, GTR1-3. The ORF5 of GTR1-3 and GTR1-2 is completely homologous and comparison of symptom development in plants, induced by pBINSN-e35S-GVA118-pA and pBINSN-e35S-GVA118ΔORF5-1-2-pA, revealed that the insertion of foreign nucleotides between ORF4 and the 3'-UTR, did not influence infectivity.

This study resulted in the deletion of the ORF5 of GTR1-3 in GVA118 which was replaced with two unique restriction enzyme sites. The ORF5 of the three South African variants, GTR1-1, GTR1-2 and GTG11-1, as well as GFP- and GUS-genes were cloned into these restriction enzyme sites. The resultant full-length cDNA clones were evaluated for infectivity and symptom development in *N. benthamiana* plants, and the effect of the individual ORF5's could be determined.

The construct pLitmus38-118 was used to obtain the templates for the overlap extension PCR, which was used to delete and replace the ORF5 with the unique restriction enzyme sites, *PdiI* and *Mph11031*. Overlap extension PCR was chosen as the method of choice in comparison with the digestion of ORF5 and re-ligation of the full-length clone, because of the size of the clone and the lack of unique restriction enzyme sites within this region of GVA118. The unique restriction enzyme sites incorporated by overlap extension PCR, were chosen with careful consideration to downstream cloning steps and are unique within GVA118 and the pBluescript-backbone (pSKM). The restriction enzyme, *PdiI* is compatible to the blunt-end restriction enzymes used during this study, *SnaBI* and *SmaI*. The enzyme is also an isoschizomer of *NgoMIV*, which creates 'sticky ends'.

Two template fragments were generated from pLitmus38-118 in individual PCR reactions and served as the templates in the overlap extension PCR (figure 3.1). The first PCR fragment contained the *NcoI*-site and 3'-terminal of ORF3, the complete ORF4 and an overlap fragment with the two unique restriction enzymes separated by three random nucleotides. The second fragment included the overlap fragment, complete 3'UTR of GVA118 and the poly-A tail followed by a SalI restriction enzyme site. The overlap sequence was designed to be completely homologous between the two overlapping fragments, and was incorporated with an overlap extension PCR reaction. This technique offered an easy and precise manner for the deletion of ORF5 and replacement with restriction enzyme sites.

Once the overlap PCR fragment was confirmed to be correct with sequencing, it was referred to as PCRΔORF5 and cloned into the original vector, pLitmus38-118, in which it replaced the homologous fragment that contained ORF5 (figure 4.11). The full-length GVA118 clone was re-assembled with the partial GVA118ΔORF5-pA within the subclone, pSKM-e35S-GVA118-pA (figure 4.12), and was referred to as pSKM-e35S-GVA118ΔORF5-pA. This

construct was used for the subsequent replacement of ORF5 and introduction of the GUS and GFP genes before the constructs were cloned into the binary vector for infiltration into plants. The complete viral cassettes of the constructed subclones containing the respective ORF5's, GFP and GUS genes, were cloned into the pBIN19-derived binary vector, pBINSN (figure 4.16).

The pBINSN-e35S-GVA118ΔORF5-pA constructs are summarised in table 4.1 and were screened with restriction enzyme digestion reactions, because of the size of the constructs and the ability to distinguish between successful ligations (figure 4.17). The respective constructs were electroporated into the *A. tumefaciens* strain, C58CI with the helper plasmid, pCH32, which can also be infiltrated into grapevine for future characterisation of these constructs.

The six respective constructs, pBINSN-e35S-GVA118ΔORF5, -1-1, -1-2, -11-1, -GFP and GUS were agro-infiltrated into *N. benthamiana* with the strong suppressor, P0, also in pBINSN under 35S control. A previous study has found that agro-infection of viral replicons are enhanced with the co-infiltration of silencing suppressors Chiba *et al.* (2006), and in light of the lack of suppression activity observed previously for the ORF5 of GTR1-1, GTR1-2 and GTG11-1, P0 was co-infiltrated with the pBINSN-constructs. The positive controls included for the plant infiltration experiments were pBINSN-e35S-GVA118-pA and pBINSN-e35S-GR-5.

The ORF5 of GVA has been shown to be the pathogenicity determinant within GVA (Galiakparov *et al.*, 2003b; Goszczynski *et al.*, 2008; Zhou *et al.*, 2006). The deletion of the ORF5 within the infectious clone would not affect the replication of the virus within the plant, but will restrict viral movement and render the virus asymptomatic within infected plants(Galiakparov *et al.*, 2003c). Similar results were expected for the replacement of ORF5 with a GFP or GUS gene, as these genes do not have any effect on symptomology or movement of a virus, and are only used as marker genes. Symptoms of differing severity have been noted for GTR1-1, GTR1-2 and GTG11-1 (Goszczynski and Jooste, 2003a; Goszczynski, 2007), and it was expected that divergent symptoms, in correspondence to results found in literature, should be observed for the constructed full-length clones. The hypothesis was that the ORF5 of GTR1-1 should induce mild symptoms and ORF5 of GTG11-1 more severe symptoms, similar to those observed in plants infiltrated with pBINSN-e35S-GVA118-pA and pBINSN-e35S-GVA118ΔORF5-1-2-pA.

Two infiltration experiments were performed with all the final full-length clones (Table 4.1). The first co-infiltration experiment included the positive control, GR-5 and was only evaluated for viral replication with TPIA and TEM. The second infiltration experiment utilised the positive control, GVA118 (pBINSN-e35S-GVA118-pA), and tested and for viral replication and symptom development over a period of 30 dpi.

Replication of GR-5 was confirmed during the first infiltration experiment with TPIA at 5 dpi which revealed that the virus was present at a high viral titer within the mesophyll leaf cells of the infiltrated *N. benthamiana*. GVA is a phloem-limited virus and is therefore restricted to the vascular tissue of the plant, and can result in irregular distribution of infected cells. Co-infiltrated

leaves were specifically tested with TPIA and could not give an indication to the systemic movement of the virus, but could show replication of the virus within the infiltrated patch.

Although high background was observed in the tissue print for the first infiltration experiments, a clear difference in colour could be seen for pBINSN-e35S-GVA118ΔORF5-1-2-pA, which indicated that this construct was replicating when compared to GR-5 in figure 4.18A. To confirm that this full-length clone was replicating and that the insertion of foreign nucleotides did not influence the infectivity of the full-length cDNA clone, the leaf samples were subjected to TEM. Scanning electron microscopy (figure 4.20) revealed that GR-5 was present at a high viral titer (A) whilst only one virus particle was observed in the leaf sample from the plant infiltrated with pBINSN-e35S-GVA118ΔORF5-1-2-pA (B). Even though only one virus particle was found on that specific leaf sample, it did indicate that the full-length cDNA clone, pBINSN-e35S-GVA118ΔORF5-1-2-pA is infectious and replicating.

Fluorescence microscopy was also performed on leaf samples infiltrated with the pBINSN-e35S-GVA118ΔORF5-GFP-pA to determine whether the GFP gene was being expressed within the cDNA clone, which would also indicate replication. These results are shown in figure 4.19, which shows that, when compared to the positive control (transgenic *N. benthamiana*, line 16c) and the negative control (non-infiltrated *N. benthamiana*), no GFP fluorescence was observed at 5 dpi. The same result was found for the marker gene, GUS. It could be concluded from these results that the full-length clones were not expressing GFP or GUS, which indicates that these constructs are not replicating, that the genes are not being expressed, or if the genes are being expressed, it is at a level that is not detectable with the techniques used in this study.

The lack of replication observed with TPIA for the other full-length cDNA clones could have been due to a very low viral titer that could not be detected with TPIA, or could indicate that these constructs are not infectious, in contrast to pBINSN-e35S-GVA118ΔORF5-1-2-pA. This prompted a second infiltration experiment, with pBINSN-e35S-GVA118-pA as positive control for the infiltration. An optimized TPIA, to reduce the high background observed during the first TPIA, was performed at 5 dpi. A high viral titer was observed for GVA118, and once again leaf tissue infiltrated with pBINSN-e35S-GVA118ΔORF5-1-2-pA seemed positive when compared to leaf tissue infiltrated with GVA118. The full-length clone, pBINSN-e35S-GVA118ΔORF5-11-1-pA also seemed positive when compared to pBINSN-e35S-GVA118ΔORF5-1-2-pA and GVA118, although this has not yet been confirmed with repeat experiments. None of the other constructs seemed positive in this experiment. Confocal fluorescence microscopy could not reveal any GFP fluorescence for the second infiltration experiment at 5 dpi (results not shown).

Furthermore, the plants were evaluated for symptom development, which was observed at 10 dpi for the plant infiltrated with pBINSN-e35S-GVA118-pA. Initial symptoms were leaf curling in newly developed leaves and mild vein clearing. At 14 dpi, leaf curling was observed in plants infiltrated with pBINSN-e35S-GVA118-pA, pBINSN-e35S-GVA118ΔORF5-1-1-pA, pBINSN-

e35S-GVA118ΔORF5-1-2-pA and pBINSN-e35S-GVA118ΔORF5-11-1-pA. The plant that was infiltrated with pBINSN-e35S-GVA118ΔORF5-1-1-pA started showing early symptoms of leaf curling and vein clearing, whilst interveinal chlorosis, necrosis and severe leaf curling were observed in plants infiltrated with pBINSN-e35S-GVA118-pA, pBINSN-e35S-GVA118ΔORF5-1-2-pA and pBINSN-e35S-GVA118ΔORF5-11-1-pA. Symptom development at 14, 20 and 30 dpi in the respective infiltrated plants, are shown in figure 4.21, figure 4.22 and figure 4.24.

These results are contradictory to the previously reported results of the TPIA, which suggested that none of the other full-length cDNA clones were replicating, with the exception of pBINSN-e35S-GVA118ΔORF5-1-2-pA. The development of symptoms between 10 and 14 dpi in plants infected with these constructs suggests otherwise, and might indicate that the viral titer of pBINSN-e35S-GVA118ΔORF5-1-1-pA and pBINSN-e35S-GVA118ΔORF5-11-1-pA was too low to detect with TPIA at 5 dpi.

At 14, 20 and 30 dpi no symptoms were observed in plants infiltrated with the respective constructs, pBINSN-e35S-GVA118ΔORF5-pA, pBINSN-e35S-GVA118ΔORF5-GFP-pA and pBINSN-e35S-GVA118ΔORF5-GUS-pA (figure 4.21, figure 4.22 and figure 4.23). These results correspond to previously published results which showed that the deletion of ORF5 from GVA rendered the virus asymptomatic (Galiakparov *et al.*, 2003c,b). The deletion of ORF5 should also restrict viral movement, and it was expected that a rapid direct-one-tube RT-PCR of the upper leaves infected with these variants should not show amplification, whilst infiltrated patches should. No conclusive results supporting the restriction of viral movement could be obtained with this method, and will have to be optimised. The construct, pBINSN-e35S-GVA118ΔORF5-pA, which is infectious when the ORF5 from GTR1-2 is inserted, could thus be infectious but not display any symptoms. This is also true for the full-length clones in which the ORF5 have been replaced with the marker genes, GFP and GUS. If it is assumed that these full-length clones are infectious, the lack of GFP fluorescence and GUS accumulation observed at 5 dpi can be due to the position of the genome. Controller elements are observed adjacent to the 5'-terminal of each of the 3'-ORF's except for ORF5 (Galiakparov *et al.*, 2003a), which led to the suggestion that ORF5 is expressed with bi- or polycistronic mRNA (Galiakparov *et al.*, 2003a). This could influence the translation of foreign genes. The remarkable size difference between the ORF5 and the genes, GFP and GUS could also affect the effective expression of these genes. If the genes were being expressed it might have been in concentrations to low to detect with the techniques used in this study. More sensitive detection of the mRNA transcripts in Northern blots or protein detection with Western blots might give a better indication of the transcription and translation of these genes in future studies.

Rapid direct-one-tube RT-PCR's were performed to confirm that the constructed full-length clones are responsible for the different symptoms observed in the infiltrated plants, but failed to show consistent results (results not shown). A draw-back of this technique is the inability to distinguish between

ssRNA viruses and the cDNA from the recombinant *A. tumefaciens* constructs, which has the ability to move systemically. This can be followed up by DNAase treatment. Further analysis will have to be performed on extracted viral RNA from visually infected plant material, to confirm that the observed symptoms are due to the replication of the different full-length constructs within the plant genome, and not an infiltration artifact of the *A. tumefaciens* constructs or contamination from the original GVA118.

Preliminary results showed that the replacement of the ORF5 of GVA118 with the ORF5 of GTR1-1, induces less severe symptoms within the full-length infectious clone. This can significantly contribute towards the construction of an expression vector that does not induce severe symptoms within the host, but can still move systemically within the plant. The replacement of ORF5 with marker genes such as GFP and GUS was not successful, which indicates that this position within the genome is not ideal for the expression of foreign genes. The infectious full-length clone, GVA118 has previously been used for the successful expression of GFP, GUS and the coat protein of CTV, but induces severe symptoms in the host plant that can mask protein expression and detection (Haviv *et al.*, 2006a). A marked decrease in the symptom severity of this vector by the replacement of the ORF5 can contribute to foreign gene expression within the current cloning site situated between two subgenomic promoters at the 3'-terminal of ORF2 and 5'-terminal of ORF3 within GVA118 as a VIGS vector.

5.3 Future work

This study failed to demonstrate silencing suppression activity encoded by ORF5 of the three South African variants, GTR1-1, GTR1-2 and GTG11-1, but did establish all three of the respective ORF5's in the binary vector, pBIN61S. Recombinant *A. tumefaciens* C58C1 cell cultures containing these constructs have been established and the constructs with which suppression activity for ORF5 of Is151 have been demonstrated previously (Zhou *et al.*, 2006), have been obtained (established within the *A. tumefaciens* strain, C58C1). The current construction of temperature controlled glasshouse facilities at Welgevallen experimental farm, University of Stellenbosch, will greatly improve future suppression assay experiments with the plasmids constructed by Zhou *et al.* (2006) as true controls for suppression activity encoded by the ORF5's of GTR1-1, GTR1-2 and GTG11-1. Molecular experiments will have to be performed in order to determine suppression activity at the RNA level. A previous study within the laboratory have found that northern blots performed with digoxigenin labeled GFP probes are not sensitive enough to detect siRNA's of the virus(Engelbrecht, 2006) and more sensitive techniques such as radio-active labeling of probes could be explored for future corroboration of suppression activity by p10 with RNA analysis. RNA analysis will also contribute towards revealing suppression activity by determining the effect of the respective ORF5s on the accumulation of siRNA's.

The deletion and replacement of ORF5 from GVA118 with ORF5 from

GTR1-1, GTR1-2 and GTG11-1 was successful and preliminary results suggested a decreased symptom severity induced by the construct, pBINSN-e35S-GVA118ΔORF5-1-1-pA. This still needs to be confirmed with RNA analysis. If RNA analysis can confirm that the full-length construct is replicating within the plant genome, future experiments can determine the effect of decreased symptom severity on foreign gene expression. The constructs can also be characterised within *Vitis vinifera*, and constructs have already been established in C58CI *Agrobacterium* cells containing the helper plasmid, pCH32 which is needed for *V. vinifera* agroinfiltration, for this purpose. The determination as to why GFP and GUS aren't translated within the ORF5 position could also contribute towards a better understanding of the expression of ORF5.

Chapter 6

Conclusion

Grapevine virus A, is a positive single-stranded RNA virus with a small genome of 5 ORF's (approximately 7 800 nts in length) that can infect both a herbaceous host such as *N. benthamiana* and its natural host *V. vinifera*. These characteristics makes the virus a good candidate to be used as a virus-based vector, and such a herbaceous expression vector (GVA118) has been constructed by Haviv *et al.* (2006a). The vector, GVA118 has been characterised within in *N. benthamiana* but is limited as an expression and VIGS vector due to the severity of the symptoms induced and the low gene expression by the vector. Many pathogenecity determinants have been identified as suppressors proteins (Voinnet *et al.*, 1999) within viral genomes. The characterisation of the ORF5 of GVA revealed that the ORF encodes for a nucleic-acid binding protein (p10), which acts as a suppressor protein, affects pathogenicity and can restrict viral movement. This study aimed to determine the suppression activity of the ORF5 of the South African variants of GVA, GTR1-1, GTR1-2 and GTG11-1, with a transient expression assay in transgenic *N. benthamiana* (line 16c).

Three respective binary vector clones were constructed with the ORF5's in pBIN61S. Preliminary results failed to demonstrate any suppression activity for the ORF5 of GTR1-2 and GTG11-1 in transient expression assays. Environmental factors could have influenced or masked suppression activity, and GFP mRNA will have to be extracted to determine whether this was the case. The constructs with which suppression activity was demonstrated for ORF5 of Is151 have also been obtained to serve as positive controls for future transient expression assays.

The ORF5's, as the pathogenicity determinants of GTR1-1, GTR1-2 and GTG11-1, were studied within the infectious full-length cDNA clone, GVA118. The aim of this part of the study was to delete the ORF5 of GVA118, and study the effect of the three respective ORF's on pathogenicity when replaced within the infectious full-length clone. Symptom development was evaluated over a period of 30 days in the various plants with the different full-length cDNA clones, and suggested that all of these clones are infectious. It still remains to be confirmed at a molecular level that these symptoms are in fact induced by the respective full-length clones that were infiltrated into the plants, and not by *A. tumefaciens* artifacts or other variants of the virus.

This study has succeeded in the establishment of an infectious full-length cDNA clone, pBINSN-e35S-GVA118ΔORF5-1-1-pA, that seem to induce much milder symptoms in the herbaceous host, *N. benthamiana*. Once confirmed, this construct can be further characterised as a possible expression vector of foreign proteins or VIGS vector in herbaceous hosts and grapevine.

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Chapter 7

Vector Maps

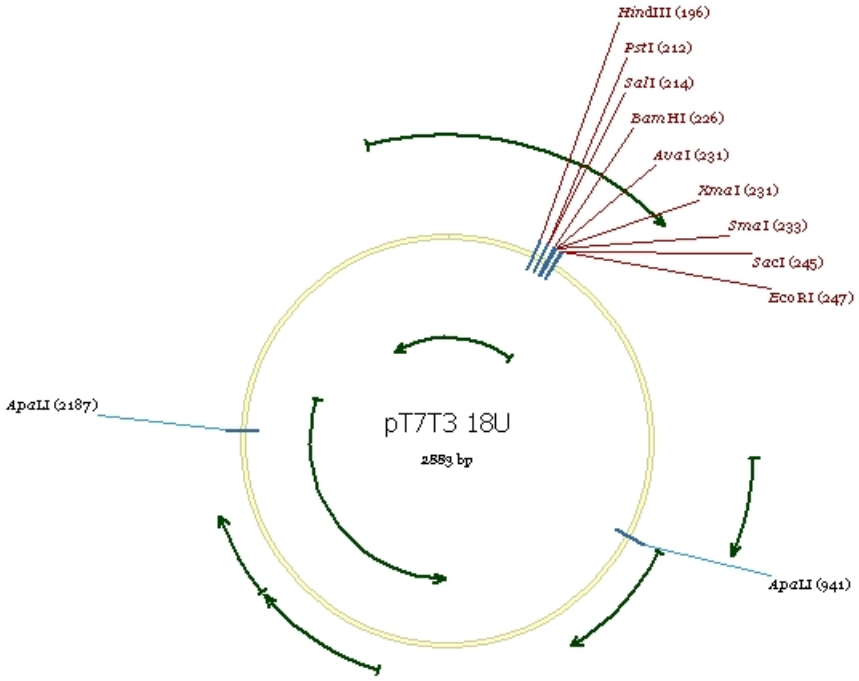

Figure 7.1: The cloning vector, pT7T318U. This vector contains an ampicillin resistance gene (not shown), and is 2553 bp in length . A multiple cloning site can be observed on the map, and includes the restriction enzyme sites, SacI and BamHI, into which ORF5 was cloned during this study.

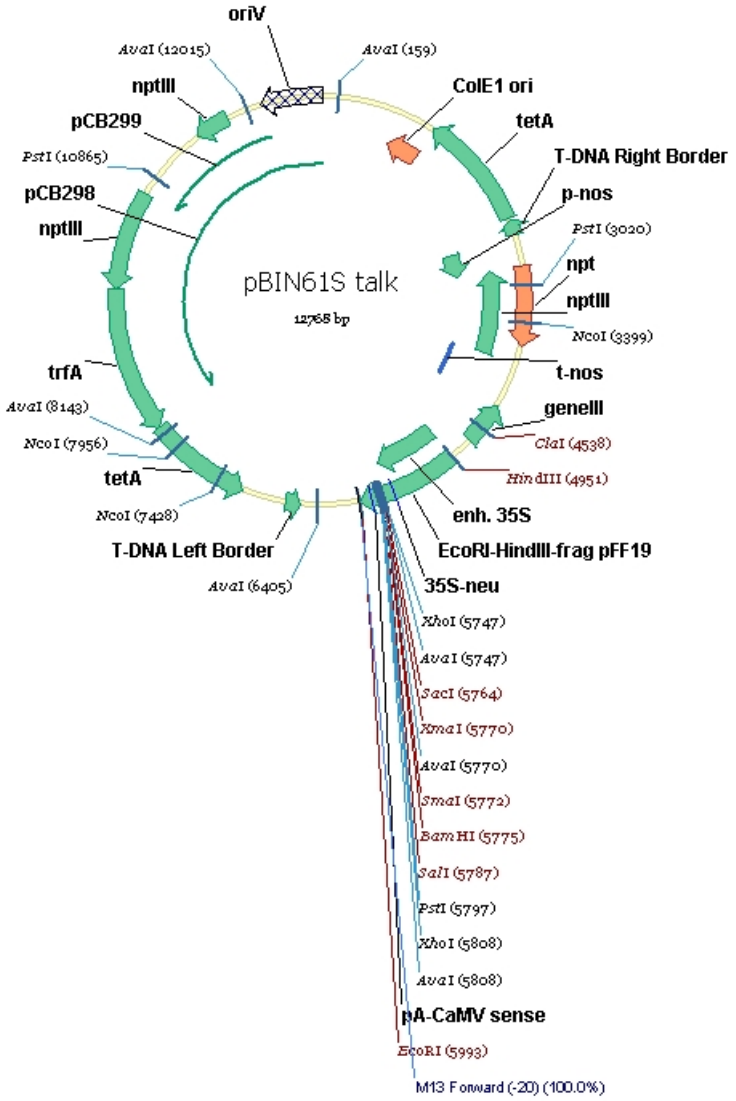

Figure 7.2: pBIN61S-talk is a binary vector with enhanced 35S control. Open reading frame 5 from GTR1-1, GTR1-2 and GTG11-1 was cloned into this vector during this study to determine suppression activity in line 16c plants. This binary vector was used for infiltration of plants with the plant pathogen, Agrobacterium tumefaciens. The vector is 12 765 bp in length and the restriction enzyme sites, SacI and BamHI can be seen within the multiple cloning site within the left and right border of the vector, just after the enhanced 35S promoter. The sequence between the left and right border is referred to as T-DNA and will be integrated and expressed in the plant genome after successful infiltration into plants. The origin of replication is indicated in the blocked arrow, annotated as OriV. A nopaline synthetase promoter is indicated as p-nos an the nopaline synthetase terminator is indicated as t-nos. This vector was derived from pBIN 19 (Bevan, 1984) and have kanamycin resistance.

Figure 7.3: The binary vector, pBIN_SN. This is a plant binary vector that is derived from pBIN19 (Bevan, 1984). The full, length modified GVA118 constructs was cloned into this binary vector for expression in N. benthamiana plants. The left and right border includes the T-DNA, with a multiple cloning site indicated within the LacZ gene. Cloning into this multiple cloning site results in disruption of the lacZ gene, which disrupts colour selection after cloning. The full-length modified GVA118 constructs was cloned into the restriction enzyme sites, XhoI (which is compatible with SalI) and SnaBI (which is also compatible with SmaI) (See section 3.3.6) within the multiple cloning site. The nopaline synthetase promoter and terminator, as well as the neomycin phosphatase II gene is indicated on the map. This vector has Kanamycin resistance (not indicated on the map).

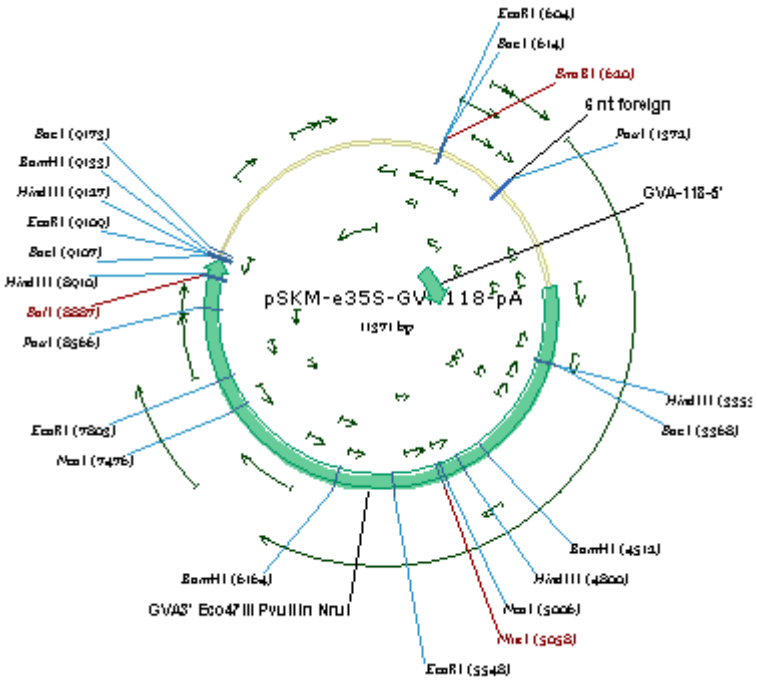

Figure 7.4: This figure shows a schematic representation of the construct, pSKM-e35S-GVA118-pA. This is the infectious full-length cDNA clone of Grapevine Vitivirus A that was under control of a T7-promoter (GVA118, Haviv *et al.*, 2006a) and was kindly provided by Dr. M Mawassi (Robert H. Smith Institute of Plant Science and Genetics in Agriculture, The Hebrew University of Jerusalem, Israel). The viral sequence including the p(A) was brought under the control of an enhanced CaMV 35S promoter and introduced in the plant binary vector pBINSN (7.3 on the previous page), a derivative of pBIN19 (Bevan, 1984), by Mr. J du Preez (Department of Genetics, University of Stellenbosch, South Africa). This clone is referred to as pBIN-e35S-GVA118-pA. This figure shows the subclone in pBluescript II SKM, containing the complete viral cassette and regulatory elements. The full-length clone is indicated as the green arrow, with the GVA118-5' UTR is indicated as a smaller green arrow. All the restriction enzyme sites used during the construction of the modified GVA118 constructs are indicated within the sequence of the full-length clone. This vector has kanamycin selection and is 11 371 bp in size.

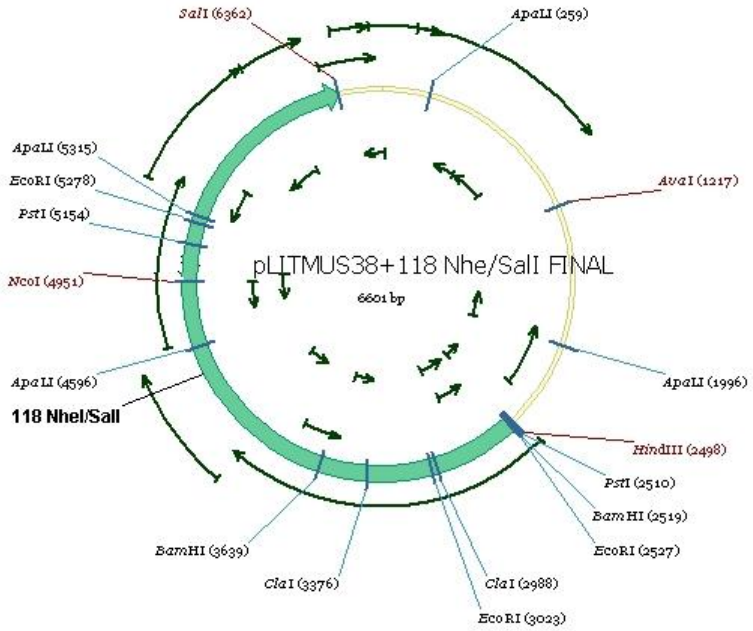

Figure 7.5: pLitmus38-118 was constructed from pSKM-e35S-GVA118-pA. The full-length clone was digested within ORF1 at the NheI site and after the poly(A) tail at the SalI site. These sites are unique within the construct and resulted in a partial full-length clone. This partial full-length clone was ligated into the vector, pLitmus38 at the restriction enzyme sites, NheI and Sal1. The clone is indicted in the green arrow as shown by the tag '118 NheI/SalI' on , and the pLitmus38 backbone as a double black line. This clone was constructed as an intermediary construct effort in which the NcoI site within ORF1 (7.4) was eliminated and resulted in a single NcoI site within ORF3. This construct could then be used to replace the original ORF5 with the PCRΔORF5 fragment (section 3.3.3).

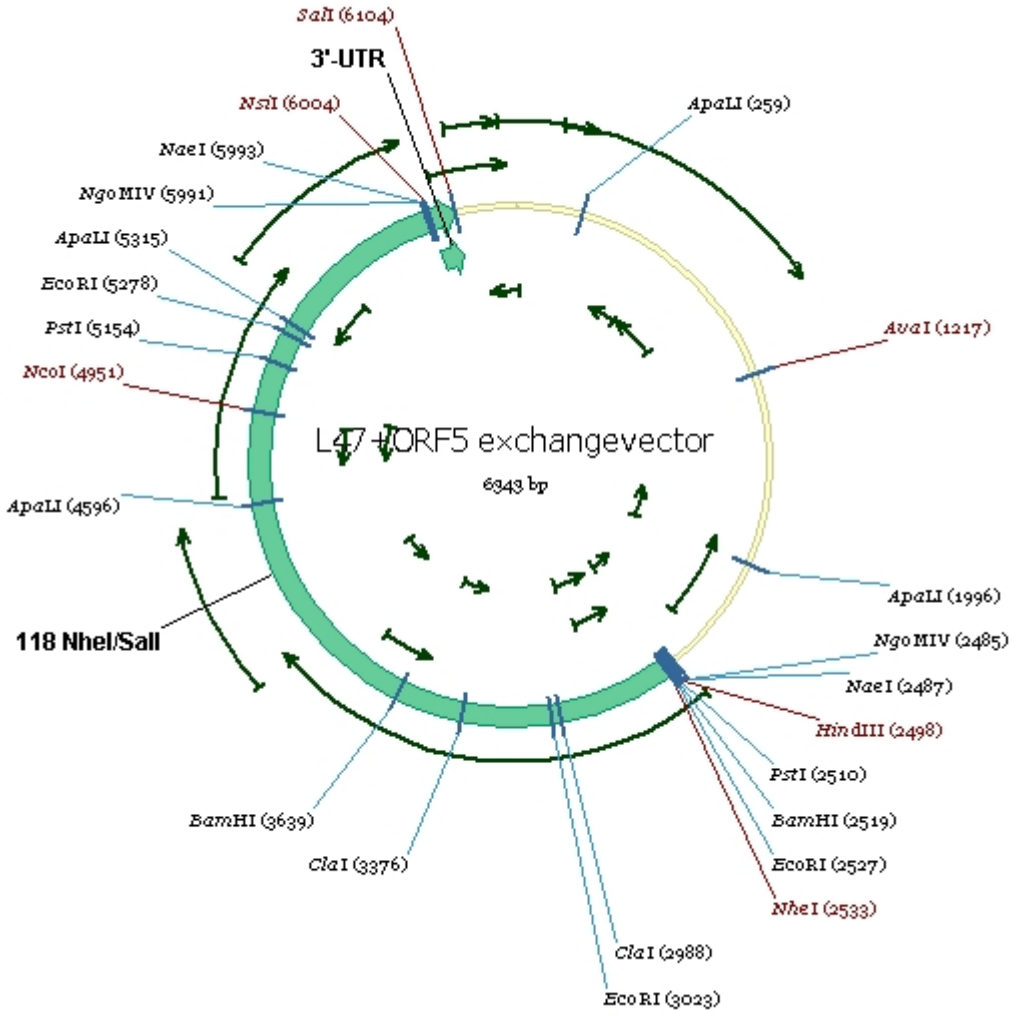

Figure 7.6: This figure is a schematic representation of pLitmus38-118ΔORF5. This plasmid was constructed to create a partial full-length clone of GVA118, lacking the ORF5. The fragment was constructed by replacing the original GVA118-fragment containing the ORF5, with the overlap extension PCR product, which does not contain the ORF5, at the NcoI and Sal1 restriction enzyme sites, within ORF3 and after the 3'-UTR respectively. The construct was digested at the NcoI and SalI sites and replaced with the PCR-ΔORF5 fragment as described in section 3.3.3. The tag '118 NheI/SalI' indicates the green arrow on the figure which is the partial full-length clone described in (7.5). The NgoMIV/NaeI site and the NsiI site is indicated just left of the 3'-UTR and SalI site, and are the restriction enzyme sites that replaced ORF5 through the construction of the PCRΔORF5 fragment.

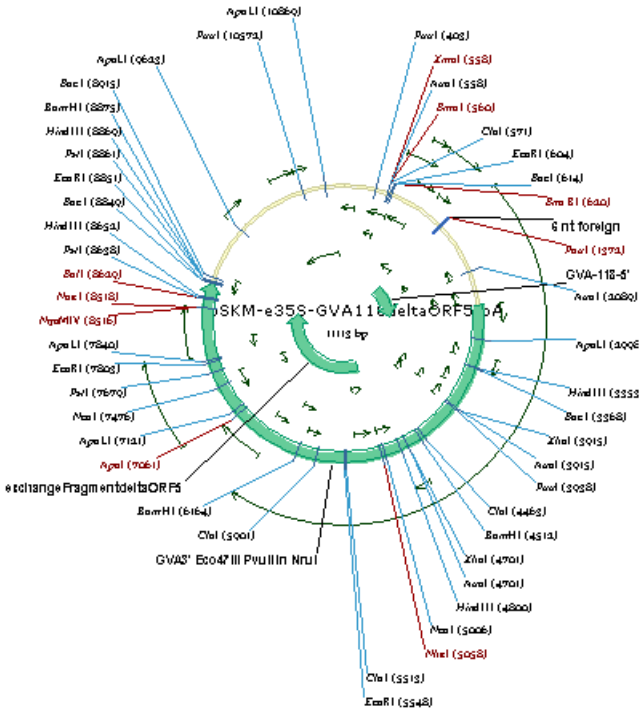

Figure 7.7: This figure shows a vector map of pSKM-e35S-GVA118ΔORF5-pA. This clone was constructed by replacing the original sequence form the NheI and SalI site in the construct, pSKM-e35S-GVA118-pA, with the partial full-length clone described in (7.6) with digestion and ligation described in section 3.3.4. The construct, pLitmus38-118ΔORF5, was digested out of the pLitmus38 backbone at the NheI and SalI sites, and ligated into pSKM-e35S-GVA118-pA, subjected to the same digestion. The unique restriction enzyme site, NaeI is shown is red. The NsiI is not shown. These restriction enzyme sites replaced ORF5 within the full-length clone, GVA118.

Figure 7.8: This figure is a vector map of pBINSN-GVA118deltaORF5. This vector was created by replacing the original fragment of the full-length clone of GVA118-pA in pSKM-e35S-GVA118-pA, with the partial full-length clone in pLitmus38-118deltaORF5, which does not contain ORF5. The result was that the full-length clone in pSKM-e35S-GVA118-pA, was returned to its original condition, but lacked the ORF5, which were replaced by the enzyme sites, PdiI (NaeI) and Mph1103 (NsiI).

Chapter 8

General Reagents and Media

All reagents have been prepared to the manufacturer's instructions or according to Sambrook *et al.* (2001).

LB medium (Biolab, Merck)

Per liter:
Tryptone 10.0 g
Sodium Chloride 10.0 g
Yeast Extract 5.0 g
Suspend 25 g per liter of LB broth in 1 liter distilled water according to manufacturer's instructions. Autoclave the broth to sterilise.

LB/agar plates

Prepare LB media as suggested by the manufacturer, and add 15g/l Bacto Agar (Biolab) to the LB media. Sterilise the media by autoclaving. Let the media cool to approximately 60°C before adding appropriate selection antibiotics, X-gal or IPTG.

SOB medium

Per liter (1000 ml):
LB medium (Merck) 950 ml
KCl (250 mM) 10 ml
Add 10 ml KCl to 950 ml LB medium. Adjust the pH of the medium to 7.0 with 5 M NaOH. Adjust the volume of the media to 1 liter with deionized sterile water. Sterilise the media by autoclaving. Add 5 ml sterile 2 M $MgCl_2$ to the media just before use.

SOC medium

SOC medium is identical to SOB medium with the exception of the addition of 20 mM sterile glucose before use.

Tissue-print immunoassay:

1X PBS: Per liter (1000 ml):
8.0g NaCl
0.2g KH_2PO_4
0.2g KCl
1.44g Na_2HPO_4*$2H_2O$
Make volume up to 1000 ml and adjust pH to 7.4. Autoclave to sterilise.
For PBS-T:
Add 0.05% Tween 20

AP-NBT-BCIP colour reaction

AP-buffer: 100 mM Tris, pH 9.5
100 mM NaCl
5 mM $MgCl_2$

NBT-stock solution (store at 4°C) 75 mg/ml in 70% DMF

BCIP-stock solution (store at 4°C) 50 mg/ml in 100% DMF

AP-substrate solution for the colour reaction (prepare just before use): 10 ml AP-buffer
66 μl NBT stock solution
33 μl BCIP stock solution

Chapter 9

Antibiotic selection for different plasmids, *E. coli* and *Agrobacterium* strains

Construct/cell strain	Antibiotic selection and concentration
DH5α	None
JM109	None
NM522	None
C58CI (pGV2260)	50 μg/ml Rif
pDRIVE-ORF5 constructs (all variants)	50 μg/ ml Kn; 100 μg/ml Amp
pBIN61S-ORF5 constructs (all variants)	50 μg/ml Kn
pT7T318U-ORF5 constructs (all variants)	100 μg/ml Amp
C58CI :: pBIN61S constructs	50 μg/ml Rif, 50 μg/ ml Kn
pLitmus38-118	100 μg/ml Amp
pLitmus38-118ΔORF5	100 μg/ml Amp
pSKM-e35S-GVA118-pA	100 μg/ml Amp
pSKM-e35S-GVA118ΔORF5-pA	100 μg/ml Amp
pBINSN-e35S-GVA118ΔORF5-pA constructs	50 μg/ml Kn
C58CI :pCH32:: pBINSN constructs	50 μg/ml Rif, 5 μg/ ml Tet