Screening the genome of grapevine leafroll associated virus-2 for proteins with RNA silencing suppressor activity and the construction of a tandem silencing vector to induce simultaneous silencing of two genes

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Verklaring

Ek, die ondergetekende, verklaar hiermee dat die werk in hierdie tesis vervat, my eie oorspronklike werk is en dat ek dit nie vantevore in die geheel of gedeeltelik by enige universiteit ter verkryging van 'n graad voorgelê het nie.

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Summary

Grapevine is one of the oldest food plants and was first exploited from in the wild and later cultivated by man. Grapevine viruses are among the most important pathogens of grapevine and one of these viruses termed Grapevine Leafroll-associated Virus (GLRaV) is regarded as one of the most harmful grapevine viruses. The virus is responsible for the disease called leafroll disease which affects the South African wine industry causing losses of millions of Rands annually. Existing control measures focus on prevention by utilising virus-free propagation material and integrated control of the insect vectors. Virus resistant grapevine by means of genetic modification seems to be a realistic approach in solving grapevine diseases, especially leafroll disease.

A natural occurring plant mechanism called post transcriptional gene silencing (PTGS) can be exploited to help in the process of obtaining transgenic virus resistant grapevine. PTGS is a sequence–specific defence system of the plant that targets alien RNA (transgenes, endogenous genes and cytoplasmically replicating viruses) for degradation (Dunoyer *et al.*, 2002; Vanitharani *et al.*, 2003; Waterhouse *et al.*, 2001a). However viruses have evolved a counter defence mechanism against PTGS by encoding suppressor proteins able to suppress RNA silencing (Thomas *et al.*, 2003). Very few suppressor proteins have been identified in grapevine viruses.

In this study the GLRaV-2 genome was screened for a suppressor protein able to reverse or to prevent the onset of PTGS. A constitutively expressed green fluorescent protein (GFP) gene was silenced in transgenic *Nicotiana benthamiana* plants (line 16c) by agro-infiltration, using a second GFP-construct. The GFP-silenced plants were inoculated with a strain of GLRaV-2 to screen for a suppressor able to reverse PTGS. Individual GLRaV-2 genes were isolated and cloned into an intermediate PCR cloning vector, followed by subsequent cloning into a plant expression vector. These constructs were transformed into *Agrobacterium tumefaciens* strains GV3101 and C58C1 and were agro-infiltrated into silenced transgenic or co-infiltrated into transgenic *N. benthamiana* plants (16c) in two different expression assays.
It was found in both the silencing reversal assay and the transient assay that the p24 protein of GLRaV-2 possessed suppressor activity. An attempt was made to corroborate the fluorescence assays by screening infiltrated plants for the presence of GFP siRNAs, which would be a tell-tale sign that silencing has occurred. Unfortunately (and probably due to technical problems) these experiments failed to yield signals in the Northern blot analysis.

The second part of this study was to construct a tandem silencing vector to serve as "proof of concept to show that two genes can be silenced simultaneously in a plant. The primary

construct, pHanViralGFP-SAS was constructed by performing a rapid direct reverse transcription reaction (RDOT-RT-PCR) with primers containing 5' -extension restriction sites to facilitate subsequent cloning and to amplify a gene fragment from the GLRaV-2 genome. A portion of the GFP was obtained by a polymerase chain reaction (PCR) from the plasmid vector pBIN mGFP5-ER, also using primers containing restriction sites. The fragments obtained in the individual reactions were ligated into an intermediate PCR cloning vector, followed by the subsequent cloning into corresponding sites in the pHannibal vector, in sense and anti-sense orientations. The silencing cassette was removed from the pHannibal vector and ligated into pART27. The final construct, pSilencer-SAS, was transformed into *A. tumefaciens* strains GV3101 and C58C1 and transgenic (16c) and non-transgenic *N. benthamiana* plantlets, of which some were infected with GLRaV-2, were agro-infiltrated with these *Agrobacterium* strains.

Results obtained showed that the tandem silencing vector was successful in silencing two genes simultaneously, justifying the construction of a tandem vector. The effectivity of the vector can now be tested by inserting genes from two different viruses.

Opsomming

Wingerd is een van die oudste plante wat al benut word vandat dit wild gegroei het tot en met vandag wat dit verbou word. Wingerdvirusse is van die mees belangrike patogene van wingerd en een van die virusse, bekend as Wingerd rolblaar geassosieerde virus (GLRaV) word beskou as een van die mees vernietigende virusse van wingerd. Die virus is verantwoordelik vir die siekte bekend as rolblaar en veroorsaak jaarliks verliese van miljoene Rande vir die Suid-Afrikaanse wynbedryf. Bestaande beheermeganismes maak gebruik van virusvrye voortplantingsmaterial en van die chemiese beheer van insekvektore verantwoordelik vir die oordrag van siektes. Genetiese manipulasie van wingerd lyk op die oomblik na een van die mees realistiese benaderings om virusbestande wingerd te verkry en so wingerdsiektes, soos rolblaar, te bekamp.

'n Meganisme wat natuurlik in plante voorkom en wat post transkripsionele geen uitowing (PTGS) genoem word kan gebruik word in die proses om virusbestande transgeniese wingerd te maak. PTGS is 'n volgordespesifieke beskermingssisteem in die plant wat vreemde RNA (transgene, endogene gene en sitoplasmiese repliseerende virusse) teiken vir degradasie (Dunoyer *et al.*, 2002; Vanitharani *et al.*, 2003; Waterhouse *et al.*, 2001a). Virusse het deur middel van evolusie onderdrukkerproteïene geproduseer as n teenvoeter teen PTGS (Thomas *et al.*, 2003). Baie min uitdowings gene is al geidentifiseer in wingerd virusse.

In hierdie studie is die genoom van die GLRaV-2 virus deursoek vir 'n onderdrukkersprotëine wat PTGS kan omkeer of dit heeltemal kan verhoed. 'n Konstitatiewe uitgedrukte groen fluoreseerende proteïen (GFP) is uitgedoof in transgeniese *Nicotiana benthamiana* plante (lyn 16c) deur middel van agro-infiltrasie met 'n tweede GFP konstruk. Die GFP uitgedoofde plante is geïnokuleer met die GLRaV-2 virusras om te toets vir die teenwoordigheid van 'n onderdrukkersproteïen wat PTGS kan omkeer. Individuele GLRaV-2 gene is geïsoleer uit die virus uit en gekloneer in 'n intermediêre vektor gevolg deur die klonering in 'n plant uitdrukkingsvektor. Hierdie konstrukte is getransformeer in die *Agrobacterium tumefaciens* rasse, GV3101 en C58C1 en is in uitgedoofde transgeniese plante deur middel van agro-infiltrasie ingesit of is in transgeniese *N. benthamiana* plante (16c) getransformeer afhangende van die uitdrukkings toets sisteem gebruik.

Dit is gevind in beide die "reverse" en "transient" uitdowings toets sisteem dat die p24 proteïen van die GLRaV-2 onderdrukkings aktiwiteit besit. Daar was probeer om die fluoreseerende toets resultate in die geinfiltreerde plante met die teenwoordigheid van siRNAs te ondersteun. Ongelukkig kon geen seine verkry word in die Noordelike kladtegniek nie en was dit hoogs waarskynlik as gevolg van tegniese probleme.

In die tweede deel van die studie is 'n tandem uitdowingsvector gemaak wat as 'n bewys van beginsel sal dien. Die vektor wys dat twee gene gelyktydig uitgedoof kan word in 'n plant. Die primêre konstruk, pHannibalGFP-SAS, bestaan uit 'n GLRaV-2 fragment tesame met 'n GFP fragment in 'n sin en anti-sin oriëntasie. Die GLRaV-2 fragment is geamplifiseer deur 'n vinnige direkte tru-transkriptase reaksie uit tevoer met inleiers wat 'n spesifieke restriksie ensiem snyplek op die 5' kant dra om klonering later te vergemaklik. Die GFP fragment is verkry vanaf die pBIN mGFP5-ER vektor deur 'n polimerase ketting reaksie (PCR) te doen met inleiers wat weereens spesifieke restriksie-ensiem snyplekke dra. Die individuele fragmente verkry in die reaksies is geligeer in 'n intermediêre PCR klonerings vektor, wat gevolg is deur die klonering van die fragmente in ooreenstemmende plekke in die pHannibal vektor in sin en anti-sin oriëntasies. Die uitdowingskasset is verwyder uit die pHannibal vektor en is geligeer in die pART27 plant transformasievektor. Die finale konstruk, pSilencer-SAS, is getransformeer in die *A. tumefaciens* rasse GV3101 en C58C1. Transgeniese (16c) en nie- transgeniese *N. benthamiana* plante, waarvan sommige met GLRaV-2 geinfekteer was, is met die finale *Agrobacterium* rasse geinfekteer.

Resultate het gewys dat die tandem uitdowingsvektor wel gewerk het om twee gene gelyktydig uit te doof. Die effektiwiteit van die vektor kan nou getoets word met gene van twee verskillende virusse.

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Abbreviations

α:	alpha
2-5A:	2'-5'-oligoadenylate synthetase
abRNA:	aberrant RNA
Ago:	ARGONAUTE
Amp:	ampicillin
ARC-PPRI:	Plant Protection Research Institute of the Agricultural Research Council
AS:	anti-sense
β:	beta
bp:	base pair
BSA:	bovine serum albumin
CAF:	Carpel factory
CHS:	chalcone synthase gene
cm:	centimetre
CP:	coat protein
CPd:	diverged coat protein / coat protein analogue
CPMR:	coat protein mediated resistance
CSIRO:	Commonwealth Scientific and Industrial Research Organisation
DCL:	dicer like
DI:	defective interfering virus
DIG:	digoxigenin
dNTPS:	deoxynucleosides triphosphate
DPI:	days post infiltration
ds:	double stranded
dsRNA:	double-stranded RNA
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetate
ELISA:	enzyme-linked immunosorbent assay
ER:	endoplasmic reticulum
EtBr:	ethidium bromide
GFP:	green fluorescent protein
GM:	genetically modified
GUS:	β-glucuronidase
ha:	hectare
HC-Pro:	helper component proteinase

HDGS:	homology dependant gene silencing
hpRNA:	hairpin RNA
HSP70:	heat shock related protein
ICTV:	International Committee on Taxonomy of Viruses
ICVG:	International Council for the Study of Virus and Virus-like Diseases of the Grapevine
ihpRNA:	intron-containing hairpin RNA
IPTG:	isopropyl Thio-β-D-Galactoside
IR:	inverted repeat
ISEM:	immunosorbent electron microscopy
Kan:	kanamycin
kb:	kilobase
kDa:	kilo Dalton
LB:	Luria-Bertani
mA :	milli ampère
McAbs:	monoclonal antibodies
MCS:	multiple cloning site
MES:	2-[N-Morpholino]ethanesulfonic acid
MET:	methyl transferase
miRNAs:	microRNAs
ml:	millilitre
MP:	movement protein
mRNA :	messenger RNA
NCBI:	National Center for Biotechnology Information
ng:	nanogram
nm :	nanometre
NOS:	nopaline synthase
NPTII:	neomycin phophotransferase II
nt	nucleotide
OCS:	octopine synthase
ORF:	open reading frame
PAPs:	pokeweed antiviral proteins
PAZ:	piwi/ARGONAUTE/zwille
PCR:	polymerase chain reaction
Pdk:	pyruvate orthophosphate dikinase
PDR:	pathogen-derived resistance
pg:	picograms

PPD:	Paz and Piwi domain
PTGS:	post-transcriptional gene silencing
R genes:	natural resistance genes
RDE	RNAi-DEFECTIVE
RdRp:	RNA-dependant RNA polymerase
RdDM:	RNA directed DNA methylation
RDOT-RT-PCR:	rapid direct one-tube RT-PCR
RdRs:	RNA dependant RNAs
Rif:	rifampicin
RISC:	RNA-induced silencing complex
RMVR:	RNA mediated virus resistance
RNAi:	RNA interference
rpm:	revolutions per minute
RT-PCR:	reverse transcription polymerase chain reaction
S:	sense
SAP:	shrimp alkaline phosphatase
SAS:	systemic acquired silencing
SAWIS:	South African Wine Industry Information & Systems
Sde:	silencing defective
Sgs:	suppressor of gene silencing
siRNAs:	small interfering RNAs
ss:	single stranded
T_A:	annealing temperature
TAE:	tris, acetic acid and EDTA
T-DNA:	transfer DNA
Tet:	tetracycline
TGS:	transcriptional gene silencing
T_m:	melting temperature
Tris:	tris(hydroxymethyl)aminomethane
µg:	microgram
USDA:	United States Department of Agriculture
UTRs:	untranslated regions
UV:	ultraviolet light
VIGS:	Virus-induced gene silencing
Vir-:	transfer defective strain
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Virus acronyms

AIMV:	*Alstroemeria mosaic potyvirus*
ArMV:	*Arabis mosaic nepovirus*
BYV:	*Beet yellows closterovirus*
CaMV:	*Cauliflower mosaic caulimovirus*
CMV:	*Cucumber mosaic cucumovirus*
GFLV:	*Grapevine fanleaf nepovirus*
GLRaV:	*Grapevine leafroll associated Closterovirus*
GLRaV-1:	*Grapevine leafroll associated Closterovirus 1*
GLRaV-2:	*Grapevine leafroll associated Closterovirus 2*
GLRaV-3:	*Grapevine leafroll associated Closterovirus 3*
GLRaV-9:	*Grapevine leafroll associated Closterovirus 9*
GVA:	*Grapevine trichovirus A*
GVB:	*Grapevine trichovirus B*
LIYV:	*Lettuce infectious yellows Closterovirus*
PPV:	*Plum pox potyvirus*
PVY:	*Potato potyvirus Y*
PVX:	*Potato potexvirus X*
RW:	*Rugose wood disease*
TBSV:	*Tomato bushy stunt tombuvirus*
TCV:	*Turnip crinkle carmovirus*
TEV:	*Tobacco etch potyvirus*
TMV:	*Tobacco mosaic tobamovirus*
ToMV:	*Tomato mosaic tobamovirus*
TRSV:	*Tobacco ringspot nepovirus*
TRV:	*Tobacco rattle tobravirus*
TSWV:	*Tomato spotted wilt tospovirus*

During the first half of the twentieth century, plant virology gradually evolved from its initial status as an agricultural science of simple concepts to its present place in the front rank of biological sciences.

Francis O. Holmes, 1968

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Times gone by

The word 'virus' originates from Latin, meaning poison. In the modern sense, the term refers to minute infectious particles which can only be seen in an electron microscope at a magnification of at least 20 - 40000 times. Although the field of virology is only about 100 years old, viruses have probably been present in living organisms since the origin of life. One could conclude that every species on this planet carries viruses. While many viruses are harmful to their host, others are symbiotic, and some viruses may even be advantageous to their infected hosts.

THE "CLASSICAL" DISCOVERY PERIOD (1883 -1951)

This period started in the sixteenth century, with the discovery that plant diseases, were caused by viruses. This discovery was followed by the acceptance that viruses can be transmitted by insects, via seed and that certain viruses could even be transmitted through soil and viral vectors (fungi and nematodes). The first plant virus list was released in 1939 and it already constituted 129 viruses including grapevine leafroll virus.

The phenomenon of cross protection was discovered and it was followed by the development of a bioassay for *Tobacco mosaic tobamovirus* (TMV) in 1927 and the purification of the virus in 1936. This led to the publication of its structure and other viruses later in the period.

THE EARLY MOLECULAR ERA (1952 -1983)

This era confirmed that genetic information was also carried in the RNA. Sequencing, viral chemotherapy and the seeking for the basis of symptomatology were on the foreground of the studies in 1950. A breakthrough came with the technology to translate RNAs *in vitro*. This led to the discovery of viral satellites (1969), defective interfering viruses (DI), viroids and protoplasts. *In vitro* replication and the first purification of plant replicases also saw the light in the molecular era. All these discoveries led to the improvement of virus diagnosis, reducing the time of assays.

RECENT PERIOD (1984 -)

This period is characterised by the ability to modify plant virus genomes, to detect non-structural gene products, to determine the functions of viral gene products, and to modify plants using viral-derived sequences to provide novel forms of resistance. The development and utilisation of technologies that allow viruses to be manipulated is what differentiates the current period from the earlier periods, where plant virology was predominantly a science of observation and description (Zaitlin & Palukaitis, 2000).

Chapter 1
General Introduction

1.1 Introduction

The grapevine is a very old food plant, at first foraged from in the wild state, and later cultivated by man. It is generally accepted that the first vines were cultivated in Asia Minor, south of the Caspian and Black seas. The grape varieties cultivated in South Africa were originally imported from Europe and belong to the species *Vitis vinifera*. The wine history of South Africa began in the middle of the 1600s and was pioneered by the first governor of the Cape, Jan van Riebeeck, who is considered as the father of the viticulture and wine making in South Africa. He ordered vines from France (Chenin Blanc and Muscat de Alexandrie) and planted the first vineyard in 1655 at the Dutch East India Company's refreshment station in the Cape.

The first grapes were harvested in 1659 and on 2 February 1659 Jan van Riebeeck wrote in his diary: "Today, praise be to God, for the first time we pressed Cape's grapes and made wine." Simon van der Stel succeeded Van Riebeeck in 1679, and established a prestigious winery in 1685 called Constantia, and which is still producing some of the finest wines in the world today. In 1886 the country's vineyards were devastated by the parasite phylloxera. The disease lasted for 20 years, and only in the beginning of the 1900s local producers started planting vineyards again (Wines of South Africa; http://www.wosa.co.za/SA/history.htm).


Jan van Riebeeck

According to a study, commissioned by the South African Wine Industry Information & Systems (SAWIS), the wine industry contributes R14 557 million to the annual Gross Geographical Product of the country and provides employment opportunities for 348 500 people. South Africa has 5000 wine producers and currently 108 000 hectares of vines producing wine grapes are under cultivation in South Africa spanning an area of some 800 kilometres in length. The South African wine industry has its roots in the Western Cape. This area has 95% of South Africa's vineyards and it is estimated that 60% of the industry's activities have a direct impact on the Western Cape's economy. A rough estimate shows that of the R14.5 billion of the Gross Geographical Product which the industry creates, about R8.7 billion eventually would remain in the Western Cape to benefit its residents.

Although local vineyards account for just 1.6% of the world's vineyards, South Africa ranks as number nine in volume production of wine and produces 3% of the world's wine (SAWIS; http://www.sawis.co.za).

Grapevine viruses are among the most important pathogens of grapevine and cause severe losses by substantially reducing yield, affecting fruit quality and shortening the lifespan of infected plants in the vineyard. Viral diseases are widespread in grapevine and although only some, out of the 53 viruses (from 20 different genera) found in *Vitis* have practical importance; their presence in grapevine is generally considered detrimental. *Grapevine fanleaf nepovirus* (GFLV), *Grapevine leafroll associated closterovirus* (GLRaV) (from this point forward the word leafroll disease will be used) and Rugose wood complex (RW) are regarded as the most harmful and widespread grapevine viral diseases. Leafroll disease especially affects the South African wine industry greatly with losses running into millions of Rands.

A complex of nine known GLRaVs belonging to the group of long filamentous viruses, which falls in the *Closteroviridae* family, is associated with leafroll disease. These viruses live in the phloem tissue, and can be transmitted by mealybugs. They block the phloem and thus carbohydrates are prevented from moving into the fruit and this delays the development of pigments and sugars. This is especially problematic in short season areas, since the virus can add weeks to the maturation process. At present the most significant GLRaVs associated with leafroll disease are GLRaV-1, GLRaV-3 (which poses the biggest problem in South African vineyards), and GLRaV-2, which is beginning to draw attention too.

Existing control measures against leafroll are primitive and inefficient and vineyards infected with leafroll cannot be returned to a healthy state. A realistic long-term approach in solving leafroll disease is to introduce genetic resistance into vines by genetic engineering, although the anti-genetically modified (GM) movement does not welcome it. Progress in this field has been made by the introduction of protein-mediated resistance or RNA-mediated virus resistance (RMVR). The underlying mechanism of RMVR is a natural plant response mechanism called post-transcriptional gene silencing (PTGS).

PTGS is ubiquitous in both the animal and the plant kingdoms and acts like a defence system for the plant, protecting the genome against invasion by transgenes, endogenous genes and cytoplasmically replicating viruses, which produce abRNA or dsRNA in the host cell when they become active (Cogoni & Macino, 2000; Finnegan *et al.*, 2001). The PTGS mechanism is initiated by dsRNA. The dsRNA is processed into small 21-23 nucleotide RNAs by an RNase-III nuclease

(Dicer) and is then incorporated as guide RNAs into a RNA-induced silencing complex (RISC). After an ATP-dependent activation step the small RNAs would bind with homologous RNA after which an endonuclease cleavage of the target RNA occurs (Smith, 1999).

Post-transcriptional gene silencing has great potential for the introduction of resistance against viruses in plants. This potential is hampered by the fact that plant viruses have evolved to produce suppressor proteins that can counter the PTGS mechanism. More than a dozen silencing suppressors (Thomas *et al.,* 2003) have been identified from different types of viruses by utilizing silencing assays (Roth *et al.*, 2004). Similar silencing assays can be utilised to assess and identify suppressor activity from different grapevine viruses, especially GLRaV-3, and thus transgenic virus resistant grapevine can be the ultimate answer to the grapevine virus problem in South African vineyards and even worldwide!

In designing strategies for the control of GLRaV-3, the genome of GLRaV-2 were screened for suppressor activity. This was done because GLRaV-2 has a broad host range which comprises the experimental tobacco, *N. benthamiana* which GLRaV-3 does not infect. Moreover *N. benthamiana* plants grow extremely fast and are easy to work with in laboratory conditions while grapevine is on the other side of the spectrum with its long maturation period.

1.2 Project Proposal

The first part of this study will be undertaken to screen the GLRaV-2 genome for the presence of a suppressor protein. A constitutively expressed green fluorescent protein (GFP) gene will be silenced in transgenic *N. benthamiana* plants (line 16c) by agro-infiltration, using a second GFP-construct. Ultra violet (UV) illumination will be used to distinguish between silenced (red chlorophyll fluorescence) and non-silenced plants (green GFP fluorescence). The GFP-silenced plants will be inoculated with a strain of GLRaV-2 to screen for suppressor activity. To pinpoint the individual gene/genes responsible for the suppressor activity, individual GLRaV-2 genes will be isolated and cloned into an intermediate Polymerase chain reaction (PCR) cloning vector, followed by subsequent cloning into a plant expression vector. The vectors will be transformed into *Agrobacterium tumefaciens* (*A. tumefaciens*) strains GV3101 and C58C1 and will be agro-infiltrated into silenced transgenic or transgenic *N. benthamiana* (16c) plants in two different expression assays (reverse- or transient expression assay).

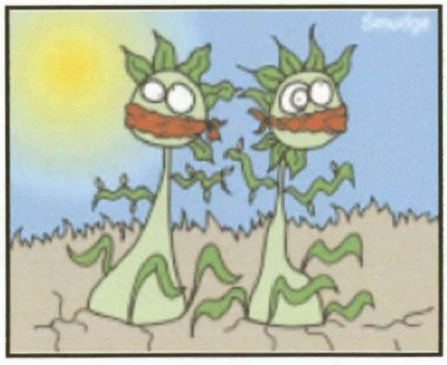
The second part of this study will be to construct a tandem silencing vector, based on the generic plant gene silencing vector pHannibal. This vector will serve as a "proof of concept" to show that two genes can be silenced simultaneously in a plant. Expression of this gene silencing vector yields an mRNA containing sense and anti-sense copies of the genes to be silenced, separated by a plant intron. Nucleotide homology will favour the formation of a stem loop structure, while the loop will be removed during post-transcriptional processing of the RNA, yielding dsRNA to trigger gene silencing. A Rapid Direct One-Tube RT-PCR reaction (RDOT-RT-PCR) using primers with 5'-extensions containing restriction sites to facilitate subsequent cloning will be used to amplify a gene fragment from the South African isolate of the GLRaV-2 genome. A portion of the GFP will be isolated from the plasmid vector pBIN mGFP5-ER, also using primers containing restriction sites. Individual PCR products will be ligated into an intermediate PCR cloning vector, followed by the subsequent cloning into corresponding sites in the pHannibal vector, in the sense and anti-sense orientations. The silencing cassette will be removed from pHannibal and ligated into a plant expression vector. The vector will be transformed into *A. tumefaciens* strains GV3101 and C58C1 and transgenic and non-transgenic *N. benthamiana* plantlets, of which some will be infected with GLRaV-2, will be agro-infiltrated with the tandem silencing vector and results will be observed non-invasively by UV illumination of plants.

Chapter 2

Literature Review

O, I die, Horatio;
The potent poison quite o'er-crows my spirit:
I cannot live to hear the news from England;
But I do prophesy the election lights On Fortinbras: he has my dying voice;
So tell him, with the occurrents, more and less, which have solicited.
The rest is silence.

Hamlet, Act V Scene II

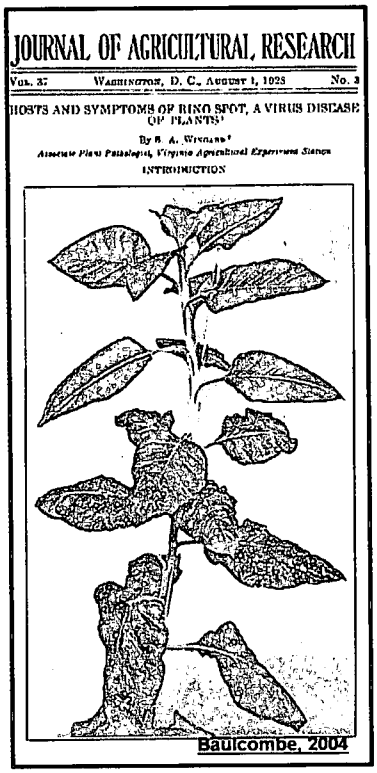


Introduction

Since it was known as the "White emperor", leafroll disease has had detrimental effects on vineyards all over the world and substantial damage has been reported in South Africa. To date nine serologically different clostero-like viruses all belonging to the *Clostoviridae* family have been associated with this disease (GLRaV-1 to GLRaV-9). Of these, GLRaV-3 is the most important pathogen of virus-infected grapevines worldwide. The disease is characterised by a downwards rolling of leaves and an interveinal reddening or yellowing of leaf laminas. These symptoms are especially conspicuous in late summer and early autumn and are accompanied by significant reduction in crop quantity, pigmentation and vine growth.

Leafroll is transmitted by phloem feeding mealybugs and can also spread through secondary transmission between neighbourly vines. Different detection and control measures are employed for early leafroll detection and to control the virus once present in the vineyards, but no existing control measure has been 100% effective to control or prevent leafroll disease. Engineered virus resistance to plant viruses seems to be one of the answers to virus free grapevine in the future. A natural occurring phenomenon termed RNA silencing may hold the key to the future of virus free grapevine.

The first encounter with RNA silencing was witnessed by plant pathologist, S.A. Wingard. In a paper he published in the "Journal of Agricultural Research" in 1928. He described an unsuspected outcome in which only the initially infected leaves of a tobacco plant was necrotic and diseased in response to infection with *Tobacco ringspot nepovirus* (TRSV) (Figure 2.1). This was due to RNA silencing but it was still unknown to the world at that time (Baulcombe, 2004).


JOURNAL OF AGRICULTURAL RESEARCH

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HOSTS AND SYMPTOMS OF RING SPOT, A VIRUS DISEASE OF PLANTS[1]

By S. A. Wingard,

Associate Plant Pathologist, Virginia Agricultural Experiment Station

INTRODUCTION

Baulcombe, 2004

Figure 2.1: Cover page of S.A. Wingard's published paper in 1928 describing his unsuspected outcomes

RNA silencing was officially discovered in the petunia plant and was initially considered a strange phenomenon limited to plant species only. It is now regarded as one of the newest fields of molecular biology and is present in both the animal and the plant kingdom as a type of defence system which acts at the nucleic aid level against foreign invasions which produce aberrant RNA (abRNA) or double stranded (dsRNA) (Simón-Mateo *et al.*, 2003; Tuschi, 2001).

Extensive studies have been undertaken in organisms such as nematodes, insects, fungi and mammals (Matzke *et al.*, 2001; Yu & Kumar, 2003) to determine the precise mechanism of RNA silencing, but it is still not yet fully understood. It is proposed that the cascade of events is initiated by dsRNA which is processed into small 21-23 nucleotide RNAs (siRNAs) by an RNase-III nuclease (Dicer). The siRNAs are incorporated as guide RNAs in a RNA-induced silencing complex (RISC). The siRNAs bind with homologous RNA and this results in the cleavage of the target mRNA (Cogoni & Macino, 2000; Dalmay *et al.*, 2001).

By selectively silencing single or whole gene families RNA silencing can be used as a tool for studying gene expression and function. It can also be used to introduce virus resistance in plants and holds a promising future.

2.1 History of leafroll

Leafroll disease has been present in grapevine since the 1920s under the names rougeau, flavescence and brunisure in France, Rollkrankheit in Germany and red-leaf and "White Emperor" in California, but was only identified in the late 1950s on the red table grape cultivar Emperor.

Emperor plantings usually known for its dark, red fruit started producing pale grapes in Californian vineyards. Field trails were conducted under Harold Olmo and Al Rizzi to establish the cause of the colour problem in the Emperor plantings. Olmo found that healthy rootstock could not cause the disease and that the disease could be transmitted to progeny by propagation and speculated that a latent virus could be involved (Olma & Rizzi, 1943).

In 1946, two United States Department of Agriculture (USDA) breeders Harmon and Snyder reported that the "White Emperor" disease was graft transmissible and that the discoloration of the grapes was definitely caused by some type of virus present in unhealthy rootstock (Harmon & Snyder, 1946). The plant pathologist William B. Hewitt followed in 1951 with the declaration that the "White Emperor" virus was present in the Thompson Seedless, Colombard and Salvador cultivars (Hewitt, 1951) and in 1952 that it occurred in common rootstocks (1202,1613) used. These observations were followed by the announcement by Herman and Snyder that the virus was widespread in the USDA bred table grapes (Cordinal and Red Malaga) and that it was the result of virus infected rootstock.

By 1959 it was common knowledge that California's vineyards were affected with "White Emperor" disease and a connection between the leafroll virus described in Germany in 1936 by Schen and the "Emperor" virus was made by Goheen. Thereafter "White Emperor Disease" became known as leafroll (Hewitt, 1951).

2.2 Leafroll and the *Closteroviridae* family

Leafroll is widely acknowledged as the most important virus disease of *Vitis vinifera,* and it was only in the late 1970's that a breakthrough in the etiology of the disease was made. Virions were recovered from infected vines in Japan that had the typical structure and outward appearance of closteroviruses (Namba *et al.*, 1979).

At least five different clostero-like viruses were associated with the disease in the following few years. At the 10th meeting of the International council for the study of virus and virus-like diseases of the grapevine (ICVG), the five different clostero-like viruses were confirmed and assigned the

name Grapevine leafroll associated virus followed by Roman numerals I to V (i.e. GLRaV-I to GLRaV-V).

But, in 1995 the International committee on Taxonomy of viruses (ICTV) changed this method by deciding that the numbers in the virus acronyms must be written with Arabic numerals separated by a hyphen from the letters (i.e. GLRaV-1 to GLRaV-5) (Fauquet & Martelli, 1995).

Two decades after the purification and serological characterization of the first five grapevine leafroll associated viruses, we are now confronted with at least nine viral types associated with the disease (GLRaV-1 to GLRaV-9). The different GLRaV types are serologically different from one another and are regarded as different species although they all belong to the *Closteroviridae* family (Gugerli, 2003).

These GLRaV types are all phloem-limited viruses, because they are found most consistently in phloem companion and parenchyma cells in infected plants. This usually results to low virus titers in infected plants, poor extractability of the viruses and low yields during purifications, which make this group of viruses very difficult to study.

2.2.1 Taxonomy and classification of the *Closteroviridae* family

In earlier days the widely accepted morphological approach divided Closteroviruses into a "long" subgroup, with particles of 1200-2000 nanometre (nm), and "short" subgroup, with particles in the range of 700-950 nm. But, several unrelated filamentous viruses were included into the "short" subgroup and these viruses were later removed to form three separate genera called capillo-, tricho-, and vitiviruses.

Vector specificities were also used to divide the Closteroviruses but to a lesser extent because vectors for Closteroviruses were often unknown or poorly studied (Bar-Joseph *et al.*, 1979).

By 1998, the family *Closteroviridae* consisted of two genera, a monopartite genus Closterovirus with the type member *Beet yellows closterovirus* (BYV) and a bipartite genus Crinivirus ("short" subgroup) with the type member *Lettuce infectious yellows closterovirus* (LIYV). As more molecular information accumulated, Karasev, (2000) argued that closteroviruses be classified by type of insect vector rather than by the number of genomic RNAs. He proposed a further refinement by adding a third genus to the family, with the name Viniviruses from *Vitis vinifera* (the natural host of the type species) and named the mealybug transmitted GLRaV-3 as a type member. Bipartite Criniviruses are transmitted by whiteflies, monopartite Closteroviruses are transmitted by aphids and the new Viniviruses by mealybugs (Karasev, 2000).

Karasev's proposal was reviewed by the ICTV and the name of the Vinivirus was changed to Ampelovirus in order to avoid confusion with the already existing Vitivirus genus (Martelli *et al.*,

2002). The revised version of the family *Closteroviridae* was approved by the ICTV in July 2002 (Figure 2.2).

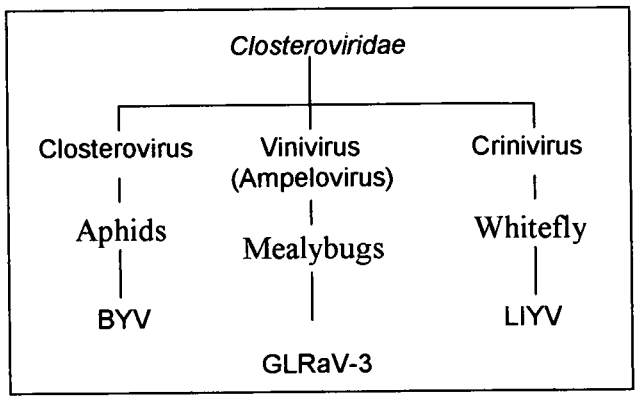


Figure 2.2: The *Closteroviridae* family with its respective genera, type members and vectors associated with the generas.

2.2.1.1 Genus: Closterovirus

The name of the genus is derived from a Greek word, which means, thread. The type species of the genus closterovirus is BYV and members of this genus affect several crops of major economic importance like citrus, tomato, potato and grapevine. Change in agricultural practises and invasions and spread of new vectors have led to the recognition of new closteroviruses (Karasev, 2000). GLRaV-2 is the only GLRaV belonging to this group and can be mechanically transmitted to herbaceous plant species (Goszczynski *et al.*, 1996b).

Virus morphology. Virions are filamentous, not enveloped, very flexuous and in the region of 1250-2200 nm in length with a distinct virion tail (Figure 2.3).

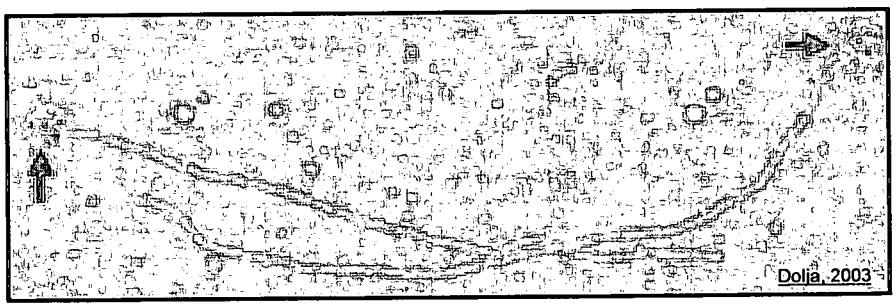


Figure 2.3: Electron micrograph of the *Beet yellows Closterovirus* (BYV) with arrows indicating the distinct virion tail.

Genomic organization. Closteroviruses have a monopartite, linear, single-stranded positive sense RNA 15.5-19.3 kilobase (kb) in size. The 5'-terminus has a methylated nucleotide cap and the 3' terminus has no poly (A) tract and sequences with potential hairpin structures are present near the 3'-end.

The viral genes are translated from a large number of open reading frames (ORFs). There is a large replicase present near the 5'-end of the genome and the remaining ORFs are translated from a set of nested messenger RNAs (mRNAs) (Dolja, 2003). The major coat protein (CP) subunit (22-25 kiloDaltons [kDa]) coats most of the virion length while the divergent CP analogue (CPd) (24-27 kDa) coats a shorter segment at one end of the particle. The closterovirus genus differs from the other two genera in the family (Ampelovirus and Crinivirus), by means of the gene encoding the CPd, which is situated upstream of the CP gene and not downstream (Russell, 1970; Martelli *et al.*, 2002; Dolja, 2003) (Figure 2.4).

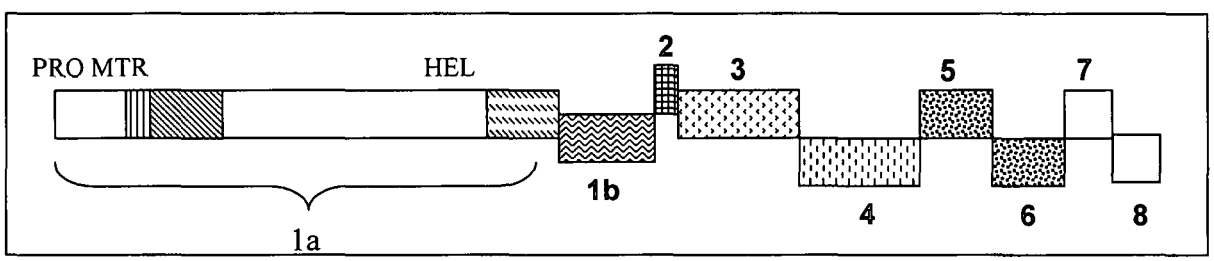


Figure 2.4: The genome structure of *Beet yellows closterovirus* (BYV), the type species of the genus Closterovirus, showing boxes representing ORFs with their expression products. **ORF 1a:** 295 kDa containing papian-like protease (PRO), methyltransferase (MTR) and RNA helicase (HEL) motifs; **ORF 1b:** 53 kDa RNA polymerse (RdRp) expressed as a 1a/1b fusion protein of 348 kDa by +1 frame shift; **ORF 2:** 6 kDa small hydrophobic membrane protein; **ORF 3:** 65 kDa heat-shock-related protein (HSP70); **ORF 4:** 64 kDa; **ORF 5:** 24 kDa divergent coat protein (CPd) analogue; **ORF 6:** coat protein (CP); **ORF 7:** 20kDa long distance transport protein; **ORF 8:** 21 kDa replication protein.

2.2.1.2 Genus: Ampelovirus

The name of the genus is derived from the Greek word ampelos which means grapevine; the plant host of the type member. The genus contains only viruses that infect dicotyledonous hosts and none of these viruses are transmissible by sap inoculation. The type species of this genus is GLRaV-3 and all of the GLRaVs are found in this genus except GLRaV-2.

Virus morphology. Virions are again filamentous, not enveloped, very flexuous and of size > 1000 nm in length (Figure 2.5).

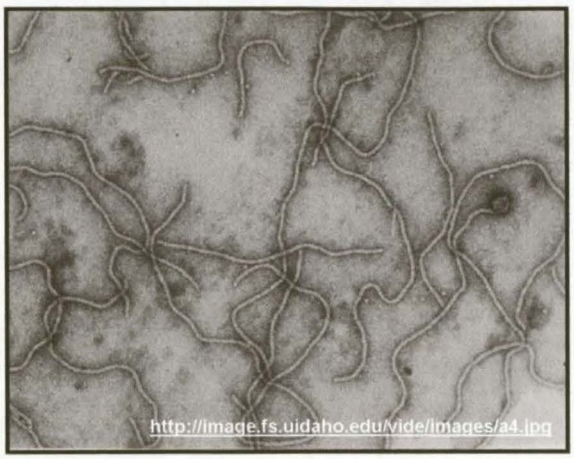


Figure 2.5: Electron micrograph of the *Grapevine leafroll-associated closterovirus* 3 (GLRaV-3).

Genomic organization. Virions contain a single molecule of linear, single-stranded positive sense RNA 16.9-19.5 kb in size. Sequences with potential hairpin structures are also present near the 3'-end. The 3'-terminus has no poly (A) tract but the 5'-terminus has a methylated nucleotide cap.The RNA is translated from up to 12 reading frames and a large replicase is present near the 5'-end of the genome. With GLRaV-3 and several other sequenced members of the genus, the CPd gene follows the CP gene. In GLRaV-1 the CPd gene is duplicated (Martelli *et al.*, 2002) (Figure 2.6).

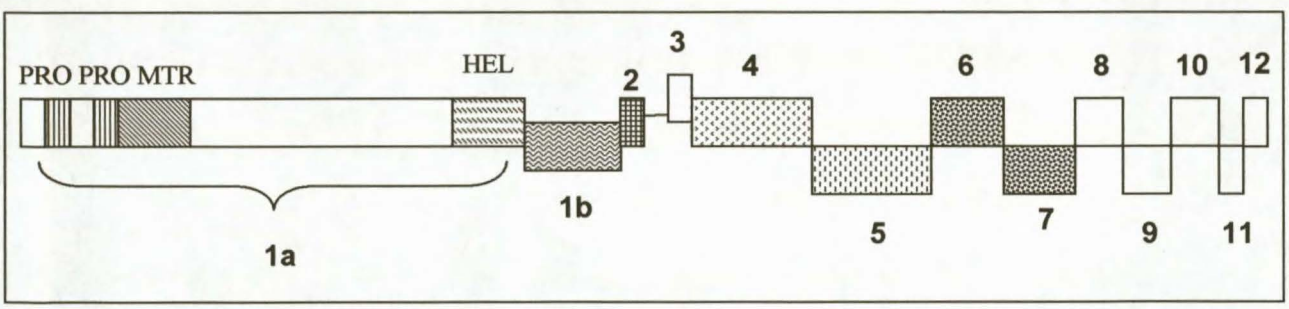


Figure 2.6: The genome structure of *Grapevine leafroll associated virus 3* (GLRaV-3), the type species of the genus Ampelovirus, showing boxes representing ORFs with their expression products. **ORF 1a**: 245 kDa containing a double papian-like protease (PRO), methyltransferase (MTR) and RNA helicase (HEL); **ORF 1b**: 61 kDa RNA polymerse (RdRp); **ORF 2**: 6 kDa unknown protein; **ORF 3** 5 kDa unknown protein; **ORF 4**: 59 kDa heat-shock-related protein (HSP70); **ORF 5**: 55 kDa cell-to-cell movement protein; **ORF 6**: 35 kDa coat protein (CP); **ORF 7**: 53 kDa divergent coat protein analogue (CPd); **ORF 8**: 21 kDa unknown protein; **ORF 9**: 20 kDa unknown protein; **ORF 10**: 20 kDa unknown protein; **ORF 11**: 4 kDa unknown protein; **ORF 12**: 7 kDa unknown protein.

2.2.1.3 Genus: Crinivirus

The name of the genus is derived from the Latin word *crinis* which means hair. The type species of this genus is LIYV.

Virus morphology. Virions are filamentous, not enveloped, very flexuous and have two modal lengths (650-850 nm and 700-900 nm) (Figure 2.7).

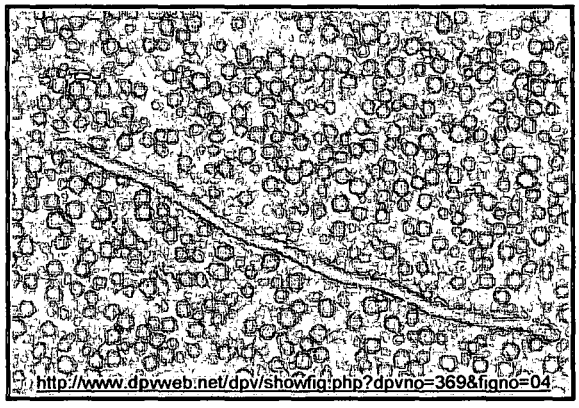


Figure 2.7: Electron micrograph of the *Lettuce infectious yellows Closterovirus* (LIYC).

Genomic organization. It has a bipartite, linear, single-stranded positive sense RNA 15.3-19 kb in size. Sequences with potential hairpin structures are also present near the 3'-end. The 3'-terminus has no poly (A) tract and the 5'-terminus has a methylated nucleotide cap.

The genome is divided into two separately encapsulated molecules that are needed for infectivity. RNA-1 has three or four ORFs and is a bicistronic molecule that encodes replication related proteins (ORF1) as well as those involved in virus replication. RNA-2 has seven ORFs. It contains the five-gene module typical of the family, which, however differs from that of the Closterovirus and Ampelovirus genera because of the insertion of an extra small gene (ORF6) upstream of the CP gene. In all members of the genus, the CPd ORF is downstream of the CP gene (Martelli, 2002). The CP size is rather uniform ranging from 28 to 33 kDa, but the size of the CPd can be double the size of the CP. The ORFs are translated from a set of overlapping, 3'-coterminal subgenomic RNAs (Falk & Tian, 1999) (Figure 2.8).

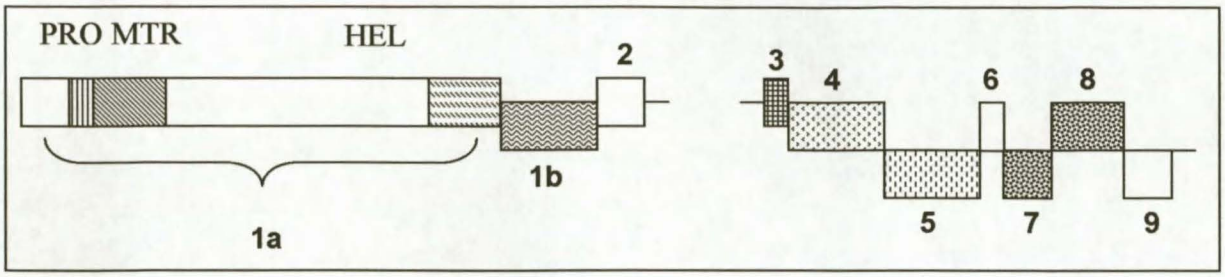


Figure 2.8: The bipartite genome structure of *Lettuce infectious yellows closterovirus* (LIYV), the type species of the genus Crinivirus, and showing boxes representing ORFs with their expression products. RNA 1-**ORF 1a:** 214 kDa containing putative papian-like protease (PRO), methyltransferase (MTR) and RNA helicase (HEL); **ORF 1b:** 55 kDa RNA polymerse (RdRp); **ORF 2:** 32 kDa unknown protein p32; RNA 2-**ORF 3:** 4 kDa hydrophobic protein; **ORF 4:** 62 kDa heat-shock-related protein (HSP70); **ORF 5:** 56 kDa unknown protein p59; **ORF 6:** 4.8 kDa unknown protein p9; **ORF 7:** 25 kDa coat protein (CP); **ORF 8:** 74 kDa divergent coat protein analogue (CPd); **ORF 9:** 26 kDa unknown protein p26.

2.2.2 Sequencing information

More molecular information has come available in the last few years by cloning and sequencing of the GLRaVs. The following Genebank identification numbers with genes sequenced are available in the National Centre for Biotechnology Information (NCBI) sequence database (http://www.ncbi.nlm.nih.gov/) (**Appendix D**).

2.3 Biology of Leafroll disease

2.3.1 Symptoms of leafroll

Grapevine leafroll associated virus causes varying degrees of disease symptoms depending on environmental conditions as well as different grape varieties. Generally, symptoms occur that resemble potassium deficiency, low temperatures and leafhopper damage i.e. yellowing or reddening, vein-clearing, stunting, wilting and in some cases collapse of the plant (Wisler, 1998). Mechanical damage to the trunk, shoots, poor graft unions, girdling can also mimic leafroll symptoms. Vine symptoms are thus not a reliable indicator of the presence of leafroll virus (Carstens, 2002).

In woody perennials abnormalities in the phloem and xylem results in leaf rolling, stem pitting, reduced vigour and reduced quantity and quality of fruit. Symptoms vary from one cultivar to the

next and usually appear first at the base of the shoot. The disease is predominantly caused by GLRaV-3 in Western Cape vineyards and is best observed in the period between harvesting and shedding of leaves. In some cases GLRaV-3 are accompanied by *Grapevine trichovirus A* (GVA) and in rare circumstances by GLRaV-1 and GLRaV-2.

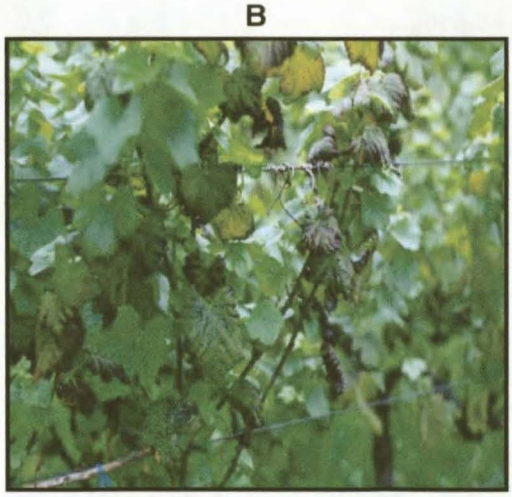
Red-or black-fruited cultivars redden in colour in autumn and this is often accompanied by the downward curling of the leaf margins, which stay green (hence the disease name "leafroll") (Figure 2.9A & 2.9C). White-fruited cultivars may exhibit a light green to yellowish colour between the downward curling leaf margins at this time of summer (Figure 2.9B). The extents to which the leaves curl differ from cultivar to cultivar. Some varieties can be infected with the virus and suffer no apparent negative effects. In cultivars such as Chardonnay and Sauvignon Blanc, the leaf margins roll down severely, where as cultivars such as Sultana and Chenin Blanc display only minor rolling of the leaves. Once symptoms show up, they recur every year.

Leafroll disease also affects the grapes on infected vineyards. By the degeneration of phloem tissue in young shoots, leaves, petioles and rachis, fewer and smaller clusters of grapes are produced. These grapes are lower in soluble solids and are poorer pigmented than normal. The disease also delays the accumulation of sugar and lowers the production of anthocyanin in red wine cultivars. Some studies estimate yield losses caused by leafroll disease to as much as 40% to 60%.

A

http://winegrapes.tamu.edu/grow/diseases/leafroll.shtml

B



www.wynboer.co.za/imagesart/1002grape2.jpg

Figure 2.9: (A) Red fruited cultivar displaying the discolouration and downward curling of the leaves and **(B)** White cultivar displaying the downward curling of the leaf margins associated with grapevine leafroll.

C

Figure 2.9: (C) Typical discolouration of a leaf of leafroll infected grapevine.

2.3.2 Occurrence and transmission of leafroll

Leafroll occurs in all major grape-growing regions of the world, causing reductions in productivity and quality of both wine and table grapes. One of the most worrying aspects of the disease is the rate of spread or the "re-infection problem". It is estimated that a newly establish vineyard can become completely infected (80% plus) in five to six years (Jordan *et al.,* 1993).

In 1936 Germany suffered an 80% leafroll infection and data collected in the eighties pointed out that grapevine from Germany, northern France and eastern Switzerland were essentially affected by GLRaV-1. Vineyards further south and west, including western and southern Switzerland, Italy and France (80% infected) were generally infected with GLRaV-3 and some with GLRaV-2 (Gugerli, unpublished data) (Weber, 1993). Recently, leafroll was also seen in the Czech Republic (10% of their vineyards are infected with GLRaV-1 & GLRaV-3) and in Spain 54% of their grapevine diseases is represented by GLRaVs (Gugerli, 2003).

In 1993, 6% of vines in Nuriootpa in Southern Australia, displayed leafroll symptoms, which escalated to 36% in 1995 (Habilli *et al.,* 1995). The incidence of leafroll infection in New Zealand has increased from a low 11% to 100% in a five year period (Jordan *et al.,* 1993).

Commercial wine cultivars in the Western Cape have been tested in 1970 for a variety of viruses. An extremely high percentage (99.9%) of vines had been infected with viruses of which leafroll represented 68,4% of these virus infections (Nel *et al.*, 1972). In a recent study published by Pietersen (2004) the status of the vineyards had improved remarkable and disputed the results obtained in 1970.

It was first thought that leafroll could only spread by grafting healthy stock onto infected stock and could not spread naturally in vineyards (Goheen, 1989). Therefore, healthy and diseased vines

were planted in the same vineyard blocks. The assumption was soon proven wrong when secondary transmission of the disease occurred between neighbouring vines. The most natural form of transmission of the disease is down and across vineyard rows and the second most common form of transmission of the disease is between two adjacent infected vineyards (Pietersen, 2004).

Internationally GLRaV is being transmitted mainly by infected propagation material. It has also been found that insect vectors can transmit the disease. Viruses belonging to the *Closteroviridae* family are transmitted by insects belonging to the order Homoptera, suborder Sternorrhyncha and have been found in three families, Aphididae (aphids), Aleyrodidae (whiteflies) and Pseudococcidae (mealybugs) (Bar-Joseph *et al.*, 1979; Golino *et al.*, 1998). These insects feed on phloem tissue and their piercing-sucking mouthparts generally cause little damage to a plant, they transmit the virus in a semi-persistent manner, with a minimum acquisition period of 30 minutes to one hour and may retain infectivity in an insect for up to nine days, depending on the virus-insect combination (Wisler, 1998).

One of the most important vectors in the transmission of leafroll disease world wide is a variety of mealybug species, which are as follows: The vine mealybug, *Planococcus ficus* (GLRaV-3), that is commonly found, the long-tailed mealybug, *Pseudococcus longispinus* (GLRaV-2 & GLRaV-3), that is found to a lesser extent in South African vineyards, *Pseudococcus affinis* (GLRaV-2 & GLRaV-3), *Planococcus citri* (GLRaV-3), *Pseudococcus calceolaria* (GLRaV-3), *Phenacoccus aceris* (GLRaV-1) and *Ceroplastes rusci* (GLRaV-3). Mealybugs had also recently been found on grapevine roots up to 60 cm below the ground surface (Carstens, 2002)

2.3.3 Detection methods of leafroll

Biological Indexing. The standard procedure for the detection of grapevine viruses is classical indexing. An indicator vine, usually Cabernet franc or Pinot Noir, is chip-budded (grafted) with one or two buds from a vine with unknown virus status. The vines are planted in the field and monitored for two seasons (+/- two years) for the occurrence of diseases symptoms (van der Merwe, 2001; Golino *et al.*, 2002).

Another type of indexing called green grafting is a faster type of biological indexing, which is performed in a greenhouse with micro-propagated and acclimatised indicator plants. Extracts from leaf petioles or secondary shoots from test vines are mechanically inoculated onto the sensitive greenhouse indicator plants and symptoms can be observed within two to three months on the indicator plants, if the test vines were positive (van der Merwe, 2001; Golino *et al.*, 2002).

Both of these indexing methods are not ideal, since it requires time for the symptoms to appear (considerable less with green-grafting), its labour intensive and large amounts of field/greenhouse space is needed. Another negative is that these tests can only detect if a vine is positive or negative for a virus and can not identify the specific virus causing the disease (van der Merwe, 2001).

Serological testing. Enzyme-linked immunosorbent assay (ELISA) utilises antibodies and/or antiserum produced against a specific virus to detect its presence. During the 1980s, commercially manufactured polyclonal and monoclonal antibodies (McAbs) suitable to detect the GLRaVs were made (Martin *et al.*, 2000; Monis, 2000). The ELISA test is very sensitive, fairly simple, are up scalable and results can be obtained within one to two days. The downfall is that high concentrations of purified virus are needed for the production of the antiserum and testing must be done when virus titers are at their peak and even then results are not reproducible. ELISA can detect if a virus is present and can differentiate between serologically distinct types (as with GLRaVs), but needs several different antibodies. Currently, monoclonal antibodies are available for GLRaV-1 to 6 and recently J. Monis (2000) developed an antibody for GLRaV-8.
Another type of testing is Immunosorbent electron microscopy (ISEM) which entails the trapping of virus particles onto grids, which have been coated with specific virus antibodies and observing the grids under an electron microscope. The test is rapid and sensitive but requires skill, is labour intensive, expensive and requires an electron microscope.

Molecular methods. Double-stranded RNA (dsRNA) is extracted from infected plants and purified. The purified dsRNA are loaded onto a gel matrix, after running the gel certain band patterns appears which signals the presence of a virus. The disadvantage of the test is that large amount of virus-infected tissue is needed; it is time consuming and not many samples can be processed at one time.
Another molecular method, nucleic acid hybridization, uses the efficient base pairing between a viral template (virus genetic material), immobilised on a solid support and a detecting, complementary labelled copy (probe) for detection of the molecules.

A more sensitive and strain specific detection method was presented in the 1990s, which targeted the nucleic acid (genome) of the virus, namely the reverse transcription polymerase chain reaction (RT-PCR) technique. Primers are designed to copy specific regions in the genome. Because the GLRaVs are all members of the *Closteroviridae* family, primers representing conserved sequences of the heat shock protein (signature feature of this family) were designed for use in the RT-PCR

reaction (Martin *et al.*, 2000). This is an expensive but very sensitive method and can be used to detect the presence of viruses throughout the year with results available within one day. The disadvantages are that viral genome sequences are needed for the designing of the primers to detect all the viruses of a family (conserved region) and for the detecting of serologically different isolates of a family (Golino , 2002; Hu *et al.*, 1991; Weber *et al.*, 2002). The test is also more expensive than ELISA.

2.3.4 Control measures for leafroll

Existing vineyards infected with viruses cannot be restored to a healthy state!

Plant viruses are still a major problem in the cultivation of many crops throughout the world. These pathogens have been controlled for centuries by using conventional measures like crop rotation, early detection and destruction of infected source plants, cross-protection, breeding for resistance and chemical control of their vectors (Goldbach *et al.*, 2003). Control strategies haven't much improved at present and are still essentially preventive but include sanitary selection programs which are directed to eradicate virus vectors with agrochemicals. These strategies are effective at limiting the presence of viruses in propagation material and reducing the infectious potential of vectors, but cannot eliminate the virus once it is present in the vineyard. The toxicity of the agrochemicals and their effect on the environment is also raising concerns about its usage to control virus vectors (Fuchs, 2003).

2.3.4.1 Existing control measures

Existing control measures are largely inefficient and the most promising approach to controlling GLRaV in grapevines is to use disease-free tested grapevine nursery stock. Current techniques to control GLRaV are as follows:

Plant: The introduction of natural resistance by conventional breeding against GLRaV-3 in *Vitis vinifera* is an unattainable prospect and no such breeding programmes exists in South Africa (or anywhere in the world) at present. The most effective measure is that of sanitation. Sanitation measures include the use of virus-free propagation material, insect control (to eliminate possible vectors) and weed control (to eliminate possible alternative virus hosts). These are only preventative measures and offer no real resistance to the virus but delays the onset of the disease at least for five to six years (Pietersen, 2004; Burger, 1999).

Virus: Viruses, unlike many other pathogens, cannot be killed by agro-chemicals. The application of chemicals can only inhibit insect vectors and thus at best can only prevent the spread of the virus. Valuable material is subjected to heat therapy and meristem tip-culture, or somatic embryogenesis to eliminate viruses (Pietersen, 2004; Burger, 1999).

Vector: As mentioned above, the application of pesticides can control possible insect vectors. But the uncertainty about the insects that play a role in virus transmission makes this approach only a precautionary measure and not a control against the virus (Burger, 1999).

2.3.4.2 Engineered resistance to plant viruses

In the mid 80s, with the advent of biotechnologies and especially the concept of pathogen-derived resistance (PDR), and the increased knowledge of the molecular traits of grapevine viruses' new avenues were opened for the introduction of transgenic resistance into vines such as the use of natural resistance genes and RNA silencing techniques (Grunet, 1994).

Pathogen-derived resistance (PDR). PDR was first used by Sanford and Johnson in 1985 and entails the use of viral genes or part of a gene to transform plants with the purpose to block certain virus multiplication steps. Molecular characterisation of virus genomes revealed that these simple pathogens possess a few primitive genes which can be used in PDR (Goldbach *et al.*, 2003).
After the pioneering work of M.G. Mullins and colleagues who were the first to stable transform grape plants (Mullins *et al.*, 1990), several reports described the development of transgenic grapevine and *Nicotiana* plants with virus-derived gene constructs.
The most promising of these viral genes are those involved in genome replication and encapsidation and genes responsible for virus spread. Success has also been obtained by transforming plants with cDNA copies of symptom-suppressing satellites and with mutagenized movement protein (MP) or replicase subunits. Plant viral CP genes are at present presenting the most positive results and thus accomplishing CP-mediated resistance (CPMR). The CP gene was used in the first experiment to prove the viability of PDR, when Powell-Abel *et al.*, (1986) transformed and expressed the tobacco mosaic virus CP gene in tobacco plants and obtained resistance against the virus. This CPMR mode of introducing virus resistance in plants has over the years proven to be very effective (Grunet, 1994). CPMR have been used successfully in greenhouse conditions against *Tobacco mosaic tobavirus* (TMV), *Potato potexvirus* (PVX), *Alstroemeria mosaic potyvirus* (AlMV), *Cucumber mosaic* cucumovirus (CMV) and *Tobacco rattle*

tobravirus (TRV) by blocking the virus due to the presence of the transgenically expressed CP (Goldbach *et al.*, 2003).

The latest developments is the transformation of *Vitis vinifera* cultivars with the CP sequences of *Grapevine fanleaf nepovirus* (GFLV), *Arabis mosaic nepovirus* (ArMV), *Grapevine trichovirus A* (GVA) and *Grapevine trichovirus B* (GVB) (Goelles *et al.*, 2000) and of *Vitis rupestrsis* and other rootstock with different genes of GFLV, GLRaV-2 and GLRaV-3 (Krastanova *et al.*, 2000). The CP sequence of GLRaV-2 has been used to obtain transgenic *N. benthamiana* plants that show resistance to GLRaV-2 mechanically inoculated onto the *N. benthamiana* plants. The resistance was also passed through several generations (Gonsalves, 2000).

Natural resistance (R) genes. An alternative to PDR is to use transformation of natural resistance (R) genes into plants, and thus obtaining virus resistance. This technique has had success against TMV as described by Whithman *et al.*, (1996) and against PVX and *Tomato spotted wilt tospovirus* (TSWV) as described by Bendahmane *et al.*, 1997; Spassova *et al.*, 2001).

Anti-sense RNA transgenes. Anti-sense RNA has the opposite sense to mRNA, and thus forms a complex with the mRNA which could interfere with gene expression at the level of transcription, RNA processing or even at translation. It is used as a natural mechanism to regulate gene expression in prokaryote systems and anti-sense constructs have been used in plants to some success for gene inhibition (Primrose *et al.*, 2002). The expression of anti-sense RNA (CP and replicase genes) complementary to viral sequences has been used to suppress expression of certain gene from the invading virus and thus improving resistance (Melander, 2004).

Post-transcriptional gene silencing (PTGS). A high level of transgenic expression of viral genes is needed for PDR. This drawback has been overcome by introducing transgenic virus resistance, based on PTGS (Goldbach *et al.,* 2003). PTGS involves the suppression of viruses, through a sequence specific RNA degradation mechanism and was first observed in transgenic petunias and later identified as the answer to these phenomena in PDR plants by Lindbo *et al.*, (1993) and Dougherty *et al.*, (1994).

The suppression is achieved by expressing genes that are part of the pathogen's genome in transgenic plants. The transgenic RNA as well as all the RNA complementary to the transgene is degraded in a sequence specific manner (Goldbach *et al.*, 2003). The specificity of this method is high and is only functional against homologous virus and transgenic plants expressing PTGS to a specific virus are sometimes still susceptible to other viruses which pose little or no threat to the plant. But, if any of the important viruses against which PTGS are incorporated encode a PTGS

suppressor protein it could cause that the resistance to the virus be broken. Thus, studies to identify as many suppressor proteins as possible from all major viruses are of great importance for successful transgenic virus resistant plants (Di Serio *et al.*, 2002). A large proportion of tobacco plants have been transformed using PTGS to obtain immunity against *Potato potyvirus* (PVY) (Waterhouse *et al.*, 1998).

2.4 What is RNA silencing

RNA silencing is known as co-suppression or PTGS in plants, quelling in fungus (*Neurospora crassa)*, (Cogoni & Macino, 2000; Hammond *et al.*, 2001), RNA interference (RNAi) in nematodes (Fire, 1999), insects (Kennerdell & Carthew, 1998) and mammals (Wianny & Zernicka-Goetz, 2000), and is also present in Trypanosoma brucei (Ratcliff *et al.,* 2001; Simón-Mateo *et al.*, 2003; Simpson, 2002). It is seen as an ancient defence system which protects the genome by degrading transgenes, endogenous genes and transposons. It also plays an important role in antiviral genome defence by degrading viral RNA which produces abRNA or dsRNA in the host cell when they become active (Simón-Mateo *et al.*, 2003; Tuschi, 2001; Vanitharani *et al.*, 2005). Some of the components of RNA silencing also interferes with the regulation of developmental timing by means that the components are interconnected with gene regulatory mechanisms (Bernstein *et al.*, 2001a)

Double stranded RNA (dsRNA). All of the different silencing pathways are connected by one common aspect – dsRNA. The first hint that dsRNA played an important role in RNA silencing came from the experiments of Guo & Kemphues (1995). They attempted to use an anti-sense RNA approach to inactivate a *Caenorhabditis elegans* gene, instead they found that injecting sense RNA to the *par-1* gene in the gonad of the nematode induced par-1 null phenocopies at the same frequency as injection of anti-sense RNA and was actually as effective as the anti-sense RNA at inhibiting gene function (Mello & Conte, 2004). The mystery of the involvement of dsRNA in *C. elegans* was solved in 1998 by Fire and Mello who showed that the injection of dsRNA and not single stranded, anti-sense RNA or unrelated dsRNA for specific genes, caused the disappearance of the specific gene products from both the somatic cells and the F1 progeny in *C. elegans* (Coburn & Cullen, 2003; Denli & Hannon, 2003; Geley & Müller, 2004; Kusaba, 2004; Simpson, 2002).

In 1998 Waterhouse and co-workers provided the first evidence that dsRNA might be a trigger of RNA silencing in plants. He reported that plants carrying both a sense and an anti-sense transgene homologous to a viral genome were much more resistant to that virus than were plants

carrying either a sense or an anti-sense transgene alone (Jorgensen, 2003). He also demonstrated that constructs producing RNAs capable of duplex formation, either via hairpin or panhandle constructs were more potent inducers of gene silencing than constructs that produce RNAs of only sense or anti-sense polarity (Jorgensen, 2003; Waterhouse *et al.*, 1998). Complete sequence homology between the dsRNA and the target RNA is not required for degradation (Hutvágner *et al.*, 2000) and the minimum length of dsRNA necessary to induce silencing is about 21 nucleotides.

Sources of double stranded RNA (dsRNA). The two main factors inducing the formation of dsRNA in plants are viruses and transgenes. dsRNA can be produced from invader DNA in the nucleus (transgene, transposons, rearranged genes or DNA virus), cytoplasm (RNA viruses) (Covey, 2000) or the dsRNA can be naturally derived from the transcription of inverted-repeat loci or replicating exogenous RNAs by host-or viral-encoded RNA-dependant RNA polymerase (RdRp) (Finnegan *et al.*, 2001; Geley & Müller, 2004; Tuschi, 2001; Vanitharani *et al.*, 2003; Yu & Kumar, 2003). dsRNA can also be transiently delivered by biolistic bombardment of plants with nucleic-acid-coated beads or by infiltrating plant cells with transgene-carrying *A. tumefaciens* (agro-infiltration). Plants can also be infected with a virus or by making use of a virus vector (virus-induced gene silencing), *in vitro* transcription vectors or by a stem-loop expression system to induce the formation of dsRNA (Hammond *et al.*, 2001; Sijen & Kooter, 2000; Yu & Kumar, 2003).

2.4.1 Various appearances of RNA silencing

Transgene silencing. Transgene silencing is initiated by the introduction of foreign nucleic acids (transgenes) into plant cells, if the transgenes is homologous to an endogenous gene the endogenous gene can also be silenced by a process called co-suppression as seen in the petunia plant (Primrose *et al.*, 2002). The transgene does not have to encode a functional protein in order for co-suppression to occur, but the dose of RNA / DNA is important, as RNA silencing occurs more often in plants homozygous for a transgene locus. Three different classes of transgenes such as sense, inverted-repeat (IR) and amplicons have been shown to trigger RNA silencing, as well as abRNA. With sense transgenes the plant cell recognise the abRNA and converts it into dsRNA by the activity of a plant encoded RdRp (Primrose *et al.*, 2002; Sijen & Kooter, 2000). The RNA silencing is especially high if sense and anti-sense transcripts are expressed simultaneously, or when the sense transgenes anneal with anti-sense transcripts derived from IR by read-through transcription and form dsRNA (Kubota *et al.*, 2003; Sijen & Kooter, 2000; Tuschi, 2001; Wang & Waterhouse, 2001). The expression of the affected transgene is reduced or totally abolished. The

frequency and extent of RNA silencing is also increased when the transgenes are expressed using strong promoters or when the RNA is more stable (Sijen & Kooter, 2000).

RNA silencing by ectopically delivered DNA molecules. RNA silencing can be initiated by DNA molecules homologous to a transgene that are delivered by leaf infiltration of *A. tumefaciens,* by DNA Geminivirus vectors or via bombardment of DNA-coated gold particles (biolistics). The introduced DNA interacts with the homologous transgenes or endogenous genes to initiate RNA silencing (Bruening, 1998; Sijen & Kooter, 2000).

RNA-directed DNA methylation (RdDM). Plant RNA silencing is frequently accompanied by dense DNA methylation at the cytosines in transcribed regions of the silenced genes (Wang & Waterhouse, 2001; Waterhouse *et al.*, 2001a). RdDM is responsible for this sequence-specific methylation associated with RNA silencing (Matzke *et al.*, 2003) and requires dsRNA that is cleaved into small RNAs (~20 nt in length). DNA sequences complementary to the guide RNA become modified, suggesting direct RNA-DNA interactions (Matzke *et al.*, 2001). Although the phenomenon of RdDM is well established in plants, the identity of the DNA methyltransferase that catalyses it and the RNA species that triggers it, is still unknown (Matzke *et al.*, 2003; Sijen & Kooter, 2000).

Homology-dependant gene silencing (HDGS). HDGS is caused by the integration of multiple copies of a transgene in the plant and homologous endogenous genes are often co-suppressed. The suppression of the transgene can occur at the transcriptional or post transcriptional level (Fire, 1999; Sijen & Kooter, 2000).

Transcriptional gene silencing (TGS). TGS is generally observed in plants but has also been seen in animals. Gene expression is reduced by a blockade at the transcriptional level. Evidence indicates that TGS usual occurs when more than one copy of a transgene is inserted and is sometimes accompanied by chromatin modification, such as condensation, or DNA methylation (Himber *et al.*, 2003; Vaucheret *et al.*, 1998).

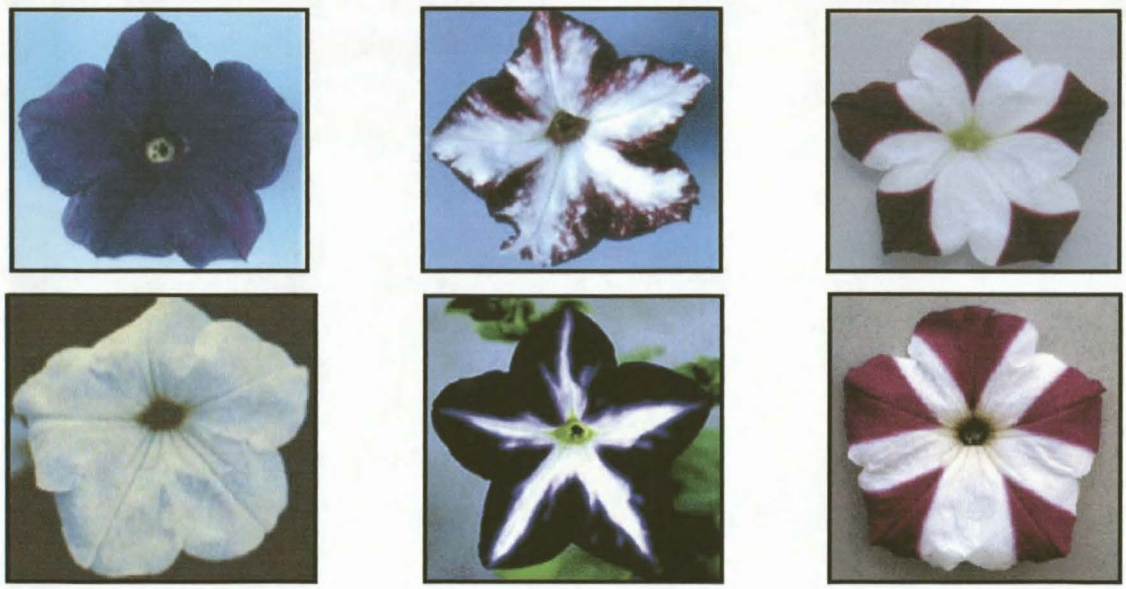
Post Transcriptional gene silencing (PTGS). PTGS is the silencing of an endogenous gene caused by the introduction of a homologous dsRNA, transgene or virus and leads to the degradation of homologous RNA in the cytoplasm and no mRNA accumulates although transcription has occurred (Himber *et al.*, 2003; Vaucheret *et al.,* 1998). PTGS was first identified

in *C. elegans* (Ramaswamy & Slack, 2002) and leads to the specific degradation of mRNAs with complementary sequence to the dsRNA.

The purpose of this project was to utilise PTGS as a possible virus resistance mechanism in *N. benthamiana* plants against leafroll disease.

2.5 Post-transcriptional gene silencing (PTGS)

PTGS was first observed in 1990 but was actually already present in plants since 1828 when breeders making crosses between petunia plants produced flowers with a star pattern of white stripes. This was achieved by accidental duplication of the chalcone synthase gene (CHS) (Day, 2000). The same happened with plant biotechnologists, Rich Jorgensen and his team when they attempted to construct transgenic plants with more desirable characteristics by over expressing endogenous genes. The aim was to try to deepen the hue of purple in the colour of petunias by inserting an extra copy of the CHS behind a strong promoter. Unexpectedly, many plants failed to express the introduced transgene and the results were white, variegated or/and purple flowers (Baulcombe, 2000; Hammond *et al.*, 2001; Jorgensen, 2003; Marathe *et al.*, 2000) (Figure 2.10). These observations showed the first demonstration of PTGS, and were originally termed co-suppression, because the introduced transgenes and the homologous endogenous genes were simultaneously switched off, resulting in the loss of pigmentation (Voinnet, 2001; Wang & Waterhouse, 2001).



http://ag.arizona.edu/pls/faculty/jorgensen.html; www.bbc.co.uk/.../frontiers_20032303.shtml; Matzke & Matzke, 2004

Figure 2.10: Different petunia flowers obtained after insertion of an extra CHS gene behind a strong promoter to deepen the purple hue of the flowers.

PTGS is a complex, sequence-specific surveillance system that targets alien RNA, for degradation. The alien RNAs can be genomes of RNA/DNA viruses, transgenes or endogenous genes and can be double (ds) or single-stranded (ss) molecules that have homology to the target RNA present in the cell (Dunoyer *et al.,* 2002; Vanitharani *et al.*, 2003; Waterhouse *et al.*, 2001a). Once activated, it can virtually eliminate all RNA with homology to the inducer sequences from all tissues (Thomas *et al.,* 2003). The specific mRNA is reduced by sequence-specific degradation of the transcribed mRNA without an alteration in the rate of transcription of the target gene itself (Tuschi, 2001). The silencing occurs at single cell level (suppression of homologous genes in the cells in which dsRNAs accumulated) and generates a mobile silencing signal through the whole plant (systemic silencing). Silencing of the mRNA is maintained even after removal of the source of the initiator RNA (Jorgensen, 2003).

PTGS is widely used as a tool for inactivating gene expression (Vanitharani *et al.*, 2003) and to create novel traits in crop plants. It has been used to alter the ripening time in tomatoes to produce fruits that are easier to process and store and which are more resistant to specific pathogens (Angell & Baulcombe, 1999).

2.5.1 Mechanism of PTGS

Several models have been proposed to explain the sequence-specific degradation system in plants, considering mainly the roles of RNA thresholds, DNA repeats, ectopic pairings and abRNA. At first it was suggested that the high copy number of the transgenes produced excessively high levels of transgene mRNA and that this level induced the degradation system. Other opinions were that the methylation of the transgene made them produce abRNA and that this aberrance induced the system. A current model and the most convincing one, proposes that the system is induced and directed by dsRNA, since dsRNA is not common in plant cells. The model suggests that dsRNA forms a complex with an enzyme called Dicer, which cleaves fragments of ~21 nucleotides of sense and anti-sense RNA called short interfering RNAs (siRNAs). Some of these siRNAs hybridize with homologous single-stranded RNAs, which bind more Dicer complexes. The other siRNAs assemble with one or more protein components to form an RNA-induced silencing complex (RISC). After an ATP-dependent conversion to a single strand conformation, siRNAs recognise and bind to homologous RNA after which an endonuclease cleavage of the target RNA occurs. This model also suggests that PTGS can be divided into three distinct steps (1) initiation, (2) effector and (3) spread and maintenance (Eckardt, 2002; Hammond *et al.*, 2001; Matzke *et al.*, 2001; Waterhouse *et al.,* 2001b).

2.5.1.1 Initiation phase (dsRNA processed into siRNAs)

DsRNA, which can be introduced experimentally or arise from endogenous transposons, replicating RNA viruses (Figure 2.11A), or the transcription of transgenes (Waterhouse *et al.*, 1999) triggers the silencing pathway. The initiation phase involves the cleavage of the dsRNA into siRNAs of 21-23 nucleotides with 2-nucleotide 3' overhangs, which corresponds to both sense and anti-sense strands of the target gene (Figure 2.11B) (Yu & Kumar, 2003). The cleavage of dsRNA occurs mainly from the ends of both blunt-ended dsRNAs and proceeds in ~ 21-23-nt steps, to generate double-stranded siRNAs in an ATP-dependant step (Figure 2.11C). This cleavage is mediated by an RNase-III-like dsRNA-specific ribonuclease, named Dicer (Dykxhoorn *et al.*, 2003; Tuschi, 2001; Zamore, 2002) which is evolutionary conserved in nematodes, insects, plants, fungi and mammals (Cerutti, 2003).

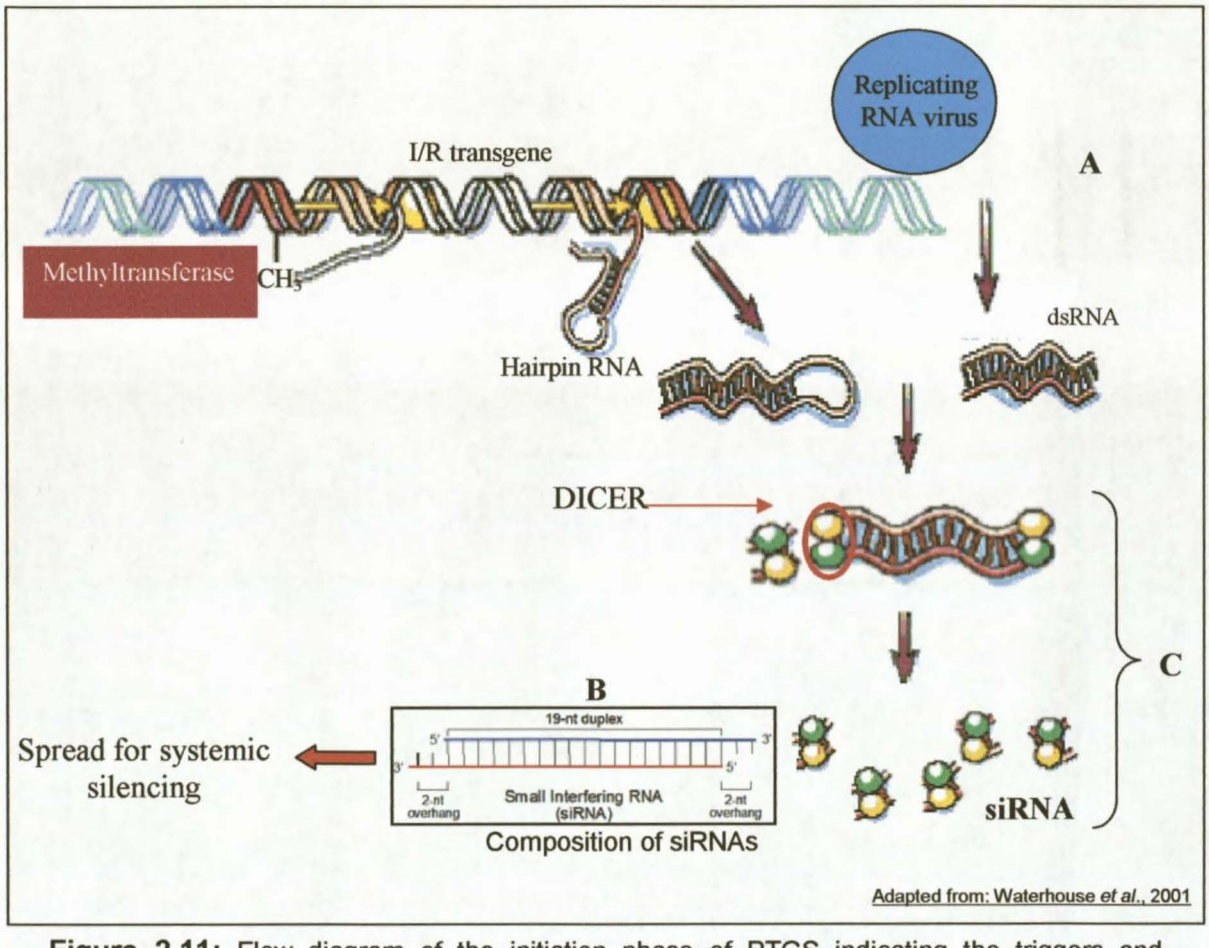


Figure 2.11: Flow diagram of the initiation phase of PTGS indicating the triggers and components involved in the process.

Dicer was discovered in 2001 by Berstein and colleagues when they analysed the genomes of *Drosophila* and *C. elegans* for RNase-III-like activity since similarities was observed between siRNAs and the products of RNase-III nucleases. Three nucleases that had dsRNA specificity; RNase-III, Drosha and CG4792 were found. To prove the involvement of RNase-III from *Drosophila*, it was depleted from cells which resulted in the loss of the ability to silence an endogenous transgene and the RNase-III nuclease was named Dicer.
The members of the Dicer protein family have a distinctive structure, which includes an ATP-dependant, N-terminal RNA helicase domain, a C-terminal dsRNA-binding domain, a PAZ (Piwi/Argonaute/Zwille) protein-protein interaction domain as well as two RNase-III domains (Berstein *et al.*, 2001b; Hutvágner & Zamore, 2002; Melander, 2004; Moss, 2001).

Baulcombe and Hamilton discovered in 1998 a small duplex RNA species, of ~21 nucleotides (nt) long containing two nucleotide 3'-overhangs with a 5'-monophosphate group and a free 3'-hydroxyl group in plants undergoing either co-suppression or virus-induced gene silencing, but was absent in plants that did not display silencing. These small duplexes was short interfering RNAs (siRNAs) formed by Dicer (Hamilton & Baulcombe,1999; Hammond *et al.*, 2001; Hutvágner *et al.*, 2000; Hunter, 2000; Kusaba, 2004; Simpson, 2002; Waterhouse *et al.*, 2001a).
It was later found by Voinnet *et al.*, (2000) that two size classes of siRNAs exist in plants. These are, 'short siRNA' of 21-22 nt and 'long siRNA' of 24-26 nt in length. The 21-nt species correlates with sequence-specific degradation of target mRNAs (intracellular silencing) and is sufficient for RISC-mediated cleavage of target transcripts. The 24-nt species is essential for triggering systemic gene silencing and correlates positively with DNA methylation (Denli & Hannon, 2003; Hunter & Poethig, 2003; Waterhouse, 2001).
Over 50% of the siRNAs formed by Dicer are exactly 21-nt in length and corresponds to both the sense and anti-sense sequences of the specific co-suppressed genes. After being processed by Dicer the duplex siRNAs are unwound, leaving the anti-sense strand to guide a RISC to its homologous target mRNA (Figure 2.12). Only siRNAs which are phosphorylated on the 5' region can enter the RISC complex. The target mRNA is cleaved at the centre of the siRNA within the relevant RISC, resulting in over 90% inhibition of target gene expression (Dykxhoorn *et al.*, 2003; Hunter & Poethig, 2003; Pélissier & Wassenegger, 2000; Silhavy & Burgyán, 2004; Yu & Kumar, 2003).

Another class of small interfering RNAs called micro-RNAs (miRNAs) have recently been identified. They are similar to siRNAs and comprises of 2-nucleotide-long 3' overhangs and 5'-phosphate/3'-hydroxyl ends and are most likely processed in the nucleus. Most pre-miRNAs are

encoded in intergenic regions and are probably transcribed from autonomous promoters (Cerutti, 2003; Silhavy & Burgyán, 2004). The miRNAs are generated from endogenous dsRNA hairpin precursors by a Dicer homologue, Carpel Factory (CAF), referred to as Dicer-like 1 (DCL1) (Szittya *et al.*, 2003; Voinnet, 2003).

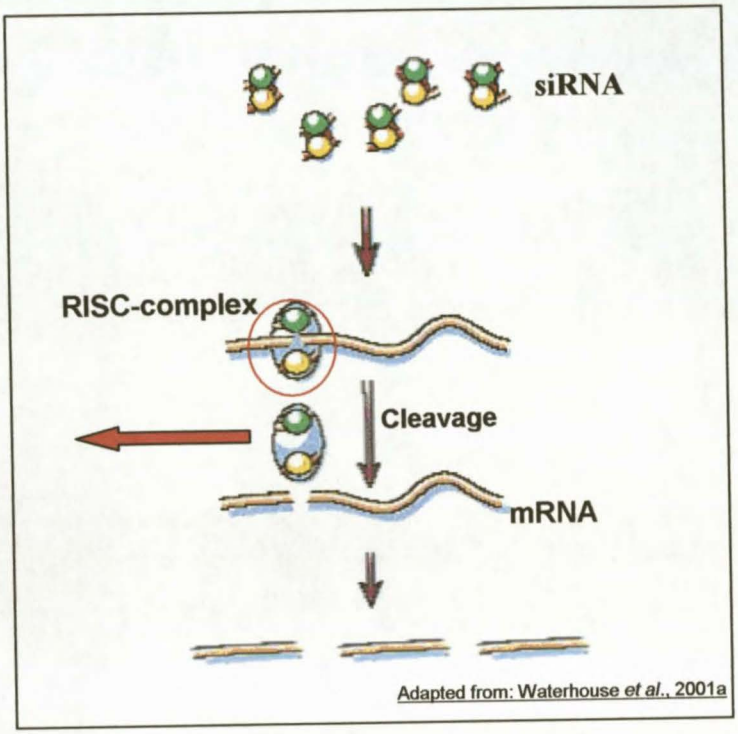


Figure 2.12: Flow diagram depicting the effector phase of the silencing pathway showing the degradation of mRNA by the RISC-complex with integrated siRNA.

2.5.1.2 Effector Phase (assembly of siRNAs)

In the effector step, the double-stranded siRNAs are recruited into the multi-protein complex RISC (Szittya *et al.*, 2002) and undergoes an ATP-dependant activation step that results in the unwinding of the double-stranded siRNAs. Each RISC appears to include a single siRNA, an endonuclease, an exonuclease, a helicase and an mRNA-homology-searching activity (Yu & Kumar, 2003). Once, activated RISC uses the single-stranded siRNA as a guide to identify complementary mRNA by Watson-Crick base pairing and cleaves the target RNA across from the centre of the region paired with anti-sense siRNA, which results in the complete degradation of the target mRNAs (Figure 2.12) (Cerutti, 2003; Coburn & Cullen, 2003; Elbashir *et al.*, 2001; Yu & Kumar, 2003).

The way in which the incorporated siRNA mediate the cleavage and destruction of the target RNA is still unclear but two competing models have been proposed. The 'random degradative PCR model' proposes that the siRNAs act similar to PCR primers to produce dsRNA from the single stranded template. The "guide" molecules on the target mRNA is extended by the plant RdRp in a type of polymerization reaction to form dsRNA. However it has been shown that plant RdRp is not required for virus-induced gene silencing; the viral RdRp synthesizes its own complementary viral RNA without guide RNAs (Baulcombe, 2004; Bernstein *et al.*, 2001b; Matzke *et al.*, 2001; Mello & Conte, 2004; Plasterk, 2002; Ramaswamy & Slack, 2002; Simpson, 2002; Silhavy & Burgyán, 2004; Sijen & Kooter, 2000).
The 'endonucleolytic cleavage' model suggest that the siRNAs combines with proteins in an ATP dependant step in which the siRNAs are unwound and guide the activated RISC to cleave the target. This model is supported by many *in vitro* studies (Ramaswamy & Slack, 2002).

RISC also exploits ribosomal localization as a mechanism to improve the efficiency with which it scans for potential substrates (Coburn & Cullen, 2003; Silhavy & Burgyán, 2004). After degradation RISC is released to seek additional mRNA targets, while the two fragments of the mRNA are degraded by cellular exonucleases (Cullen, 2004).

Within the initiation and effector phases there are also genes and proteins that play an important role in the silencing process. *Arabidopsis* genes involved in PTGS were discovered by screening *Arabidopsis* mutants that were defective in the silencing response (Covey, 2000; Hammond *et al.*, 2001)(Figure 2.13).

Argonaute (Ago) 1 and Ago4. PAZ and PIWI domain (PPD) proteins (also called Argonaute (Ago) proteins) are components of complexes associated with siRNA and miRNA generation and are required for developmental events in plants and other organisms (Zilberman *et al.*, 2003). The *Arabidopsis* genome encodes ten AGO proteins all identified by the presence of a central PAZ domain and a C-terminal PIWI domain (Hunter & Poethig, 2003). AGO1 is the type member of this group of proteins and mutants in the *Ago*1 gene shows severe developmental abnormalities, including sterility and is PTGS deficient. It has been suggested that the gene may play a role in the initiation step of RNA silencing by affecting the formation, stabilization or localization of dsRNA prior to siRNA formation (Hammond *et al.*, 2001; Tuschi, 2001) but recent studies have shown that AGO1 is involved in the transfer of siRNAs from DICER to RISC (Herr, 2004). Dicer interacts with the RISC complex through their shared PAZ domains present in the amino acid sequence of AGO2 found in both complexes, to incorporate the siRNAs into RISC (Hannon & Zamore, 2003;

Matzke *et al.*, 2001; Voinnet, 2001). Another PPD protein recently characterised, AGO4 is required for PTGS and DNA methylation and reduces the levels of long siRNAs (Hunter & Poethig, 2003).

Suppressors of gene silencing (sgs) 1, sgs2 and sgs3 mutants. *SGS* genes were discovered by Elmayan *et al.* in 1998 and it was proposed that these proteins play a role in PTGS by turning RNA triggers into dsRNA. The potency and systemic effects of RNA silencing has led to the proposal that an RdRp protein must be active in the triggering and amplification of the silencing effect. The RdRp recognizes abRNAs and converts them to a double-stranded form. *Sgs2* mutant plants fail to accumulate small RNAs corresponding to the sense transcript of an ectopically expressed gene and thus cannot silence a transgene but, can accumulate siRNAs to an endogenous replicating virus. This can be explained by the presence of a viral RdRp that substitute the cellular RdRp in the silencing mechanism (Maine, 2000; Hammond *et al.*, 2001; Tuschi, 2001). *SGS3* is a novel gene, unique to plants, and contains a nucleic acid binding domain, which has no significant similarity to other known proteins in plant or other kingdoms (Finnegan *et al.*, 2001). *Sgs3* mutants are PTGS deficient and this may be accomplished by the gene which interferes with the synthesizing of the dsRNA initiator of silencing (Finnegan *et al.*, 2001; Hunter & Poethig, 2003).

Silencing defective gene (sde) 1, sde3 and sde4 mutants. In 2000, Dalmay and co-workers discovered the *SDE1* gene. *SDE1* encodes the same protein (RdRp) as *SGS2* and mutant plants fails to accumulate siRNAs. The *SDE3* gene encodes a putative RNA helicase gene with sequence similarity to RNA helicase-like proteins conserved in all kingdoms and is necessary for the initiation of PTGS (Zilberman *et al.*, 2003). This is probably possible because the RNA helicase contributes to the production of dsRNA by creating additional single-stranded RNA templates by RNA unwinding for an RdRp (Hunter & Poethig, 2003; Willmann, 2001). Another gene *SDE4* is responsible for PTGS viral defence but also plays a role in chromatin silencing (Herr, 2004).

Carpel Factory (CAF1) and methyl-transferase (met1) gene. Carpel Factory (CAF) is predicted to be a nuclear protein, a putative equivalent of the Dicer homolog found in *Arabidopsis*, and has been implicated in the generation of miRNAs. It has an RNaseIII motif, RNA helicase domain and a PAZ domain. Dramatic developmental defects are observed in mutants for this gene. The maintenance *MET1* gene encodes a methyl-transferase that drives the methylation of the coding sequences of PTGS-inducing transgenes (Bernstein *et al.*, 2001b; Waterhouse *et al.*, 2001b).

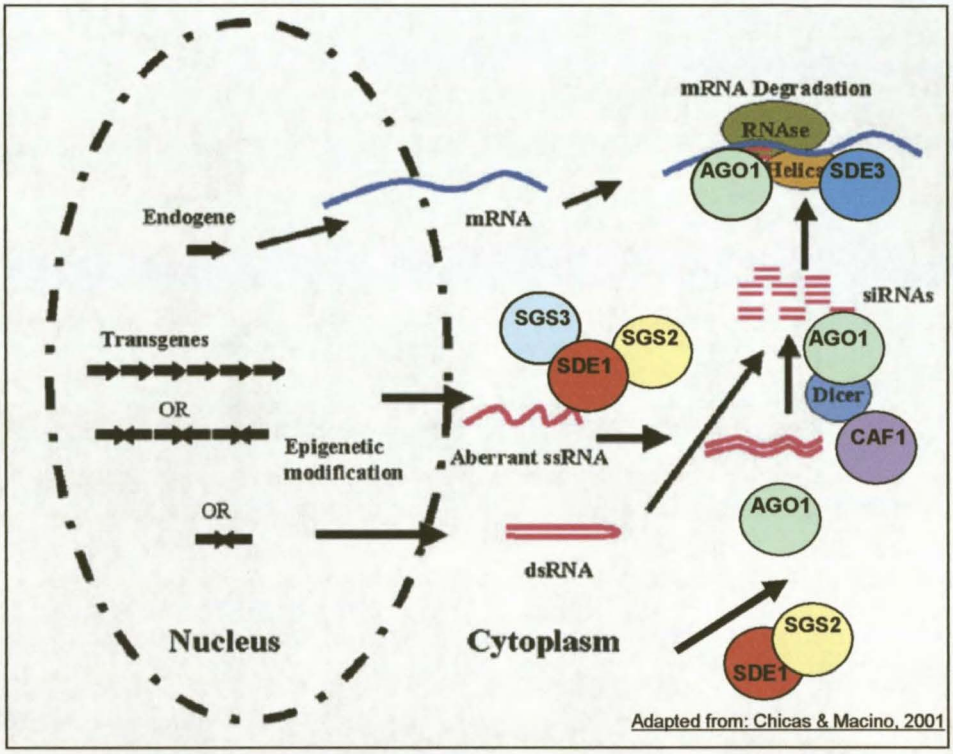


Figure 2.13: A presentation of an *Arabidopsis* plant cell showing the locations of genes and proteins involved in the silencing pathway identified by screening *Arabidopsis* mutants that were defective for silencing.

2.5.2 Maintenance and Systemic propagation of PTGS

PTGS generates a mobile silencing signal that is non-cell autonomous, thus it travels through the plant from the site of initiation to other tissue to induce sequence-specific RNA silencing (Figure 2.14). This was most convincingly demonstrated by grafting experiments in tobacco plants. Non-silenced plants became silenced when grafted onto silenced stocks but non-silenced stocks do not become silenced when a silenced scion is grafted onto the plants. This indicated the signal was unidirectional. The experiments also showed that the signal had 100% efficiency between a silenced stock and a grafted target scion expressing the corresponding transgene and even persisted after the graft union had been broken but had no effect on target scions with a non-homologous transgene (Palauqui *et al.*, 1997; Vaucheret *et al.*, 1998). *Agrobacterium* infiltration experiments also indicated the presence of a mobile silencing signal since top leaves of a plant also showed silencing after being transiently infiltrated on the bottom leaves (Dalmay *et al.*, 2000; Vaucheret *et al.*, 2001; Waterhouse *et al.*, 2001a). It appears that an interaction between the signal and the target gene facilitates the spread and maintenance of PTGS (Waterhouse *et al.*, 1999).

This ability of the silencing agent to move within the plant was named systemic acquired silencing (SAS) (Palauqui *et al.*, 1997). The diffusible signal is capable of travelling both between cells via plasmodesmata and long distances via the vascular system (phloem) and could move either with or ahead of the virus to silence the viral RNA before or at the same time as the virus moves into a cell (Baulcombe, 2004).

The sequence specificity of the signal strongly implies that it is a nucleic acid, most likely dsRNA, siRNAs or abRNA (Baulcombe, 2004; Eckardt, 2002; Tuschi, 2001). siRNAs have been excluded from the list since studies have found that eliminating siRNAs does not affect the spread of silencing (Mallory *et al.*, 2001).

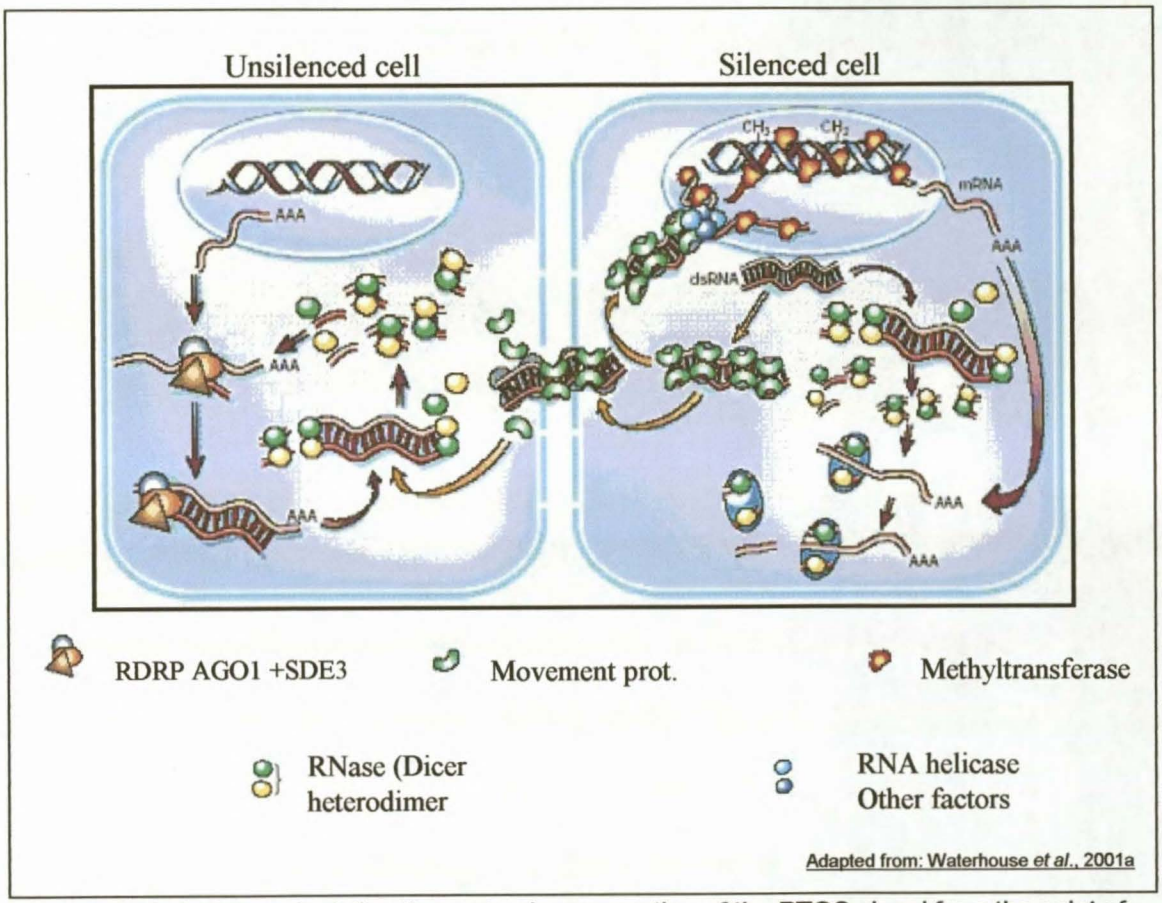


Figure 2.14: A picture depicting the systemic propagation of the PTGS signal from the point of initiation in the silenced cell to the unsilenced plant cell (yellow arrows) and the different genes, proteins and components involved.

2.6 How plants and viruses deal with PTGS

For decades, the use of mild stains of viruses to protect plants from infection by more severe virus strains or closely related viruses has been used to control viral diseases in plants. The rationale for this form of vaccination, called 'cross-protection', came from classic observations that many infections in plants prevent the secondary accumulation of viral strains that are closely related to the primary-infecting virus. For many plant viruses, the mechanism underlying cross-protection is a post-transcriptional and RNA-mediated process that targets the secondary-challenged virus in a nucleotide-sequence specific manner. After the discovery of dsRNA as a potent inducer of PTGS and that most plant viruses replicate via a dsRNA intermediate it was certain that PTGS is a part of a defence system in plants against viruses (Linbo *et al.*, 2001; Lu *et al.,* 2003).

The RNA defence system is remarkable in its ability to adapt to any virus, because its specificity is not genetically programmed by the host but, instead, is dictated by the genome sequence of the viral intruder itself (Voinnet, 2001). All genomes of complex organisms are potential targets of invasion by viruses and transposable elements. In plants, cytoplasmically replicating viruses are not just potential targets of RNA silencing; they can also induce RNA silencing.

2.6.1 PTGS as a natural viral defence mechanism

A series of observations in plants led to the conclusion that PTGS plays an important role as a natural viral defence mechanism with the function of protecting the genome against foreign RNA, especially viruses (Cogoni & Macino, 2000; Finnegan *et al.*, 2001). The concept of pathogen-derived resistance (PDR) provided one of the first indications that PTGS plays a role in viral defence by showing that transgenic plants expressing a region of a specific plant virus genome were resistant to that virus, and that the same kind of resistance could be obtained against non-viral transgenes or untranslatable virus proteins in a process called RNA-mediated virus resistance (RMVR) (Fire, 1999; Waterhouse *et al.*, 1999). The natural interactions between plants and viruses in which a plant 'recovers' from a viral infection, displaying PTGS-like activity in the 'recovered' tissue as well as the use of cross-protection in which wild virus strains are inoculated into plants to control viral diseases, also strengthened the idea of PTGS as a viral defence mechanism (Elbashir *et al.*, 2001; Linbo *et al.*, 2001; Vance & Vaucheret, 2001). It was also observed that plants defective in PTGS were also hypersensitive to the infection of certain viruses. The final clue for PTGS as a virus-defence mechanism came from the viruses themselves. It was shown that several different plant viruses encode proteins that suppress RNA silencing suggesting that they

have evolved to overcome the PTGS mechanism which hinders them from infecting plants (Vance & Vaucheret, 2001; Voinnet, *et al.*, 1999).

2.6.2 Viruses as Targets, Inducers and Inhibitors of PTGS

Targets. Plants target viruses and can exhibit two different types of resistance to the viruses. When transgenes, expressing part of a virus genome are introduced into a plant it can lead to resistance of the plant to infection by the same virus. If the transgene is silenced by PTGS before infection, the plant will have immunity against the virus and no symptoms will appear. If the transgene is silenced after infection has already occurred the plant will show an initial phase of infection but will recover from the virus (Vaucheret *et al.*, 1998; Vaucheret *et al.*, 2001).

Inducers. Plant viruses enter the plant at a small wound, replicate within the cells, and then move cell-to-cell until they reach the vascular tissue. The majority of plant viruses (>90%) are single-stranded RNA viruses. These viruses are strong inducers of RNA silencing since they replicate by a virus-encoded RdRp, which produces a dsRNA intermediate and thus triggers the sequence-specific degradation of the viral RNA (Eckardt, 2002; Ratcliff *et al.*, 2001; Waterhouse *et al.*, 2001a). In the later stages of the infection, as the rate of viral RNA replication increases, the viral dsRNA and siRNA would become more abundant. Eventually the viral siRNA would be targeted intensively and virus accumulation would slow down. If the virus moves faster, and reaches the vascular tissue before the silencing signal it can establish a systemic infection. If not, the virus will enter the vascular tissue to find RNA silencing already established, and the infection will be aborted (Vance & Vaucheret, 2001).

Virus induced gene silencing (VIGS) can also be exploited to induce PTGS of endogenous or transgenes (Vaucheret *et al.*, 2001). VIGS is a virus vector technology that exploits PTGS, by silencing the expression of a plant transgene or an endogenous gene by infection of a plant with a recombinant virus carrying part of the gene sequences. Only replication competent viruses can be used for VIGS, since the virus RdRp is necessary to produce the activator, dsRNA (Baulcombe, 1999; Bruening, 1998; Hammond *et al.*, 2001; Primrose *et al.*, 2002; Vance & Vaucheret, 2001; Vanitharani *et al.*, 2005). The dsRNA replication intermediate are processed in a sequence-specific manner so that the siRNA in the infected cell would correspond to parts of the viral vector genome, including any non-viral insert, this would lower the titer of the invading virus and thus leading to viral RNA degradation. If the insert is from a host gene, the siRNAs would lead the RNase complex

to the corresponding host mRNA and the symptoms in the infected plant would reflect the loss of the function in the encoded protein (Faivre-Rampant *et al.*, 2004; Lu *et al.*, 2003).

Inhibitors. Viruses can also inhibit the onset of PTGS by encoding suppressor proteins to silence PTGS. The suppressor proteins can silence PTGS without the presence of the virus (Vaucheret *et al.*, 2001).

2.6.3 Viral-encoded Suppressors

Since PTGS is an anti-viral defence mechanism in plants and virus infected plants are still present in our community, viruses must have evolved a counter defence mechanism to defend themselves against PTGS (Voinnet, 2001). In 1998 an important discovery was made by three independent research groups that exposed the viruses' secret; they encode proteins that suppress PTGS, to ensure their survival. This was observed in experiments in which silenced transgenes in plants were reactivated by the infection of certain viruses (Brigneti *et al.,* 1998; Kasschau & Carrington, 1998; Savenkov & Valkonen, 2002; Silhavy & Burgyán, 2004; Yamashita *et al.*, 1998). Studies of synergistic viral diseases showed that the co-inoculation of two independent viruses of which one was a potyvirus led to more extreme symptoms and the accumulation of the normal innocuous virus. This synergism was the result of suppression by a suppressor protein present in the potyvirus (Marathe *et al.*, 2000; Voinnet, 2001).

More than a dozen silencing suppressors have been identified from different types of viruses, by using different gene silencing assays and are shown in Table 2.1 (Thomas *et al.,* 2003). Suppressor proteins are often found to be pathogenicity determinants, and thus enhances pathogenicity and act directly or indirectly at any step of the silencing pathway (Dong *et al.*, 2003). They sometimes bind to and sometimes inactivate siRNAs so that they don't target the corresponding viral RNAs and thus siRNAs are prevented form incorporating into the active RISC (Baulcombe, 2004).

Table 2.1: Showing the various suppressors identified from different DNA and RNA viruses (Silhavy & Burgyán, 2004).

Genus	Virus	Suppressors
Begomovirus	Tomato yellow leaf curl virus	C2
Carmovirus	Turnip crinkle virus (TCV)	P38
Closterovirus	Beet yellow virus (BYV)	p21
	Beet yellow stunt virus (BYSV)	P22
Cucumovirus	Cucumber mosaic virus (CMV)	2b
	Tomato aspermy virus (TAV)	2b
Furovirus	Beet necrotic yellow vein virus (BNYVV)	P14
Geminivirus	African cassava mosaic virus (ACMV)	AC2
	Tomato yellow leaf curl virus-China (TYLCV-C)	C2
Hordeivirus	Barley stripe mosaic virus (BSMV)	b
	Poa semilatent virus (PSLV)	
Pecluvirus	Peanut clump virus (PCV)	P15
Poleovirus	Beet western yellows virus (BWYV)	PO
	Cucurbit aphid-borne yellows virus (CABYV)	
Potexvirus	Potato virus X (PVX)	p25
Potyvirus	Potato virus Y (PVY)	HC-PRO
	Tobacco etch virus (TEV)	HC-PRO
	Turnip mosaic virus (TMV)	HC-PRO
Sobemovirus	Rice yellow mottle virus (RYMV)	P1
	Cocksfoot mottle virus	P1
Tenuivirus	Rice hoja blanca virus (RHBV)	NS3
Tobamovirus	Tomato mosaic virus	P130
Tombusvirus	Tomato bushy stunt virus (TBSV)	P19
	Cymbidium ringspot virus (CymRSV)	P19
	Artichoke mottled crinkle virus	P19
	Carnation Italian ringspot virus	P19
Tospovirus	Tomato spotted wilt virus (TSWV)	NS_S

Do all plant viruses have viral suppressor proteins of silencing? Although many different viral suppressors have been identified, a lot of viruses do not effectively suppress silencing. Such viruses may have evolved other ways to try to avoid silencing, such as by replicating within spherules in the endoplasmic reticulum (ER), where the dsRNA is hidden, or by replicating and moving rapidly enough to outrun the mobile silencing signal. Plants may also have other ways of protecting themselves against viruses and thus viruses can possess suppressors of other pathways or systems (Roth *et al.*, 2004). The only way to ensure that all known suppressors are identified is to screen the genomes of all the major important viruses for the presence of suppressor proteins.

Diversity and evolution of silencing suppressors. An aspect of the virus-encoded suppressors is their high diversity in structure. No sequence homology, no similarities on nucleic acid level or protein level are shared (Silhavy & Burgyán, 2004; Voinnet, 2001). They are also functionally diverse, as revealed by their distinct effects in overcoming the different stages of PTGS, including synthesis or movement of the systemic signal (Hamilton *et al.*, 2002). It is speculated that the suppressors have evolved as additional features of unrelated proteins that already had diverse functions and this can reveal an evolutionary convergence (Voinnet, 2001). Some of the well known suppressors are pathogenicity determinants of their respective viruses and thus might lead us to think that more suppressors can be identified by isolating the pathogenicity determinants of respective viruses (Voinnet *et al.*, 1999).

2.6.3.1 Identification of suppressors by silencing assays

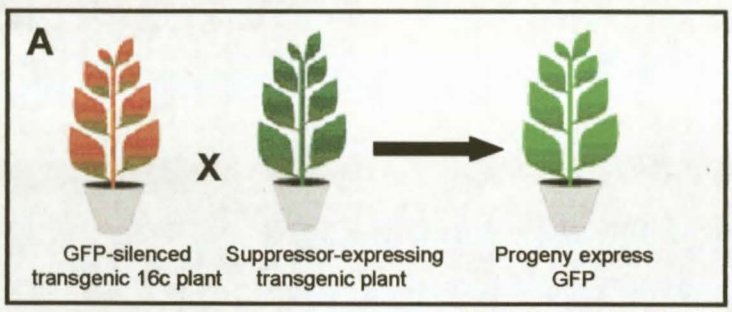
Three major assays have been used to identify plant viral suppressors of RNA silencing: (1) Stable expression assay, (2) Reversal of silencing assay, and (3) Transient expression assay (Roth *et al.*, 2004).

All of these assays require a few basic components. The key component is a reporter transgene that undergoes silencing. The reporter can be silenced by the candidate suppression protein by a replicating virus vector, constitutively (genetic crosses) or by infiltration (agro-infiltration) with an *A. tumefaciens* strain carrying a Ti plasmid encoding the same transgene (Li & Ding, 2001). Other components include transgenic plants expressing the reporter gene and/or transgene silenced transgenic plants. An important consideration in identifying suppressors is to use more than one silencing assay since suppressor proteins may not all have the same effect on PTGS. If a suppressor protein is expressed after RNA silencing of a transgene is complete, suppression of the

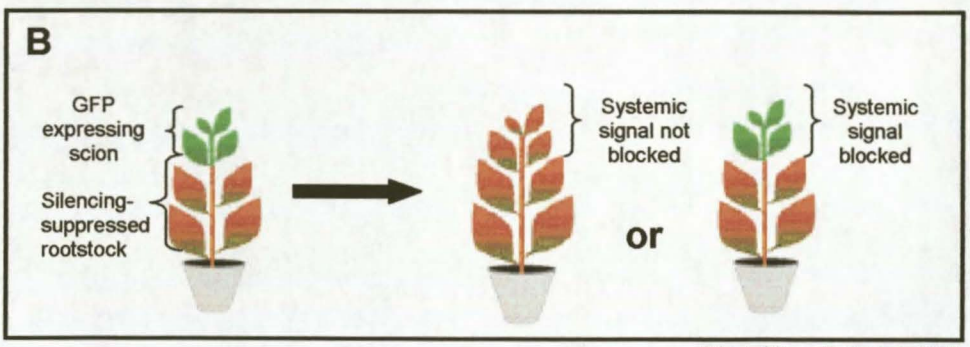
transgene occurs only if this protein is able to reverse RNA silencing (reverse silencing assay). A suppressor that targets the early stages of RNA silencing will be inactive in this assay and identification will require their expression before or during the initiation of the transgene RNA silencing such as in a transient silencing suppression assay (Li & Ding, 2001).

The first suppressor protein identified, by three independent research groups (Brigneti *et al.,* 1998; Kasschau & Carrington, 1998; Yamashita *et al.*, 1998) in a reverse silencing assay, was the helper component-proteinase (HC-Pro) from the potyvirus family. A second suppressor, the 2b protein of the CMV was later found, through a reverse silencing assay by Baulcombe's research group (Brigneti *et al.,* 1998).

Stable expression assay. In this approach a stable transgenic line expressing a candidate suppressor of silencing is crossed to a series of well-characterised transgenic lines silenced for a reporter gene (Figure 2.15A). This approach offers the opportunity to examine the effect of the suppressor on different well-defined types of transgene-induced RNA silencing, and thus providing information about the mechanism of suppression. The assay is also suited to investigate the role of suppressors in systemic silencing by using grafting techniques (Figure 2.15B).



Adapted from: Roth *et al.*, 2004



Adapted from: Roth *et al.*, 2004

Figure 2.15: Illustration of the stable expression assay showing: **(A)** Genetic crosses between a stable transgenic line and silenced transgenic lines and **(B)** Grafting assay to inspect the role of suppressors in systemic silencing.

Reversal of silencing assay. This assay was used to identify some of the first suppressors and was developed in the Baulcombe lab (Brigneti *et al.*, 1998; Marathe *et al.*, 2000; Voinnet *et al.*, 1999). It can be used to identify candidate viruses and individual viral genes that may suppress silencing as well as provide information about the mode of suppression present. The strategy is to infect a silenced plant with the candidate virus and determine whether the silenced phenotype is reversed. The most common version of this assay make use of GFP transgenic *N. benthamiana* 16c plants that are agro-infiltrated with an *A. tumefaciens* expressing a GFP transgene to obtain a silenced 16c plantlet (red in UV light) (Figure 2.16A). After silencing has been established in the entire plant, the plant is inoculated with the candidate virus. If the virus carries a suppressor the plantlet will express GFP (green fluorescence) under UV illumination (Figure 2.16B1). If not the plant will remain red under UV illumination (Figure 2.16B2).The identification of individual viral genes that suppress silencing can be done by making use of a PVX vector in a process called VIGS (Roth *et al.*, 2004). In this approach individual viral genes are cloned into a plant expression vector based on the PVX virus and are agro-infiltrated onto the silenced-GFP plants (Figure 2.16B1).

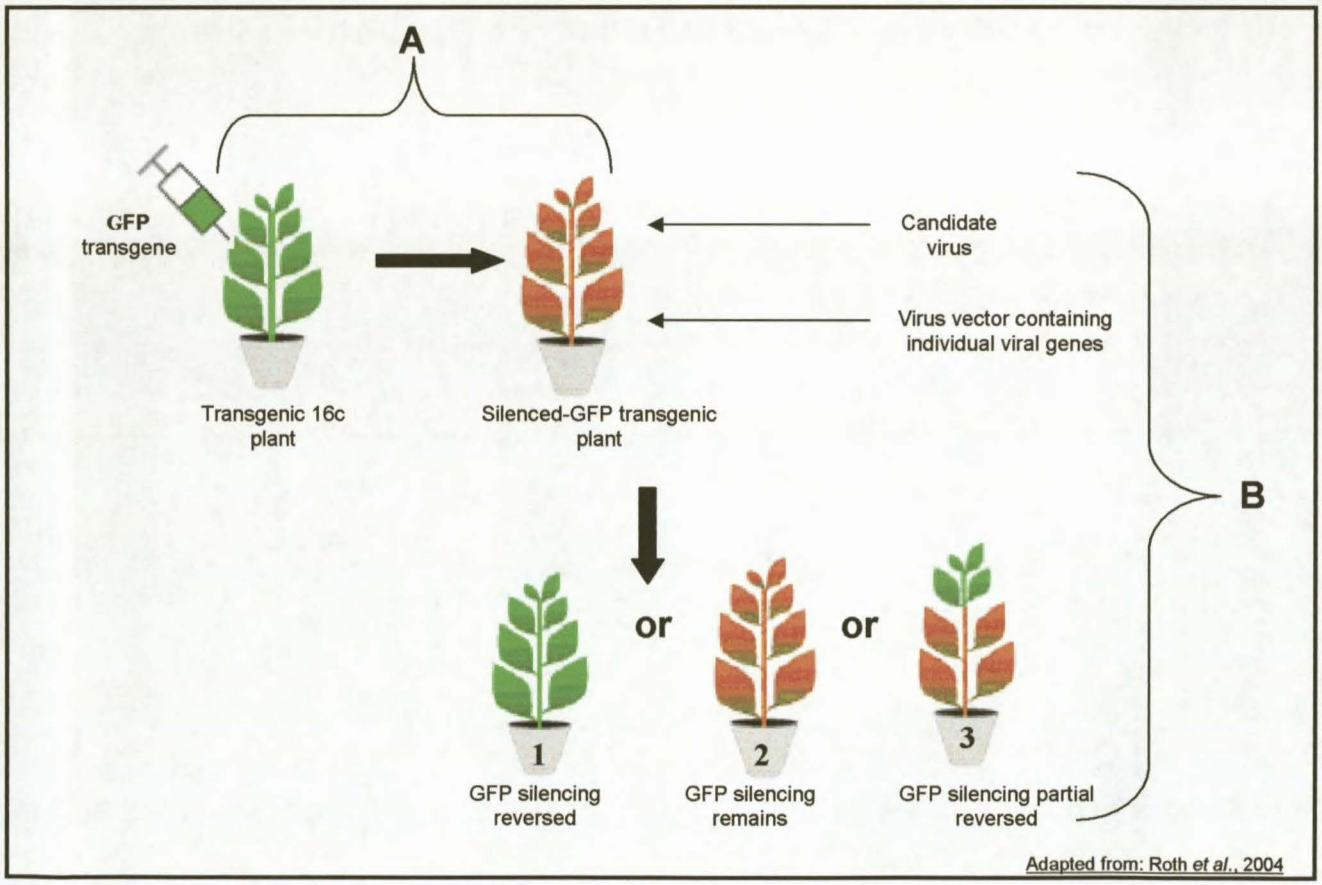


Figure 2.16: Illustration of the reversal of silencing assay showing: **(A)** *Agrobacterium*-induced systemic silencing of a reporter gene and **(B)** The reversal of silencing assay indicating the presence of a suppressor able to reverse (1) not able to reverse (2) and partially able to reverse PTGS (3).

Transient expression assay. This approach is a rapid and easy test to identify viral suppressors and makes use of a commonly used bacterial pathogen of plants, *A. tumefaciens*. The Agrobacterium serves two purposes: one strain is used to induce RNA silencing of a reporter gene, and another strain is used to express the candidate suppressor protein. Transgenic and non-transgenic plants can be used. The two bacterial strains are simultaneously introduced by agro-infiltration as a mixture into a plant and then examined over time for silencing of the reporter gene (Figure. 2.17A) (Li & Ding, 2001). With transgenic plants (GFP-silenced 16c plants; green under UV light) (Figure 2.17B) the plants are agro-infiltrated with the two strains simultaneously and after three to five days a red or green patch is visible on the infiltrated leaves (Figure 2.17C), if the candidate protein has suppressor activity the patch will be very green and will fade to normal GFP expression levels under UV illumination after a few days. If no suppressor activity is present the patch will be red and after a few days the whole plant will turn uniformly red under UV illumination. The effect of the silencing suppressors on the mobile silencing signal can also be investigated with this assay if suppression activity is present (Figure 2.17D) (Roth *et al.*, 2004).

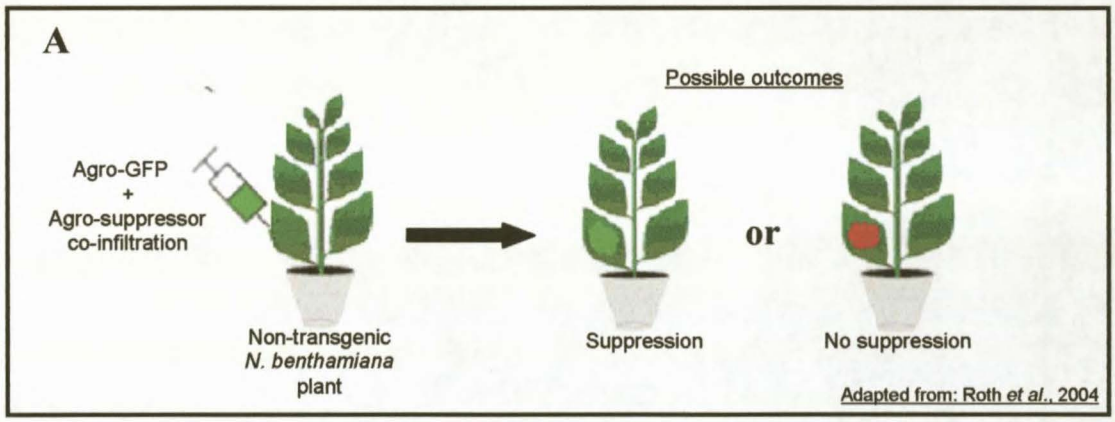


Figure 2.17: (A) Illustration of the transient expression assay in non-transgenic plants, showing the co-infiltration of two bacterial strains containing the reporter and the candidate suppressor protein.

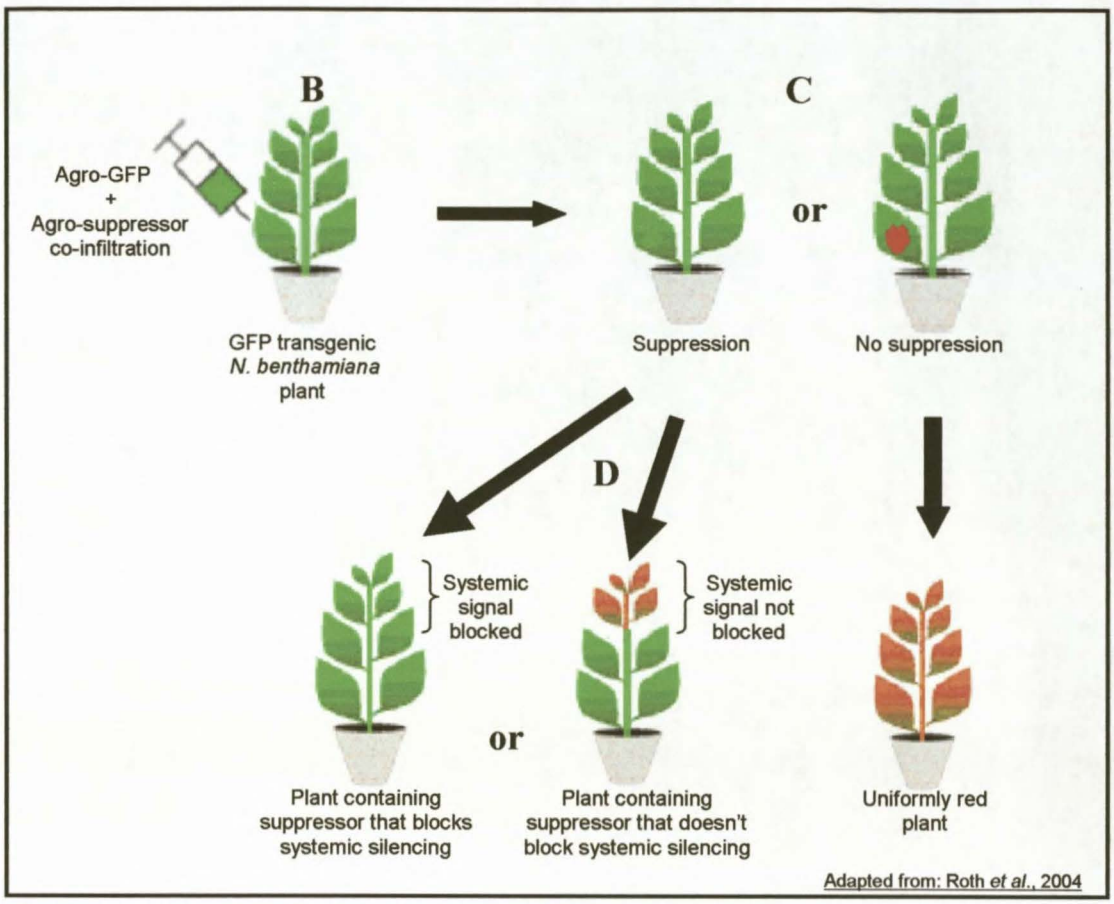


Figure 2.17: Illustration of the transient expression assay in transgenic plants showing: **(B)** Co-infiltration of the two bacterial strains into a GFP silenced plant, **(C)** Activity of a candidate suppressor protein on local RNA silencing and **(D)** The effect of the silencing suppressors on the mobile silencing signal, showing the two possible outcomes.

2.6.3.2 Mechanism and Targets of viral suppressor proteins in PTGS

Suppressors can target the RNA components (dsRNA, siRNAs) or the protein components (RISC inactivation) of the silencing machinery or they could modify the expression of endogenous genes involved in silencing by turning off transcription by host genes or by turning on expression of genes that inhibit silencing (Silhavy & Burgyán, 2004). Some of the known suppressors were identified as long-distance movement proteins which allow the virus entry in and out of the phloem. They may interfere with PTGS through two mechanisms. First they can enter the phloem before the PTGS and thus hinders it from entering the phloem or they can interact with plant proteins which are part of the PTGS pathway in the early stages or later in the pathway (Marathe *et al.*, 2000). The suppressor proteins have been placed into three groups based on the step of the PTGS pathway they inhibit (Chicas & Macino, 2001).

2.6.3.2.1 Suppressors that affect small RNA metabolism

These suppressors inhibit PTGS in all tissues of the plant, reversing PTGS to areas where it has been established. Suppression of PTGS by this group is associated with inhibition of the accumulation of siRNAs, and thus blocking the pathway at the step where Dicer slices the dsRNA into siRNAs. This can be achieved by either blocking the production of siRNAs or binding them to block their function (Roth *et al.*, 2004).

Potyviral helper-component protease (HC-Pro). HC-Pro was the first suppressor to be identified by using a silencing reversal assay. It suppresses both transgene- and virus-induced silencing and is able to reverse established silencing in old and new leaves. How HC-Pro accomplish this is still unknown but one common finding is that it affects small RNA metabolism and miRNA accumulation. This it achieves by altering the accumulation of the siRNAs and miRNAs, by targeting a maintenance step of the silencing pathway, upstream to the production of the 25 nt RNAs but downstream of the signal production. HC-Pro does not prevent the production of systemic silencing (Figure 2.18) (Bucher *et al.*, 2003; Simón-Mateo *et al.*, 2003).

Tombusvirus P19. P19 is a relative weak suppressor in the reversal of silencing assay, by only reversing silencing in the region of the veins. In transient expression assay, P19 is the only suppressor that is able to suppress all size classes of siRNAs and subsequently blocks both systemic and local silencing, and thus preventing them from incorporating into the RISC complex (Figure 2.18) (Kubota *et al.*, 2003; Papp *et al.*, 2003; Silhavy *et al.*, 2002).

2.6.3.2.2 Suppressors that affect local silencing

The second group of PTGS inhibitors blocks the spread of PTGS to newly emerging tissue but has no effect on tissue where PTGS has already been established and includes the 2b protein of the CMV.

Cucumber mosaic virus (CMV) 2b. The CMV2b protein was one of the first identified suppressors of RNA silencing by means of a silencing reversal assay in which CMV2b prevented the initiation of silencing in new emerging leaves, but did not reverse silencing that was already established in older leaves. The suppressor prevents PTGS by either inactivating the signal, by binding the signal directly or by influencing one of the processes that affects the signal (Figure 2.18) (Coburn & Cullen, 2003; Dong *et al.*, 2003; Guo & Ding, 2002).

2.6.3.2.3 Suppressors that affect systemic silencing

The third group includes the p25 protein of PVX. These proteins prevent the spread of silencing possibly by blocking the synthesis of the diffusible silencing signal.

Potato virus X (PVX) p25. The 25K movement protein (p25) suppressor is the only suppressor characterised in potexviruses. It displays no detectable suppressor activity in the reversal silencing assay, but prevents the spread of transgene- or virus-induced systemic silencing through the inhibition of the long siRNAs (Kubota *et al.*, 2003; Voinnet *et al.*, 2000). It possibly also interferes with the initiation of PTGS by preventing the single-stranded RNA (ssRNA) from being converted to dsRNA (Figure 2.18) (Li and Ding, 2001; Qu *et al.*, 2003; Roth *et al.*, 2004; Simón-Mateo *et al.*, 2003).

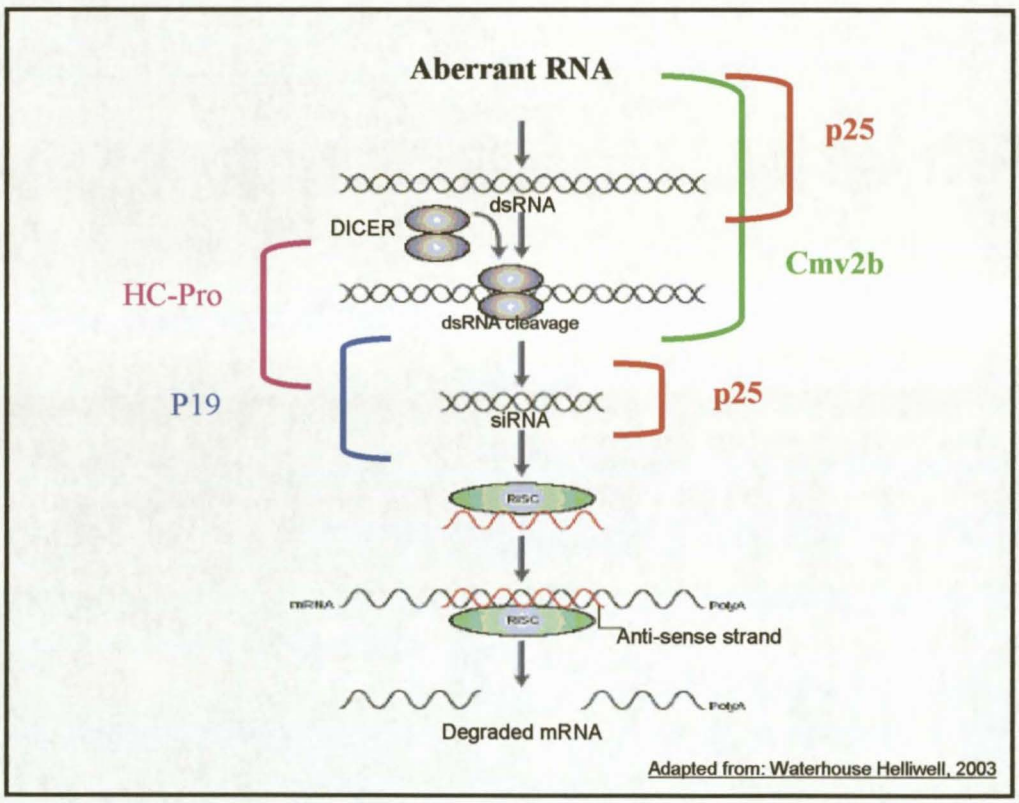


Figure 2.18: Illustration of the steps in the silencing pathway affected by the four common suppressors.

2.6.4 Application of PTGS, RNA viruses and Suppressor proteins

In genetics and molecular biology it is very difficult to relate genes with certain phenotypes and vice versa. The main goal of plant biotechnology is to over express transgenes which encode important products and to silence harmful ones to obtain the perfect phenotype or resistance against a certain virus. PTGS can be utilised to switch specific genes on or of or to determine gene function, it can be used to alter traits by slowing the production of certain proteins and it can help to unravel development and thus can be used with great success in crop enhancements (Baulcombe, 2000; Day, 2000).

RNA viruses can be utilised as vectors for transgene (VIGS) and can be used as a tool in reverse genetics to determine gene function from specific phenotypes by interfering with genes or whole gene families. A gene can be silenced quicker and more efficiently as with sense and anti-sense techniques and the function of the gene can be determined. This is especially important if the gene is harmful for in mutant or transgenic plants (Ruiz *et al.*, 1998).

Perfect crops and phenotypes can be rendered futile by viruses encoding suppressor proteins. If suppressors are known they can be used in conjunction with desirable transgenes to obtain high levels of a particular gene may it be foreign or endogenous to obtain increased yields in biotechnology applications. Suppressor proteins can also help to understand gene functions in functional genomic studies and to provide us in the future with "fool proof" virus resistant transgenic crops (Marathe *et al.*, 2000).Thus it is important to identify as many as possible viruses that carry these suppressor proteins.

Chapter 3 Screening the GLRaV-2 genome for suppressor activity - Materials and Methods

Introduction

Since PTGS is seen as a defence system against viruses in some but not all plants, plant viruses have evolved to counter-act this resistance mechanism by encoding proteins that suppress PTGS. These proteins are diverse with no sequence similarity and act at different steps of the PTGS pathway. Some of the functions assigned to suppressors are long distance movement, genome amplification and virus accumulation. Most of the suppressors have been identified by their ability to interfere with local or systemic silencing of GFP in GFP transgenic or GFP-silenced transgenic *N. benthamiana* plants (Brigneti *et al.,* 1998).

GLRaV-2 is a member of the *Closteroviridae* family, genus Closterovirus and is a single molecule of linear, positive sense single-stranded RNA (15528 nts) transmitted by mealybugs. The GLRaV-2 genome (Figure 3.1) consists of nine open reading frames (ORFs). The 5'–proximal ORF is an incomplete ORF1a followed by eight ORFs that encode proteins of 52 kDa (ORF1b), 6 kDa (ORF2), 65 kDa (ORF3), 63 kDa (ORF4), 25 kDa (ORF5), 22 kDa (ORF6), 19 kDa (ORF7) and 24 kDa (ORF8) respectively, and 216 nucleotides of the 3' untranslated region (Zhu *et al.*, 1998). GLRaV-2 has a broad host range which also includes *N. benthamiana,* which in this study will be exploited to determine the nature of any potential silencing suppressor activity from GLRaV-2. The whole GLRaV-2 genome was surveyed for suppressor activity and five different genes were targeted individually for potential suppressor activity. The five genes were selected on the basis of their proven or predicted functions. The five genes were the heat-shock-related protein (HSP70) (GeneA) coat protein analogue (CPd) (GeneB), coat protein (CP) (GeneC), long distance transport protein (P19) (GeneD) and the replication protein (P24) (GeneE). A coat protein was identified as a suppressor in *Turnip crinkle carmovirus* (TCV), (Thomas *et al.*, 2003), a replication protein was identified as a suppressor in *Tomato mosaic tobamovirus* (ToMV), (Kubota *et al.*, 2003) a long-distance movement protein was identified as a suppressor in *Potato potyvirus Y* (PVY), (Kasschau & Carrington, 2001) and movement proteins have been identified as suppressors in more than one virus (Brigneti *et al.*, 1998; Dunoyer *et al.*, 2002; Vargason *et al.*, 2003). Recently the 21 kDA protein (ORF8) of BYV, which functions as an enhancer of RNA accumulation, was identified as a suppressor protein (Chapman *et al.*, 2004; Reed *et al.*, 2003; Ye & Patel, 2005). Since BYV is also a member of the *Closteroviridae* family, p21 was compared to the five isolated GLRaV-2 genes.

Based on amino acid sequence similarities between p21 and the p24 replication protein (GeneE) of GLRaV-2 it was predicted that GeneE could be the suppressor protein of GLRaV-2 (Reed *et al.*, 2003).

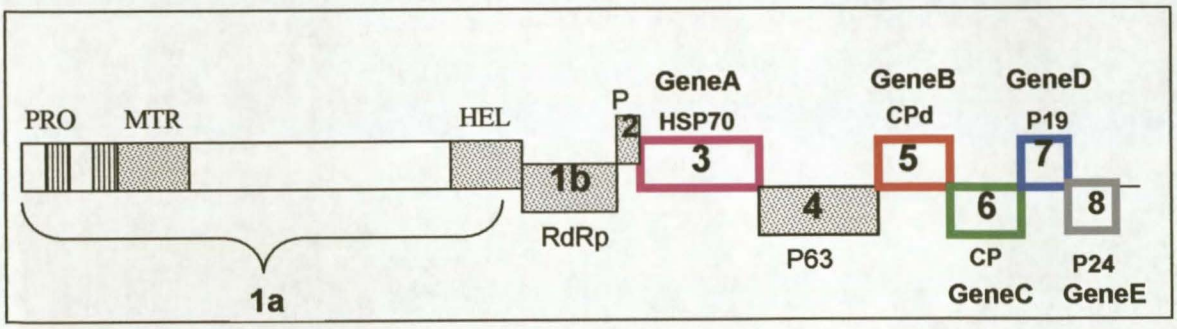


Figure 3.1: The genome structure of Grapevine leafroll associated virus 2 (GLRaV-2) showing boxes representing ORFs with their expression products. **ORF 1a:** 245 kDa containing a double papian-like protease (PRO), methyltransferase (MTR), and RNA helicase (HEL); **ORF 1b:** 53 kDa RNA polymerse (RdRp); **ORF 2:** 6 kDa small hydrophobic membrane protein; **ORF 3:** 65 kDa heat-shock-related protein (HSP70); **ORF 4:** 63 kDa P63 **ORF 5:** 24 kDa divergent coat protein analogue (CPd) **ORF 6:** coat protein (CP); **ORF 7:** 19 kDa long distance transport protein (P19); **ORF 8:** 24 kDa replication protein (P24).

3.1 Plant material and growth conditions

Transgenic Nicotiana benthamiana plants. Transgenic *N. benthamiana* seed (line 16c), homozygous for the GFP transgene, were germinated in trays and individual plantlets (1 cm-2 cm) were replanted into plastic pots. The seed were obtained from The John Innes Centre, Sainsbury Laboratory, Colney, Norwich, UK, courtesy Dr D. Baulcombe.

Non-transgenic Nicotiana benthamiana plants. Normal *N. benthamiana* seed were germinated and replanted into pots. These plants were used for the maintenance of GLRaV-2. The seed were locally obtained.

Transgenic and non-transgenic *N. benthamiana* plants were grown in a plant growth room under a 16-h light and 8-h dark regime at 24°C.

3.2 Plasmid and Virus vectors

3.2.1 pBIN m-gfp5-ER

This vector was used to insert a second copy of the *gfp* gene into transgenic *N. benthamiana* 16c plants by *Agrobacterium*-infiltration (agro-infiltration). The *gfp* reporter gene present in pBIN m-gfp5-ER originates from the jellyfish, *Aequorea victoria* and is under the control of a *Cauliflower mosaic caulimovirus* (CaMV), 35S promoter and nopaline synthase (NOS) terminator (Figure 3.2). The vector is derived from the pBI121 vector in which the β-glucuronidase (GUS) reporter gene has been replaced with the m-gfp5-ER gene and was obtained from Dr. D. Baulcombe from The John Innes Centre, Sainsbury Laboratory, Colney, Norwich, UK. The vector was maintained in *Escherichia coli* (*E. coli*) under Kan (50µg/ml) selection.

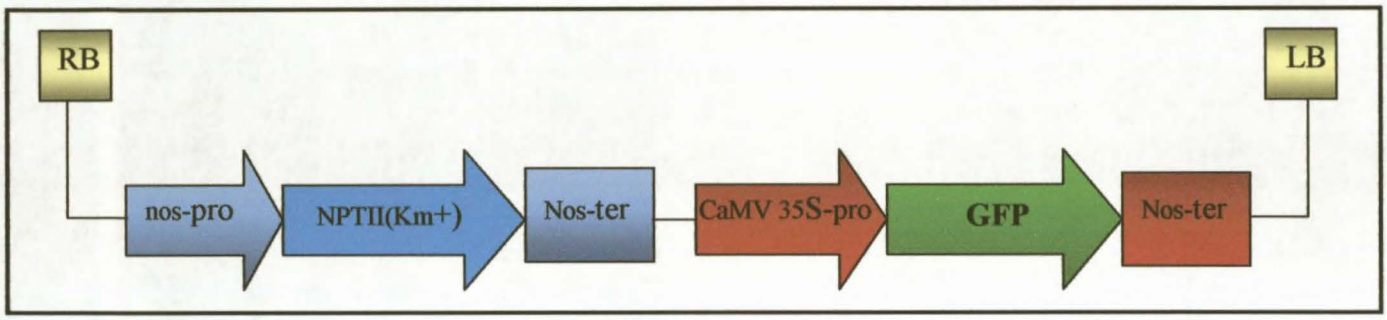


Figure 3.2: Schematic representation of the plant expression vector pBIN m-gfp5-ER

3.2.2 pgR106 PVX-based plant expression vector

The pgR106 plant expression vector is based on the vector pGreen0000 and has a size of about 10 kb. It was obtained form The John Innes Centre, Sainsbury Laboratory, Colney, Norwich, UK, courtesy Dr D. Baulcombe. pgR106 is a binary expression vector and provides replication origins for both *E. coli* and *A. tumefaciens*. The PVX genome integrated into pgR106 contains the multiple cloning site *Cla*I – *Asc*I – *Not*I – *Sal*I which is under control of a CaMV 35S promoter and NOS terminator and is surrounded by two sub-genomic RNA CP promoters between a triple gene block and the CP coding sequence (Figure 3.3). This virus-based vector makes it possible to inoculate mature plants and thus bypasses possible problems of phytotoxicity as sometimes found with permanent insertions in plants (Shivprasad *et al.*, 1999). It has also been optimised to work with in the host plant *N. benthamiana*. The vector was maintained in *E. coli* under Kan (50 µg/ml) selection. In *A. tumefaciens* it needs the helper plasmid pJIC Sa_Rep which carries Tet (5 µg/ml) as selection marker, for replication.

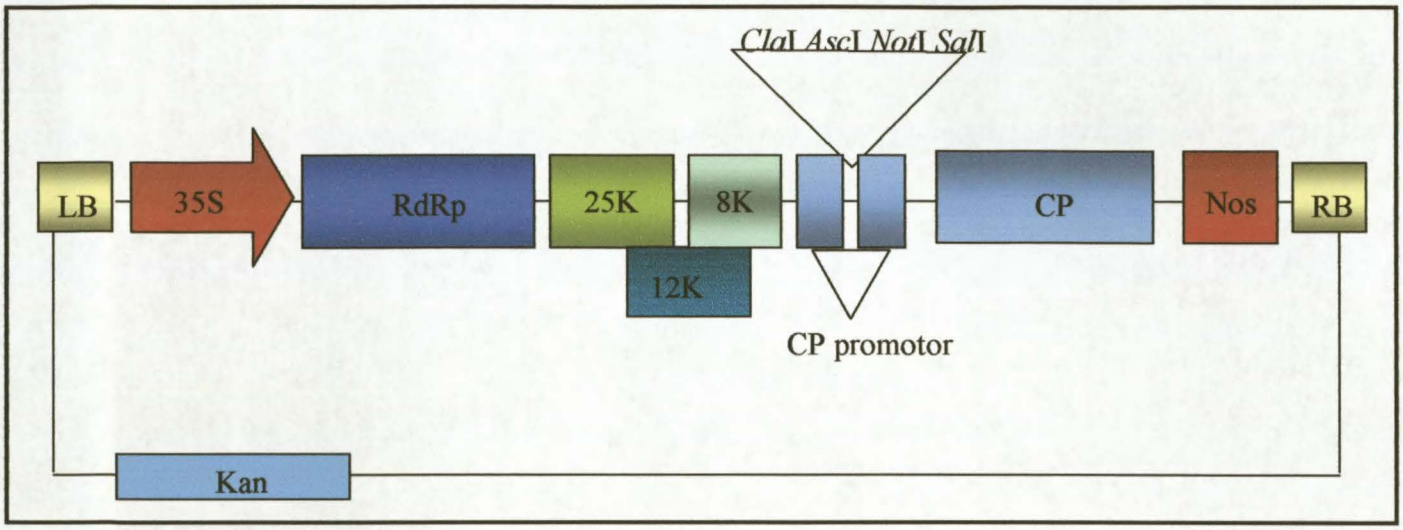


Figure 3.3: Schematic representation of the pgR106 binary vector.

3.2.3 PBIN6-p25, pBIN61-p19, pBIN61-2b

The viral suppressor's p25 from PVX, p19 from *Tomato bushy stunt tombuvirus* (TBSV) and 2b from CMV were incorporated into a pBIN61 vector consisting of a pBIN19 backbone and was maintained in *E. coli* under Kan (50 µg/ml) selection (Figure 3.4). The suppressors were obtained from The John Innes Centre, Sainsbury Laboratory, Colney, Norwich, UK, courtesy Dr D. Baulcombe.

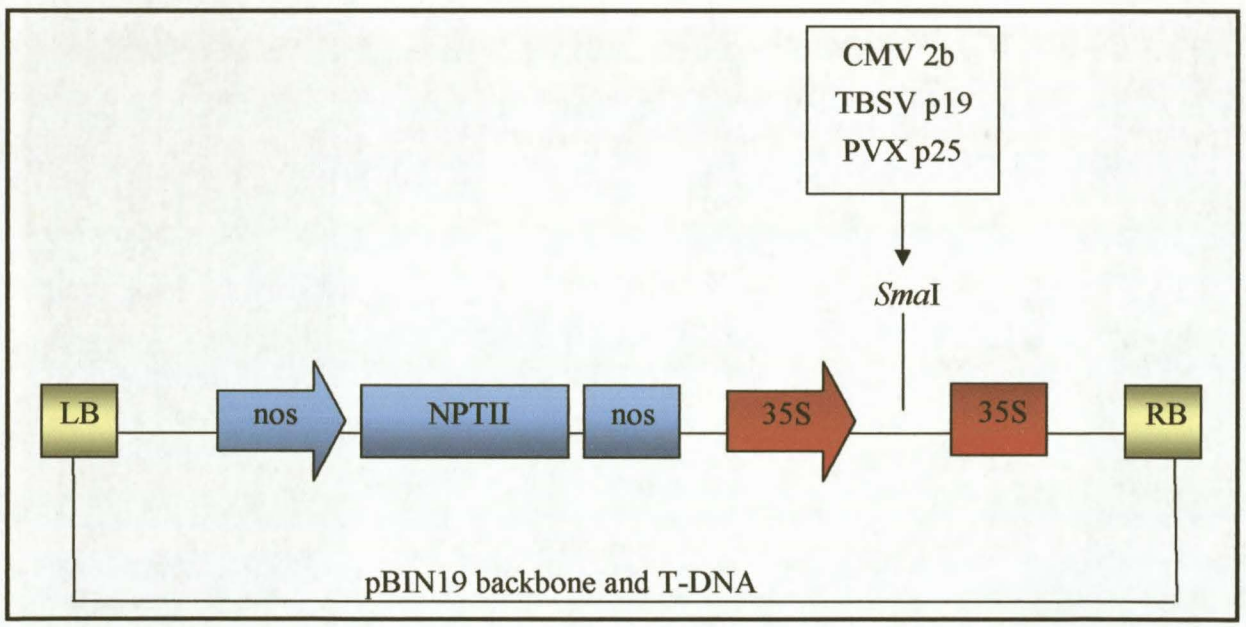


Figure 3.4: Schematic representation of the viral suppressors incorporated into the *Sma*I restriction site of the pBIN61 vector with the pBIN19 backbone.

3.2.4 pGEM®-T-Easy

The pGEM®-T-Easy vector was supplied by Promega and was used for the cloning of PCR products. The pGEM®-T-Easy vector was originally created by cutting the pGEM-5Zf (+) vector with the restriction enzyme *Eco*RV and adding a 3' terminal thymidine to both ends. The vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase (Figure 3.5). The pGEM®-T-Easy vector was maintained in *E. coli* under Amp (100 µg/ml) selection.

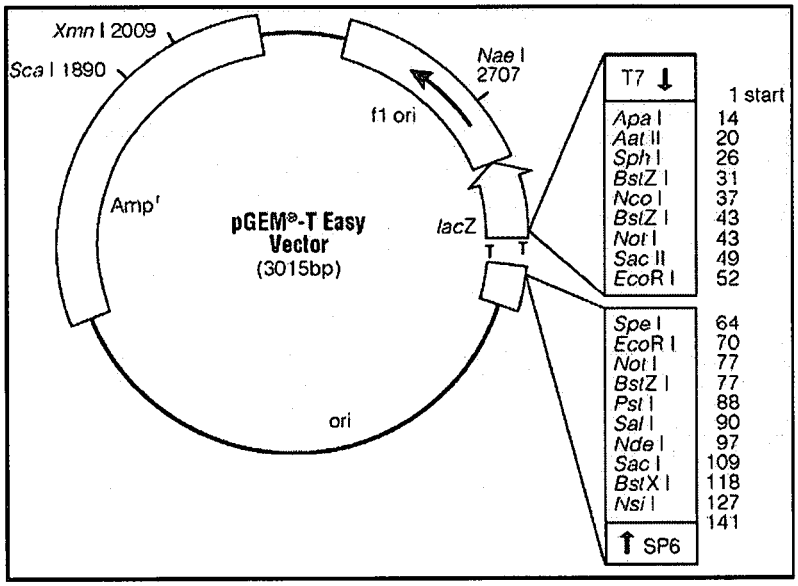


Figure 3.5: Schematic representation of the PCR cloning vector, pGEM®-T-Easy.

3.3 Virus isolates and sap inoculation

Two strains of a South African GLRaV-2 isolate were obtained from Dr. D.E. Goszczynski of the Plant Protection Research institute of the Agricultural Research Council (ARC-PPRI), South Africa. The strains designated 94/970 and 93/955, differs with regard to symptom development. The 93/955 strain is more virulent and was the preferred strain to continue the work with (Boscia *et al.*, 1995; Goszczynski *et al.*, 1996a). This strain was maintained in *N. benthamiana* plants and grown under a 16-h light and 8-h dark regime at 24°C.

Lower leaves of young *N. benthamiana* plants at five to six leaf stage were inoculated with the 93/955 strain of the South African GLRaV-2 isolate. Infected leaves, celite and inoculation buffer (0.01 M Cysteine HCL (pH 7.2), 0.01 M K_2HPO_4 and 3% (w/v) Nicotine) were added together in a mortar on ice and ground to a sap. Sap was inoculated onto plants by using latex gloves and gentle rubbing of the upper surfaces of the leaves. The inoculated leaves were washed gently by

spraying the leaves with normal tap water. The plants were kept at glasshouse temperature (20-25°C) with a 16-h light and 8-h dark regime. Symptoms (curling of top leaves, vein clearing and necrotic lesions) developed after approximately two to three weeks.

This technique was also used to inoculate 20 days old GFP-silenced *N. benthamiana* 16c plants.

3.4 Primer design for isolation of genes

Primers to isolate individual genes from the GLRaV-2 genome (Table 3.1) were designed using a primer design program (PRIMER version 1.01 Serial number 50132, Copyright 1990, Scientific & Educational software). The melting temperatures (T_m) of the individual primers were calculated using the Oligonucleotide Analyzer tool on the website http://www.rnature.com. Annealing temperatures (T_A) for the PCR reactions were set at 5°C below the T_m of a particular primer set.

Table 3.1: Primers used to obtain genes from the GLRaV-2 genome and to obtain GFP fragments for RNA probe preparation. The primer pair in bold font was used to screen for GLRaV-2 positive plants.

GLRaV-2 gene	Name	Sequence (5' - 3')	Fragment size (bp)	T_m (°C)	T_A (°C)
HSP 70 -For	**GeneA**	**TTCAACCGTAGTGTAGTCAA**	**1839**	**57.7**	**53**
HSP 70 -Rev		**GCTATGCTCCTAGAGAGGTA**		**58.8**	
Diverged coat protein - For	GeneB	TATGAGTTCCAACACAAGCGTGC	681	64.9	59
Diverged coat protein - Rev		ACACCGTGCTTAGTACCTCC		63.7	
Coat protein -For	GeneC	GCAGAAGAAAACGCTATGGA	649	59.5	54
Coat protein -Rev		CAGTATGAGCCATTCGGATT		58.7	
P19 -For	GeneD	TGTAATGGAAGATTACGAAG	490	53.5	49
P19 -Rev		CTTAACGATTTCTTTCTTCG		53.5	
P24 -For	GeneE	GATGAGGGTTATAGTGTCTCCTT	618	61.1	56
p24 -Rev		TTAACATTCGTCTTGGAGTTCG		60.4	
GFP - For	--------	ATAGGATCCGGTGAAGGTGATG CAACATACG	452	70.9	64
GFP - Rev		GCCAAGCTTTTGATAATGATCAG CGAGTTGC		68.6	
M13-For	---------	GTTTTCCCAGTCACGAC	714	52.4	47
M13-Rev		CAGGAAACAGCTATGAC		50.0	

3.5 Rapid Direct One-Tube RT-PCR procedure (RDOT- RT-PCR)

Individual GLRaV-2 genes were isolated by a Rapid Direct One-Tube RT-PCR procedure (RDOT-RT-PCR), based on the procedure performed by MacKenzie (1997). The primers used are shown in Table 3.1.

Petioles (0.3 g) of GLRaV-2 infected *N. benthamiana*, plants were ground with 12 ml Grape-ELISA Grinding buffer (0.5 M Na_2C0_3, 0.5 M $NaHCO_3$, 2% (w/v) PVP 40, 0.2% (w/v) BSA, 0.05% (v/v) Tween 20 and 1% Sodium Metabisulfite, pH 9.6) in a mortar to obtain sap at a dilution factor of 1:20. Samples were decanted into 1.5 ml microcentrifuge tubes and centrifuged for one minute at 14 000 rpm.

Four microlitres of the cleared supernatant was pipetted into microcentrifuge tubes containing 25 µl sterile 1 x GES (0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM EDTA, and 0.5% (v/v) Triton X-100). The samples were boiled at 95°C for 10 minutes in a water bath and then chilled on ice for a minimum of five minutes.

The RDOT-RT-PCR reaction mix was prepared by adding two µl of the cooled sample into 23 µl reaction mix (1 x Bioline NH_4 PCR Buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL pH 8.8 at 25°C, 0.01% (v/v) Tween-20], 1 x Sucrose/cresol red dye solution [1x: 2% (w/v) sucrose, 0.1 mM cresol red], 0.5 µM of each forward and reverse primer, 5 mM dithiothreitol (DTT), 1.5 mM $MgCl_2$, 200 µM dNTP's, 25 U Superscript II (200 U/µl Life Technologies), and 1.25 U Taq DNA polymerase [5 U/µl Bioline BIOTAQ™ DNA polymersase]). The final sample was placed into a thermal cycler (GeneAmp PCR System 9700, Perkin & Elmer/Applied Biosystems) and subjected to the following cycling conditions: One cycle of 48°C for 30 minutes; 35 cycles comprising of 94°C for 30 seconds, appropriate T_A for 45 seconds, 72°C for 60 seconds; One cycle of 72°C for seven minutes and indefinitely at 4°C.

3.6 Analytical Gel Electrophoresis

A 1.4 % (w/v) agarose (D1-LE Hispanagar) TAE gel was used for the separation of the DNA fragments in electrophoresis. A 50 x concentrated stock solution of the TAE gel electrophoresis buffer (2 M Tris base, 0.5 M EDTA (pH 8.0), 5.71% (v/v) glacial acetic acid) was prepared and used at a 1 x concentration. Ethidium bromide (EtBr) (0.25 µg/ml) was added to the agarose gel prior to electrophoresis and used for DNA band visualisation along the gel. Electrophoresis was carried out at a voltage of 80 V for approximately 45 min. Hyperladder I (5 µl/lane; Bioline) was

used as a size marker. Loading dye (40% (w/v) sucrose, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol) was mixed 1:4 with samples before electrophoresis.

3.7 DNA Extraction, Purification and Quantification

The QIAquick ® Gel Extraction Kit (QIAgen) was used for the extraction and purification of the RDOT-RT-PCR products obtained from agarose gels. The purification of the products was performed according to the manufacturer's instructions.

The DNA fragments were excised from the gel by using a clean scalpel. The weight of the gel slices was determined and three volumes of buffer QC (capture buffer) to one volume gel slice were added. The gel slices were incubated in a water bath at 50°C for 10 minutes or until the slices were completely dissolved. The process was helped along by vortexing the sample every two to three minutes.

After incubation the colour of the mixture turned yellow and one gel volume of isopropanol was added to the sample and thoroughly mixed. A QIAquick spin column was inserted into a 2 ml collection tube and the sample was added to the column and centrifuged (~13 000 rpm) for one minute, the flow-through was discarded and the column was replaced into the 2 ml tube. An additional 0.5 ml Buffer QC (wash buffer) was added to the column and re-centrifuged (~ 13 000 rpm) for one minute. This step was followed by the addition of 0.75 ml of Buffer PE to the column and centrifuged (~ 13 000 rpm) for one minute, the flow-through was discarded and centrifuged for an additional one min at ~13 000 rpm.

The QIAquick column was then placed into a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 50 µl of sterile water. The sample was placed on the bench for one minute and then centrifuged for an additional one minute at ~ 13 000 rpm to improve DNA concentration. After purification the concentration of the samples were determined in the NanoDrop spectrophotometer system (NanoDrop® ND-1000 UV-Vis, NanoDrop technologies, Inqaba Biotechnical Industries (Pty) Ltd, SA).

3.8 Cloning of PCR products

The high copy number pGEM®-T-Easy Vector System (Promega, SA) was used for the cloning of the individual GLRaV-2 RDOT-RT-PCR products after the gel purification step. The ligation reactions were performed in 10 µl (1 X Rapid Ligation Buffer, T4 DNA Ligase (3µ/µl), and 50 ng pGEM®-T-Easy vector and x ng PCR product). The amount (x) of PCR product used per individual

reaction was calculated accordingly to the manufacturer's instructions and an example is provided in **Appendix A.** The reaction was incubated overnight at 4°C for the maximum number of transformants.

3.9 Transformation and Selection of recombinants by colony PCR

Transformation of the pGEM®-T-Easy vectors containing the cloned GLRaV-2 genes was performed as per Sambrook *et al.* (1989). Competent *E. coli* DH5α cells, prepared by the $CaCl_2$ method (**Appendix B**), were used for all standard transformation reactions. Frozen competent DH5α cells were removed from -80°C and placed on ice for approximately five minutes. Two microlitres of the ligation reactions were added to 50 µl of competent cells on ice. The reaction was gently mixed by flicking the tube and incubated on ice for 20 minutes. The cells were heat-shocked at exactly 42°C for 45-50 seconds in a water bath and immediately returned to ice for a further two minutes. Nine hundred and fifty microlitres of room temperature Luria-Bertani (LB) medium (Tryptone, Yeast extract, NaCl, pH 7.5) were added to the transformation mix and incubated at 37°C with shaking (~150 rpm) for 1.5 hours. The transformation mix was centrifuged at 1 000 rpm for 10 minutes, the supernatant was discarded and the pellet was resuspended in 100 µl LB medium. A total of 100 µl of transformation mix was plated onto prepared LB/agar plates (15 g agar / litre LB medium) with appropriate antibiotics, X-gal and IPTG.
The plates were incubated overnight (16-24 hours) in a 37°C incubator and stored at 4°C the next day.

The authenticity of the putative recombinants was determined by colony PCR. Single, white colonies and a single blue colony (negative) were picked from the LB agar plates and inserted into 10 µl of PCR reaction mix (1 x NH_4 PCR Buffer, 1 x Sucrose/Cresol dye, 0.5 µm forward and reverse primer, 1.5 mM $MgCl_2$, 200 µM dNTP's and 0.5 U Taq DNA polymerase (5 U/µl Bioline Biotaq)). The final product was placed in a thermal cycler and subjected to the following cycling conditions: One cycle of 94°C for five minutes; 30 cycles comprising of 94°C for 30 seconds, T_A of a particular primer set for 30 seconds, 72°C for 30 seconds; One cycle of 72°C for seven minutes and indefinitely at 4°C. GLRaV-2 gene specific primers were used in the colony PCR reaction and the primer annealing temperatures are listed in Table 3.1. The products of the PCR reaction was run on a 1.4% (w/v) Agarose/TAE gel as explained in Section 3.6.

3.10 Plasmid DNA Extractions

PCR positive colonies were inoculated in five ml LB medium containing Amp (100 ug/ml) for selection and incubated overnight at 37°C with shaking (200 rpm). Plasmid DNA was harvested from the five ml overnight cultures using the Wizard® Plus SV miniprep DNA purification system (Promega) as prescribed by the manufacturer.

The five ml overnight culture was pelleted (10 000 rpm for five minutes) and resuspended in 250 µl cell resuspension solution (100 µg/ml RNase A, 50 mM Tris-HCl pH 7.5, 10 mM EDTA) and vortexed. Two hundred and fifty microlitres cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) was added and incubated for five minutes at room temperature. Three hundred and fifty microlitres of neutralization solution (1.32 M KoAC pH 4.8) was added, the tubes were centrifuged (13 000 rpm for 10 minutes) and the cleared lysate was decanted into the spin column. The sample was centrifuged (13 000 rpm for one minute), the flow through was discarded and 750 µl wash solution (80mM KoAC, 8.3 mM Tris-HCL pH 7.5, 40 µM EDTA, 55% (v/v) Ethanol) was added, followed by an centrifuge step. Another 250 µl wash buffer was added and centrifuged (13 000 rpm for five minutes). The spin column was transported to a sterile 1.5 ml microcentrifuge tube, 100 µl nuclease free water was added and centrifuged (13 000 rpm for one minute). The concentration of the DNA was determined spectrophotometrically and stored at -20°C for further use. Freezer cultures of the pGEM®-T-Easy vectors containing the individual GLRaV-2 genes were prepared.

3.11 Sub-cloning of individual GLRaV-2 genes

The restriction digestion of vector pgR106 was performed as prescribed by the manufacturer of the restriction enzymes. The reaction was performed in 10 µl (1 x restriction buffer O [50 mM Tris-HCL pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA], 5U *Not*I restriction enzyme (Fermentas), 500 ng DNA) and incubated in a water bath at 37°C for two hours. The blunt ended vector was dephosphorylated by Shrimp Alkaline Phosphatase (SAP); Promega, in a final volume of 30 µl (1X SAP reaction buffer [0.05 M Tris-HCL pH 9.0,10 mM $MgCl_2$], 3 U SAP(1 U/µl)).The reaction was incubated at 37°C for 12 minutes in a water bath and heat inactivated at 65°C for 15 minutes in a heating block. The final product was run on a 1.4% (w/v) agarose gel (Section 3.6) and the correctly sized fragment was excised form the gel and extracted using the QIAquick® kit (Section 3.7). The concentration of the excised fragment was determined spectrophotometrically.

The individual GLRaV-2 genes were excised from their respective pGem®-T Easy vectors by means of a *Not*I restriction digestion reaction, as described above and was visualised on a 1.4 % (w/v) agarose gel. Correctly sized fragments were excised by means of the QIAquick® kit and the concentration was determined in a NanoDrop spectrophotometer system as mentioned above. The ligation reactions were performed in 20 µl with T_4 DNA ligase and buffer (50 mM Tris-HCL, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA) supplied by New England Biolabs and 50 ng pgR106 vector and x ng PCR product. The amount of PCR product used per individual reaction was calculated according to the manufacturer's instructions (an example is provided in **Appendix A**). The reaction was incubated overnight at 16°C.

Transformation of the constructs was performed (Section 3.9) and since the pgR106 vector does not contain blue/white selection, no X-gal or IPTG was added to the LB/agar plates. Colonies containing this vector were selected on Kan (50 µg/ml) and the authenticity of the colonies obtained after 16 h of incubation, was determined by colony PCR (Section 3.9). Recombinant *E.coli* colonies were selected for on LB medium containing Kan (50 µg/ml) selection. Plasmid extractions were performed as described in Section 3.10.

A second screening of the positive recombinants was done before the plasmids were transformed into *A. tumefaciens.* The mini-prep DNA obtained was digested in a final volume of 10 µl with appropriate buffer and restriction enzyme (*Not*I, Fermentas) and incubated in a water bath at 37°C for two hours. The final product was run on a 1.4% (w/v) agarose gel (Section 3.6) to visually confirm the integrity of the plasmid.

3.12 *Agrobacterium tumefaciens* transformation and Selection

Two *A. tumefaciens* strains GV3101 and C58C1 were used for the *Agrobacterium* experiments. Both strains are transfer-defective strains (Vir-). GV3101 contains the helper plasmid pSOUP, also known as pJIC Sa-Rep which carries a Tet resistant gene. C58C1 contains the helper plasmid pCH32 which carries two virulence genes (vir E and vir G) and a Tet resistant gene.

Transformation of the plasmids (pViral106-GeneA to pViral106-GeneE) were performed with competent *A. tumefaciens* cells (C58C1 and GV3101), prepared as per Tzfira *et al.* (1997) (**Appendix B**), were used for all *A. tumefaciens* transformation reactions. Frozen competent C58C1/GV3101 cells were removed from -80°C and 500 ng of each plasmid (pViral106-GeneA to

pViral106-GeneE) were added to the cells. The cells were thawed by incubating them for five minutes in a 37°C water bath. One millilitre of room temperature LB medium (without antibiotics) were added to the microcentifuge tube and incubated at 28°C with gentle shaking (~150 rpm) for three hours. The transformation was centrifuged at 13 000 rpm for 30 seconds. The supernatant was discarded and the pellet was resuspended in 100 µl LB medium. A total of 100 µl of transformation mix was plated onto prepared LB/agar plates with appropriate antibiotics (**Appendix C**). The plates were incubated at 28°C for two to three days. After three days a single colony was re-streaked onto freshly prepared LB/agar plates with suitable antibiotics. The authenticity of the putative transformants was determined by colony PCR (Section 3.9).

Transformation of the pBIN m-gfp5-ER and pBIN61-suppressor (p25, p19 and 2b) constructs was performed as explained previously and the antibiotic selection used on the LB/agar plates is described in **Appendix C**. The authenticity of the colonies on the plates containing the GFP recombinants were determined by colony PCR and GFP specific primers were used. The primer annealing temperatures are listed in Table 3.1. The colonies on the plates containing the pBIN61 recombinants were re-streaked several times onto selection plates (**Appendix C**), since no primers were available for performing a colony PCR reaction.

3.13 Agro-infiltration experiments

Constructs containing viral genes (GeneA to GeneE) were prepared for agro-infiltration by following the protocol provided on the Sainsbury Laboratory website (http://www.jic.bbsrc.ac.uk/sainsbury-lab/dcb/Services/AgroInfiltrationHP.htm). The colonies were inoculated in five ml liquid LB medium with appropriate antibiotics (Tet [5 µg/ml final], Kan [50 µg/ml final], and Rifampicin (Rif) [30 µg/ml final]) and incubated overnight at 28°C with shaking (200 rpm). Two millilitres of the overnight cultures were re-inoculated in 20 ml LB medium containing appropriate antibiotics (Tet [5 µg/ml final], Kan [50 µg/ml final], Rif [30 µg/ml final], 10 mM 2-[Morpholino]ethanesulfonic acid (MES) and 20 µM acetosyringone) and incubated overnight at 28°C with shaking (200 rpm). The bacteria were precipitated (4 000 rpm for 5 minutes) and the pellets were resuspended in resuspension solution (10 mM MES, 10 mM $MgCl_2$, 100 µM acetosyringone). Enough solution was added to the pellets to ensure an OD_{600} of between 1 and 2. The suspensions were left at room temperature for three hours before the agro-infiltration reactions were performed.

Agro-infiltration of different constructs. The *A. tumefaciens* suspensions of the different constructs were infiltrated into the leaves of *N. benthamiana* plants (Section 3.1) by two or three small incisions, made by scalpel blade, on the adaxial side of the leaf. A syringe (without needle) was used to inject the *A. tumefaciens* suspension into the leaf by pressing the tip against the incisions and exerting a counter-pressure with the index finger on the abaxial side of the leaf. About four to five leaves per plant were infiltrated and the plants were left covered with plastic overnight with 16-h light and 8-h dark light cycles. The plastic was removed from the plants the following day.

All of the *N. benthamiana* plants agro-infiltrated were monitored for a period of 5-20 DPI and infiltrated leaves as well as new emerging growth were monitored visually under UV illumination for any colour changes.

3.14 GFP imaging

Visual detection of GFP fluorescence was performed using a 100 W, hand-held, long wave length (320 nm) ultraviolet (UV) lamp (SB-100F Series model, Spectroline). Plants were photographed with a digital camera (Canon, 300D). The images were processed by using the Microsoft Photo editor program.

3.15 RNA analysis

3.15.1 Synthesis of a digoxigenin (DIG)-labelled DNA probe

The GFP probe was labelled in a PCR reaction. Ten pg of plasmid (pBIN m-gfp5 ER) DNA was added to a final volume of 50 µl PCR-DIG labelling reaction mix (1 x NH_4 PCR Buffer, 0.5 µm forward and reverse primer, 1.5 mM $MgCl_2$, 200 µM dNTP's, 10 x concentrated DIG DNA labelling mix, and 0.5 U Taq DNA polymerase (5U/µl Bioline Biotaq)). The GFP Sense primer pair shown in Table 3.1 was used in the reaction. A normal PCR reaction was performed simultaneously under the same conditions as a control reaction to judge labelling efficiency. The final products were placed in a thermal cycler and subjected to the following cycling conditions: One cycle of 94°C for five minutes; 30 cycles comprising of 94°C for 30 seconds, T_A of a particular primer set for 30 seconds, 72°C for 30 seconds; one cycle of 72°C for seven minutes and indefinitely at 4°C. Five microliters of each reaction was visualised on a 1.4% (w/v) TAE agarose gel (Section 3.6). The sensitivity of the probe was determined by spotting a dilution series (52 ng -0.0526 pg) onto a membrane. The correct dilutions of the cold reaction (526 ng/µl) were mixed with an equal amount of loading buffer and boiled in a water bath set at 100°C for five minutes before spotting the

different concentrations on the membrane followed by UV crosslinking for five minutes on the DNA side and 45 seconds on the non-DNA side.

3.15.2 Total RNA and small RNA extraction

Total RNA were isolated from transgenic *N. benthamiana* 16c plants and normal *N. benthamiana* plants using the *mir*Vana™ miRNA isolation kit (Ambion) as prescribed by the manufacturer. A plant RNA isolation protocol (http://www.genisphere.com/array_detection_protocols_quick.html) was also used. Five millilitres extraction buffer (100 mM LiCL, 100 mM Tris pH 8.0, 10 mM EDTA and 1% (w/v) SDS) were combined with five millilitres phenol in a sterile 50 ml tube and heated at 80°C for ten minutes. Well ground tissue (1 – 2 grams) was added to the hot phenol/extraction buffer and vortexed for 30 seconds. Five millilitres chloroform/isoamyl alcohol (24:1) were added and vortexed for an additional 30 seconds. The mix was centrifuged at 9 000 rpm for 25 minutes at 4°C. After centrifugation the aqueous phase was removed to a clean 50 ml tube and an equal amount of 4 M LiCL was added and mixed by inverting the tube several times. The tube was placed at -80°C for an hour. This was followed by a centrifugation step at 9 000 rpm for 20 minutes at 4°C. The supernatant was discarded and the pellet was washed with five ml 70% (v/v) alcohol by centrifugation (10 minutes at 4°C). The alcohol was removed and the pellet was air dried for five minutes before it was resuspended in 100 µl double distilled water. The concentration of the RNA was determined spectrophotometrically.

Small RNAs were isolated from agro-infiltrated leaves using the *mir*Vana™ miRNA isolation kit (Ambion) as prescribed by the manufacturer. The concentration of the RNA was determined spectrophotometrically.

Gel analysis of total RNA. Twenty microlitres of the RNA sample was mixed with four microlitres sterile 6 x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (w/v) glycerol, 60 mM sodium phosphate [pH6.8] and 1.2% (w/v) SDS) and incubated at 75°C for five minutes. This was followed by loading the samples on a 1.5% (w/v) 10 mM sodium phosphate gel containing EtBr (0.25 µl/ml). The gel was electrophoresed at 60V for two hours in 10 mM sodium phosphate running buffer, pH 6.8 which was recirculated during electrophoresis (Pellé and Murphy, 1993).

Northern analysis of total RNA. Detection of total RNAs was performed essentially as described in the DIG application manual (Roche Molecular Biochemicals) with a few exceptions. Since a Dig-

labelled DNA probe was used to detect RNA on the northern blot the prehybridization, hybridization and high stringency wash steps were performed at 50°C. Chemiluminescent visualization of the GFP probe-target hybrids were performed with CDP-Star overnight in the GeneSnap chemiluminescent detector system from SynGene.

Gel analysis of small RNAs. A 15 µl aliquot of the extracted small RNAs was mixed with an equal amount of Gel loading Buffer II (1-2X solution of 95% (v/v) formamide, 18 mM EDTA and 0.025% (w/v) SDS, 0.025% (w/v) Xylene Cyanol and 0.025% (w/v) Bromophemol Blue) and denatured at 100°C for five minutes. Two microlitres of two size markers, a 20 bp and 30 bp, was prepared in the same manner as the small RNAs as size markers. A 10 x concentrated solution of a TBE gel electrophoresis buffer (0.9 M Tris base, 0.9 M Boric acid, 0.5 M EDTA (pH 8.0)) was prepared and used at a 1 x concentration. The samples were loaded on a denaturing 15% (w/v) polyacrylamide gel with 8 M urea and electrophoresed at 45 mA for approximately one and a half hours. The gel was stained with EtBr (0.25 µl/ml) in 1 x TBE for five minutes and destained in 1 X TBE for 2 minutes before visualization.

Northern analysis of GFP siRNAs. Detection of GFP siRNAs by Northern Blot was performed as per Patterson and Guthrie (1987). The denaturing 15 % polyacrylamide gel was stained in EtBr (0.25 µl/ml) and visualised to determine good RNA separation. The RNA was transferred to a nylon membrane by electroblotting in a semi-dry apparatus using filter paper soaked in 0.25 X TBE which was placed above and below the gel/membrane sandwich. The transfer was carried out at 200 mA for approximately 1 h. The membrane was UV crosslinked (five minutes on RNA side and 45 seconds on non-RNA side). A 50 x Denhardt's solution (10 g Ficoll 400, 10 g bovine serum albumin and 10 g polyvinylpyrrolidone in 1 litre H_2O) and a 20 x SSC solution (3 M NaCl, 300 mM Sodium citrate, pH7.0) were prepared for the prehybridization and hybridization steps. The membrane was prehybridized in 10 ml prehybridization solution (6x SSC, 10x Denhardt's solution and 0.2% (w/v) SDS) for one hour at 65 °C. The prehybridization solution was discarded and the membrane was hybridized in 10 ml hybridization solution (6x SSC, 5x Denhardt's solution, 0.2% (w/v) SDS) containing the labelled probe for overnight with gentle shaking at room temperature. The prehybridization solution was discarded and the membrane was washed three times with 10 ml wash solution (6 x SSC, 0.2% (w/v) SDS) at room temperature with gentle shaking for five minutes allocated for each wash. The wash step was rounded of with one wash at 42°C with gentle shaking for 10 minutes. The wash solution was removed and discarded. Chemiluminescent detection was performed with CDP-*Star* in the GeneSnap chemiluminescent detector system from SynGene.

Chapter 4 Screening the GLRaV-2 genome for suppressor activity - Results and Discussion

Introduction

When inoculated to a host plant containing a post-transcriptionally silenced transgene, a virus that possesses anti-PTGS activity can reverse silencing that is already established and/or prevent its onset in the new growth (Béclin *et al.*, 1998; Brigneti *et al.*, 1998). In this work GLRaV-2 was investigated for suppressor activity by using GFP-constitutive (16c) and GFP-silenced transgenic *N. benthamiana* plants in two different silencing suppression assays, the reverse silencing assay and the transient silencing assay. 16c plantlets accumulate high levels of GFP mRNA and GFP, so that the leaves and stems appear green under UV illumination (Ruiz *et al.*, 1998; Voinnet & Baulcombe, 1997).

Infection of GFP-silenced 16c plants with GLRaV-2 resulted in the reversal of silencing in the new growth. Among the five genes tested of the GLRaV-2 genome only GeneE (p24 gene) displayed silencing suppressor activity in the reverse/transient silencing suppressor assay based on its ability to suppress the onset of PTGS of the GFP in transgenic 16c *N.* benthamiana plants or to reverse established PTGS of the GFP by agro-infiltrating/ co-infiltrating experiments. PTGS is induced in the infiltrated "patch" and this is followed by systemic spread of the signal throughout the plant (Voinnet & Baulcombe, 1997; Voinnet *et al.*, 1998).It is also known that PTGS is associated with the accumulation of siRNAs of 21-25 nt in length (Hamilton & Baulcombe, 1999; Hamilton *et al.*, 2002) and leaves infiltrated with the x protein showed increased GFP siRNA levels as the suppression of silencing increased. The level of the GFP mRNA was also monitored to verify the visual observations.

4.1 Isolation and cloning of individual GLRaV-2 genes (GeneA-GeneE)

N. benthamiana plant material infected with the South African isolate of GLRaV-2 was received and used to sap inoculate new *N. benthamiana* plants to maintain the virus in active form. The five GLRaV-2 genes (designated GeneA to GeneE) were isolated by the RDOT-RT-PCR reaction method with GLRaV-2 specific primers (Table 3.1) and visualized (Figure 4.1). The purified products were cloned into the intermediate pGEM® -T-Easy vector and transformed into competent DH5α cells. Recombinants were selected by colony PCR, with appropriate primers (Table 3.1) and visualised (not shown).

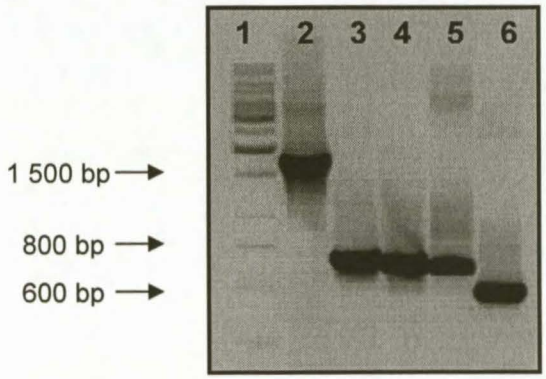


Figure 4.1: Isolated GLRaV-2 genes (GeneA-GeneE) by RDOT-RT-PCR. **Lane 1:** Hyperladder I. **Lane 2:** GeneA, 1839 bp. **Lane 3:** GeneB, 681 bp. **Lane 4:** GeneC, 649 bp. **Lane 5:** GeneD, 618 bp. **Lane 6:** GeneE, 490 bp.

4.2 Construction of plant expression vectors pViral106-GeneA to pViral106-GeneE

The pgR106 plant expression vector was used to transiently express the GLRaV-2 genes (GeneA to GeneE) in the plant. The vector was digested with the restriction enzyme *Not*I, dephosphorylated and visualized (Figure 4.2).

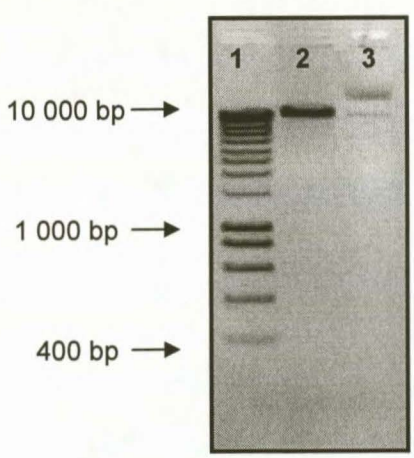


Figure 4.2: Restriction digestion of pgR106 with *Not*I for subsequent cloning. **Lane 1:** Hypperladder I. **Lane 2:** *Not*I digested pgR106. **Lane 3:** Undigested pgR106 as control.

The digested pgR106 vector was excised form the gel and purified for the cloning of the individual GLRaV-2 genes. The genes, GeneA to GeneE, were digested form their respective pGem®-T-Easy vectors by means of a *Not*I restriction digestion, visualized (not shown) and correctly sized fragments were excised and purified. The purified genes were ligated into the digested pgR106 vector (Figure 4.3) under the control of a strong sub-genomic promoter and transformed into competent DH5α cells. Recombinants were selected by colony PCR, with appropriate primers (Table 3.1).

The final constructs (pViral106-GeneA to pViral106-GeneE) were re-verified for authenticity by a *Not*I restriction digestion. pgR106 vector without an insert was also digested as a control. This restriction digestion reaction was supposed to excise the incorporated GLRaV-2 genes from their respective pgR106 vectors and thus verify their presence in the vector. The pgR106 vector without an insert was expected to produce a linarised band. Correctly sized fragments were obtained with each of the digestions as shown in Figure 4.4.

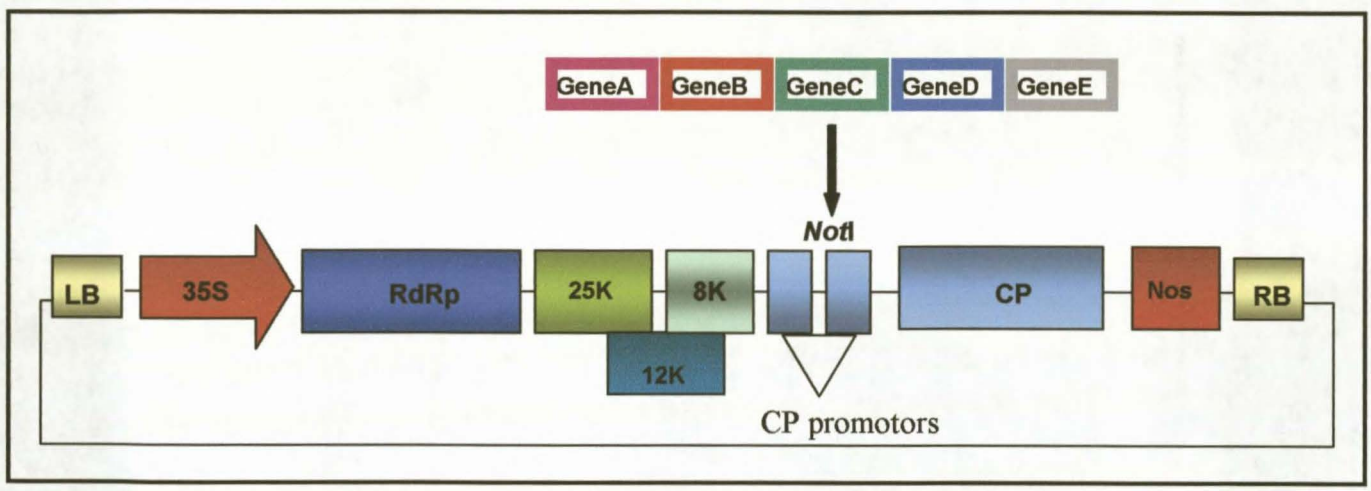


Figure 4.3: Schematic representation of the pgR106 plant expression vector with incorporated genes pViral106-GeneA to pViral106-GeneE in the *Not*I restriction site of the vector.

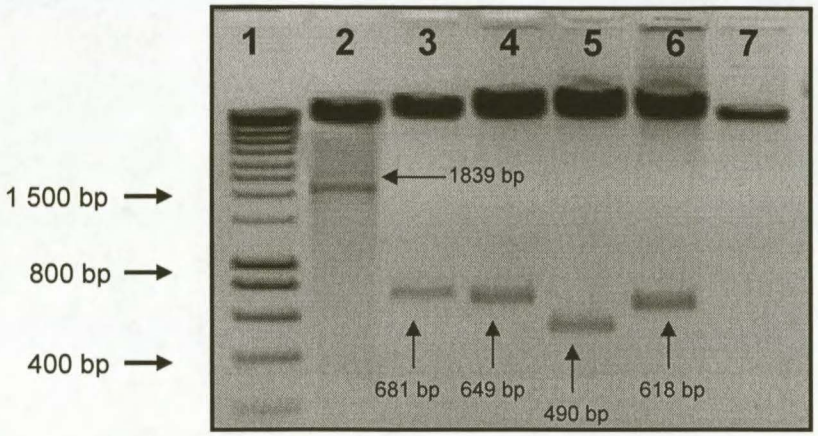


Figure 4.4: *Not*I restriction digestion of final constructs pViral106-GeneA to pViral106-GeneE. **Lane 1:** Hyperladder I. **Lane 2:** pViral106-GeneA, 1839 bp. **Lane 3:** pViral106-GeneB, 681 bp. **Lane 4:** pViral106-GeneC, 649 bp. **Lane 5:** pViral106-GeneD, 490 bp. **Lane 6:** pViral106-GeneE, 618 bp. **Lane 7:** Digested pViral106.

4.3 *A. tumefaciens* transformation of constructs

When a liquid culture of *A. tumefaciens* is agro-infiltrated into leaves, the transferred DNA (T-DNA) of the bacterial Ti plasmid is transferred into the plant cells, where transient expression of the T-DNA encoded genes proceeds (Hamilton *et al.*, 2002). Thus, *A. tumefaciens* competent cells, GV3101 and C58C1 were directly transformed with the constructs (pBIN-GFP, pBIN61-p25, p19, 2b and pViral106-GeneA to pViral106-GeneE) and were re-streaked onto fresh LB/agar plates with appropriate selection (**Appendix C**) after three days. The transformants containing constructs pViral106-GeneA to pViral106-GeneE were verified by colony PCR using appropriate primers (Table 3.1), not shown and transformants containing the pBIN-GFP and pBIN61-p25, p19, 2b constructs were re-streaked onto fresh selection plates, since no primers were available to perform colony PCR reactions with. Positive recombinants were grown to log phase, collected by centrifugation and resuspended for the agro-infiltration experiments. We anticipated that the virus based vectors harbouring the GLRaV-2 genes would be transferred into the cells of the infiltrated region and will be transcribed by viral replication in the cytoplasm, which will generate many transcripts of the genes of interest and thus suppress PTGS (Fischer *et al.*, 1999).

4.4 Screening the GLRaV-2 genome for suppressor activity

This experiment was performed to determine if the GLRaV-2 genome possesses suppressor activity. A virus possesses suppressor activity if it can reverse silencing already established and/or prevents the onset of silencing in the infiltrated leaf or in newly emerging leaves. This experiment was based on a previously described experimental system (Brigneti *et al.*, 1998) which utilized transgenic *N. benthamiana* 16c plants and GFP-silenced *N. benthamiana* 16c plants. Transgenic *N. benthamiana* 16c plants contains a GFP transgene (Ruiz *et al.* 1998; Voinnet & Baulcombe,1997) and expresses high levels of GFP and thus fluoresces bright green under UV illumination (Figure 4.5A).

Lower leaves of transgenic *N. benthamiana* 16c plants were agro-infiltrated with an extra copy of the GFP transgene already present in the plant (C58C1::GFP) to induce GFP silencing and thus obtain GFP-silenced *N. benthamiana* 16c plants. Two days post inoculation (DPI) the infiltrated patches on the leaves were bright green (Figure 4.5B). This was due to the expression of the ectopic and stably integrated GFP transgene. The colour faded (Figure 4.5C) and at seven DPI PTGS was visible in the infiltrated leaves as red dots under UV illumination (Figure 4.5D). This was followed by systemic spread of the PTGS signal through the plant to non-infiltrated leaves, starting along the veins, spreading through the leaf laminas (Figure 4.5E-H) until the whole plant appeared uniformly red under UV illumination due to fluorescence of chlorophyll at 20 DPI(Figure 4.5I-J).

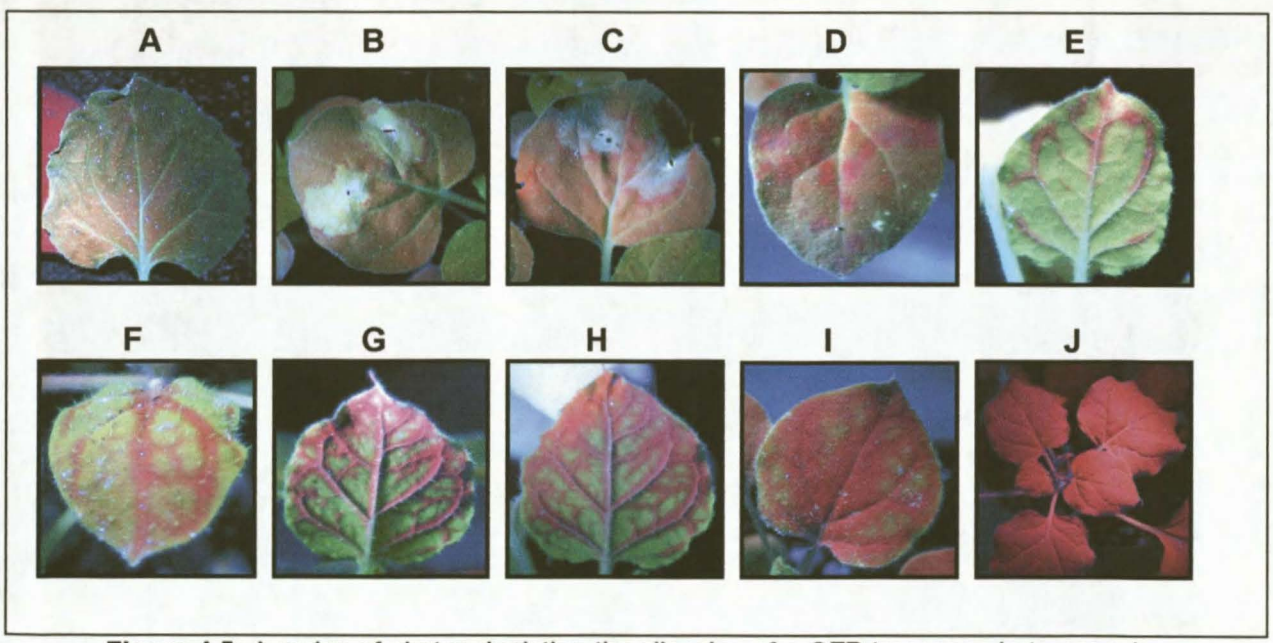


Figure 4.5: A series of photos depicting the silencing of a GFP transgene in transgenic *N. benthamiana* 16c plants.

Lower leaves of the GFP-silenced *N. benthamiana* 16c plants were sap inoculated with a South African GLRaV-2 isolate to screen for suppressor activity in the virus genome. The plants were monitored closely for the development of viral symptoms in old and new leaves.

GFP fluorescence was monitored using a hand held long wavelength UV light source. Three weeks after GLRaV-2 inoculation viral symptoms were visible and suppression of GFP silencing was not visible in the whole plant. New emerging leaves were green under UV illumination and were slower to change colour (Figure 4.6). This could show that GLRaV-2 might possess a suppressor protein or it could be that the new growth was still young and thus displayed a greenish colour before silencing was fully established. If the discolouration was due to suppressor activity it could be that GLRaV-2 contains a suppressor protein similar to the 2b suppressor protein. Since the infiltration of the 2b suppressor protein also produces green new emerging leaves. GLRaV-2 may also encode a suppressor protein that can inhibit local or systemic silencing as will be determined by another silencing assay.

Figure 4.6: Picture indicating the new emerging leaves on a GFP silenced *N. benthamiana* plant inoculated with GLRaV-2.

4.5 Identification of individual GLRaV-2 gene responsible for silencing reversal

In the previous experiment it was observed that the GLRaV-2 genome might possess a suppressor protein able to reverse PTGS as seen by the GFP fluorescence of the new emerging leaves. To identify the specific GLRaV-2 gene responsible for this reversal, lower leaves of GFP-silenced transgenic *N. benthamiana* 16c plants were agro-infiltrated with clones of the different viral genes

(GV3101::pViral106-GeneA to GV3101::pViral106-GeneE) suspensions. In parallel three known suppressor proteins (p19, p25 and 2b) were agro-infiltrated onto GFP-silenced transgenic *N. benthamiana* 16c plants, as positive controls and a binary vector with no insert was agro-infiltrated as a negative control.

GFP fluorescence was monitored using a hand held long wavelength UV light source. UV illumination of the infiltrated plants at 20 DPI displayed the following results:
Plants infiltrated with the known suppressor protein 2b were red and only the new emerging leaves were greenish in colour, thus confirming previous findings by Li & Ding, (2001); Béclin *et al.*, (1998) and Brigneti *et al.,* (1998) (Figure 4.7-1). This discolouration is the result of the inactivation of the signal by either binding it, or some of the processes which affects the signal. The p19 suppressor had almost no effect on the plants and only a slight green fluorescence was visible in the new emerging leaves but only around the veins of the leaves (Figure 4.7-2). This discolouration is due to the binding of the long siRNAs by the p19 protein. Plants agro-infiltrated with the p25 suppressor protein had no effect on PTGS and remained red under UV illumination and no green fluorescence were visible (Figure 4.7-3). These observations confirmed previous findings (Brigneti *et al.*, 1998; Guo & Ding, 2002; Voinnet *et al.,* 1999) and showed that some of the suppressor proteins were able to reverse silencing but only in and around the veins of new emerging leaves and was not able to reverse established PTGS.

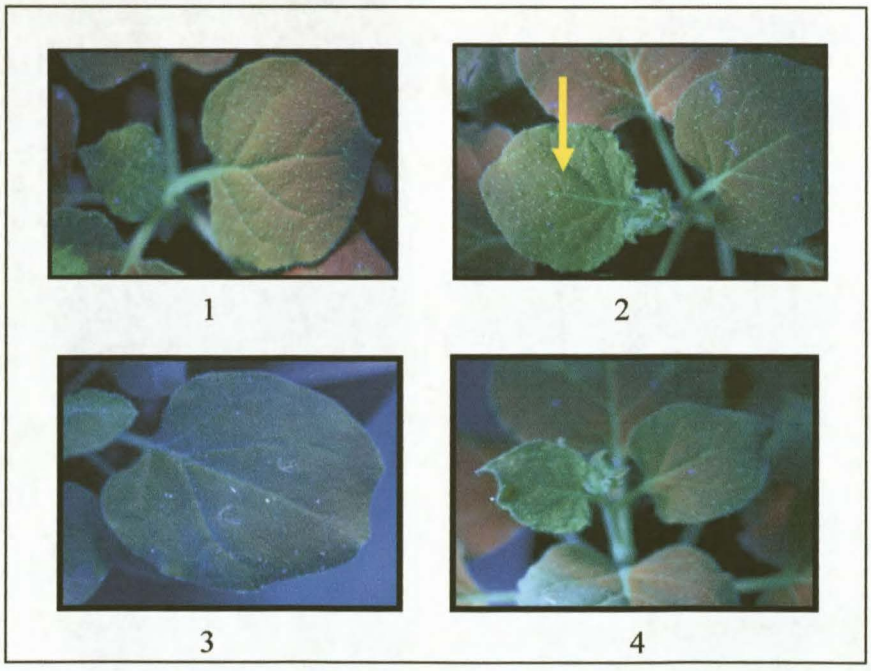


Figure 4.7: Picture indicating the effect of the known suppressors 2b **(1)** p19 **(2)** and p25 **(3)** and pViral106-GeneE **(4)** on silenced transgenic *N. benthamiana* plants. Arrow indicates green veins visible under UV illumination.

PTGS were not influenced in plants agro-infiltrated with all but one of the GLRaV-2 genes and plants still displayed a uniformly red colour under UV illumination. Plants agro-infiltrated with pViral106-GeneE, showed signs of silencing reversal in the new emerging leaves (Figure 4.7-4). The leaves remained green for a period of time but turned red at a later stage. This could mean that GeneE had similar suppressor activity to the 2b protein but not the same since the top leaves of plants infiltrated with the 2b protein remained red. This could mean that GeneE suppresses the PTGS signal partially. The negative control plant was also still uniformly red with no green fluorescence under UV illumination.

4.6 Identification of a suppressor protein in GLRaV-2 of GFP silencing

It has been shown that silencing of a GFP transgene in transgenic *N. benthamiana* 16c plants can be blocked by the transient expression of a known silencing suppressor protein (Voinnet *et al.*, 2000; Johansen & Carrington, 2001). This can be achieved by co-infiltration of an *A. tumefaciens* strain containing the binary plasmid 35S-GFP with a culture of *A. tumefaciens* containing a binary plasmid containing the known suppressor protein into a *N. benthamiana* plant expressing GFP. This type of assay was used to determine if the GLRaV-2 genome contained of a suppressor protein which can inhibit local or systemic PTGS.

The *A. tumefaciens* strain carrying the binary plasmid 35S-GFP and the *A. tumefaciens* strain carrying one of the GLRaV-2 gene constructs were agro-infiltrated into young leaves of transgenic *N. benthamiana* 16c plants. Equal volumes of the GFP cassette (C58C1::GFP) and *A. tumefaciens* expressing each of the individual GLRaV-2 viral genes were mixed prior to agro-infiltration of the young leaves. In parallel, known suppressor proteins (p25, 2b and p19) were co-infiltrated with equal volumes of the GFP cassette onto transgenic 16c *N. benthamiana* plants as positive controls. Transgenic *N. benthamiana* 16c plants agro-infiltrated with only the GFP cassettes were used as a negative control.

4.6.1 GFP imaging

GFP fluorescence was monitored using a hand held long wavelength UV light source. All of the patches infiltrated with GLRaV-2 gene constructs and the GFP cassette were bright green under UV illumination at one DPI (Figure 4.8A1 and A2). This intense fluorescence is due to the expression of the transgene GFP as well as the expression of the *A. tumefaciens* delivered GFP.

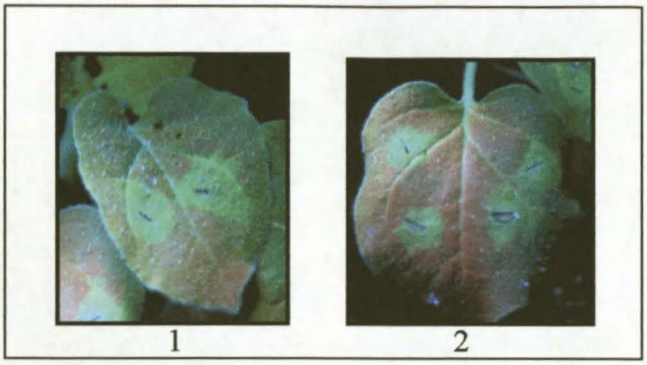


Figure 4.8A: Picture indicating the co-infiltration of transgenic *N. benthamiana* plants with pViral106-GeneE and the GFP construct **(1)** and the GFP construct **(2)** at 1 DPI.

Inoculation with the GFP construct. Examination of the infiltrated patches on the leaves under UV illumination at five DPI confirmed previous findings (Voinnet & Baulcombe, 1997; Dunoyer *et al.*, 2002), patches infiltrated with the GFP construct alone showed decreased green fluorescence and local and systemic GFP silencing was induced. The systemic silencing is observed by the formation of a red fluorescent front (indicated by yellow arrow) around the infiltrated area (Figure 4.8B).

Figure 4.8B: Picture indicating the co-infiltration of a transgenic *N. benthamiana* plant with the GFP construct at 5 DPI. Arrow indicates systemic silencing front.

Inoculation with the GFP construct and known suppressor proteins. Leaf patches co-infiltrated with the known suppressor protein 2b showed similar discolouration as the patches infiltrated with the GFP construct with the exception that no red front was visible around the infiltrated patches at five DPI (Figure 4.8C1). The fact that the patches did not display a brighter green fluorescence was an indication that the initiation of PTGS was not suppressed. Patches

infiltrated with the suppressor protein p25 displayed a dull green with little green fluorescence and no red front. It was still too early to determine if the suppressor was able to suppress local silencing. The decreased green fluorescence could have been the result of the inactivation of the conversion of some of the ssRNAs to dsRNAs as described by Li & Ding, (2001) (Figure 4.8C2) and thus the slight decrease in green fluorescence. In contrast, the leaf patches infiltrated with the p19 suppressor protein were bright green under UV illumination (Figure 4.8C3). This discolouration was due to the p19 protein blocking all size classes of siRNAs as described by Kubota *et al.* (2003, Papp *et al.* (2003) and Silhavy *et al.* (2002).

Figure 4.8C: Picture indicating the co-infiltration of transgenic *N. benthamiana* plants with the known suppressor proteins 2b **(1)** p25 **(2)** and p19 **(3)**. The difference in the brightness of the infiltrated patches is clearly visible between photos 1, 2 and 3.

Inoculation with the GFP construct and GLRaV-2 viral gene constructs. Co-infiltrated leaf patches of the GFP construct with GLRaV-2 viral gene constructs (GeneA to GeneD) showed decreased green fluorescence very similar to the plants infiltrated with the GFP construct. No red front was visible at five DPI, indicating that none of the GLRaV-2 genes could suppress local silencing (Figure 4.8D1-5). Since my focus was on pViral106-GeneE it was monitored more closely for colour changes. The patches infiltrated with pViral106-GeneE were green but not the bright green as seen with the p19 suppressor protein and no red front was visible around the patches (Figure 4.8D5). This led us to conclude that GeneE does not have the same kind of suppressor activity as p19 and thus is not able to suppress all size classes of siRNAs. Since the discoloration of the patches was similar to the patches infiltrated with the suppressor protein p25, GeneE may suppress silencing weakly by interfering with the conversion of ssRNAs to dsRNA or by affecting the siRNAs.

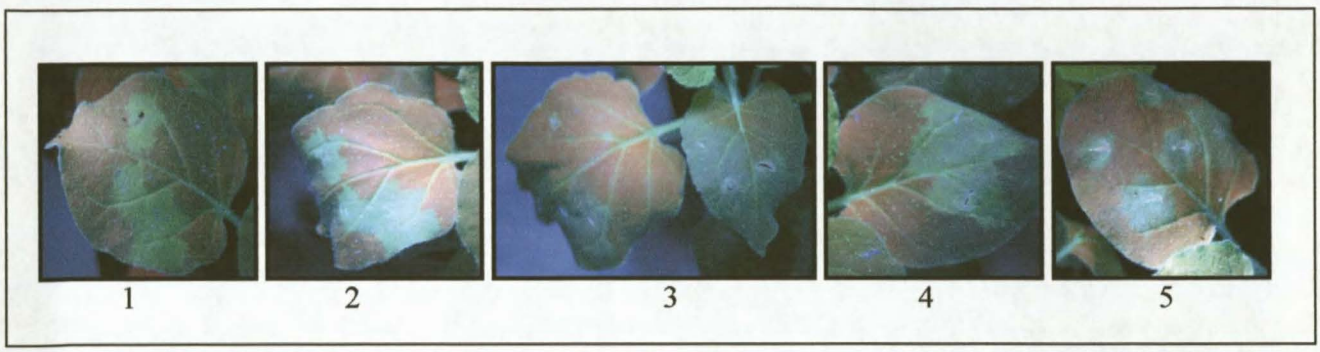


Figure 4.8D: Pictures indicating the co-infiltration of transgenic *N. benthamiana* plants with the constructs pViral106-GeneA **(1)**; pViral106-GeneB **(2)**; pViral106-GeneC **(3)**; pViral106-GeneD **(4)**; pViral106-GeneE **(5)**.

Inoculation with the GFP construct. Further examination of the plants at 12 DPI showed the following results and confirmed previous findings (Voinnet & Baulcombe, 1997; Brigneti *et al.*, 1998). Plants infiltrated with the GFP cassette alone displayed systemic silencing in the upper leaves and the infiltrated leaves were almost completely red under UV illumination with the red front still clearly visible around the patches (Figure 4.8E, indicated by arrow).

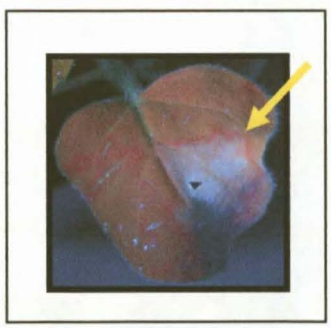


Figure 4.8E: Picture indicating the co-infiltration of a transgenic *N. benthamiana* plant with the GFP construct at 12 DPI. Arrow indicates systemic silencing front.

Inoculation with the GFP construct and known suppressor proteins. Plants infiltrated with the 2b construct showed no more green fluorescence at 12 DPI in the infiltrated patches and thus local silencing was not suppressed (Figure 4.8F1). The 2b suppressor protein is only able to suppress PTGS in other leaves and does not prevent the initiation of PTGS. This is probably due to blocking the systemic transport of the signal into the growing points of the plant (Guo & Ding, 2002). Upper leaves of the plants co-infiltrated with the p25 suppressor also stayed green since p25 is only able to block the systemic spread of the silencing signal as mentioned by Voinnet *et al.*, (2000) by

targeting either the production or the transport of the silencing signal. The leaves infiltrated with the p25 suppressor was still a dull green, almost white (Figure 4.8F2) and confirmed that local silencing was fully suppressed. The bright green patches infiltrated with the p19 suppressor stayed bright green till the leaves started to necrotize (indicated by the arrow) at 15 DPI (Figure 4.8F3). This confirmed previous findings by Silhavy *et al.*, (2002) which found similar results in GFP transgenic plants agro-infiltrated with the p19 protein of TBSV. The p19 suppressor blocks the production of long and short siRNAs and thus prevents the onset of local silencing (Hamilton *et al.*, 2002).

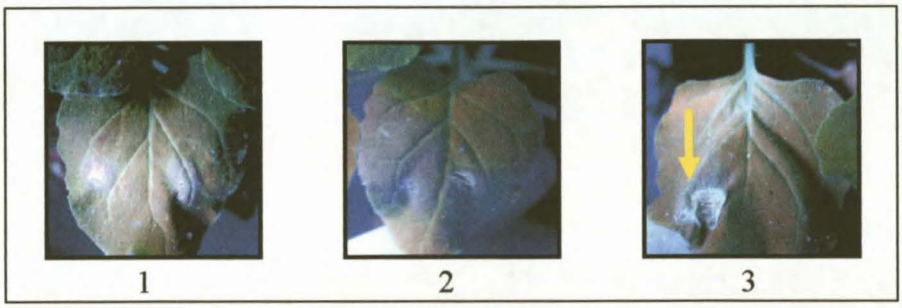


Figure 4.8F: Pictures indicating the co-infiltration of transgenic *N. benthamiana* plants with the known suppressor proteins 2b **(1)** p25 **(2)** and p19 **(3)** at 15 DPI. A necrotic lesion is clearly visible on the leave infiltrated with p19 (indicated by the arrow).

Inoculation with the GFP construct and GLRaV-2 viral gene constructs. Infiltrated leaf patches on the plants co-infiltrated with combinations of the GFP cassette and GLRaV-2 viral genes (GeneA to GeneD) were completely red at 12 DPI (Figure 4.8G1). The infiltrated patches of pViral106-GeneE were also almost completely red (Figure 4.8G2). This was an indication that GeneE was not able to suppress local silencing in the same manner as the p25 suppressor protein. Systemic silencing was also present since the new emerging leaves were turning red under UV illumination (Figure 4.8G3). This showed that the gene was not able to suppress systemic silencing as with the suppressor proteins p25 and 2b.

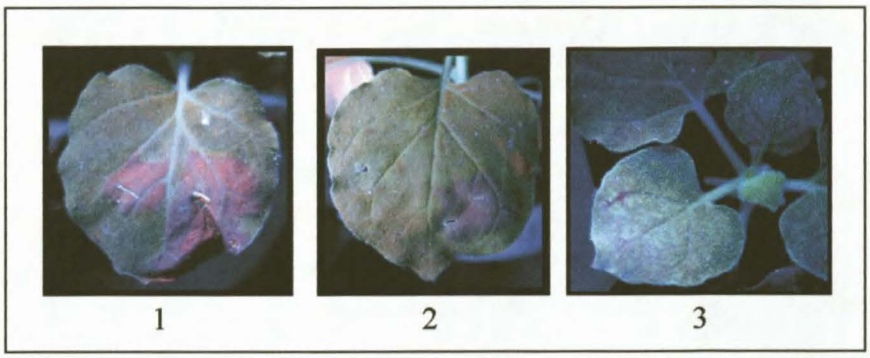


Figure 4.8G: Pictures indicating the co-infiltration of transgenic *N. benthamiana* plants with pViral106-GeneB **(1)** pViral106-GeneE **(2)** pViral106-GeneE top leaves **(3)** at 15 DPI. Systemic silencing is visible on picture 3.

4.6.2 RNA analysis

Total RNA extraction. Total RNA was extracted from transgenic *N. benthamiana* 16c plants and normal *N. benthamiana* to determine if the constructed GFP probe would detect the GFP present in the transgenic plants. Total RNA were visible on the gel with only minor degradation visible (Figure 4.9).

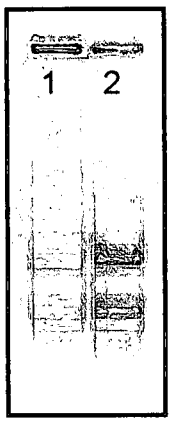


Figure 4.9: Ethidium bromide-stained 10 mM sodium phosphate gel of total RNA isolated from non-transgenic (lane 1) and transgenic (lane 2) *N. benthamiana* plants.

DIG-labelled GFP DNA probe. The labelling of the probe was successful since the labelled PCR product had a greater molecular weight than the control reaction as can be seen in (Figure 4.10). The concentration of the probe was determined spectrophotometrically.

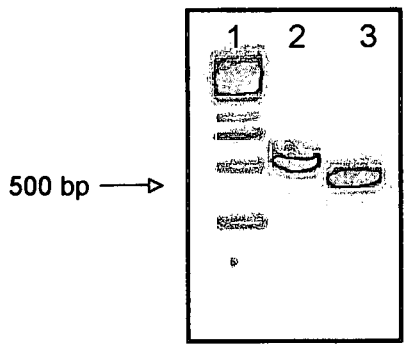


Figure 4.10: Agarose gel displaying the obtained PCR-labelled probe (lane 2) and the control reaction (lane 3). A 1kb ladder is present in lane 1.

The sensitivity of the probe was determined by performing a dilution series with the DIG-labelled GFP DNA probe and GFP DNA. The DIG-labelled GFP DNA probe dilution series ranged from 16 ng to 1.6 pg in a series of five steps and the DNA dilution series ranged from 52 ng to 0.052 pg. The dilution series were spotted on a membrane and visualised by chemiluminescence assay (Figure 4.11). The fifth DNA probe spot (1.6 pg) in the dilution series was very light and showed that the probe was not adequately labelled. The inefficient probe labelling was compensated for by adding five times more of the probe to the hybridization reaction.

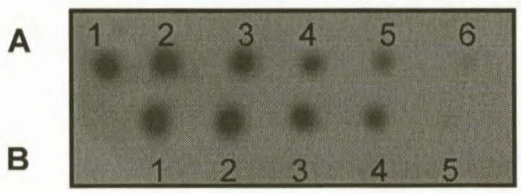


Figure 4.11: DNA and PCR-labelled DNA probe dilution series. **Top lane: (A)** DNA dilution series **(1)** 52 ng, **(2)** 5.2 ng, **(3)** 0.52 ng, **(4)** 52 pg, **(5)** 5.2 pg, **(6)** 0.52 pg, **(7)** 0.052 pg (not visible). **Bottom lane: (B)** PCR-labelled DNA probe dilution series **(1)** 16 ng, **(2)** 1.6 ng, **(3)** 0.16 ng, **(4)** 16 pg, **(5)** 1.6 pg.

Detection of GFP probe-target hybrids. Since probe labelling was inefficient five times the estimated amount of probe was added in the hybridization step of the Northern analysis of the total RNA. The chemiluminescent visualization of the probe-target hybrids was obtained overnight. No signal was detected. The transgenic *N. benthamiana* plants were expected to show a signal since a copy of the GFP gene is present in the plants. This result could be due to the fact that the probe was not efficiently labelled to detect the GFP present in the total amount of RNA. A new probe was synthesised and the same procedures as above were performed but still no signal was detected.

Small RNA extraction. To confirm the GFP fluorescence studies in the transgenic *N. benthamiana* plants in the transient silencing assay, small RNAs were isolated at six DPI from leaves agro-infiltrated with the GFP cassette, the GFP cassette in combination with pViral106-GeneE and with the GFP cassette in combination with suppressor proteins 2b and p19. Small RNAs were also extracted from normal *N. benthamiana* leaves as a control for the detection of the GFP siRNAs. DNA oligonucleotides of 20 bp and 30 bp were also run on the gel as a size estimates. An aliquot (15 µl) of each sample was analysed on a 15% (w/v) polyacrylamide gel with 8 M urea. The high quality small RNAs had a clearly visible tRNA, 5S rRNA and 5.8S rRNA bands (Figure 4.12).

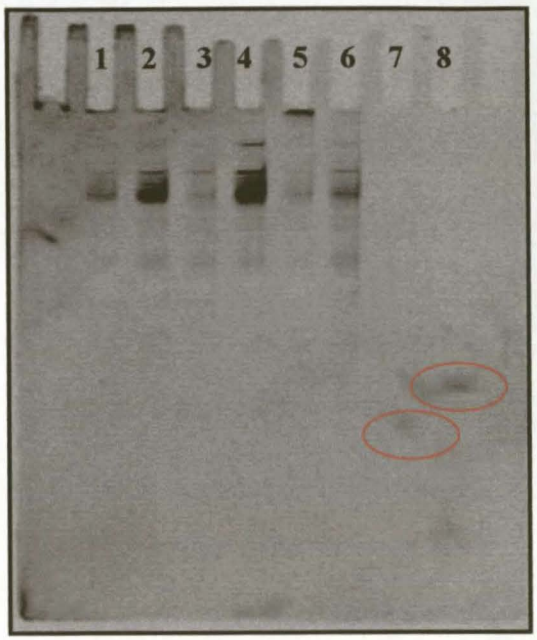


Figure 4.12: Ethidium bromide-stained 15% (w/v) polyacrylamide gel with 8 M urea of small RNAs isolated from leaves agro-infiltrated with the following constructs: **Lane 1:** non-transgenic *N. benthamiana* plants, **Lane 2:** GFP cassette with suppressor protein 2b, **Lane 3:** GFP cassette with suppressor protein p19, **Lane 4:**. GFP cassette, **Lane 5**: non-transgenic *N. benthamiana* plants, **Lane 6**: GFP cassette with pViral106-GeneE, **Lane 7**: 20 bp primer, **Lane 8**: 30 bp primer.

The RNA blot analysis was unsuccessful and thus the levels of the GFP siRNAs could not be compared between the different plants infiltrated. What I expected was that the non-transgenic plants would display no GFP siRNAs since PTGS was not initiated in the plant and thus no dsRNA was cleaved into siRNAs. The leaves infiltrated with the suppressor protein 2b could have displayed GFP siRNAs if the signal of silencing was blocked after the siRNAs was formed. If it didn't display GFP siRNAs the signal was blocked at initiation as described (Coburn & Cullen, 2003; Dong *et al.*, 2003; Guo & Ding, 2002). No siRNAs were expected with the suppressor protein p19 since it is the only suppressor able to block all types of siRNAs (Kubota *et al.*, 2003; Papp *et al.*, 2003; Silhavy *et al.*, 2002). Plants infiltrated with the GFP cassette should have produced the brightest signal since PTGS was initiated against the GFP and no suppressor was present to suppress the process. With the infiltration of the pViral106-GeneE construct, I was unsure what to expect. It was not clear where in the silencing process GeneE played a role and if it would influence the amount of siRNAs formed. Of the two size markers visible on the EtBr-stained gel,

the 30 bp oligonucleotide should have hybridised with the probe since 21 bp on the 3'-end of the oligonucleotide was homologous to the GFP sequence.

4.7 Conclusion

Results of experiments showed that the top leaves of GFP silenced transgenic *N. benthamiana* plants were slower to develop PTGS symptoms suggesting that the genome of GLRaV-2 possesses a suppressor protein. To identify the possible suppressor protein, a silencing reversal assay was performed with five GLRaV-2 genes. Theses genes were designated GeneA to GeneE and were agro-infiltrated into the GFP silenced transgenic *N. benthamiana* plants. Of these, only GeneE seems to show any suppressor reversal ability. Plants infiltrated with GeneE showed signs of silencing reversal in the new emerging leaves appearing after the infiltration of the construct pViral106-GeneE. To see if any of the genes affected local and systemic silencing a second silencing assay was performed.
Leaves of transgenic *N. benthamiana* plants were co-infiltrated with the GFP construct and the five GLRaV-2 genes.

Results from this assay suggested that the pViral106-GeneE was able to suppress local silencing for a while. It was seen that the leaf patches stayed green for longer than the plants infiltrated with only the GFP construct. This suggests that GeneE (p24 gene) may interfere with the some of the processes of PTGS initiation and not with the siRNAs. The patches did not remain fluorescent green until they became necrotic like the p19 suppressor protein and thus PTGS was not fully suppressed. The top leaves of the infiltrated plants displayed systemic silencing and thus GeneE was not able to suppress systemic silencing. Molecular analysis of the siRNAs associated with gene silencing was attempted by Northern blots. Unfortunately no hybridization signals could be detected in any of these experiments. The reason for this is probably of a technical nature (inefficient probe labelling or RNA blotting) and not an indication of the absence of a suppressor in the GLRaV-2 genome.

Visual observations of GFP fluorescence suggested that GeneE, p24 gene of GLRaV-2, has suppressor activity. This result needs to be corroborated with RNA analysis.

Chapter 5 The construction of a tandem-silencing vector Materials and Methods

Introduction

Viruses are among the most important pathogens of grapevine. Leafroll disease is widely acknowledged as the most important virus disease of grapevines, especially in South Africa. A complex of viruses belonging to the family of *Closteroviridae* is associated with leafroll disease, of which Grapevine Leafroll associated Virus 3 (GLRaV-3) is suspected to cause leafroll disease in vineyards all over the world. This disease affects the South African wine industry greatly with losses running into millions of rands.

The only realistic long-term approach in solving the leafroll disease problem is to introduce genetic resistance into vines by genetic engineering. However, when multiple copies of a transgene are inserted in a plant, a phenomenon called post-transcriptional gene silencing (PTGS) often shuts down the expression of these transgenes and the plant loses the beneficial effect of genetic modification.

Designing a "tandem" silencing vector, in which two gene fragments can be simultaneously silenced, could exploit the PTGS phenomenon and be utilized in the field of virus resistant to produce transgenic grapevine.

It has been shown that dsRNA is an effective trigger of PTGS in plants (Waterhouse *et al.,* 2001a), even more efficient than sense or anti-sense RNA separately. Thus to effectively silence an endogenous gene, a plant can be transformed with a transgene which is able to express dsRNA. It was with this in mind that Waterhouse and colleagues discovered that transgenes designed to express single stranded self-complementary (hairpin) RNA have a similar effect on PTGS as dsRNA and if the hairpin contains an intron (ihpRNA), an even higher level of PTGS could be obtained (Wang & Waterhouse, 2000). A generic vector pHannibal, based on the ihpRNA technology, were designed by Helliwell and Waterhouse and allowed the conversion of a PCR product, from a gene of interest, into a highly effective ihpRNA silencing construct (Helliwell & Waterhouse, 2003; Wesley *et al.*, 2001).

In this study, this pHannibal vector will be used to silence two genes simultaneously by inserting two copies of each of the target genes as inverted repeats. The one arm of the hairpin will contain the gene fragments in the sense orientation and the other arm will contain the gene fragments in

the anti-sense orientation. The two arms of the hairpin are separated by an intron, which is a functional spacer and lends stability to the DNA. RNA in a hairpin conformation with a loop of 30-50 bases will be formed after transcription and the intron will be excised from the stemloop construct and the sense and anti-sense fragments will form a complementary double helix RNA (dsRNA) which will be detected by the plants defence system and PTGS will be initiated against any homologous genes (Burch-Smith *et al.*, 2003; Hammond *et al.*, 2001, Wesley *et al.*, 2001).

5.1 Growth conditions of *N. benthamiana* plants

Transgenic *N. benthamiana* seed (line 16c) and non-transgenic seed were germinated in trays and individual plantlets were replanted into plastic pots.

Transgenic and non-transgenic *N. benthamiana* plants were grown in a plant growth room under a 16-h light and 8-h dark regime at 24°C.

5.2 Sap inoculation

Lower leaves of *N. benthamiana* plants at five to six leaf stage were inoculated with the 93/955 GLRaV-2 isolate. Symptoms (curling of top leaves, vein clearing and necrotic lesions) developed after approximately two to three weeks.
This technique was also used to inoculate 15 to 20 days old GFP-silenced, *N. benthamiana* 16c plants with the GLRaV-2 isolate.

5.3 Silencing vectors

5.3.1 pHannibal-generic ihpRNA vector

The pHannibal vector was obtained from the Commonwealth Scientific and Industrial Reasearch Organisation (CSIRO), Canberra, Australia, courtesy P. Waterhouse. It contains two multiple cloning sites (MCS) (5'-*Xho*I.*Eco*RI.*Kpn*I and 3'-*Cla*I.*Hind*III.*Bam*HI.*Xba*I) separated by a pyruvate orthophosphate dikinase (pdk) intron, under the control of a CaMV 35S promoter and octopine synthase (OCS) terminator (Figure 5.1). Desired gene fragments are cloned into the MCS in the sense and anti-sense conformation and the silencing cassette can be excised as unit by a *Not*I

restriction digestion (Hammond *et al.*, 2001; Wesley *et al.*, 2001). The vector was maintained in *E. coli* under Amp (100 µg/ml) selection.

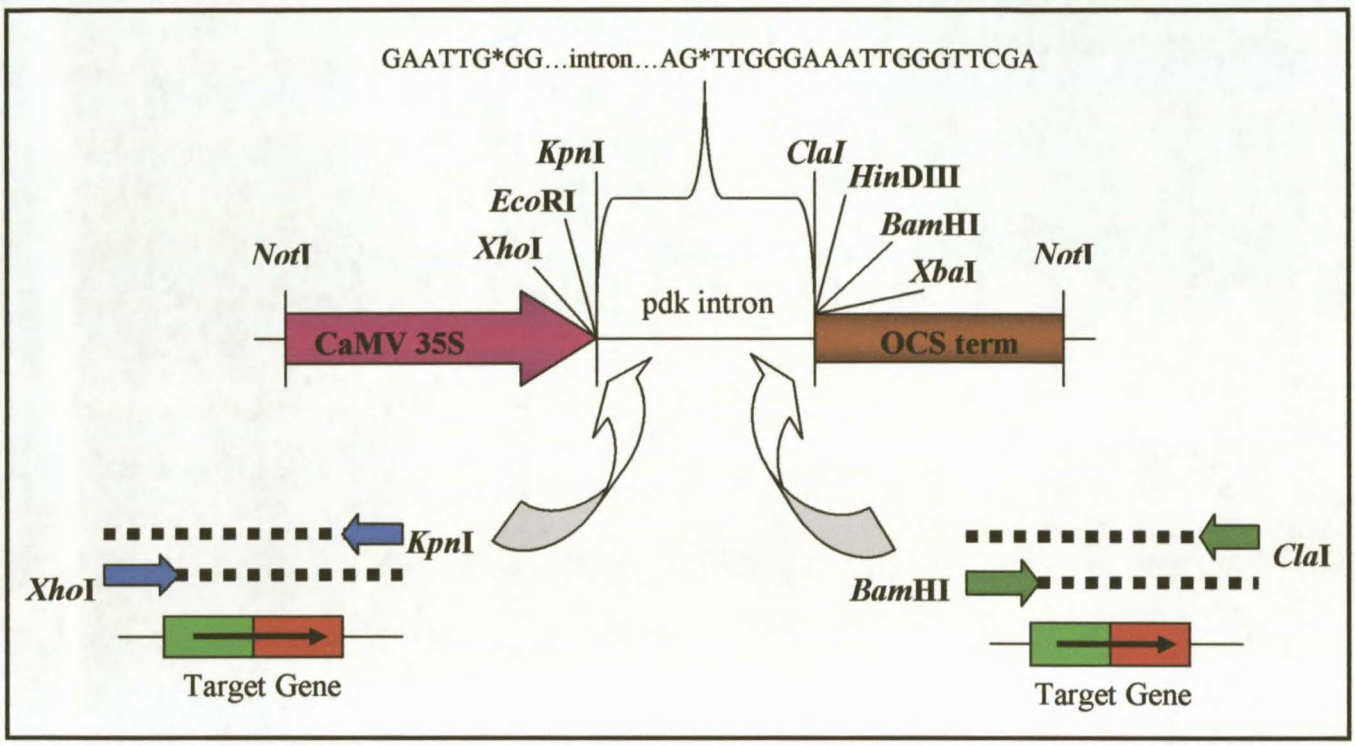


Figure 5.1: Schematic representation of the pHANNIBAL-generic ihpRNA vector which was utilised to obtain sense and anti-sense orientated genes of interest. The MCS and intron is depicted in the representation.

5.3.2 pART27

The pART27 vector (Figure 5.2) was obtained from the Molecular Genetics Group, Horticulture and Food Research Institute of New Zealand (HortReasearch), Auckland, courtesy A.P. Gleave. The backbone of the binary vector pART27 is derived from the binary vector pMON530 (Rogers, 1978) and provides a RK2 minimal replicon for maintenance in *A. tumefaciens,* and the ColE1 replicon for high-copy maintenance in *E.* coli and the Tn7 spectinomycin/streptomycin resistance gene for bacterial selection. The T-DNA of pART27 contains a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase which is under control of a NOS promoter and terminator. It also carries in its T-DNA the chimaeric Kanamycin gene NPTII (nopaline synthase promoter-neomycin phosphotransferase-nopaline synthase terminator) and was maintained in *E.coli* under Kan (20 µg/ml) selection (Gleave, 1992).

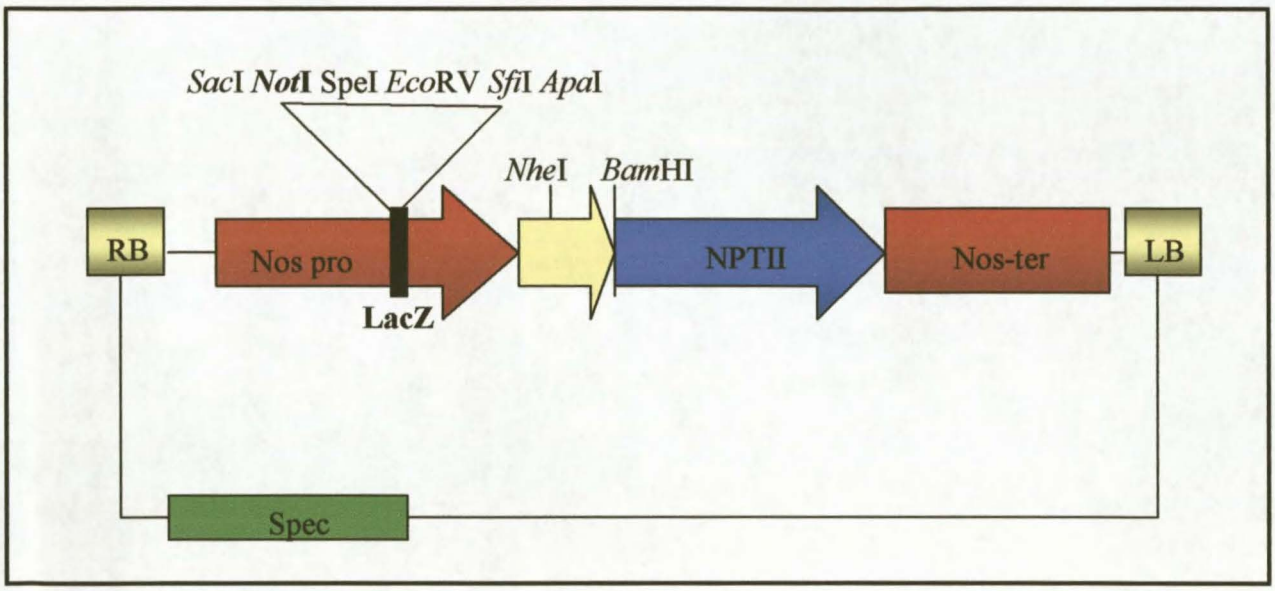


Figure 5.2: Schematic representation of the plant transformation vector pART27. The MCS is located within the LacZ gene.

5.4 Isolation and cloning of GLRaV-2 and GFP genes

Primers to isolate the sense and anti-sense fragments from the *Hsp70* gene of the GLRaV-2 genome and the GFP gene from the pBIN m-gfp5-ER vector (Table 5.1) were designed using the same primer design program as mentioned in Chapter 3, Section 3.4. The primers were made with incorporated restriction sites to facilitate cloning and the T_m of the individual primers was calculated using the Oligonucleotide Analyzer tool. The T_A for the PCR reactions was set at 5°C below the T_m of a particular primer set.

Table 5.1: Primers used to obtain sense and anti-sense fragments from the GLRaV-2

Primer	Incorporated RE site	Sequence (5'-3')	Fragment size (bp)	Tm (°C)
GLRaV-2 Sense-For	*Kpn*I- ATT **GGT ACC**	CGG TGT GTG TGT ACA AGG ATG G	487	72.4
GLRaV-2 Sense-Rev	*Eco*RI-GCC **GAA TTC**	TAC GCA CTG ATA ACC GCT GAG T		72
GFP Sense-For	*Eco*RI-CGG **GAA TTC**	GGT GAA GGT GAT GCA ACA TAC G	452	72.6
GFP Sense-Rev	*Xho*I-CGG **CTC GAG**	TTG ATA ATG ATC AGC GAG TTG C		71.8
GLRaV-2 Ant-sense-For	*Hin*DIII-AGA **AAG CTT**	CTT CGG TGT GTG TGT ACA AGG AT	487	68.7
GLRaV-2 Anti-sense-Rev	*Cla*I-CAT **ATC GAT**	TAC GCA CTG ATA ACC GCT GAG T		68
GFP Anti-sense-For	*Bam*HI-ATA **GGA TCC**	GGT GAA GGT GAT GCA ACA TAC G	452	70.9
GFP Anti-sense-Rev	*Hin*DIII-GCC **AAG CTT**	TTG ATA ATG ATC AGC GAG TTG C		68.6

The sense (S) and anti-sense (AS) orientated fragments of the GLRaV-2 gene were isolated by the RDOT-RT-PCR procedure. The primers used are shown in Table 5.1. The procedure was performed as described in Chapter 3, Section 3.5.

The sense and anti-sense orientated fragments of the GFP gene were isolated from the plasmid vector pBIN mgfp5-ER, by a PCR using the appropriate primers shown in Table 5.1. The reaction was performed in 20 µl of PCR reaction mix (1 x NH_4 PCR Buffer, 1 x Sucrose/Cresol dye, 0.5 µm forward and reverse primer, 1.5 mM $MgCl_2$, 200 µM dNTP's and 0.5 U Taq DNA polymerase (5 U/µl bioline Biotaq)).The final product was placed in a thermal cycler and subjected to the following cycling conditions: One cycle of 94°C for five minutes; 30 cycles comprising of 94°C for 30 seconds, T_A (T_m-5) of a particular primer set for 30 seconds, 72°C for 30 seconds; One cycle of 72°C for seven minutes and indefinitely at 4°C.

Purification and quantification of sense and anti-sense orientated gene fragments. A 1.4% (w/v) agarose (D1-LE Hispanagar) TAE gel was used for the separation of the DNA fragments in the electrophoresis experiments. A 50x concentrated stock solution of the TAE gel electrophoresis buffer (2 M Tris base, 0.5 M EDTA (pH 8.0), 5.71% (v/v) glacial acetic acid) was prepared and used at a 1 x concentration. EtBr (0.25 ug/ml) was added to the agarose gel prior to electrophoresis to visualise DNA fragments. The electrophoresis experiments were carried out at a voltage of 80V for approximately 45 minutes. Hyperladder I (5 µl/lane; Bioline) was used as a size marker. Loading dye (40% (w/v) sucrose, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol) was mixed 1:4 with samples before electrophoresis.
The QIAquick ® Gel Extraction Kit (QIAgen) was used for the extraction and purification of the RDOT-RT- PCR and PCR products obtained from the agarose/TAE gel. The purification of the products was performed according to the manufacturer's instructions and as described in Chapter 3, Section 3.7.

Cloning, Transformation and Selection of Recombinants. The high copy number pGEM®-T-Easy Vector System (Promega, SA) was used for the cloning of the individual sense and anti-sense products obtained after the gel purification step. The ligation reactions were performed as described in Chapter 3, Section 3.8. The amount of PCR product used per individual reaction was calculated accordingly to the manufacturer's instructions and an example is provided in **Appendix A.** The reaction was incubated overnight at 4°C for the maximum number of transformants.

Transformation of the pGEM®-T-Easy vectors containing the cloned sense and anti-sense orientated fragments was performed as per Sambrook *et al.* (1989). Competent *E.coli* DH5α cells, prepared by the $CaCl_2$ method (**Appendix B**), were used for all standard transformation reactions. The transformation reactions were performed as described in Chapter 3, Section 3.9.

The authenticity of the putative recombinants obtained after 16 h of incubation, was determined by colony PCR (Chapter 3, Section 3.9).

PCR positive colonies were inoculated in five ml LB medium containing Amp (100µg/ml) for selection and incubated overnight at 37°C with shaking (200 rpm). Plasmid DNA was harvested from the five ml overnight cultures using the Wizard® Plus SV miniprep DNA (Promega) as described by the manufacturer (Chapter 3, Section 3.10).

Freezer cultures of the pGEM®-T-Easy vectors containing the S and AS fragments were prepared.

5.5 Linking of sense and anti-sense fragments of the GLRaV-2 and GFP genes.

The restriction digestions of the pGEM®-T-Easy vectors containing the sense and anti-sense fragments were performed as prescribed by the manufacturer. Each of the four fragments was excised from their respective pGEM®-T-Easy vectors by utilising the restriction sites incorporated in the RDOT-RT-PCR and PCR reactions respectively. The reactions were performed in 10 µl (1 x restriction buffer [50 mM Tris-HCL pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA], 5 U appropriate restriction enzyme (Fermentas), and 500 ng DNA) and incubated in a water bath at 37°C for two hours. The final products were run on a 1.4% (w/v) Agarose/TAE gel (Chapter 3, Section 3.6) and the correctly sized fragments were excised by means of the QIAquick® kit (Chapter 3, Section 3.7). The concentrations of the newly excised fragments were determined spectrophotometrically.

The GLRaV-2–Sense and GFP-Sense orientated fragments were ligated together using the mutual restriction enzyme *Eco*RI to form a new sense fragment termed **ViralGFP-S**. The anti-sense orientated fragments of the two genes were ligated using the mutual restriction enzyme *Hin*DIII to form a new anti-sense fragment termed **ViralGFP-AS**.

The ligation reactions were performed in 20 µl with T4 DNA ligase and buffer (50mM Tris-HCL, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA) supplied by New England Biolabs and 50 ng of each fragment. The reaction was incubated overnight at 16°C.

The sub-cloning of ViralGFP-S and ViralGFP-AS into pGEM®-T-Easy, the transformation, selection of the recombinants and plasmid extraction was performed as described in Chapter 3, Section 3.9 and Section 3.10.

5.6 Construction of the plant expression vector, pHanViralGFP-SAS

The restriction digestion of the pHannibal vector was performed as prescribed by the manufacturer of the restriction enzymes. The vector was digested in two restriction reactions with the following restriction enzymes *Kpn*I and *Xho*I for the incorporation of the sense fragment and *Bam*HI and *Cla*I for the incorporation of the anti-sense fragment, after the sense fragment had been incorporated. The reactions were performed in 10 µl (1 x restriction buffer [50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA], 5 U of each restriction enzyme (Fermentas), 500 ng DNA) and incubated in a water bath at 37°C for two hours. The final products were run on a 1.4% (w/v) Agarose/TAE gel (Chapter 3, Section 3.6) and the correctly sized fragments were excised by means of the QIAquick® kit (Chapter 3, Section 3.7). The concentrations of the newly excised fragments were determined spectrophotometrically.

The sense and anti-sense fragments were excised from their individual pGEM®-T-Easy vectors by means of restriction digestion of their respective incorporated restriction sites *Kpn*I and *Xho*I (ViralGFP-S) and *Bam*HI and *Cla*I (ViralGFP-AS) and was visualised on a 1.4% (w/v) Agarose/TAE gel (Chapter 3, Section 3.6). Correctly sized fragments were excised from gels and extracted using the QIAquick® Gel Extraction Kit (Chapter 3, Section 3.7) and the concentrations were determined spectrophotometrically. The ligation reactions were performed in 20 µl as described in Section 5.5 with the following exception. The ViralGFP-S fragment was first ligated into the pHannibal vector followed by the ligation of the ViralGFP-AS fragment into the vector already containing the ViralGFP-S fragment.

The pHanViralGFP-SAS transformation reaction was performed as described in Chapter 3, Section 3.9. Since the pHannibal vector does not contain blue/white selection, no X-gal or IPTG was added to the LB/agar plates. The authenticity of the putative transformants was determined by colony PCR (Chapter 3, Section 3.9. The primer pair GLRaV-2 Sense-For and GFP Sense-Rev listed in red in Table 5.1 were used in the colony PCR reaction. The products of the PCR reaction was run on a 1.4% (w/v) Agarose/TAE gel (Chapter 3, Section 3.6).

Recombinant *E. coli* colonies were selected for on LB/Agar plates containing Amp selection. Plasmid DNA extractions were performed as described in Chapter 3, Section 3.10. A second screening of the positive recombinants (containing the final plasmid pHanViralGFP-SAS) was done before the plasmids were transformed into *A. tumefaciens.* The mini-prep DNA obtained above was subjected to eight restriction digestion reactions, all performed in a final volume of 10 µl. The final products were run on a 1.4% (w/v) Agarose/TAE gel (Chapter 3, Section 3.6) to visually confirm the integrity of the plasmid.

5.7 Construction of the plant expression vector, pSilencer-SAS

The pART27 vector was digested with the restriction enzyme *Not*I and the reaction was performed as described in Section 5.5 with the following exception. The blunt ended vector was dephosphorylated by SAP treatment, in a final volume of 30 µl (1X SAP reaction buffer [0.05 M Tris-HCl pH 9.0, 10 mM $MgCl_2$], 3 U SAP (1U/µl)). The reaction was incubated at 37°C for 12 minutes in a water bath and heat inactivated at 65°C for 15 minutes in a heating block.

The silencing cassette (ViralGFP-*SAS*) was excised from the pHannibal vector as a *Not*I fragment in a restriction digestion reaction as described above and was visualised on a 1.4% (w/v) Agarose/TAE gel. A correctly sized fragment was excised form the gel and extracted using the QIAquick® kit and the concentration was determined spectrophotometrically. The ligation reactions were performed in 20 µl with T4 DNA ligase and buffer (50 mM Tris-HCL, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA) supplied by New England Biolabs and 50 ng pART27 vector and x ng PCR product. The quantity of PCR product used per individual reaction was calculated accordingly to the manufacturer's instructions and an example is provided in **Appendix A**. The reaction was incubated overnight at 16°C.

The transformation of pSilencer-SAS was performed (Chapter 3, Section 3.9) and colonies containing this vector were selected on Kan (20 µg/ml). The authenticity of the putative transformants were determined by restriction digestion of the colonies with the restriction enzymes *Not*I, *Sal*I, *Eco*RV and *Sac*I, *Spe*I as described in Section 5.5. Recombinant *E. coli* colonies were selected for on LB medium containing Kan (20 µg/ml) selection. Plasmid extractions were performed as described in Chapter 3, Section 3.10.

The final construct pSilencer-SAS was sequenced at the Core DNA Sequencing facility at Stellenbosch University.

5.8 *Agrobacterium tumefaciens* Transformation and Selection

Transformation of the pSilencer-SAS construct was performed with competent *A. tumefaciens* cells (C58C1 and GV3101), prepared as per Tzfira *et al.* (1997) (**Appendix B**) and as described in Chapter 3, Section 3.12. The authenticity of the colonies on the plates, obtained after three days of incubation, was determined by colony PCR (Chapter 3, Section 3.9) and the primer pair GLRaV-2 Sense-For and GFP Sense-Rev listed in (Table 5.1) were used in the reaction.

5.9 Transformation of *Nicotiana tabacum* cv. Petit Havana (SR1)

The final construct pSilencer-SAS incorporated into *A. tumefaciens* strains Gv3101 and C58C1 was constitutively transformed into Petit Havana plants by L. Watts at ARC-Infruitec/Nietvoorbij, Stellenbosch.

5.10 Agro-infiltration of the silencing construct

Positive colonies GV3101::pSilencer-SAS were prepared for agro-infiltration by following the protocol provided on the Sainsbury Laboratory website (http://www.jic.bbsrc.ac.uk/sainsburylab/dcb/Services/AgroInfiltrationHP.htm) and as described in Chapter 3, Section 3.14. The final Kan selection used for incubation was 20 µg/ml. The *A. tumefaciens* suspensions of the different constructs were infiltrated into the leaves of *N. benthamiana* plants as described in Chapter 3, Section 3.13. All of the *N.benthamiana* plants agro-infiltrated were monitored for a period of 5-20 DPI and infiltrated leaves as well as new emerging growth were monitored visually under UV illumination for any colour changes.

5.11 GFP imaging

Visual detection of GFP fluorescence was performed using a 100 W, hand-held, long wave ultraviolet (UV) lamp (SB-100F Series model, Spectroline). Plants were photographed with a digital camera (Canon, D300). The images were processed by using the Microsoft Photo editor program.

Chapter 6 The construction of a tandem-silencing vector Results and Discussion

Introduction

It is generally accepted that the South African wine industry loses millions of rands annually, as a result of infected vineyards, and that this ultimately impacts negatively on the economy of the Western Cape. Existing control measures focus on prevention by utilising virus-free propagation material and integrated control of the insect vectors. The introduction of transgenic resistance into vines has increased greatly since the mid 1990s and *Vitis rupestrsis* and other rootstocks have been transformed with genes of GLRaV-2 and GLRaV-3, (Krastonova *et al.*, 2000), but it is still in its early stages and much needed work must still be done.

A natural genetic mechanism found in most plants can help protect plants against virus attacks. PTGS forms a natural defence mechanism against foreign RNA, especially viruses and degrades them in a sequence-specific manner. Some viruses replicate in the cytoplasm by means of a dsRNA intermediate and this dsRNA is perceived as foreign and triggers the degradation of itself and homologous RNA within the cell. PTGS can be induced in plants by transformation of plants with transgenes that encode ds or self-complementary hairpin RNA (hpRNA) containing sequences homologous to the target genes (Waterhouse *et al.*, 1998; Wang & Waterhouse, 2000; Smith *et al.*, 2000). This type of resistance has been successfully obtained against *Plum pox potyvirus* (PPV) in *N. benthamiana* (Pandolfini *et al.*, 2003)

A tandem silencing vector, based on the generic gene-silencing vector pHannibal was constructed to be used for proof of concept for the introduction of virus resistance by genetic modification into grapevine and will utilise the natural occurring PTGS method to prevent the onset of virus infection. The complimentary regions of the vector (pSilencer-SAS) contain a GLRaV-2 Hsp70 gene sequence and a reporter protein GFP sequence separated by an intron to lend stability to the plasmid (Smith *et al.*, 2000). Gene fragments' ranging from 50 bp to 1.6 kb have been used successfully in this type of vector but it is recommended that fragments are between 300 and 1000 bp in length to maximise the efficiency of silencing. Translated as well as untranslated regions (UTRs) have been used with equal success.

Once the construct is transformed into the *N. benthamiana* plant it will be transcribed, and because of their complimentary design of the two arms dsRNA will be formed. Upon infection of the plant with the homologous virus and or GFP transgene the dsRNA will trigger a post transcriptional gene silencing mechanism which will recognise and destroy the viral RNA in its intermediate replication form, soon after infection.

6.1 Isolation and linking of sense and anti-sense fragments

N. benthamiana plant material infected with the South African isolate of GLRaV-2 was received from the Plant Protection Research Institute of the Agricultural Research Council SA (ARC-PPRI). The infected material was used to sap inoculate new *N. benthamiana* plants to maintain the virus in active form. Sense and anti-sense orientated fragments from the Hsp70 gene of GLRaV-2 were isolated by a RDOT- RT-PCR reaction using primers with 5'-extensions containing restriction sites to facilitate subsequent cloning (Table 5.1). GFP sense and anti-sense orientated fragments of similar size were isolated from the vector pBIN mGFP5-ER by a PCR reaction using appropriate primers with 5'-extensions to facilitate subsequent cloning (Table 5.1). The amplified products were visualized (Figure 6.1) and were 452 bp (GFP) and 487 bp (Hsp70) in size respectively.
Purified products were cloned into the intermediate vector pGEM®-T-Easy (**Appendix E-1 & E-2**) and transformed into competent DH5α cells. Positive recombinants were selected by colony PCR, with appropriate primers (Table 5.1).

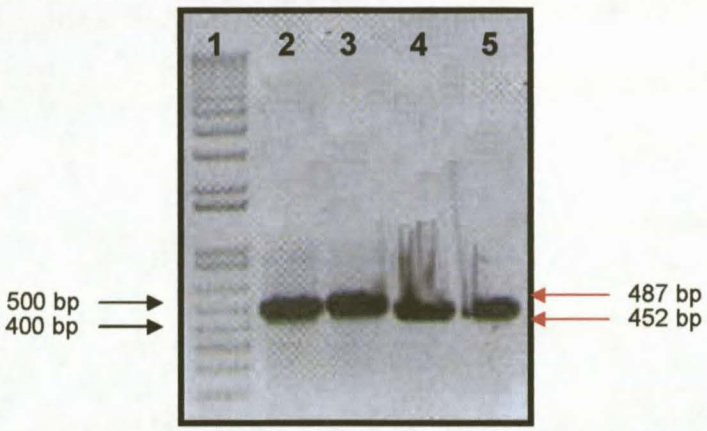


Figure 6.1: Fragments obtained from RDOT-RT-PCR and PCR reactions. **Lane 1:** 1 kb+ ladder. **Lane 2:** Sense orientated Hsp70 fragment. **Lane 3:** Anti-sense orientated Hsp70 fragment. **Lane 4:** Sense orientated GFP fragment. **Lane 5:** Anti-sense orientated GFP fragment.

Sense and anti-sense orientated gene fragments of GLRaV-2 (487 bp) and GFP (452 bp) were excised from their respective pGEM®-T-Easy vectors by utilizing the restriction sites incorporated in the RDOT-RT-PCR and PCR reactions respectively. The final products were visualized (not shown) and gel-purified. The GLRaV-2 and GFP sense orientated fragments were ligated using the mutual restriction enzyme *Eco*RI and in parallel, the GLRaV-2 and GFP anti-sense orientated fragments were ligated using the mutual restriction enzyme *Hin*DIII. To obtain the final sense and anti-sense fragments (ViralGFP-S and ViralGFP-AS) (**Appendix E-3**), one microlitre of each ligation reaction were added to a PCR reaction and amplified using the primer pairs GLRaV-2 sense-For/Rev and GFP anti-sense-For/Rev. This was done to incorporate the restriction sites *Kpn*I and *Xho*I on the ends of the sense fragment and the restriction sites *Bam*HI and *Cla*I on the ends of the anti-sense fragments to ensure subsequent cloning of the fragments. The amplified PCR products were visualized (Figure 6.2), gel purified, cloned into the intermediate vector pGEM®-T-Easy and transformed into competent DH5α cells. Recombinants were selected by colony PCR, with appropriate primers (Table 5.1) inoculated and purified.

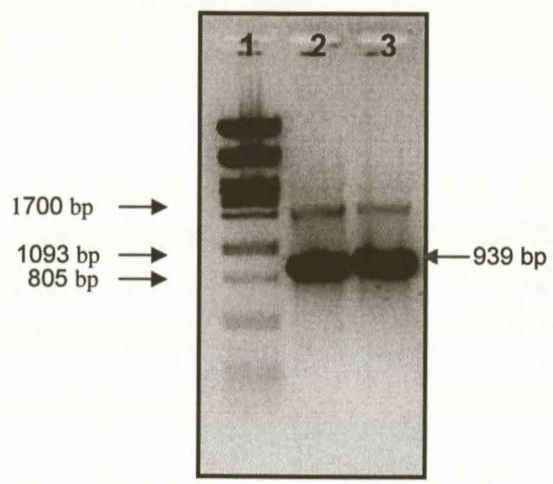


Figure 6.2: Amplified PCR products to obtain final ViralGFP-S and ViralGFP-AS fragments. **Lane 1:** λ *Pst*I DNA ladder. **Lane 2:** ViralGFP-S. **Lane 3:** ViralGFP-AS

6.2 Construction of plant expression vector pHanViralGFP-SAS

The pHannibal vector was first digested with the restriction enzymes *Kpn*I and *Xho*I for the incorporation of the ViralGFP-S fragment. After the incorporation of the sense fragment the vector was digested with the restriction enzymes *Bam*HI and *Cla*I for the incorporation of the ViralGFP-AS

fragment. The ViralGFP-S and ViralGFP-AS fragments were excised from their individual pGem®-T-Easy vectors by means of their incorporated restriction sites *Kpn*I and *Xho*I (ViralGFP-S) and *Bam*HI and *Cla*I (ViralGFP-AS) in a restriction digestion reaction. Correctly sized fragments were gel-purified after visualization of the gel.

The ViralGFP-S fragment was first ligated into the corresponding sites of the MCS of the pHannibal vector in sense orientation followed by the cloning of the ViralGFP-AS fragment in the anti-sense orientation into the pHannibal vector already containing the ViralGFP-S fragment. The final construct, pHanViralGFP-SAS (**Appendix E-4**) containing the ViralGFP-S and ViralGFP-AS fragments were transformed into competent DH5α cells. Recombinants were selected by colony PCR, with primers GLRaV-2 Sense-For and GFP Sense-Rev (Table 5.1) and PCR positive colonies were inoculated and purified. The final construct pHanViralGFP-SAS was sequenced for final confirmation where an internal *Cla*I restriction site was discovered in the GFP fragments, 167 bp from the *Hin*DIII and *Xho*I restriction sites.

A second screening was incorporated to confirm the sequencing results and to authenticate the positive recombinants. The internal *Cla*I site was kept in consideration for this second screening step. Mini-prep DNA was subjected to multiple restriction digestion reactions with different restriction enzymes as shown in Figure 6.3. In lane 2 the *Bam*HI – *Xho*I digest excised two bands. The band of 2619 bp represents the ViralGFP-S (969 bp) fragment, ViralGFP-AS (939 bp) fragment and the intron (741 bp). The 5083 bp band represents the backbone of the pHanViralGFP vector. The *Bam*HI – *Cla*I digest in lane 3 should have excised the intact ViralGFP-AS fragment (939 bp), similar to the digest in Lane 8. But because of the internal *Cla*I site four bands were generated. The bands represents the following fragments of the pHanViralGFP vector, 285 bp (Figure 6.4A), 654 bp (Figure 6.4B), 1513 bp (Figure 6.4C) and 5250 bp (Figure 6.4D) and a backbone of 6763 bp. In lane 4 the *Bam*HI – *Hin*DIII digest excised the GFP-AS fragment (452 bp) with a backbone of 7250 bp. The *Hin*DIII – *Cla*I digest in lane 5 should have excise the GLRaV-2-AS fragment (487 bp) but generated four bands because of the extra *Cla*I site. The bands represents the following fragments of the pHanViralGFP vector, 167 bp (Figure 6.4E), 487 bp (Figure 6.4F), 1513 bp (Figure 6.4G) and 5535 bp (Figure 6.4H). The *Kpn*I – *Xho*I digest in lane 8 excised the ViralGFP-S fragment (939 bp) with a backbone of (6763 bp). In lane 9 the *Kpn*I – *Eco*RI digest excised the GLRaV-2-S fragment (487 bp) with a backbone of 7215 bp. The *Eco*RI – *Xho*I digest in lane 10 excised the GFP -S fragment (452 bp) with a backbone of 7250 bp.

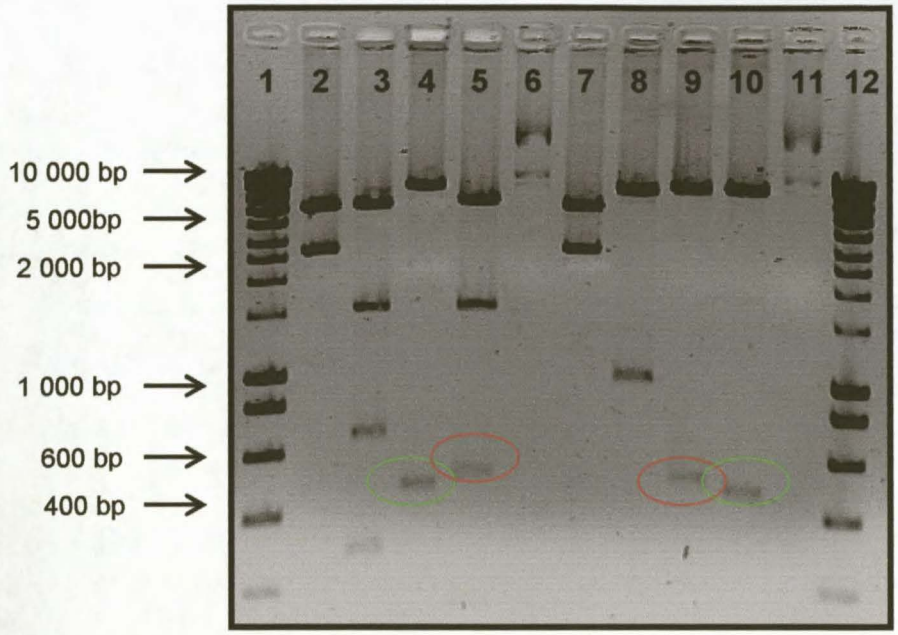


Figure 6.3: Multiple digest of final construct pHanViralGFP-SAS to confirm authenticity. **Lane 1** and **12:** Hyperladder 1. **Lane 2:** *Bam*HI – *Xho*I digest (2619 bp & 5083 bp). **Lane 3:** *Bam*HI – *Cla*I digest (285 bp & 654 bp & 1513 bp & 5250 bp). **Lane 4:** *Bam*HI – *Hin*DIII digest (452 bp & 7250 bp). **Lane 5:** *Hin*DIII – *Cla*I digest (167 bp & 487 bp & 1513 bp & 5535 bp). **Lane 6:** Undigested vector pHannibal. **Lane 7:** *Bam*HI – *Xho*I digest (2619 bp & 5083 bp). **Lane 8:** *Kpn*I – *Xho*I digest (939 bp & 676 3bp). **Lane 9:** *Kpn*I – *Eco*RI digest (487 bp & 7215 bp). **Lane 10:** *Eco*RI – *Xho*I digest (452 bp & 7250 bp). **Lane 11:** Undigested vector pHannibal.

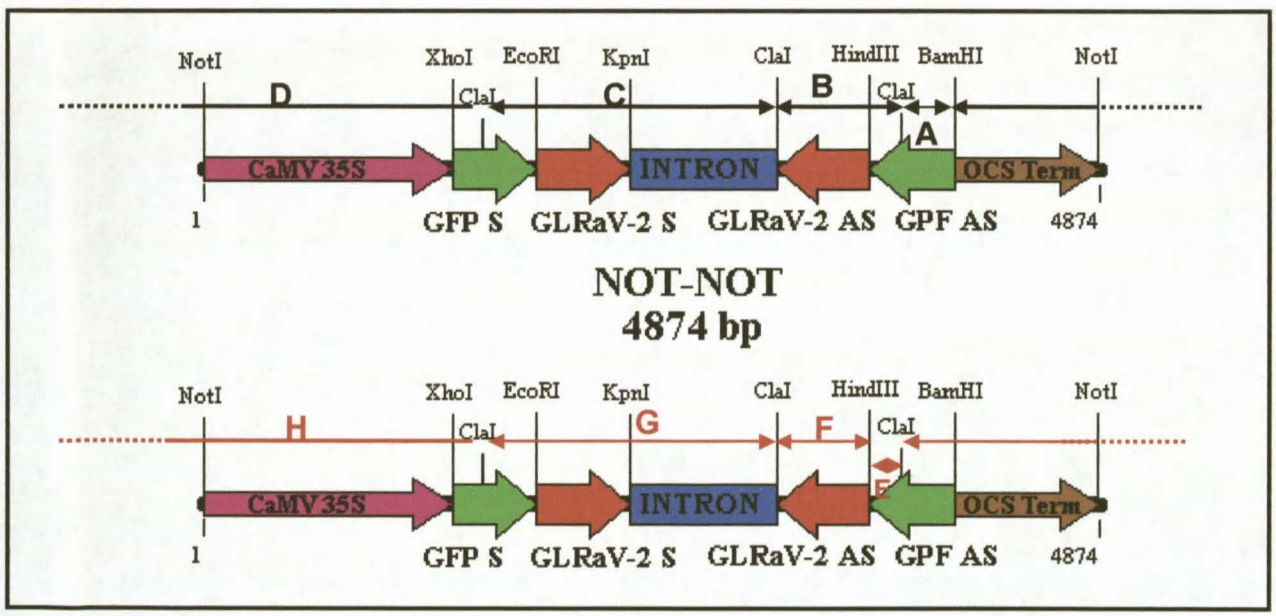


Figure 6.4: Representation of the silencing cassette of the pHanViralGFP construct with indicated restriction sites as observed after multiple restriction digestion.

6.3 Construction of tandem silencing vector pSilencer-SAS

The T-DNA of pART27 carries a Kanamycin resistance gene (*npt*II) for the selection of pART27 in both *E.coli* and *A. tumefaciens* and a nopaline synthase (NOS) promoter and terminator. The T-DNA will be inserted into the plant by agro-infiltration. The pART27 vector was digested with the restriction enzyme *Not*I and dephosphorylated by SAP treatment for the cloning of the silencing cassette from pHanViralGFP-SAS. The silencing cassette was removed as a *Not*I fragment (**Appendix E-5**) from pHanViralGFP-SAS and ligated into the *Not*I site located in the Lac Z region between the left and right border of the plant expression vector pART27. The ligation reaction was visualized (not shown) and the correctly sized fragment was excised from the gel and purified. The final construct pSilencer-SAS (**Appendix E-6**) was transformed into competent DH5α cells.

Recombinants were authenticated by multiple restriction digestion with the following restriction enzymes *Not*I, *Sal*I, *Eco*RV and *Sac*I, *Spe*I (Figures 6.5-Figure 6.7). Two blue colonies (pART27 vector without insert) were digested as negative controls to support the positive results. Recombinants providing correctly sized fragments where inoculated and purified. In the *Not*I restriction digestion the silencing cassette (4843 bp) was excised from the pSilencer-SAS construct and left a backbone of 11667 bp. Negative colonies were linarised by this digestion, since no silencing cassette was present. The *Sal*I restriction digestion excised the fragment form the RB to the LB (8507 bp) of the pSilencer-SAS construct with a backbone of 8003 bp. Because the band sizes are so close to each other only one band is visible on the gel. The negative colonies generated the same fragment but without the silencing cassette (4843 bp) and yielded a band of 3664 bp with a backbone of 8003 bp. Since *Eco*RV cuts the pART27 vector in two places two bands were generated of sizes 7483 bp and 9027 bp for the positive colonies and for the negative colonies 7483 bp and 4184 bp (9027 bp without the silencing cassette(4843 bp)). *Sac*I and *Spe*I restriction sites are located closely to the *Not*I restriction site and positive colonies yielded a band of ~ 4843 bp the size of the inserted silencing cassette and a backbone of ~ 11 667 bp. The negative colonies were linarised because of the close proximity of the two restriction sites. Recombinants were inoculated, plasmid DNA were purified and sequenced at the Core DNA Sequencing facility at Stellenbosch University.

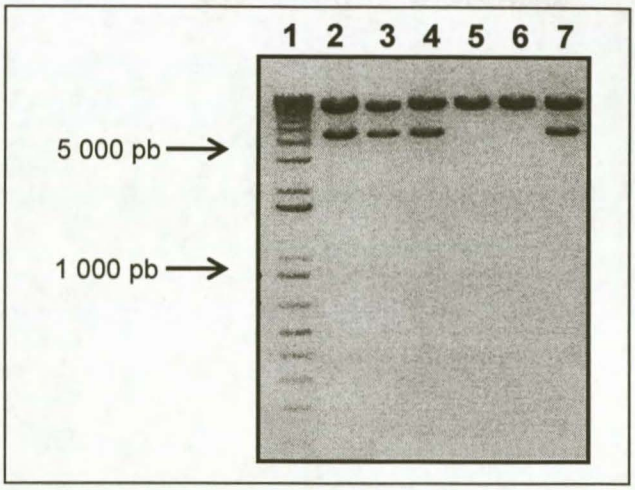


Figure 6.5: Restriction digestion of final construct pSilenser-SAS with the restriction enzyme *Not*I. **Lane 1:** 1kb+ ladder. **Lane 2, 3, 4, 7:** Positive colonies (4843 bp & 11667 bp). **Lane 5, 6:** Two negative colonies (linerised pART27)

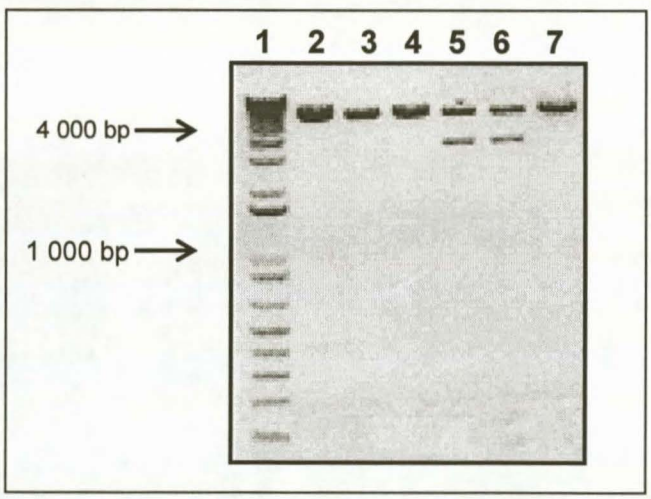


Figure 6.6: Restriction digestion of final construct pSilenser-SAS with the restriction enzyme *Sal*I. **Lane 1:** 1kb+ ladder. **Lane 2, 3, 4, 7:** Positive colonies (8507 bp & 8003 bp). **Lane 5, 6:** Two negative colonies (3664 bp & 8003 bp)

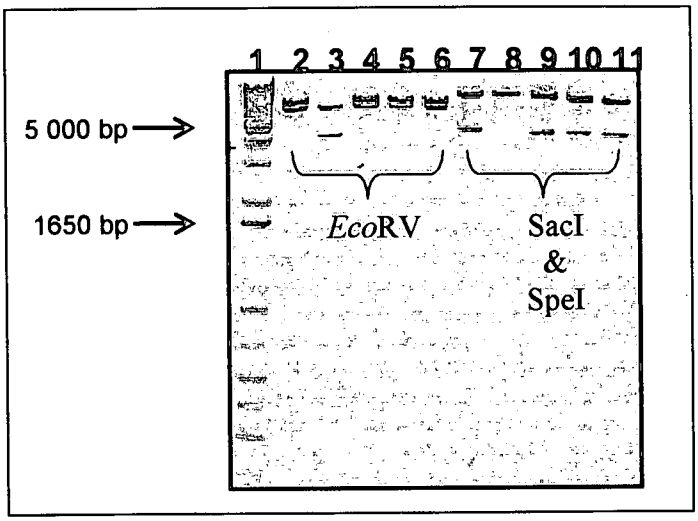


Figure 6.7: Restriction digestion of construct pSilenser-SAS with the restriction enzyme *Eco*RV & *Sac*I, *Spe*I. **Lane 1:** 1kb+ ladder. **Lanes 2, 4, 5, 6:** Positive colonies (7483 bp & 9027 bp) of *Eco*RV digestions. **Lanes 7, 9, 10, 11:** Positive colonies (4843 bp & 11667 bp) of *Sac*I and *Spe*I digestions. **Lane 3:** Negative colony (7483 bp & 4184 bp). **Lane 8:** Negative colony (linerised pART27).

6.4 *A. tumefaciens* transformation with pSilencer-SAS

A. tumefaciens C58C1 and GV3101 competent cells were directly transformed with the tandem silencing construct, pSilencer-SAS. Single colonies obtained were re-streaked onto LB/agar plates with suitable antibiotics to ensure that no *E. coli* contamination was present. Recombinants were authenticated by colony PCR using the GLRaV-2 Sense-For and GFP Sense-Rev primers listed in Table 5.1. All plants displaying virus symptoms as well as symptom-free plants were screened for GLRaV-2 presence by a RDOT-RT-PCR reaction using GLRaV-2 specific primers (Table 5.1). The GV3101::pSilencer-SAS construct was infiltrated into the list of plants indicated in Table 6.1 and results obtained (after three weeks) are also listed in Table 6.1.

Table 6.1: Table indicating the appearance of infiltrated plants under UV illumination before and after agro-infiltration with the construct GV3101::pSilencer-SAS

Plants infiltrated with construct (GV3101::pSilencer-SAS)	**Description of plants before agro-infiltration with the construct (GV3101::pSilencer-SAS)**	**Description of plants after agro-infiltration with the construct (GV3101::pSilencer-SAS)**
Transgenic *N. benthamiana* (16c) plants	• Green under UV illumination ❖ No GLRaV-2 viral symptoms present	• Red under UV illumination ❖ No GLRaV-2 viral symptoms present
Transgenic *N. benthamiana* (16c) plants	❖ Green under UV illumination ❖ GLRaV-2 viral symptoms present	• Red under UV illumination ❖ No GLRaV-2 viral symptoms present
GFP-silenced transgenic *N. benthamiana* (16c) plants	• Uniformly red under UV illumination ❖ No GLRaV-2 viral symptoms present	• Red under UV illumination ❖ No GLRaV-2 viral symptoms present
GFP-silenced transgenic *N. benthamiana* (16c) plants	• Uniformly red under UV illumination ❖ GLRaV-2 viral symptoms present	• Red under UV illumination ❖ No GLRaV-2 viral symptoms present
Non-transgenic *N. benthamiana* plants	• Uniformly red under UV illumination ❖ No GLRaV-2 viral symptoms present	• Red under UV illumination ❖ No GLRaV-2 viral symptoms present
Non-transgenic *N. benthamiana* plants	• Uniformly red under UV illumination ❖ GLRaV-2 viral symptoms present	• Red under UV illumination ❖ No GLRaV-2 viral symptoms present

Transgenic N. benthamiana (16c) plants. These plants expressed high levels of GFP and thus fluoresces bright green under UV illumination. Some of these plants were sap inoculated with a South African GLRaV-2 isolate to obtain GLRaV-2 infected plants expressing the viral target gene Hsp70. Viral symptoms were visible on the inoculated plants at 20 DPI. Plants that tested positive for GLRaV-2 were agro-infiltrated with the suspension containing the GV3101::pSilencer-SAS construct as well as plants not sap inoculated. After infiltration the plants appeared red under UV illumination and no GLRaV-2 symptoms were present at this time. This was expected since the tandem silencing construct would induce PTGS of the *gfp* transgene, because of the presence of dsRNA GFP which is a potent initiator of PTGS. This would make the plant appear red under UV illumination. It was also expected that the plant would not develop GLRaV-2 symptoms because of the presence of the dsRNA of the GLRaV-2 HSP70 gene which would induce PTGS against the viral gene.

GFP-silenced transgenic N. benthamiana (16c) plants. These plants were agro-infiltrated with an extra copy of the GFP transgene by utilizing a previously mentioned construct (C58C1::GFP) to induce silencing of the *gfp* gene present in the plants and thus obtain GFP-silenced 16c *N. benthamiana* plants. PTGS of GFP was manifested throughout the whole plant at 20 DPI and the plant appeared uniformly red under UV illumination due to fluorescence of chlorophyll (Figure 6.8). Some of these plants were sap inoculated with a South African GLRaV-2 isolate to obtain GLRaV-2 infected plants expressing the viral target gene *Hsp*70. Viral symptoms were visible on the inoculated plants at 20 DPI. Plants that tested positive for GLRaV-2 were agro-infiltrated with the suspension containing the GV3101::pSilencer-SAS construct as well as plants not sap inoculated. The infiltrated plants displayed a uniformly red colour under UV illumination and no viral symptoms were visible on the plants at this time. This was expected because PTGS was already established in the plant. No viral symptoms were present because of the presence of the dsRNA of the viral GLRaV-2 *Hsp*70 gene which will induce PTGS against the viral genome.

Figure 6.8: Picture indicating GFP silenced transgenic *N. benthamiana* plant at 20 DPI.

Non-transgenic N. benthamiana plants. These plants were uniformly red under UV illumination since chlorophyll fluoresces red under UV illumination. Some of these plants were sap inoculated with a South African GLRaV-2 isolate to obtain GLRaV-2 infected plants expressing the viral target gene Hsp70. Viral symptoms were visible on the inoculated plants at 20 DPI. Plants that tested positive for GLRaV-2 were agro-infiltrated with the suspension containing the GV3101::pSilencer-SAS construct as well as plants not sap inoculated. The two different plants both showed bright green patches under UV illumination for the first two DPI (Figure 6.9) where the construct was infiltrated. The green patches disappeared and the plants again appeared red under UV illumination with no virus symptoms present at this time. This indicated that the construct had no effect on the plant. This outcome was expected since PTGS would be initiated by the dsRNA GFP present in the construct, but because no GFP is present in the plant it would remain red under UV illumination. No viral symptoms would be present since the dsRNA of the GLRaV-2 gene would induce PTGS against the viral gene.

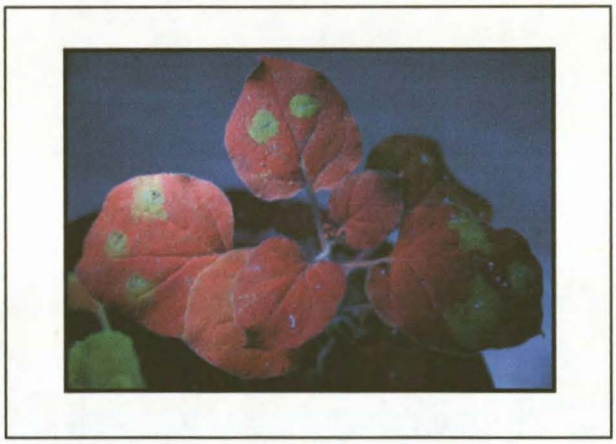


Figure 6.9: Picture indicating green fluorescent patches under UV illumination at 2 DPI on the leaves where the construct (GV3101::pSilencer-SAS) was infiltrated

The final construct pSilencer-SAS was also constitutively transformed by L. Watts at ARC-Infruitec/Nietvoorbij, Stellenbosch with the *A. tumefaciens* Gv3101 and C58C1. These results are not available yet.

6.5 Conclusion

Results of the experiments described here suggested that the vector pSilencer-SAS silenced two genes simultaneously. In all of the infiltration experiments the expected visual results were observed under UV illumination. PTGS of the *gfp* gene were obtained when the transgenic *N. benthamiana* plants (16c) were infiltrated with the vector. PTGS of the *gfp* gene remained in GFP silenced transgenic plants and the red plants remained red under UV illumination because of the persistence of PTGS by the vector. Non-transgenic *N. benthamiana* plants also remained red under UV illumination after infiltration because no GFP was present to silence. This indicated that the incorporated GFP sense and anti-sense fragments did form a dsRNA complex after transcription, which initiated the PTGS mechanism and thus the changes were observed under UV illumination.

The presence of symptoms is not a reliable proof of infection. Here, it was used as an indication of virus infection, and thus the presence of the Hsp70 gene. The disappearance of symptoms could thus be attributed to gene silencing, but could also be because of masking as a result of sub-optimal growth conditions.

Chapter 7
General Conclusion

Grapevine leafroll disease is one of the most destructive diseases of grapevine and causes severe financial losses to the wine industry. A complex of nine grapevine leafroll viruses (GLRaV 1-9) is associated with the disease, of which GLRaV-3 seems to be the most important at the moment.

Existing control measures against leafroll are outdated and inefficient. The introduction of genetic resistance by genetic engineering is a realistic long term approach and success has been obtained by the introduction of RNA mediated virus resistance (RMVR). An underlying mechanism of RMVR is a natural plant response called PTGS) which acts like a defence system for the plant against invasions by transgenes, endogenous genes and replicating viruses which produce dsRNA in the host cell.

PTGS as great potential for the introduction of resistance against viruses in plants. This potential is hampered by the fact that some plant viruses have evolved to produce suppressor proteins able to counter the PTGS mechanism.

The aim of this project was to screen the GLRaV-2 genome for suppressor activity by utilising two silencing assays. Of the nine GLRaVs only GLRaV-2 is capable of infecting tobacco. This virus was therefore chosen to conduct this study. Potential suppressor genes in the genome of the economically more important GLRaV-3 could possibly be identified using a parallel approach. In the second part of this project a tandem silencing vector was constructed as a proof of concept, to prove that two genes can be simultaneously silenced in a plant from a single construct.

Two silencing assays, a silencing reversal and a transient expression assay identified the p24 gene (GeneE) of GLRaV-2 as a putative suppressor protein. This was concluded after visual examination of fluorescence in silenced and non-silenced transgenic *N. benthamiana* (16c) plants. In the silencing reversal assay, transgenic *N. benthamiana* (16c) plants, which appear green under UV illumination because of the presence of a *gfp* transgene, were agro-infiltrated with an additional copy of the *gfp* gene to obtain GFP silenced plants. Silenced plants appear red under UV illumination since the extra copy of GFP triggers PTGS by forming dsRNA in the plant. These plants were sap inoculated with a South African GLRaV-2 isolate to determine if the genome possesses suppressor activity. No silencing reversal was visible in the old tissue but new emerging

leaves remained green over a longer period of time and were slow in changing colour. This was an indication that the virus might possess a suppressor protein since; if no suppressor activity was present, the new emerging leaves would have been red under UV illumination as observed with the control plants.

To identify the individual gene of GLRaV-2 responsible for the silencing reversal, five genes of GLRaV-2 were isolated on the basis of their predicted functions. The main focus was on the p24 gene (GeneE) of the virus because of amino acid sequence similarities between this and another suppressor protein, p21 from *Beet yellows Closterovirus* (BYV) also a member of the *Closteroviridae* family. GFP silenced transgenic *N. benthamiana* plants (red under UV illumination) were agro-infiltrated with the pViral106-GeneE construct and displayed similar results than obtained before. New emerging leaves remained green over a longer period of time and in a similar manner to plants infiltrated with the known suppressor protein 2b from *Cucumber mosaic cucumovirus* (CMV). The exception was that the leaves turned red at a later stage while still no red was visible with the 2b protein. Thus, it was concluded that GeneE may play a role in the initiation of the PTGS signal but not at the same stage as the 2b protein.

In the transient expression assay, transgenic *N. benthamiana* plants (16c) were co-infiltrated with a GFP construct and the GLRaV-2 viral gene (GeneA to GeneE) constructs. It was observed that the leaf patches infiltrated with the pViral106-GeneE construct remained green in the same manner as the patches infiltrated with the known suppressor proteins p25 and p19. The intensity of the patches was different from that of the p19 protein, which showed bright green patches on the infiltrated leaves and thus it was concluded that GeneE did not affect the same stage of the silencing pathway as the p19 protein. The infiltrated patches remained green, similar to the known suppressor p25 but at 12 DPI the patches faded in colour and started to turn red. This was not observed in the p25 infiltrated patches which were still a dull green and thus GeneE did not affect the same step of the silencing pathway as p25 either.

The transient expression silencing assays thus indicated that the GeneE mode of action was not by inactivating all size classes of siRNAs as p19 and it was also not able to inactivate all the conversions of ssRNAs to dsRNAs like the suppressor p25. In the silencing reversal assay GeneE showed similar reversal discolorations as the 2b protein and thus might affect the PTGS mechanism by interfering with the signal of systemic silencing. If both of these assays are taken in consideration it may show that GeneE interferes with stages of the silencing pathway differently

from the three known suppressor proteins. It seems that GeneE affects the silencing process between the initiation of PTGS and the production of siRNAs.

Molecular analysis of the GFP siRNAs to confirm the GFP fluorescence observations was attempted by Northern blots. Total small RNAs (>200 nt) were isolated, visualised and probed with a DIG-labelled DNA probe in a Northern blot but unfortunately no hybridization signals could be observed in any of these experiments. The reason for this is probably of a technical nature since not even the positive control produced a signal. This may be because a DNA probe was used to detect very small (20-23 nt) RNAs in a large amount of non-target RNAs. It may also be that the probe was not sensitive enough since it was labelled with DIG instead of radioactivity or just inefficient labelled in the PCR labelling reaction.

In the second part of this project a tandem silencing vector was designed to prove that two genes can be simultaneously silenced. It has been shown that dsRNA is an effective trigger of PTGS, even more so than sense or anti-sense RNA separately. Thus to effectively silence an endogenous gene a plant can be transformed with a transgene which is able to express dsRNA. To obtain dsRNA, the pHannibal vector was used to produce a sense and anti-sense arm separated by an intron. Each arm consisted of a GFP fragment and an Hsp70 gene fragment from GLRaV-2 in the sense or anti-sense orientation. In the plant cell an RNA hairpin with a loop will be formed and after transcription the intron will be spliced out and the sense and anti-sense arms will form a dsRNA fragment which should activate PTGS.

The tandem silencing vector, pSilencer-SAS, was constructed and infiltrated into transgenic *N. benthamiana* plants (16c), into non-transgenic *N. benthamiana* plants and into GFP silenced plants. Some of these plants were also sap inoculated with a South African GLRaV-2 isolate to obtain GLRaV-2 infected plants expressing the viral target gene Hsp70. The effect of the tandem silencing vector, pSilencer-SAS, on the different infiltrated plants produced the expected visual results under UV illumination. It showed that PTGS of the *gfp* gene was obtained when the transgenic *N. benthamiana* plants (16c) were infiltrated with the vector. This was probably achieved by the formation of GFP dsRNA which silenced the GFP transgene and thus the green plants changed to red under UV illumination. PTGS of the *gfp* was maintained in GFP silenced transgenic plants and the red plants remained red under UV illumination because of the persistence of PTGS by the vector. As expected, non-transgenic *N. benthamiana* plants also remained red under UV illumination after infiltration because no GFP was present to silence. The disappearance of the GLRaV-2 symptoms could be attributed to gene silencing, but could also be

because of masking as a result of sub-optimal growth conditions. Symptoms are not a reliable proof of infection but were used as an indication of virus infection, and thus the presence of the Hsp70 gene.

The next goal of this project is to confirm the biological observations obtained with molecular data such as the presence of the GFP siRNAs in the different plants. The mode of action of the p24 suppressor can also be determined. This project also opens avenues for research on the active molecular dissection of the suppressor protein once identified and confirmed by molecular analysis. This dissection of the suppressor protein can lead to the identification of the active site of suppressor activity and thus ease the identification of suppressors in other grapevine viruses.
The tandem silencing vector can be used for the molecular characterisation of transgenic plants and its efficiency can be tested with two functional viral genes.

This study had two main objectives. The first was to identify a potential suppressor gene form the GLRaV-2 genome. Although results obtained here need to be corroborated by RNA analysis, I believe GFP fluorescent observations suggested that p24 from GLRaV-2 has suppressor activity. The second part of the project was to construct a tandem silencing vector. This was achieved and preliminary results in *N. benthamiana* plants suggested that both GFP and the Hsp70 gene was silenced in agro-infiltration experiments performed.

Appendices

A: Example of a calculation to obtain amount of PCR product to use in ligation reactions

A molar ratio of 1:1, 1:3 or 3:1 of vector: insert DNA is recommended for cloning into a plasmid vector. The following equation was used to calculate the appropriate amount of PCR product (insert) to include in a ligation reaction between the different GLRaV-2 genes and the vectors pGEM®-T Easy and pgR106.

$$\frac{\text{ng of vector X kb size of insert}}{\text{kb size of vector}} \text{ X insert: vector molar ratio = ng of insert}$$

Equation for calculating amount of insert needed

B: Competent cell preparation

Preparation of competent *E.coli* DH5α cells

DH5α cells were plated on LB/agar plates (without antibiotics) and incubated at 37°C overnight. A single colony was inoculated in five ml LB medium and incubated at 37°C overnight with shaking (225-250 rpm). Five microlitres of the overnight starter culture were inoculated in 500 ml fresh LB media to achieve a 1:1000 dilution. The culture was incubated at 37°C with vigorous shaking until OD_{600} = 0.6 was achieved (about 2-3 hours). The culture was incubated on ice for 10 minutes and the cells were harvested by centrifuging at 4°C, at low speed (3-5 000 rpm) for 5 minutes. The pellet was resuspended in a 1/4 culture volume of ice cold 0.1 M $MgCl_2$ and incubated on ice for 5 minutes. The cells were centrifuged (4 000 rpm for 10 minutes) at 4°C at and the pellet was resuspended in a 1/4 culture volume of ice cold 0.1 M $CaCl_2$. The cells were gently mixed by tapping the side of the microcentrifuge tube lightly and incubated on ice for 20 minutes and centrifuged for 10 minutes at 4 000 rpm. The pellet was gently resuspended in 1/20-1/40 culture volume of ice cold buffer (0.1M $CaCl_2$, 14% (v/v) glycerol). Desired aliquots of cells were dispensed into sterile, prechilled microcentrifuge tubes on ice and the tubes were flash frozen in ice cold ethanol for a few seconds before they were placed at -80°C.

Preparation of *Agrobacterium* competent cells

Two *Agrobacterium* strains (C58C1 & GV3101) were streaked out on LB/agar plates containing Tet (5µg/ml) and incubated at 28°C for three days. A single colony was inoculated in 5 ml liquid LB medium and incubated at 28°C overnight with vigorous shaking (250 rpm).

Two millilitres of the overnight culture was inoculated in 50 ml liquid LB medium in a 250 ml flask and incubated at 28°C with vigorous shaking until the culture had reached an OD of between 0.5-0.6 (4-5 hours). The cultures were chilled on ice for 10 minutes and centrifuged at 4°C for 5 minutes at 3 000 g. The supernatant was discarded and the pellets were resuspended in one ml sterile, ice-cold, 20 mM $CaCl_2$ solution. Desired aliquots (100 µl) were dispensed into pre-chilled 1.5 ml microcentrifuge tubes on ice and the tubes were flash frozen in ice cold ethanol for a few seconds before they were placed at -80°C for later use.

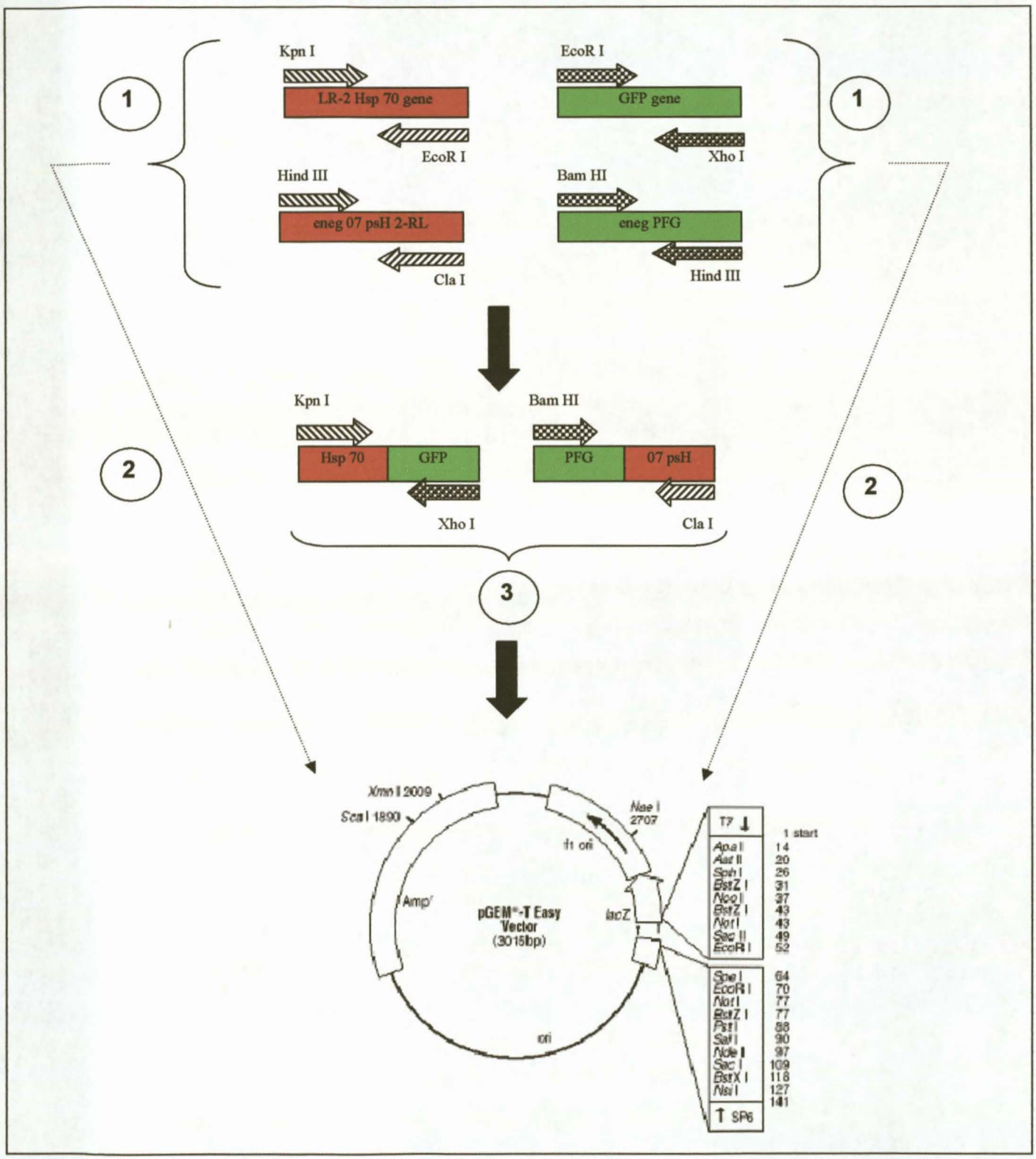
C: Antibiotics selection for different constructs and *Agrobacterium* strains

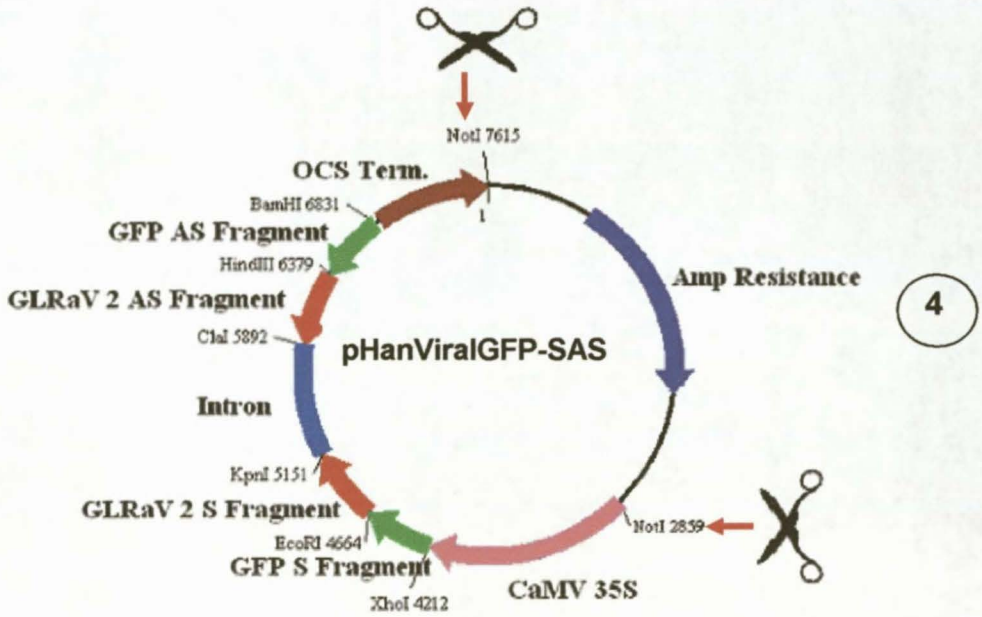
Construct	Name	Selection	Selection Concentration
pBIN m-gfp5-ER binary vector	GFP	Kan	50 µg/ml
pGEM®-T-Easy vector	—	Amp	100 µg/ml
pgR106PVX-based plant expression vector	—	Kan	50 µg/ml
pBIN61 containing the suppressors (p25, p19, 2b)	pBIN61- suppressors	Kan	50 µg/ml
pGEM®-T-Easy Vector containing the individual GLRaV-2 genes	—	Amp IPTG X-gal	100 µg/ml 20 mM 20 mg/ml.
pgR106 vector containing the individual GLRaV-2 genes	pViral106-GeneA to pViral106-GeneE	Kan	50 µg/ml
GV3101	—	Tet Rif	5 µg/ml 30 µg/ml
C58C1	—	Tet Rif	5 µg/ml 25 µg/ml
GV3101 containing the pgR106 vector containing the individual GLRaV-2 genes	GV3101::pViral106-GeneA to pViral106-GeneE	Tet Rif Kan	5 µg/ml 25 µg/ml 50 µg/ml
C58C1 containing the suppressors (p25, p19, 2b)	C58C1::pBIN61-suppressors	Tet Rif Kan	5 µg/ml 25 µg/ml 50 µg/ml
pHannibal vector	—	Amp	100 µg/ml
pHannibal vector containing the sense and anti-sense constructs	pHanViralGFP-SAS	Tet Rif Amp	5 µg/ml 30 µg/ml 100 µg/ml
pART27 vector	—	Kan	20 µg/ml
pART27 vector containing the silencing cassette	pSilencer-SAS	Kan	20 µg/ml
A. tumefaciens C58C1 containing pSilencer-SAS	C58C1::pSilencer-SAS	Tet Rif Kan	5 µg/ml 30 µg/ml 20 µg/ml

D: NCBI ID numbers and genes sequenced of the grapevine leafroll associated viruses

Virus	ID	Genes sequenced
GLRaV-1	**AF195822**	Grapevine leafroll associated virus 1 helicase (HEL) and RNA-dependant RNA polymerase (POL) genes, partial cds; and p7, HSP70-like protein, p55, coat protein (CP) p55 (CPd1), p50 (CPd2), p22, and p24 genes, complete cds. 12394 nts.
	AF233935	Grapevine leafroll associated virus 1HSP70 gene, partial cds. 1383 nts
	AJ404738	Grapevine leafroll associated virus 1 partial mRNA for HSP70 protein. 511 nts
	U58335	Grapevine leafroll associated virus 1 gene, partial cds. 588 nts.
	Y15890	Grapevine leafroll associated virus 1 hsp70 gene, partial. 620 nts.
GLRaV-2	**AF039204**	Grapevine leafroll associated virus 2 methyltransferase helicase polyprotein gene, partial cds; RdRp, Putitive hydrophobic transmembrane protein, 65K, 63K, p25, p22, p19, p24 genes complete cds
	Y14131	Grapevine leafroll associated virus 2 genes encoding RNA polymerase, CP, HSP70, HSP90, ORF2, ORF7, and ORF8
	AY456132	Grapevine leafroll associated virus 2 P6 and HSP70 homologue, Alfie strain.
GLRaV-3	**Y15891**	Grapevine leafroll associated virus 3 partial HSP70 gene. 581 nts.
	AF037268	Grapevine leafroll associated virus 3, complete genome, strain NY1. 17919 nts.
	AF438411	Grapevine leafroll associated virus 3 polymerase gene, partial cds. 340 nts.
	AY424407	Grapevine leafroll associated virus 3 RdRp gene, partial cds. 340 nts.
	AY424408	Grapevine leafroll associated virus 3 HSP70-like protein gene, partial cds. 1655 nts.
	AY424409	Grapevine leafroll associated virus 3 55 kDa protein (55k) gene, partial cds. 300 nts.
	U22158	Grapevine leafroll associated virus 3 p20 protein gene, complete cds. 1012 nts.
	U22170	Grapevine leafroll associated virus 3 RNA6 gene, partial cds. 965 nts.
	AY495340	Grapevine leafroll associated virus 3 RdRp gene partial cds, Chinese isolate. 1655 nts.
GLRaV-4	**AF030168**	Grapevine leafroll associated virus 4 ORF1 and ORF2 genes, partial cds. 581nts.
	AF039553	Grapevine leafroll associated virus 4 HSP 70 gene, partial cds. 591 nts.
GLRaV-5	**AF039552**	Grapevine leafroll associated virus 5 HSP 70 gene, partial cds.591 nts.
	AF233934	Grapevine leafroll associated virus 5 HSP 70 gene, partial cds; and HSP90, CP, and CPd genes, complete cds. 4766 nts.
GLRaV-6	**U22170**	Grapevine leafroll associated virus 6 RNA 6 gene, partial cds.
GLRaV-7	**Y15987**	Grapevine leafroll associated virus 7 HSP 70 gene, partial cds.
GLRaV-8	**AF233936**	Grapevine leafroll associated virus 8 CP gene, partial cds. 273 nts.
GLRaV-9	**AYO72797**	Grapevine leafroll associated virus 9 HSP 70 gene, partial cds. 590 nts.

E: Schematic representation of the construction of the sense and anti-sense fragments, the construct pHanViralGFP-SAS and the final construct pSilencer-SAS





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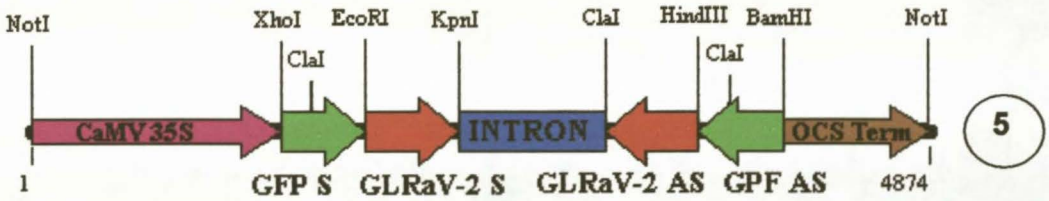
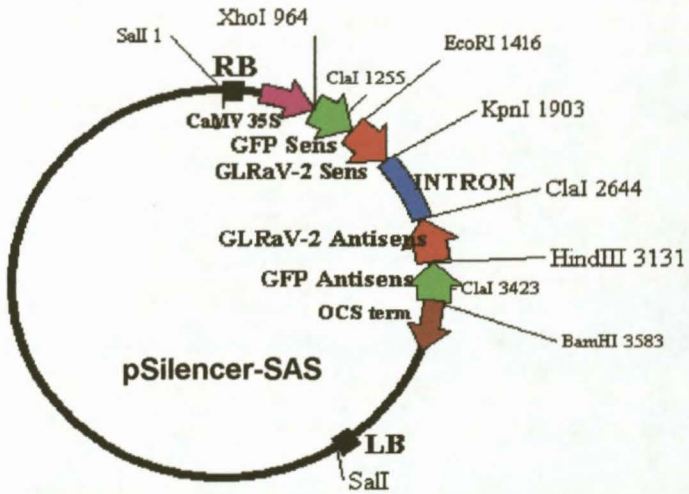


5



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