The construction of gene silencing transformation vectors for the introduction of multiple-virus resistance in grapevines.

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Declaration

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

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Summary

Viruses are some of the most important pathogens of grapevines. There are no effective chemical treatments, and no grapevine- or other natural resistance genes have been discovered against grapevine infecting viruses. The primary method of grapevine virus control is prevention by biological indexing and molecular- and serological screening of rootstocks and scions before propagation. Due to the spread of grapevine viruses through insect vectors, and in the case of GRSPaV the absence of serological screening, these methods of virus control are not always effective. In the past several methods, from cross-protection to pathogen derived resistance (PDR), have been applied to induce plant virus resistance, but with inconsistent results. In recent years the application of post-transcriptional gene silencing (PTGS), a naturally occurring plant defense mechanism, to induce targeted virus resistance has achieved great success. The Waterhouse research group has designed plant transformation vectors that facilitate specific virus resistance through PTGS. The primary focus of this study was the production of virus specific transformation vectors for the introduction of grapevine virus resistance. The Waterhouse system has been successfully utilised for the construction of three transformation vectors with the pHannibal vector as backbone. Each vector contains homologous virus coat protein (CP) gene segments, cloned in a complementary conformation upstream and downstream of an intron sequence. The primary vector (pHann-SAScon) contains complementary CP gene segments of both GRSPaV and GLRaV-3 and was designed for the introduction of multiple-virus resistance. For the construction of the primary vector the GRSPaV CP gene was isolated from RSP infected grapevines. A clone of the GLRaV-3 CP gene was acquired. The second vector (pHann-LR3CPsas) contains complementary CP gene segments of GLRaV-3. The third vector (pHann-LR2CPsas) contains complementary CP gene segments of GLRaV-2. The cassette containing the complementary CP gene segments of both GRSPaV and GLRaV-3 was cloned into pART27 (pART27-HSAScon), and used to transform *N. tabacum* cv. Petit Havana (SR1), through *A. tumefaciens* mediated transformation. Unfortunately potential transformants failed to regenerate on rooting media; hence no molecular tests were performed to confirm transformation. Once successful transformants are generated, infection with a recombinant virus vector (consisting of PVX, the GFP gene as screenable marker and the complementary CP gene segments of both GRSPaV and GLRaV-3) will be used to test for the efficacy of the vectors to induce resistance. A secondary aim was added to this project when a need was identified within the South African viticulture industry for GRSPaV specific antibodies to be used in serological screening. To facilitate future serological detection of GRSPaV, the CP gene was isolated and expressed with a bacterial expression system (pET-14b) within the *E. coli* BL21(DE3)pLysS cell line. The expressed protein will be used to generate GRSPaV CP specific antibodies.

Opsomming

Virusse is van die belangrikste patogene by wingerd. Daar bestaan geen effektiewe chemiese beheer nie, en geen wingerd- of ander natuurlike weerstandsgene teen wingerdvirusse is al ontdek nie. Die primêre metode van beheer t.o.v. wingerdvirusse is voorkoming deur biologiese indeksering, en molekulêre- en serologiese toetsing van onderstokke en entlote voor verspreiding. As gevolg van die verspreiding van wingerdvirusse deur insekvektore, en in die geval van GRSPaV die tekort aan serologiese toetsing, is dié metodes van virusbeheer nie altyd effektief nie. In die verlede is metodes soos kruis-beskerming en patogeen-afgeleide weerstand (PDR) gebruik om virusweerstand te induseer, maar met inkonsekwente resultate. In onlangse jare is post-transkripsionele geenonderdrukking (PTGS), 'n natuurlike plant-beskermingsmeganisme, met groot sukses toegepas om geteikende virusweerstand te induseer. Die Waterhouse-navorsingsgroep het planttransformasievektore ontwerp wat spesifieke virusweerstand induseer d.m.v. PTGS. Die vervaardiging van virus spesifieke tranformasievektore vir die indusering van wingerdvirusweerstand was die primêre doelwit van hierdie studie. Die Waterhouse-sisteem was gebruik vir die konstruksie van drie transformasievektore, met die pHannibal vektor as basis. Elke vektor bevat homoloë virus kapsiedproteïen (CP) geensegmente, gekloneer in 'n komplementêre vorm stroom-op en stroom-af van 'n intronvolgorde. Die primêre vektor (pHann-SAScon) bevat komplementêre CP geensegmente van beide GRSPaV en GLRaV-3, en was ontwerp vir die indusering van veelvoudige-virusweerstand. Die CP-geen van GRSPaV was vanuit RSP-geïnfekteerde wingerd geïsoleer, vir die konstruksie van die primêre vektor. 'n Kloon van die GLRaV-3 CP-geen was verkry. Die tweede vektor (pHann-LR3CPsas) bevat komplementêre CP geensegmente van GLRaV-3. Die derde vektor (pHann-LR2CPsas) bevat komplementêre CP geensegmente van GLRaV-2. Die kasset bestaande uit die komplementêre CP geensegmente van beide GRSPaV en GLRaV-3, was gekloneer in pART27 (pART27-HSAScon), en gebruik om *N. tabacum* cv. Petit Havana (SR1) te transformeer d.m.v. *A. tumefaciens* bemiddelde transformasie. Ongelukkig het potensiële transformante nie geregenereer op bewortelings-media nie; gevolglik was geen molekulêre toetse gedoen om transformasie te bevestig nie. Na suksesvolle transformante gegenereer is, sal infeksie met 'n rekombinante-virusvektor (bestaande uit PVX, die GFP geen as waarneembare merker en die komplementêre CP geensegmente van beide GRSPaV en GLRaV-3) gebruik word om die effektiwiteit van die vektore as weerstandsinduseerders te toets. 'n Sekondêre doelwit is by die projek gevoeg toe 'n behoefte aan GRSPaV spesifieke teenliggame binne die Suid-Afrikaanse wynbedryf geïdentifiseer is, vir gebruik in serologiese toetsing. Om toekomstige serologiese toetsing van GRSPaV te bemiddel, was die CP-geen geïsoleer en in 'n bakteriële uitdrukkingsisteem (pET14b) uitgedruk, in die *E. coli* BL21(DE3)pLysS sellyn. Die uitgedrukte proteïne sal gebruik word vir die vervaardiging van GRSPaV CP spesifieke antiliggame.

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Abbreviations

°C	- Degrees Celsius
β-ME	- β-Mercaptoethanol
Amp	- Ampicilin
ATP	- Adenosine triphosphate
BAP	- 6-Benzylaminopurine
bp	- Base pair
BSA	- Bovine serum albumin
cDNA	- Complementary DNA
Cef	- Cefotaxime
Chlor	- Chloramphenicol
CP	- Coat protein
CPd	- Divergent coat protein
CTAB	- N-Cetyl-N,N,N-trimethyl ammonium bromide
ddH_2O	- Double distilled H_2O
DNA	- Deoxyribonucleic acid
dNTP	- Deoxynucleoside triphosphate
dsRNA	- Double stranded RNA
DTT	- Dithiothreitol
EDTA	- Ethylene diamine tetra-acetic acid di-sodium salt
EtOH	- Ethanol
GLR	- Grapevine leafroll disease
GMO	- Genetically modified organism
GOI	- Gene of interest
h	- Hour(s)
HEL	- Helicase
hpRNA	- Hairpin RNA
HR	- Hypersensitive response
ICTV	- The International Committee on Taxonomy of Viruses
IPTG	- Isopropyl-β-D-thiogalactopyranoside
LB	- Left border
LB	- Luria-Bertani medium
Kan	- Kanamycin
kb	- Kilobase pairs
kDa	- Kilo Daltons
MCS	- Multiple cloning site
MET	- Methyltransferase

min	- Minute(s)
NAA	- 1-Napthaleneacetic acid
nt	- Nucleotide
ocs	- Octopine sythase
OD	- Optical density
ORF	- Open reading frame
PAP	- Pokeweed antiviral protein
pdk	- Pyruvate orthophosphate dikinase
PDR	- Pathogen-derived resistance
RE	- Restriction enzyme
PEG	- Polyethylene glycol
P-PRO	- Papain-like protease
RB	- Right border
RBS	- Ribosomal binding site
RdRp	- RNA-dependant RNA polymerase
RE	- Restriction enzyme
R genes	- Dominant resistance gene
Rif	- Rifampicin
RIP	- Ribosome-inactivating protein
RNA	- Ribonucleic acid
Rpm	- Revolutions per minute
PCR	- Polymerase chain reaction
RSP	- Grapevine rupestris stem pitting
RT	- Reverse transcription
RW	- Rugose wood disease
SDS	- Sodium dodecyl sulphate
SDS-PAG	- SDS-polyacrylamide gel
SDS-PAGE	- SDS-polyacrylamide gel electrophoresis
sec	- Second(s)
siRNA	- Short interfering RNA
SSA	- Silencing suppression assay
ssRNA	- Single stranded RNA
STE	- Sodium/Tris/EDTA
stRNA	- Small temporal RNA
TAE	- Tris/Acetic acid/EDTA
TB	- Terrific broth
T-DNA	- Transfer-DNA
Tet	- Tetracycline

Ti	- Tumor inducing
Tris	- Tris(hydroxymethyl)aminomethane
U	- Unit(s)
X-gal	- 5-Bromo-4-chloro-3-indocyl-β-D-galactoside

Abbreviations of virus species

APRLV	- *apricot latent virus*
ArMV	- *arabis mosaic virus*
ASPV	- *apple stem pitting virus*
BYSV	- *beet yellow stunt virus*
BYV	- *beet yellows virus*
CGRMV	- cherry green ring mottle virus
CLBV	- *citrus leaf blotch virus*
CMV	- *cucumber mosaic virus*
CNRMV	- cherry necrotic rusty mottle virus
CTV	- *citrus tristeza virus*
GFLV	- *grapevine fanleaf virus*
GLRaV-2	- *grapevine leafroll-associated virus 2*
GLRaV-3	- *grapevine leafroll-associated virus 3*
GRSPaV	- *grapevine rupestris stem pitting-associated virus*
GVA	- *grapevine virus A*
GVB	- *grapevine virus B*
GVC	- grapevine virus C
GVD	- *grapevine virus D*
LChV-2	- *little cherry virus 2*
PPV	- *plum pox virus*
PSRV	- *peach sooty ringspot virus*
PVA	- *potato virus A*
PVX	- *potato virus X*
PVY	- *potato virus Y*
RRV	- *raspberry ringspot virus*
TEV	- *tobacco etch virus*
TMV	- *tobacco mosaic virus*
TRV	- *tobacco rattle virus*
TSWV	- *tomato spotted wilt virus*
TuMV	- *turnip mosaic virus*

"Gods play games with the fates of men. But first they have to get all the pieces on the board, and look all over the place for dice."

- *Terry Pratchett, Soul Music*

Contents

Chapter 1: Introduction

1.1. GENERAL INTRODUCTION

Agriculture accounts for 3% of the gross domestic product (GDP) of South Africa (World development report, 2003). Although it seems like a small percentage when compared to those of industry (21%) and services (66%), the importance of agriculture can not be overestimated and can not be measured only by its percentage stake in the GDP. Agriculture, especially in developing countries, is extremely important due to the enormous role that it plays in the provision of basic needs and food security, and the array of other industries and fields that make use of and rely on it. One of the biggest agricultural industries in South Africa is the viticulture and grape industry, which includes the production of wine grapes, table grapes and related products for both the local and export markets.

According to South African Wine Industry and Systems (SAWIS), the South African wine industry, although not a producer of primary foods for food security, employed more than 348 500 people with wine related tourism employing a further 48 350 people in 2002, approximately 1% of the country's population. A total of 1 079 875 tons of grapes were harvested in 2002, yielding 834.156 million litres of wine and non-alcoholic products. This industry generates more than R18 billion per year in the Western Cape, the biggest wine producing region in South Africa. In 2000 the South African wine industry was the 8th largest in the world, contributing 2.5% of the total wine (Wait, 2003). A report (Abstracts of agricultural statistics, 2002) by the National Department of Agriculture provides certain values concerning viticulture and grape production in South Africa. To illustrate the importance of these industries comparative values from this report are given in **Table 1.1**.

Table 1.1. Comparison of financial values concerning the Viticulture/Grape producing agricultural industries for the midyear 2001/2002.

Horticulture		**SACU* exports**		**Gross value: Fruit**	
Potatoes	1 929 781	Sugar	2 702 216	Grapes	2 166 228
Citrus fruit	2 801 662	Wine	1 963 036	Oranges	1 830 270
Viticulture	1 744 740	Grapes	1 327 216	Apples	963 537
Other	9 156 072	Other	14 081 463	Grapefruit	349 396
Total	15 632 255	**Total**	20 074 509		
All values given are multiples of R1000.00 *South African Customs Union					

Like any agricultural organism, grapevines are susceptible to disease causing pathogens that can seriously influence their lifespan and the quantity and quality of fruit production. Examples of important grapevine pathogens and pests include bacteria (*Xylella fastidiosa* that causes Pierce's disease), fungi (*Phaeoacremonium* vine decline or Petri grapevine decline, formerly known as "Black Goo"), insects (*Daktulosphaira vitifoliae* or grape phyloxerra), nematodes (*Xiphinema index*, a vector of *grapevine fanleaf virus* (GFLV)), phytoplasmas (Grapevine yellows "flavescence doreé" phytoplasma) and viruses. Of these pathogens and pests, viruses are some of the most devastating pathogens over the long term. There are no cures or treatments and no grapevine resistance genes or other natural resistance genes have been discovered against grapevine viruses. According to a study conducted by Nel and Engelbrecht (1972), where 220 485 grapevines were visually screened in the field, followed by biological indexing of those that did not show any symptoms, only 40 plants tested negative to viruses in the Western and Northern Cape vineyards. This translates into a near saturation point of 99.98%. The situation has improved since then due to improved awareness, quarantine measures for imported grapevine material and improved grapevine indexing techniques, but no official study has been done in South Africa in recent years to elucidate the true situation.

Worldwide, plant viruses account for a significant portion of total annual crop losses. Certain sources estimate annual worldwide crop losses due to plant viruses as high as US$60 billion (Cann, 1997). Although it is extremely difficult to make a formal estimate of the annual losses caused by grapevine viral diseases, it is generally accepted that millions of Rands worth of losses are accrued by the South African wine industry due to infected vineyards. Consequently the economy of the Western Cape region, and ultimately South Africa, is negatively impacted upon.

Attempts at controlling virus infection and spread are being made by using virus free propagation material to plant new vineyards and also by spraying insecticides to control virus vectors. These are proving less than effective due to the difficulty in consistently producing 100% virus free material and the inability to kill all virus vectors in a vineyard. The control methods are time consuming, costly and have a negative environmental impact. Several molecular methods of inducing targeted virus resistance have been developed in recent years. These include functional viral coat protein-mediated resistance, transgene-mediated virus resistance, sense/anti-sense suppression and post-transcriptional gene silencing (PTGS). Of these, PTGS is proving to be the most versatile and is believed to be a naturally evolved plant defence mechanism targeted against viruses. Functional transgenic proteins need not be expressed in the genetically modified plant for PTGS to induce resistance. The PTGS signal

is systemic; therefore grapevine scions grafted onto transgenic rootstocks can potentially gain the benefit of the protection without containing any transgenic components. Of the modern molecular methods PTGS is proving to be the most revolutionary, most acceptable and most effective.

Winetech, a network of participating institutions and individuals that aims to improve the position of the South African wine industry through co-operative and participative R&D initiatives, has identified viruses, and especially *grapevine leafroll-associated virus 3* (GLRaV-3), as the most devastating pathogens of South African grapevines. Grapevine leafroll disease (GLR), with GLRaV-3 as its main component, and Rugose wood disease (RW), with grapevine rupestris stem pitting (RSP) and the RSP causing *grapevine rupestris stem pitting-associated virus* (GRSPaV) as its main components, are seen as the most economically important virus diseases in South African vineyards. Thus it is imperative that, in conjunction with current methods of virus control, effective modern methods are developed to screen for and control these grapevine viruses and the diseases they cause.

1.2. PROJECT PROPOSAL

The manipulation of the PTGS mechanism to induce targeted virus resistance in plants is at present the best available strategy for the introduction of virus resistance. Post-transcriptional gene silencing has, to date, not been used to induce virus resistance within grapevines.

The main focus of this thesis was the construction of plant transformation vectors that would impart specific multiple-virus and single-virus resistance to grapevines through the mechanism of PTGS. The virus coat protein (CP) gene was chosen as the target for PTGS. If efficient silencing of the virus CP gene could be achieved the virus would not be able to form functional copies of itself, and thus not be able to further infect the host. The CP and CP gene has effectively been used to introduce resistance to plant viruses. Three vectors were to be made. Each of these vectors would consist of a pHannibal (Wesley *et al.*, 2001) backbone and grapevine virus CP gene segments. The CP gene segments would be cloned in such a way as to induce self-complementarity, resulting in virus specific dsRNA, once transcribed within a transformed plant. The primary, and most important, vector would contain both GLRaV-3 and GRSPaV complementary CP gene segments. The second vector would contain GLRaV-3 complementary CP gene segments. The third vector would contain *grapevine leafroll-associated virus 2* (GLRaV-2) complementary CP gene segments and would have been used to provide proof of concept. This vector would have been used to transform *Nicotiana benthamiana*, a host of GLRaV-2 (Goszczynksi *et al.*, 1996b). Transformants would

subsequently have been inoculated with a GLRaV-2 isolate to determine if the construct induces efficient GLRaV-2 specific resistance. Due to the importance of the multiple-virus (GLRaV-3 and GRSPaV) construct for the viticulture industry, it was ultimately decided to use this construct to transform *N. tabacum*, rather than use the GLRaV-2 vector to transform *N. benthamiana*. The ability of the transformed multiple-virus construct to successfully silence homologous sequences within a recombinant challenging virus will be tested after the completion of this thesis.

As part of the larger purpose of grapevine virus control, a secondary aim was added during the project. The complete GRSPaV CP was to be expressed within a bacterial expression system. The resultant protein will be used to generate GRSPaV CP antibodies. Parties within the viticulture industry will use these antibodies as an additional method of screening for GRSPaV in grapevine rootstocks and scions, using an ELISA approach. GRSPaV CP specific antibodies can be used to screen for GRSPaV infection quickly, cheaply and in large quantities of plants at a time.

Chapter 2: Literature review

2.1. INTRODUCTION

Many grapevine virus diseases occur worldwide, including the economically important diseases GLR, RSP, corky bark and fanleaf degeneration. Of these diseases GLR and, to a lesser extent, RSP are the most ubiquitous in South Africa and have the largest economic impact on the viticulture industry and economy of the Western Cape. Grapevine leafroll disease is characterised by the downward rolling of leaves and interveinal discolouration. Grapevines infected with GLR are often smaller than uninfected plants and the yield and berry composition are adversely affected, with a cumulative effect every season until the infected plants have to be removed (Goheen, 1988a). Grapevine rupestris stem pitting, the primary component of the RW disease complex, is characterised by pitting and grooving of the stem in severe infections. Grapevines infected by RSP are often smaller than uninfected vines and the yield is reduced, the quality of berries affected and ripening of the berries delayed (Goheen, 1988b). Rugose wood infected plants can die within a few years of planting (Meng *et al.*, 1999a; Martelli, 1993).

Historically, grapevine virus diseases have been studied much more thoroughly than their causal viruses (Flaherty, 1992). Even when the causal virus is unknown, the physiological effects of the disease can still be studied due to the infection being present in the whole plant and all material used for propagation. Prevention of diseases has until recently relied almost entirely on avoiding the use of infected material for plant propagation and the prevention of infection (Flaherty, 1992). During the last 2 decades several new related methods of preventing virus infection have been studied and applied, with varying degrees of success.

During the last century the prevention of infection and propagation of infected material has been supplemented with cross-protection as the only other method of reducing virus infection (Ratcliff *et al.*, 1999). However, in the 1980s a study conducted by Powell-Abel *et al.* (1986) demonstrated that the expression of a functional virus CP gene within a transformed plant could lead to partial or total protection against infection by the virus. This phenomenon led to further investigations where plants were transformed with translatable and untranslatable pathogen derived sequences in both the sense and antisense conformations, which imparted varying levels of resistance (English *et al.*, 1996). It was reasoned that an RNA molecule somehow influenced the virus replication and directly led to virus resistance (Lindbo and Dougherty, 1992). These phenomena were collectively named transgene-mediated virus resistance and pathogen-derived resistance (PDR). It was later discovered by Waterhouse *et*

al. (1998) that the expression of a construct transformed into a plant, which contained complementary sense and antisense sequences, resulted in the formation of dsRNA. This could be used to silence either homologous endogenous plant genes, resulting in phenotypic changes, or homologous genes of a challenging virus, resulting in resistance. The resistance conferred by this homologous dsRNA was much more complete than that brought about by transformation using only sense or antisense constructs (Waterhouse *et al.*, 1998).

In 1990 it was discovered that the over-expression of a plant gene via the transformation of the plant with additional copies of the relevant gene, leads to reduced expression of the gene product. This phenomenon was subsequently named co-suppression (Napoli *et al.*, 1990). Initial experiments indicated that co-suppression functions at both the transcriptional and post-transcriptional levels (Hammond *et al.*, 2001b). The study of co-suppression led to the elucidation of, what today is known as, post-transcriptional gene silencing (PTGS).

Post-transcriptional gene silencing is the suppression of expression of an endogenous plant gene or transgene caused by the introduction of homologous dsRNA, a homologous virus or a complementary transgene. This process is known as RNA interference (RNAi) in animals. Together these two mechanisms have far reaching implications in the field of biotechnology. Post-transcriptional gene silencing is the basis of natural plant virus resistance and RNAi has a role in the suppression of transposon mobilisation in animals (Waterhouse *et al.*, 2001; Ruiz *et al.*, 1998). In plants the PTGS signal is also amplified by a process named transitivity, and can spread systemically through the plant, facilitating an efficient gene silencing mechanism (Himber *et al.*, 2003; Voinnet and Baulcombe, 1997). Both PTGS and RNAi have been applied with success as tools for functional genomics (Hammond *et al.*, 2001b). Perhaps the most exhilarating aspect of PTGS is the ability to induce targeted virus resistance against virtually any plant virus, including viruses that encode PTGS suppressor proteins (Savenkov and Valkonen, 2002). Post-transcriptional gene silencing has been applied to induce resistance in dicotyledonous and monocotyledonous plant species, against most of the major DNA and RNA plant viruses (Goldbach *et al.*, 2003; Ding, 2000; Waterhouse *et al.*, 1999). The discovery that certain plant viruses encode proteins that result in the suppression of PTGS is believed to be the strongest evidence that the PTGS mechanism evolved as a plant defence mechanism against viruses (Li and Ding, 2001).

Thus, the manipulation of the PTGS mechanism to target specific viruses is potentially the most powerful method for the control of plant viruses in the agriculture industry.

2.2. RUGOSE WOOD DISEASE

The RW complex was first described in 1961 (Graniti and Ciccarone, 1961). Rugose wood disease is characterised by an alteration of the woody cylinder which includes pitting, grooving and swelling of the wood (Minafra, 2000; Meng *et al.*, 1999a). In the field RW affects some grapevine varieties visibly while some seem completely unaffected. It was later found that grapevines infected with RW are often without symptoms, and that infected ungrafted rootstocks and scions only develop symptoms after grafting, qualifying RW as a disease of combination (Martelli, 1993). Rugose wood disease occurs worldwide in most grapevine cultivating countries (Meng *et al.*, 1999b; Martelli, 1993). Ultimately RW causes a reduction in yield, graft take and rooting ability and infected grapevines may severely decline and even die within a few years of planting (Meng *et al.*, 1999a; Martelli, 1993).

Initially RW was thought to be the same disease as corky bark (CB) (Goheen, 1988b), but after records of biological indexing on woody indicators were critically re-examined suspicions arose that RW was in fact a new disease complex (Minafra, 2000). It was proposed (Garau *et al.*, 1989; Savino *et al.*, 1989) that RW consisted of four diseases that could be differentiated based on the response of three woody indicators: Kober 5BB (*Vitis berlandieri* Planch. X *V. riparia* Michx.), LN 33 (Couderc 1613 X Thompson seedless) and *V. rupestris* St. George (Synonyms: Du Lot, *V. rupestris* Scheele) (Minafra, 2000; Meng *et al.*, 1999b). The four diseases are: RSP indexed on *V. rupestris* St. George, characterised by a distinctive basipetal pitting downwards from the point of inoculation in the chip budded indicator (severe strains produce ridges and pits all around the bud union); kober stem grooving (KSG) indexed on Kober 5BB, characterised by grooving in the wood of the grafted indicator; CB indexed on LN 33, characterised by internodal swelling and cracking of young shoots a few months after chip-budding onto the indicator and also stunting and grooving (in addition it induces pits and grooves on the woods of both LN 33 and St. George (Meng *et al.*, 1999b)); LN 33 stem grooving (LNSG) indexed on LN 33, characterised by grooving of the trunk of the indicator but not producing internodal swelling and phloem proliferation present in CB (Martelli, 1993).

Over several decades the etiology of the RW disease complex was studied. In 1980 in Italy the first filamentous virus from stem pitting-affected grapevines was isolated (Conti *et al.*, 1980). Several advances have been made since and the etiology of RW is slowly being unraveled.

Currently five different filamentous phloem-restricted viruses are associated with the disease. Four of these viruses have been denoted; *grapevine virus A* (GVA), *grapevine virus B* (GVB),

grapevine virus C (GVC) and *grapevine virus D* (GVD) (Minafra, 2000). *Grapevine virus A* was initially classified as a putative closterovirus, due to its particle morphology, but has since been transferred to the genus *Trichovirus* (Martelli *et al.*, 1994) and ultimately to the *Vitivirus* genus. This change in classification is due to differences in biological properties and genome organisation. *Grapevine virus B*, GVC and GVD were also assigned to the genus *Vitivirus* (Martelli *et al.*, 1997). *Grapevine virus A* has been shown to be consistently associated with RW and GVB has been consistently associated with the CB component of RW (Minafra, 2000). Although GVC and GVD were purified from CB-affected grapevines in Italy (Bonavia *et al.*, 1996) and Canada (Monette and James, 1991), they have not yet been shown to be significant contributors to RW (Minafra, 2000). Currently GVA, GVB and GVD are definitive members of the *Vitivirus* genus, and GVC is a tentative member (Büchen-Osmond, 2003).

A fifth virus was found to be associated with RW, and has been consistently associated with the RSP component of the RW disease complex (Meng *et al.*, 1999a). The *Foveavirus* that is accepted as the cause for RSP (Martelli and Jelkmann, 2000) is known by several names. The name *grapevine rupestris stem pitting-associated virus* (GRSPaV) was proposed by Martelli and Jelkmann (1998), and Zhang *et al.* (1998b). It is also known as *rupestris stem pitting associated virus 1* (Meng *et al.*, 1999a), and has been described as *rupestris stem pitting associated virus* in the official list of plant viruses of 1999 (Martelli, 2000). Due to the confusion inherent in using three names to describe a single virus species, the name *grapevine rupestris stem pitting-associated virus* (GRSPaV) will be used in this work. This name is analogous to all other plant viruses first described in grapevines and named after it, and numbers or letters can be added later to differentiate between different isolates or species if the GRSPaV situation proves to be similar to that of the grapevine leafroll associated-viruses and the grapevine Vitiviruses (Martelli, 2000).

2.2.1. Grapevine rupestris stem pitting

As the primary and most widespread component of the RW disease complex (Meng *et al.*, 1999a), RSP was discovered in 1961 when RW was first described (Ciccarone, 1961; Graniti and Ciccarone, 1961), but was only officially recognised in 1976 in California (Meng *et al.*, 1997; Goheen, 1988b). Grapevine rupestris stem pitting (RSP) is considered to be one of the major virus diseases worldwide, and is found in the USA, Europe, Australia, South Africa and many other grapevine cultivating countries (Meng *et al.*, 1997; Goheen, 1988b).

Grapevine rupestris stem pitting causes a slow decline in the growth of *V. vinifera* over several years (Goheen, 1988b). Infected vines are often smaller than uninfected vines but this

usually only becomes apparent after the fourth season. The disease causes a reduction in yield and ripening is delayed (Flaherty, 1992). The effects on fruit yield and quality are similar to those of leafroll disease (Goheen, 1988b). Symptoms, which include pitting and grooving of the stem (**Figure 2.1**), do not show on most cultivars, although leaves are slightly smaller and canes have a reduced diameter when compared with uninfected grapevines. Grapevine rupestris stem pitting is a graft- transmissible disease (Zhang *et al.*, 1998b). *V. rupestris* St. George is the best indicator for RSP infection, and chip bud grafting of the candidate onto the indicator is the best method of inoculation for indexing. When this method is used a narrow strip of small pits develops in the xylem directly below the point of inoculation on the St. George indicator, but not above. Grafting onto the top of the indicator rarely produces symptoms, even when the candidate is seriously infected, and has been the cause of incorrectly indexing infected plants as uninfected. Grapevine rupestris stem pitting is often confused with CB when the wrong grafting technique is used, resulting in pitting distributed all around the woody cylinder. No symptoms develop when RSP infected chip buds are grafted onto the indicator for CB, LN 33 (Goheen, 1988b).

Figure 2.1. Severe symptoms of grapevine rupestris stem pitting. Pitting and grooving can be seen along the stem.

Indexing on woody indicators is laborious and slow. Once indicators have been grafted with a bud from the plant that is being tested, it takes 2 seasons before the development of virus induced disease symptoms (**Figure 2.1**). Because diseased material is primarily spread due to incorrect indexing of propagation material, it is important to use rapid, effective methods of detecting the presence of the RSP causing virus. Methods like reverse transcription-polymerase chain reaction (RT-PCR), immunosorbent electron microscopy (ISEM) and

enzyme-linked immunosorbent assay (ELISA) are powerful and accurate techniques for the detection of viruses. The consistent and accurate detection of RSP in potential grapevine rootstocks and scions will ensure that no infected grapevines are planted, thus curbing the spread and prevalence of the disease.

2.2.1.1. *Foveavirus*

Grapevine rupestris stem pitting-associated virus has been classified in the novel genus *Foveavirus*, which has as its type member *apple stem pitting virus* (ASPV), also known as pear vein yellows virus. The genus *Foveavirus* has not yet been assigned to any family and is a floating genus (Martelli, G.P., Pers. Comm., Dipartimento di Protezione della Piante, University of Bari, Italy). In the sixth report (1995) of the ICTV (The International Committee on Taxonomy of Viruses) (Murphy *et al.*, 1995), none of the viruses currently classified in this novel genus were assigned to a genus. After the detailed molecular study of the ASPV genome however, enough differences with other sequenced filamentous viruses were determined to support the idea that it was a representative of a novel taxon. The establishment of the new viral genus *Foveavirus* (from *fovea*, Latin for "pit", "hole") was proposed and approved by the Executive Committee of ICTV at the 1998 mid-term meeting in La Jolla, California, USA. *Apple stem pitting virus* was chosen as the type species for *Foveavirus*. *Grapevine rupestris stem pitting-associated virus* (Zhang *et al.*, 1998b; Meng *et al.*, 1997) was also included as a member whereas cherry green ring mottle virus (CGRMV) (Zhang *et al.*, 1998a) was included as a tentative member (Martelli and Jelkmann, 1998). Currently there are five species and two species accepted by the ICTV. *Apricot latent foveavirus*, is listed by Gentit *et al.* (2001) in the genus *Foveavirus*, but has not yet been approved by the ICTV (Martelli and Jelkmann, 2002).

Foveaviruses have a limited host range. GRSPaV infects only grapevine plants whereas ASPV infects primarily pome fruit plants (apple and pear) and CGRMV infects stone fruit (cherry, peach and apricot) plants. These three viruses have a wide geographical distribution (Martelli and Jelkmann, 2002), and GRSPaV is seen as the most widespread and prevalent of all the viruses associated with RW disease (Meng *et al.*, 1999a). There are no known vectors for the Foveaviruses, and they are believed to be spread only through grafting or, in specific cases, through mechanical inoculation. It was traditionally thought that GRSPaV is only spread through grafting of a scion onto rootstock and not through mechanical inoculation, contact between plants, by seed or by pollen (Martelli and Jelkmann, 1998). However, it has recently been shown that GRSPaV might be spread through the pollen (Minafra, 2000). At present these viruses are believed to be primarily spread through infected propagation material.

The virions are flexuous and have a variable length of 723 nm (GRSPaV) (Petrovic *et al.*, 2003), 800 nm (ASPV), and 1000-2000 nm (CGRMV). *Apple stem pitting virus* particles have a tendency to aggregate end-to-end. This phenomenon might also occur with CGRMV explaining its large observed particle size which should have a length in the range of 750-800 nm based on its genome size (Martelli and Jelkmann, 1998). The virions consist of a single molecule of single stranded positive-sense RNA encapsidated by the CP. The genomes are polyadenylated at the 3' terminus with sizes ranging from 8.4-9.3 kb. GRSPaV has a genome with a size of 8.7 kb (Zhang *et al.*, 1998b; Meng *et al.*, 1999a). The CPs range in size from 28 kDa (GRSPaV) to 44 kDa (ASPV) (Martelli and Jelkmann, 1998).

The ASPV genome consists of 5 open reading frames (ORFs) (**Figure 2.2**). The 5' area starts with a non-coding sequence of 59 nts. ORF1 contains the genes that code for the replication-related proteins: the methyltransferase (MET), helicase (HEL) and the RNA-dependent RNA polymerase (RdRp) domains. ORF2 to 4 contains the triple gene block proteins (Martelli and Jelkmann, 1998), which are required for cell-to-cell and systemic movement (Carrington *et al.*, 1996). ORF5 is the CP cistron. The 3' terminus consists of a 135 nt non-coding sequence terminated by poly(A) tail. *Grapevine rupestris stem pitting-associated virus* has an identical genome structure and organisation as observed for ASPV. Most of the expressed products are of similar size, with the exception being the CP (28 kDa (GRSPaV) vs. 44 kDa (ASPV)). The 5' and 3' noncoding regions of GRSPaV are of the sizes 61 nt and 176 nt respectively (Martelli and Jelkmann, 1998). Zhang *et al.* (1998b) reported that the GRSPaV isolate they studied has an extra ORF at the 3' end. This encodes for a 14 kDa product of unknown function. The 5' and 3' non-coding areas of their Californian isolate are 60 nt and 140 nt respectively. Cherry green ring mottle virus has a capped 5' terminus and contains two extra ORFs within ORF2 and ORF5 (ORF2a and ORF5a). No homologues were found for these proteins in other virus taxa (Martelli and Jelkmann, 1998).

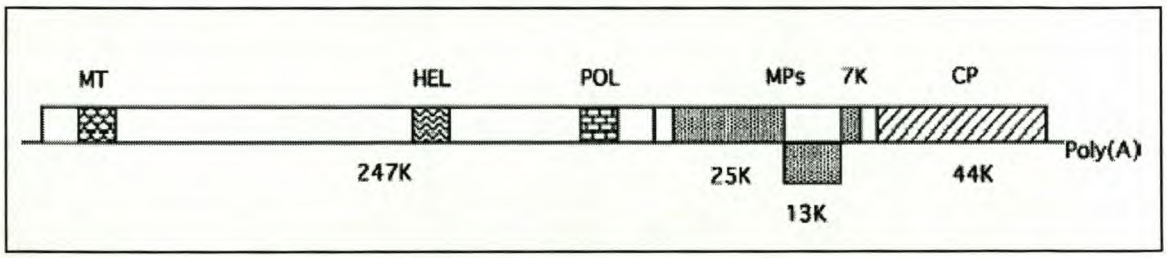

Figure 2.2. The genome organisation of ASPV, the type species of the genus *Foveavirus*. *MT*, methyltransferase; *HEL*, helicase; *POL*, RNA polymerase (RdRp); *MPs*, putative movement proteins (triple gene block); *CP*, coat protein (Martelli and Jelkmann, 1998).

There are several similarities between *Foveavirus* and other virus genera. The morphology of the virions is similar to species of the genera *Closterovirus*, *Crinivirus*, *Ampelovirus*, *Trichovirus*, *Vitivirus*, *Capillovirus* and *Allexivirus*. The genome structure and organisation strongly resembles that of the genera *Potexvirus*, *Carlavirus* and *Allexivirus* in the number and order of the genes. However, ORF1 and the CP cistron of ASPV are significantly larger than those of species in these genera. Signature sequences found within ORF1 are homologous to those found in members of the ssRNA positive-sense viruses, especially those of *Potexvirus*, *Carlavirus* and *Allexivirus*. The triple gene block contains similarities between the mentioned genera. The CP genes of all the *Foveavirus* species share homology with species of *Potexvirus* (the closest as shown by phylogenetic analysis), *Carlavirus* and *Allexivirus* (Jelkmann, 1997). *Foveavirus* species are discriminated on the following characteristics: (1) natural host range; (2) mechanical transmissibility; (3) serological relatedness; (4) CP size; (5) amino acid sequence of any gene product greater than 10% of the total gene products (Martelli and Jelkmann, 1998).

2.2.1.1.1. *Grapevine rupestris stem pitting-associated virus*

Grapevine rupestris stem pitting-associated virus was potentially first isolated in 1980 (Conti *et al.*, 1980). This research group isolated an 800 nm flexuous filamentous particle from an RSP infected grapevine plant, but its relationship to RSP could not be determined. Monette and Godkin (1995) isolated filamentous particles from shoot-tip cultured grapevine plants that were infected with GRSPaV and LN 33 stem grooving but they were not able to associate the particles with the disease due to a lack of GRSPaV specific antibodies. *Grapevine rupestris stem pitting-associated virus* was first successfully isolated, sequenced and characterised from grapevine plants exhibiting RSP symptoms by two research groups in 1998 and 1999 (Zhang *et al.*, 1998b; Meng *et al.*, 1999a). The first detection of GRSPaV was achieved when Petrovic *et al.* (2003) used antibodies, generated using the GRSPaV CP gene, to detect the virus particles in tissue culture-, greenhouse-, and field-grown grapevines that were infected with GRSPaV, but not in control plants (**Figure 2.3**). It has been shown that GRSPaV is consistently associated with RSP (Meng *et al.*, 1999a).

The virions of GRSPaV are flexuous rod shaped particles of approximately 723 nm (ca. 800 nm (Martelli and Jelkmann, 1998)) in length (**Figure 2.3**) (Petrovic *et al.*, 2003). The CP of GRSPaV is 28 kDa (Meng *et al.*, 2003; Minafra *et al.*, 2000) in size and the genome is approximately 8.7 kb in size (NCBI accessions AF026278 and AF057136). An 8.7 kb dsRNA has been found to be consistently associated with grapevines infected with RSP (Meng *et al.*, 1999a), which is thought to be the same as the 8.0 to 8.3 kb dsRNA isolated by Azzam *et al.* (1991) and Walter and Cameron (1991). The genome of GRSPaV consists of

six ORFs. ORF1 encodes for a polypeptide consisting of a MET, a papain-like protease (P-PRO), a HEL and an RdRp domain; ORFs 2-4 compose a triple gene block that respectively encodes proteins of sizes 24.4 kDa, 12.8 kDa and 8.4 kDa; ORF5 encodes the 28 kDa CP and ORF6 is situated near the 3'-terminal end and encodes for a 14 kDa protein of unknown function (Zhang *et al.*, 1998b). Meng *et al.* (1999a) sequenced and characterised a GRSPaV isolate from New York (USA) and observed that their isolate did not encode the ORF6 of the California (USA) isolate used by Zhang *et al.* (1998b).

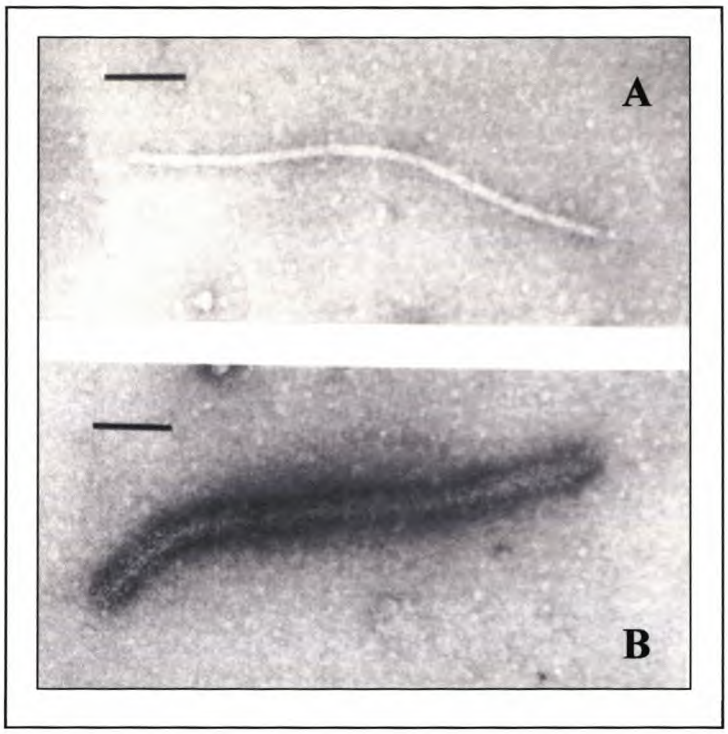

Figure 2.3. Electron micrograph of GRSPaV particles detected through immunosorbent electron microscopy. **(A)** nondecorated and **(B)** decorated particles trapped with the polyclonal antiserum to the recombinant GRSPaV coat protein. The bar represents 100 nm (Petrovic *et al.*, 2003).

Grapevine rupestris stem pitting-associated virus is a graft transmissible virus but is not mechanically transmissible through sap inoculation (Minafra, 2000). Rowhani et al. (2000) have shown through the use of the RT-PCR technique that GRSPaV is found internally in the pollen of infected plants, but they were not able to determine if the virus was present in the seeds. Traditionally GRSPaV is detected in plants through indexing on *V. rupestris* St. George, the same indicator used for RW, but it has been shown that a very efficient method of screening for the presence of the virus is the use of the RT-PCR technique with universal primers for known sequence variants of GRSPaV (Minafra, 2000).

Analysis of the GRSPaV cDNA clones of isolates from various RSP-infected grapevines revealed the presence of a family of sequence variants. Even though the genome structure

was identical, nucleotide sequence identities ranged from 75% to 93% and amino acid sequence from 80% to 99%. The CP gene and the region of ORF1 associated with the RdRp were the most highly conserved. Coat proteins from these sequence variants exhibited highly similar antigenic properties, suggesting serological relatedness. The presence of multiple sequence variants in single RSP infected grapevine plants was also observed (Meng *et al.*, 1999b).

It has to be noted that there are differences in the scientific literature as to whether GRSPaV has a negative impact on infected grapevine plants. Reynolds *et al.* (1997) observed virus-free and GRSPaV infected grapevines (infected through chip bud grafting) to study the effect of the virus on vigor, yield, berry composition and time of budburst. It is not known if they infected with a pure isolate of GRSPaV. The study was conducted at two locations, Summerland and Sidney (British Columbia, Canada) over a period of 7 and 4 years respectively, using five *Vitis* cultivars. Their results indicated that, relative to virus-free vines, GRSPaV has very little or no effect on the yield, time of budburst and grapevine size, and that it might accelerate fruit maturation through a more rapid reduction of titratable acidity and a faster increase in pH.

However, they also mention that the vines should be observed over a longer period of time to determine if other debilitating effects occur and to assess a possible delay in sugar accumulation due to GRSPaV. At the time of print they were in the process of re-indexing the GRSPaV-inoculated plants to determine if the results could be attributed to virus infection (Reynolds *et al.*, 1997). Although grapevines were infected through chip grafting, no molecular or serological tests were conducted to determine proof of GRSPaV infection during the study and no indexing was done on the RSP indicator St. George (*V. rupestris*), the accepted method for testing for RSP (Meng *et al.*, 1999a; Zhang *et al.*, 1998b). This indicates a flaw in their study seeing as the vines could have been uninfected. The results would be more complete if older plants were studied and if the virus status of all the plants had been identified by correct indexing.

2.3. GRAPEVINE LEAFROLL DISEASE

The GLR complex has been known for many years and under various names: rougeau (in France, 1850s), flavescence, brunisure, rollkrankheit, red-leaf and white-emperor disease (Over de Linden and Chamberlain, 1970). The viral nature of GLR was first described when Scheu (1936) successfully graft transmitted the virus from diseased to healthy vines. The disease has a worldwide occurrence and is a graft-transmitted virus disease that adversely

affects grape yields and fruit quality (Goheen, 1988a). The characterisation of a new virus associated with GLR, named grapevine leafroll-associated virus 9 and tentatively grouped within the *Ampelovirus* genus, brings the total of serologically distinct viruses that have been isolated from grapevines and that is seen as having a role in GLR to nine. These are designated grapevine leafroll-associated virus 1 to 9 (Alkowni and Rowhani, 2003). Due to its wide occurrence and adverse effects on grapevines, GLR is seen as one of the most important grapevine diseases (Zhu *et al.*, 1998; Goszczynksi *et al.*, 1996b).

Leafroll probably originated in the Near East and was transported to the West along with grapevine cuttings (Goheen, 1988a). The disease causes chronic damage and early estimates of yield loss ranged from 46% to 85% (Over de Linden and Chamberlain, 1970). The current estimate of yield loss due to GLR is 20% to 40% (Alkowni and Rowhani, 2003; Goheen, 1988a).

Grapevine leafroll disease causes an array of symptoms. Leaves, shoots, canes, trunks and root systems are slightly smaller in diseased plants when compared to healthy plants (Goheen, 1988a). Although no obvious symptoms can be seen during the winter and the early part of the growing season, they start to develop during early summer in water stressed vineyards, and somewhat later in well-irrigated vineyards, and become progressively worse until late summer and autumn (Flaherty, 1992; Goheen, 1988a). The symptoms of GLR include downward rolling and interveinal reddening of leaves on varieties with dark coloured fruit (**Figure 2.4 (A)**). Varieties with light coloured fruit also show downward rolling of leaves but leaves develop interveinal chlorosis. The leaf sometimes becomes necrotic as if burned (**Figure 2.4 (B)**). Infected vines have a lowered fruit production, and due to the delayed ripening of fruit, the sugar content is reduced. Pigmented berries (red or black) become greenish or whitish due to delayed maturation and anthocyanin retardation, and although the shape of berry clusters and the size of individual berries stay the same, the average size of the clusters is smaller than normal (Goheen, 1988a). Because the disease does not kill a grapevine outright and does not cause disastrous crop losses every year, farmers rarely remove infected plants from otherwise healthy and productive vineyards. This leads to a significant loss due to reduced yield and quality grapes every year over the total life of the vineyard, and the continuous spread of the disease.

Indexing for GLR is traditionally performed on woody indicators e.g. Cabernet franc, which is a sensitive indicator for GLR. Symptoms develop on indicators within 18 months and if no symptoms develop within that period the vine can be registered as a mother vine source and used for propagation of virus-free material. However, this method is slow and not always reliable. As for RSP, newer and more efficient methods of screening for the presence of

grapevine leafroll-associated viruses are available and include RT-PCR and the use of a pool of GLRaVs specific antibodies in ISEM and ELISA (Credi and Giunchedi, 1996). When used in conjunction, these methods can be very effective in controlling the spread of GLR by ensuring that only healthy propagating material is used to plant vineyards. If uninfected source material isn't selected, GLR incidence can approach 100% within a few generations of clonal propagation (Flaherty, 1992). Planting of certified "virus free" material can reduce the source of virus for vector transmission, but does not stop the spread of GLR by vectors. When the worldwide occurrence and perennial nature of the disease is taken into account, it leads to the conclusion that GLR is one of the main contributors to production loss of grapes (Flaherty, 1992).

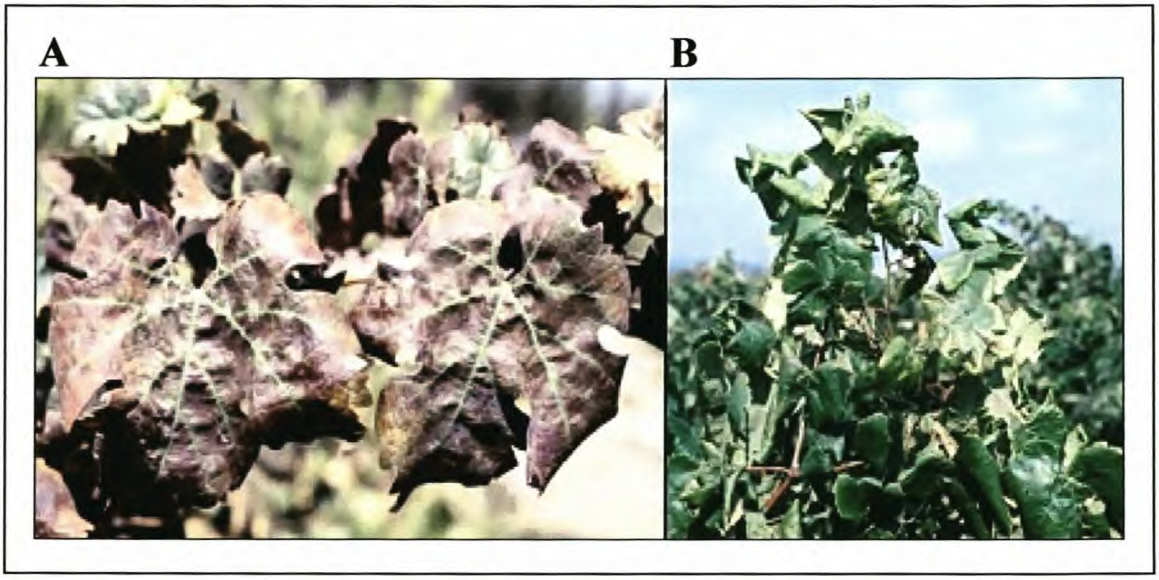

Figure 2.4. Symptoms of GLR. **(A)** Interveinal reddening and downward rolling of leaves on varieties with dark coloured fruit. **(B)** Downward rolling and necrosis of leaves on a variety with light coloured fruit (photos courtesy of G.P. Martelli).

Grapevine leafroll disease, RW and the Vitiviruses are often found in mixed infections (Credi and Giunchedi, 1996). It is still not known to what degree the causal viruses of these diseases influence each other in the expression of disease symptoms. The grapevine infecting viruses from the family *Closteroviridae* (Martelli *et al.*, 2002) have been shown, as early as 1987, to be consistently associated with GLR, which supports the evidence that these viruses are the causal agents of the disease (Kim *et al.*, 1989; Zee *et al.*, 1987).

2.3.1. *Closteroviridae*

The original floating genus *Closterovirus* was re-organised (Murphy *et al.*, 1995), which led to the establishment of the new *Closteroviridae* family in 1998 (Martelli *et al.*, 2000). This

family was again revised, resulting in the genus *Closterovirus* being split into two genera. Ultimately the *Closteroviridae* consists of three genera; *Closterovirus*, *Ampelovirus* and *Crinivirus*. Viruses in the new genera are classified according to their vector and genome organisation (**Figure 2.5**) (Martelli *et al.*, 2002; Karasev, 2000).

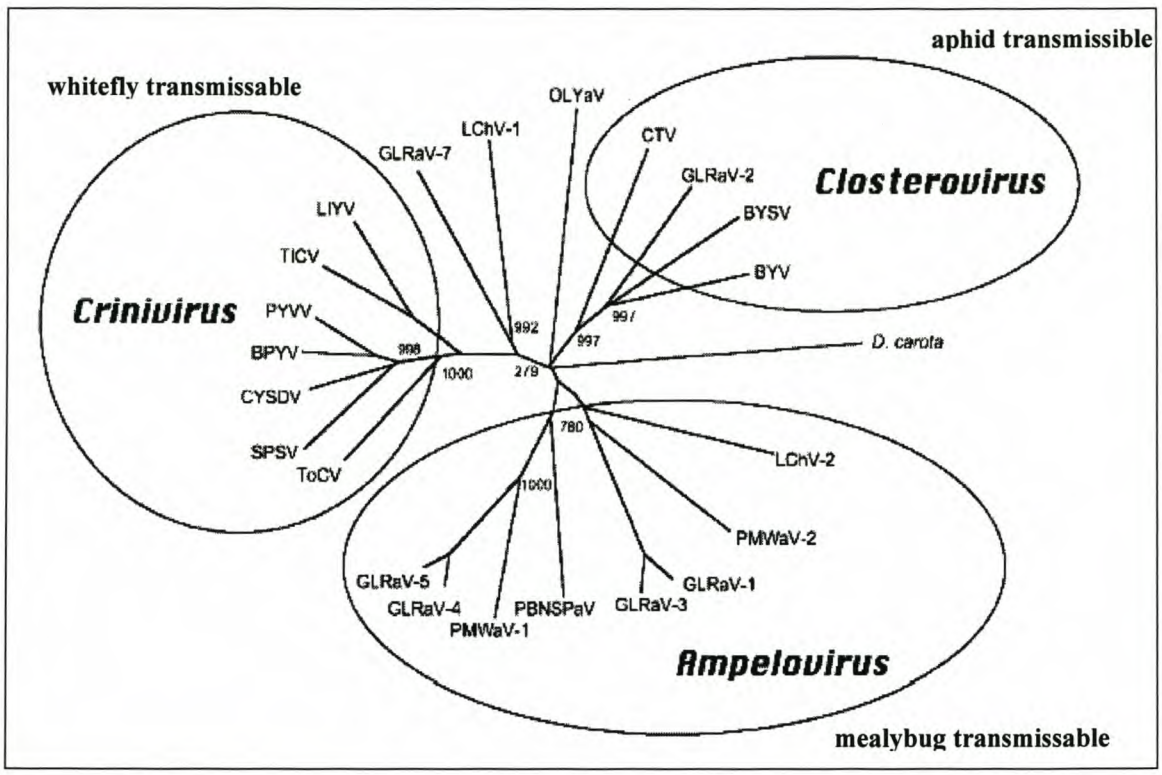

Figure 2.5. Phylogenetic tree showing the relationships between the genera and species of the family *Closteroviridae*. This neigbour-joining tree was based on the sequence of the HSP70 gene. The species GLRaV-7, LChV-1 and OLYaV are unassigned species in the family (Martelli *et al.*, 2002).

Eight of the nine grapevine-associated viruses in the family *Closteroviridae* have been assigned to 2 genera within this family. *Grapevine leafroll-associated virus 2* is the only grapevine leafroll-associated virus species in the genus *Closterovirus*. *Grapevine leafroll-associated virus 1*, *-3* and *-5* are definitive members of the *Ampelovirus* genus, and GLRaV-4, -6, -8, and -9 are tentative members of the *Ampelovirus* genus. Grapevine leafroll-associated virus 7 has not yet been assigned to any of the genera within the family *Closteroviridae* (Büchen-Osmond, 2003), however phylogenetic analysis indicates that GLRaV-7 may be assigned as a member of the *Crinivirus* genus (Martelli *et al.*, 2002). As yet none of the grapevine-associated closteroviruses have been assigned as definitive members of the *Crinivirus* genus (Alkowni and Rowhani, 2003; Büchen-Osmond, 2003). All viruses in the family *Closteroviridae* are phloem-restricted viruses.

2.3.1.1. *Closterovirus*

Grapevine leafroll-associated virus 2 is a member of the genus *Closterovirus*, which has as its type member *beet yellows virus (*BYV), in the family *Closteroviridae*. There are currently 8 assigned species and 4 tentative species assigned to this genus. Virions are 1250-2200 nm in size and the genome is a single, linear, positive sense, ssRNA molecule with a size of 15.5-19.3 kb. The CP is encoded by two subunits: the major CP subunit (22-25 kDA) which coats most of the virion length, and the divergent coat protein (CPd) analogue (24-27 kDA) which coats a short segment of 75 nm at one end of the particle. The gene encoding the CPd is upstream of the CP gene, contrasting with the other two genera of the family (*Ampelovirus* and *Crinivirus*) where the CPd gene is downstream (Martelli *et al.*, 2002).

There are three types of genome organisations in the genus *Closterovirus*, typified by BYV (**Figure 2.6**), *beet yellow stunt virus* (BYSV) and *citrus tristeza virus* (CTV). The genome of BYSV has an organisation intermediate to that of BYV and CTV suggesting three distinct stages in *Closterovirus* evolution (Martelli *et al.*, 2002).

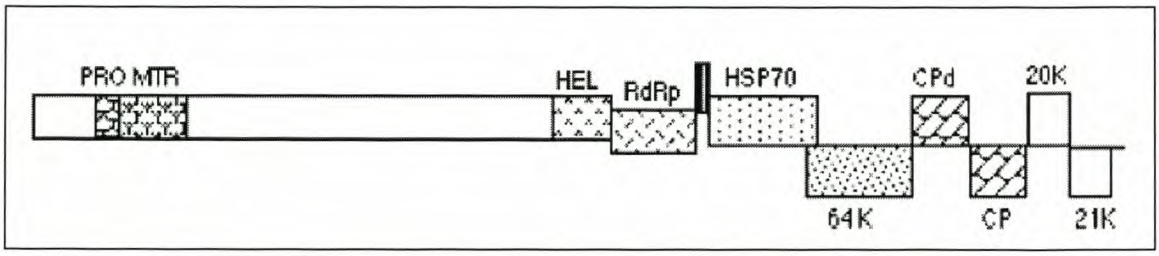

Figure 2.6. The genome organisation of *BYV*, the type species of the genus *Closterovirus*. *PRO*, papain-like protease; *MTR*, methyltransferase; *HEL*, helicase; *RdRp*, RNA polymerase; *HSP70*, heat-shock-related protein; *CPd*, divergent coat protein analogue; *CP*, coat protein (Martelli *et al.*, 2002).

Viruses from the genus *Closterovirus* primarily infect dicotyledonous plants and are transmitted in a semi-persistent manner only by aphids. Several of the species in the genus *Closterovirus* are transmissible through sap inoculation, but with difficulty (Martelli *et al.*, 2002).

2.3.1.1.1. *Grapevine leafroll-associated virus 2*

Grapevine leafroll-associated virus 2 was first reported by Gugerli *et al.* (1984), but it was later shown that the grapevine infected with GLRaV-2 was infected with a mixture of GLRaVs. These were designated GLRaV IIa and GLRaV IIb (Gugerli and Ramel, 1993). It was later shown by Boscia *et al.* (1995) that GLRaV IIb was the same as a GLRaV-2 isolated

in France (Zimmermann *et al.*, 1990). *Grapevine leafroll associated virus* IIa was designated GLRaV-6 while GLRaV IIb is now regarded as the definitive GLRaV-2 (Boscia *et al.*, 1995). Two distinct biological strains of GLRaV-2 have been described based on external symptoms, cytological alterations and differences in minor dsRNA band patterns (Goszczynski *et al.*, 1996a).

The virions of GLRaV-2 are filamentous and flexuous particles of approximately 1400-1800 nm in length (Gugerli *et al.*, 1984). The CP is 22-26 kDa (Boscia *et al.*, 1995; Gugerli and Ramel, 1993; Zimmermann *et al.*, 1990) in size, which is considerably smaller than the CPs of other GLRaVs (35-43 kDa) (Zhu *et al.*, 1998). The genome of GLRaV-2 is 15 kb in size (NCBI accession AF039204). A dsRNA of approximately 15 kb was consistently isolated from GLRaV-2 infected grapevine tissue (Zhu *et al.*, 1998; Goszczynski *et al.*, 1996a).

Grapevine leafroll-associated virus 2 was first partially sequenced and characterised by Abou-Ghanem *et al.* (1997) but the first "complete" (the exact 5' terminus was not yet cloned and sequenced) sequencing and characterisation was done by Zhu *et al.* (1998). The genome of GLRaV-2 consists of nine ORFs. Open reading frame 1 is divided into ORF1a and ORF1b. The incomplete ORF1a encodes a putative viral polyprotein containing the conserved domains characteristic of a P-PRO, a MET and a HEL. Open reading frame 1b encodes a putative RdRP. The next 5 ORFs (ORF2 to ORF6), which are in a unique five gene array conserved between Closteroviruses, encode the following: a 6 kDa hydrophobic transmembrane helical protein, a 65 kDa heatshock protein (HSP70), a 63 kDa protein of unknown function, a 25 kDa CPd and a 22 kDa CP. Open reading frame 7 encodes a 19 kDa protein of unknown function and ORF8 encodes a 24 kDa protein of unknown function. The genome also contains a 216 bp 3'-terminal untranslated region (Zhu *et al.*, 1998; Abou-Ghanem *et al.*, 1997). Further analysis of the 5' terminal region revealed a second P-PRO domain (Zhu *et al.*, 1998).

Although most of the members of the *Closterovirus* genus can be transmitted by aphids, the vector of GLRaV-2 is not yet known. *Grapevine leafroll-associated virus 2* can be transmitted by sap inoculation (Martelli *et al.*, 2002), and is the only grapevine *Closterovirus* that has been mechanically transmitted to herbaceous species (Goszczynksi *et al.*, 1996b; Boscia *et al.*, 1995; Monette and Godkin, 1993). This makes it an ideal *Closterovirus* to test methods of protection (e.g. virus targeted PTGS) against grapevine viruses, due to the relative ease of transformation and quick generation time (2-3 months) of tobacco plants, as apposed to the extremely difficult transformation and very long generation time (1 year) of grapevine plants.

2.3.1.2. *Ampelovirus*

Grapevine leafroll-associated virus 3 is the type species of the genus *Ampelovirus* in the family *Closteroviridae*. There are currently 6 species and 5 tentative species assigned to this genus. Viruses in the genus *Ampelovirus* are 1400-2200 nm in size. The genome consists of a single, linear, positive sense, ssRNA molecule with a size of 16.9-19.5 kb. The CP subunits have a molecular mass of 35-39 kDa (Martelli *et al.*, 2002).

The genus has two genome structures typified by GLRaV-3 (**Figure 2.7**) and *little cherry virus 2* (LChV-2). In the first of these genome structures, GLRaV-3 and most of the sequenced members of *Ampelovirus* has the CPd downstream of the CP. For LChV-2 the CPd gene is situated five ORFs upstream of the CP cistron, typifying the second genome structure. Another variation in the CP/CPd genome structure is that of GLRaV-1, where the CPd gene has been duplicated (Fazeli and Rezaian, 2000).

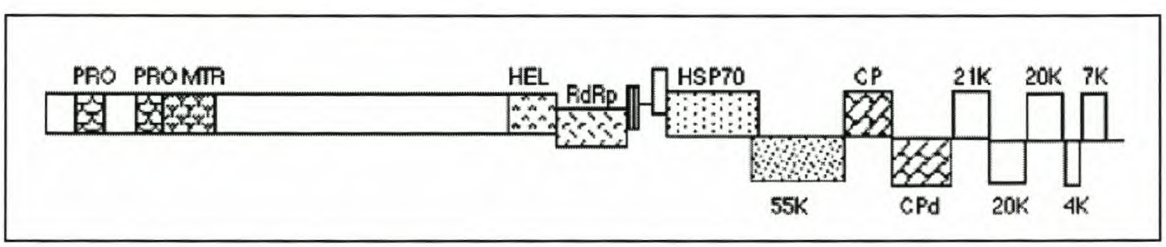

Figure 2.7. The genome organisation of GLRaV-3, the type species of the genus *Ampelovirus*. *PRO*, papain-like protease; *MTR*, methyltransferase; *HEL*, helicase; *RdRp*, RNA polymerase; *HSP70*, heat-shock-related protein; *CP*, coat protein; *CPd*, divergent coat protein analogue (Martelli *et al.*, 2002).

Viruses from the genus *Ampelovirus* only infect dicotyledonous plants and are transmitted semi-persistently by coccid and pseudococcid mealybugs. None of the species in *Ampelovirus* are transmissible through sap inoculation (Martelli *et al.*, 2002).

2.3.1.2.1. *Grapevine leafroll-associated virus 3*

Grapevine leafroll-associated virus 3 is the most well characterised virus associated with GLR and is arguably the most important component of GLR, due to its worldwide occurrence and observations that GLR apparently does not spread readily through a vineyard unless GLRaV-3 is present (Ling *et al.*, 1998). The GLRaV-3 virus was isolated for the first time by Gugerli *et al.* (1984) and named GLRaV III. It was later characterised and shown to be serologically different from GLRaV-1, GLRaV-2 and GLRaV-4 and renamed to GLRaV-3, which is the name that is currently accepted (Zimmermann *et al.*, 1990).

The virions of GLRaV-3 are filamentous and flexuous and are approximately 1800-2000 nm in length (**Figure 2.8**). The CP is 35 kDa in size, (ca. 43 kDa) which is larger than the 22-28 kDa CPs of viruses in the *Closterovirus* genus (Ling *et al.*, 1997). The genome of GLRaV-3 is 17.9 kb in size (NCBI accession AF036268). Double stranded RNA of 18 kb was isolated from GLRaV-3 infected grapevine material (Ling *et al.*, 1998/1997).

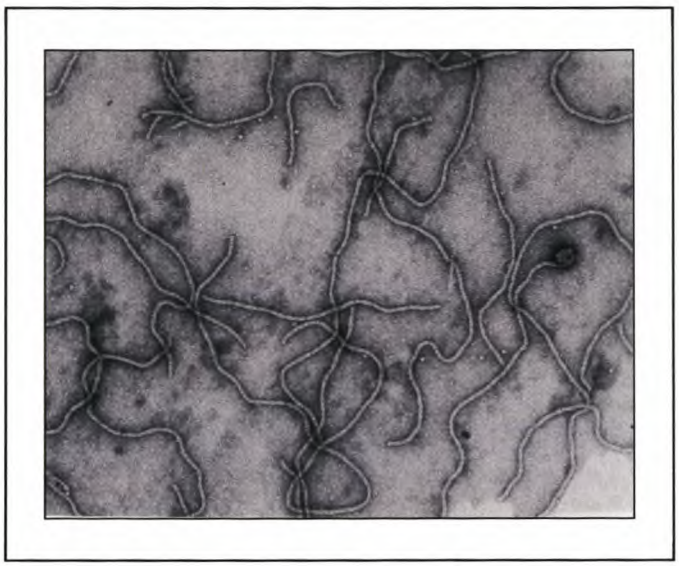

Figure 2.8. Electron micrograph of GLRaV-3 (photo courtesy of G. Kasdorf).

The GLRaV-3 genome was partially sequenced and characterised for the first time by Ling *et al.* (1997, 1998). Although a 5 kb region of the 5'-terminal portion was not sequenced, the sequence obtained contained approximately one-third of the C-terminal portion of ORF1a, which shared significant similarity with the HEL domain of positive-strand RNA viruses. It is reasonable to assume that the rest of the unsequenced ORF1a encodes the P-PRO and MET domains. Thirteen ORFs were sequenced or partially sequenced. Partial sequence of ORF1a, encoding the HEL domain, and ORF1b, encoding the RdRp, was obtained. The rest of the genome was divided as follows: a 6 kDa protein (ORF2), a ca. 5 kDa hydrophobic transmembrane helical protein (ORF3), a 59 kDa HSP70 (ORF4), a 55 kDa protein (ORF5), a 35 kDa CP (ORF6) and a 53 kDa diverged CP (ORF7). Open reading frames 8 to 12 potentially encode proteins of unknown function and of sizes: 21 kDa, 20 kDa, 20 kDa, 4 kDa, and 7 kDa. The genome of GLRaV-3 includes a 277 nt 3'-terminal untranslated region (Ling *et al.*, 1998).

Grapevine leafroll-associated virus 3 can not be transmitted through sap inoculation (Martelli *et al.*, 2002) but can be transmitted by grafting, as was shown by Scheu (1936). Various groups have shown that seven pseudococcid mealybug species (*Pseudococcus longispinus*,

Ps. viburni, *Ps. calceolariae*, *Planococcus citri*, *Pl. ficus* (Ling *et al.*, 1998), *Ps. Maritimus* (Golino *et al.*, 2002) and *Heliococcus bohemicus* (Sforza *et al.*, 2000)) can transmit the virus. There has also been a report that it can be transmitted by the scale insect *Pulvinaria vitis* L. (Belli *et al.*, 1994).

2.4. TRADITIONAL CONTROL OF PLANT VIRUSES

Various conventional methods have been used to control viral pathogens of crop and ornamental plants. These methods include crop rotation, early detection and destruction of the infected plants, resistance breeding, pesticide vector-control and cross-protection. Due to greater understanding of virus genetics, host defence mechanisms and virus/host interactions, a number of new and more effective methods have been discovered and exploited to control virus diseases in plants (Goldbach *et al.*, 2003).

Modern methods of plant virus control are exclusively based on the plant/virus interactions on the molecular level. Along with passive defence mechanisms (e.g. the rigid cell wall) plants exhibit active defence, of which the hypersensitive response (HR) is the most common. During the HR the gene products produced by the plant dominant resistance genes (*R* genes) recognise the virus and induce programmed cell death (apoptosis) in the cells surrounding the primary infection site, resulting in a visible necrotic lesion. There are 5 classes of *R* genes, but all the isolated *R* genes that confer resistance against viruses belong to the largest of these classes. This class of proteins encodes a nucleotide-binding site plus a leucine-rich repeat (NB-LRR) type of protein. No *R* genes have been found that confer resistance to grapevine viruses (Goldbach *et al.*, 2003). The expression of virus specific antibodies, otherwise known as plantibodies, within transgenic plants has been used to control plant viruses (Goldbach *et al.*, 2003). It has been shown that the expression of a single-chain antibody against the CP of *artichoke mottle crinkle virus* results in reduced susceptibility to the virus (Tavladoraki *et al.*, 1993). The mechanism by which this process occurs is unclear and little progress has been made since (Goldbach *et al.*, 2003). Specific and natural inhibitors of virus replication have been used for virus control. A promising candidate for this type of control is pokeweed (*Phytolacca americana*) antiviral proteins (PAPs), which are single-chain ribosome-inactivating proteins (RIPs). Due the inactivation of ribosomes by RIPS, they are potentially toxic to the host plant. Several less toxic and non-toxic RIPs have been used to confer virus resistance, without detrimentally affecting the plant through ribosome inactivation (Goldbach *et al.*, 2003). Broad virus resistance has been induced in plants through the expression of the mammalian 2',5'-oligoadenylate synthetase, but this system has not been explored beyond the initial experiments in the early 1990s (Goldbach *et al.*, 2003). The two remaining, and

arguably the most effective, modern methods used to induce protection against plant viruses; PDR and PTGS, will be discussed in **Section 2.5.1** and **Section 2.6** respectively.

The primary method of controlling grapevine virus spread is through the use of certified-virus free propagation material. Virus free rootstock and scion material is used to establish new vineyards. This is especially true for viruses that have no known insect vectors, e.g. GRSPaV (Flaherty, 1992; Goheen, 1988b). If virus-free material can not be obtained for a particular cultivar or clone, it is necessary to eliminate viruses from infected plants by way of heat treatment. The most commonly used method of heat treatment is known as the Goheen-method. The Goheen-method entails growing grapevine plants under optimum conditions in a heated chamber (38°C ± 2°C), for 1 to 3 months. The plants grow very fast under these conditions resulting in the apical growing points potentially being free of viruses. These apical regions are transplanted and rooted on media. Regenerated plants are indexed for viruses for three consecutive years and if they test negative for known viruses, are certified virus free. Variations of the heat treatment method exist e.g. the Galzy-method (Carstens *et al.*, 1981). Even though the diligent application of these techniques in recent years has had a positive influence on the extent of virus infection in vineyards, it has not been enough to effectively control viruses.

During the last two decades researchers have been studying a series of related phenomena that can now collectively be referred to as PTGS. It is believed that the primary function of PTGS in plants is virus control. The elucidation and application of PTGS could lead to much better protection of grapevines against their virus pathogens.

2.5. A SHORT HISTORY OF SILENCE

It has been known for more than 75 years that the inoculation of crop plants with a mild strain of a virus can delay the effects of infection by a severe strain and in some cases even prevent infection (Waterhouse *et al.*, 2001). This process, known as cross-protection, has often been used to reduce economic losses in crop plants (Powell-Abel *et al.*, 1986). Cross-protection in plants was recognised in the 1920s, but the greater mechanism is only now beginning to be unraveled (Waterhouse *et al.*, 2001).

Since the discovery of cross-protection, several phenomena have been described related to virus resistance in plants. Discovery of transgene-mediated virus resistance, co-suppression and transcriptional gene silencing (TGS) ultimately led to the discovery and elucidation of the PTGS mechanism. Post-transcriptional gene silencing is now believed to be an evolutionary

conserved mechanism, present in all eukaryotes, that could have evolved before the plant and animal kingdoms diverged (Fire *et al.*, 1998). The PTGS mechanism is believed to have evolved for the control of viruses and transposable elements, and also as a gene regulation mechanism. Today it is widely applied in the field of eukaryotic functional genomics and also for the induction of plant virus resistance. Post-transcriptional gene silencing and the related RNA interference (RNAi), are potentially the most important biotechnological "tools" that have been found to date (Hannon, 2002; Hutvágner and Zamore, 2002; Hammond *et al.*, 2001; Baulcombe, 1999).

2.5.1. Transgene-mediated virus resistance

It was shown by Powell-Abel *et al.* (1986) that the transformation of *Nicotiana tabacum* cv. Xanthi with a functional copy of the *tobacco mosaic virus* (TMV) CP gene resulted in delayed symptom development and a complete absence of symptoms in some plants, after subsequent TMV infection of the transgenic plants. It was reasoned that the expression of functional CP molecules interfered with the disassembly of the capsid of the challenging virus, thus interfering with the release and transcription of the viral RNA genome. It was also proposed that the expression of the TMV CP molecules interfered with virus movement. This type of resistance was referred to as coat protein-mediated resistance (CP-MR) (Beachy, 1999; Bendahmane *et al.*, 1997).

Related studies were performed where transgenic tobacco plants were generated to express untranslatable sense or antisense forms of the *tobacco etch virus* (TEV) CP gene, to better understand induced resistance in the protein-mediated mechanism. It was found that several transgenic plants expressing either an antisense CP gene transcript, or untranslatable sense transcripts, induced a reduction in symptoms or protection against TEV respectively (Lindbo and Dougherty, 1992). This was contrary to what was understood of the protection mechanism at the time (Waterhouse *et al.*, 2001). It was reasoned that an RNA molecule that somehow decreased the levels of virus replication, an astute observation for the time, mediated this TEV specific resistance (Lindbo and Dougherty, 1992). This type of resistance became known as transgene-mediated virus resistance. The induction of resistance in a host plant through its transformation using a pathogen gene or sequence, that blocks a specific step during virus multiplication, is also known as PDR (Sanford and Johnson, 1985). For reviews on PDR see Baulcombe (1996) and Lomonossoff (1995).

Analysis of plants exhibiting TMV CP-MR and the TEV resistant plants, that were transgenic TEV CP free, found that the transgenes were highly transcribed within the nucleus but that their mRNA levels within the cytoplasm were very low (Waterhouse *et al.*, 2001). It was

proposed that plant cells can perceive transgene mRNAs as unwanted and that they somehow degrade it. It was also believed that other homologous and complementary RNAs within the cytoplasm, including virus RNA with homology to the transgene, became degraded in a sequence specific manner through a targeted nuclease (Dougherty and Parks, 1995; Lindbo and Dougherty, 1992). This concept was supported by the findings of English *et al.* (1996) in a study that combined the principles of transgene-mediated virus resistance and co-suppression. Co-suppressed tobacco plants containing a β-glucuronidase (GUS) encoding transgene (*uidA*) were infected with wild-type *potato virus X* (PVX) and PVX-GUS (PVX containing the GUS gene). The transgenic tobacco plants were susceptible to the wild-type virus, but were resistant to the PVX-GUS. They concluded that transgenic-virus induced resistance and silencing of endogenous genes work through a similar mechanism (English *et al.*, 1996). This study played a significant role in furthering the knowledge of the mechanism of gene silencing.

2.5.2. Co-suppression

The observation in bacteria that antisense RNA could interfere with gene expression was named antisense suppression. It was thought that the hybridisation of antisense RNA to the homologous mRNA interfered with mRNA transport or translation (Hamilton *et al.*, 1990). This phenomenon directly led to the discovery of co-suppression.

Although not a method to induce virus control, in retrospect co-suppression was the first glimpse of the PTGS mechanism believed to have evolved primarily for virus control in plants. In 1990, while attempting to overexpress the Chalcone synthase (CHS) protein responsible for pigmented petals in petunias, Napoli *et al.* (1990) inadvertently stumbled upon what today is known as PTGS. Expecting highly pigmented petals in the transgenic plants, these authors instead found that 42% of the transgenic petunias had either white flowers, or mosaic flowers with a wild-type background colour and white or pale areas. They also discovered that there was a 50-fold reduction in mRNA levels of the CHS gene, and believed that there was an interaction over considerable genomic distances between homologous genes, resulting in the reduced mRNA levels. This phenomenon was named "co-suppression", due to the coordinate nature of the interaction between the homologous endogenous gene and the transgene, and its effect on expression (Napoli *et al.*, 1990).

Co-suppression has since been observed in many diverse species: *Arabidopsis thaliana*, *N. benthamiana*, *Neurospora crassa*, *Drosophila*, *Caenorhabditis elegans*, zebrafish, rodent fibroblasts, mammalian cultured cells and several others (Hammond *et al.*, 2001b; Cogoni and

Macino, 2000). Co-suppression appears to be a ubiquitous mechanism is conserved throughout all eukaryotes.

In 2001 an important observation was made. Plants that did exhibit co-suppression or transgene-mediated virus resistance usually contained multiple, methylated copies of the transgene (Waterhouse *et al.*, 2001). This observation was paramount to the elucidation of the silencing mechanism.

2.5.3. Transcriptional gene silencing

Transcriptional gene silencing involves "interacting" genes that share homology within their promoter regions. Post-transcriptional gene silencing is associated with increased promoter methylation and altered chromatin patterns (Fagard and Vaucheret, 2000; Kooter *et al.*, 1999). These alterations result in the blocking of transcription within the nucleus. Although reversible promoter methylation has been successfully used to undo TGS, it is believed that methylation assists chromatin components that induce TGS (Kooter *et al.*, 1999). A link between altered DNA methylation and chromatin remodeling has been shown in plants. In *A. thaliana* a chromomethylase gene (*cmt1*) has been identified that contains a potential DNA MET and a chromodomain (a protein module that influences transcription through chromatin remodeling) embedded within the catalytic region of the MET (Henikoff and Comai, 1998). This suggests that the same genetic elements that induce methylation can contribute to chromatin remodeling, thus regulating transcription.

Transcriptional gene silencing can occur through *cis* inactivation (e.g. when one or multiple copies of a transgene are integrated in or close to a silent hypermethylated genomic sequence), or through *trans* inactivation (e.g. when an active transgene is brought into the presence of an unlinked homologous transgene, it can become methylated and silent). There are several varying *cis* or *trans* transgene interactions that can lead to TGS, which will not be discussed here (see Fagard and Vaucheret (2000) and Vaucheret *et al.* (1998)).

It appears that highly repetitive sequences are most often targets for TGS. Transposable elements (TEs) containing highly repetitive regions, viroids and certain integrated bacterial vector DNA is often perceived as foreign and subsequently hypermethylated and silenced (Kooter *et al.*, 1999). There is also evidence of a role for chromatin modification and TGS in developmental regulation. It has been shown that transcriptionally silent transgenes acquire a condensed chromatin structure and that expression of chromodomains can lead to abnormal phenotypic development. For reviews of these aspects see Fagard and Vaucheret (2000) and Vaucheret *et al.* (1998).

Interestingly, both TGS and PTGS have developed as host defence- and expression-regulation mechanisms. It is believed that there are common stages and components between TGS and PTGS (Fagard and Vaucheret, 2000).

2.6. POST-TRANSCRIPTIONAL GENE SILENCING

Post-transcriptional gene silencing was initially discovered in plants (Napoli *et al.*, 1990). Post-transcriptional gene silencing can be defined to cover all aspects of the silencing of an endogenous gene or transgene caused by the introduction of a homologous dsRNA, complementary transgene or virus. In PTGS the transcripts of the targeted gene are synthesised, but rapidly degraded in the cytoplasm. Post-transcriptional gene silencing functions as a plant defence mechanism and developmental control system. It is adaptive, mobile and specific. During the illumination of the PTGS mechanism several terms have been used to describe different aspects of PTGS in different organisms.

Co-suppression is generally used to describe the silencing of an endogenous plant gene, due to the presence of a homologous transgene or virus (Napoli *et al.*, 1990). Co-suppression can manifest at the transcriptional level (TGS) or post-transcriptional level (PTGS) (Hammond *et al.*, 2001b). In fungi, e.g. *N. crassa*, the co-suppression observed when a transgene homologous to an endogenous gene is introduced, is named "quelling" (Romano and Macino, 1992).

A breakthrough came with the discovery of RNA interference (RNAi), which was first described in *C. elegans* (Fire *et al.*, 1998). RNA interference refers to dsRNA induced PTGS. It was observed that the introduction of dsRNA into *C. elegans* was 10-fold more effective at reducing homologous gene expression than the introduction of either the sense or antisense homologous RNA, and that the RNAi response was inherited by the progeny of the silenced nematodes. The discovery that a few molecules of dsRNA could induce complete gene silencing argued against stoichiometric interference and suggested that some kind of amplification of the interference mechanism was achieved (Fire *et al.*, 1998). It has been estimated in *Drosophila* embryos that dsRNA molecules can efficiently silence at least an order of magnitude more target mRNA molecules (Kennerdell and Carthew, 1998).

RNA interference is mechanistically related to PTGS in plants, and might be almost identical. Although it is unclear whether co-suppression at the transcriptional level is related to RNAi,

definitive links between transgene mediated co-suppression and RNAi have been found (Hammond *et al.*, 2001b; Cogoni and Macino, 2000).

To create a thorough view of PTGS it will be discussed in terms of all the organisms in which it has been significantly studied and elucidated. In an attempt to simplify this work, PTGS will be used to refer to the RNA silencing phenomenon specifically in plants, RNA interference will be used to refer to dsRNA induced gene silencing in animals and RNA silencing will be used when referring to both PTGS and RNAi.

2.6.1. Genes involved in RNA silencing

Two genes, *rde*-1 and *rde*-4 (RNAi deficient) that take part in the initiation of RNAi have been found in *C. elegans*. These genes are required for RNAi activation but not for the interference thereafter i.e. inheritance of interference by the offspring of the silenced nematodes (Grishok *et al.*, 2000). Several homologues have been found for the *rde*-1 gene family in other organisms. Mutants of the *rde*-1 homologues; the *qde*-2 (quelling deficient) gene in *Neurospora* (Hannon, 2002), and the AGO1/AGO2 (argonaute) producing genes in *A. thaliana* (Hammond *et al.*, 2001a; Bohmert *et al.*, 1998), result in deficient RNA silencing initiation (Hannon, 2002). It has been shown that AGO1 is involved in *A. thaliana* development. Mutants of AGO1 are defective for co-suppression and exhibit defects in leaf development (Bohmert *et al.*, 1998). The argonaute gene family is characterised by the presence of two domains: a PAZ domain and a Piwi domain (Hannon, 2002). The Dicer enzyme, the initiator of RNA silencing, also contains a PAZ domain, which may be necessary for the assembly of silencing complexes (Bernstein *et al.*, 2001). A mammalian protein, eIFC2, with homology to the *rde*-1 gene family, has been identified as a translation initiation factor suggesting a link between RNA silencing and translation (Cogoni and Macino, 2000).

Two RNAi effector genes have been found in *C. elegans*. Nematodes with mutations in the *rde*-2 and *mut*-7 genes have defective RNAi responses and also have an increased transposon activity. Rde-2 and Mut-7 are required downstream of the RNAi initiation site for efficient RNAi in the parent (Hannon, 2002; Grishok *et al.*, 2000). Although the *rde*-2 gene product has not yet been identified, the *mut*-7 gene product has homology to the RNase D nuclease domain and a protein indicated in a rapid aging disease, Werners syndrome, in *Homo sapiens* (Ketting *et al.*, 1999).

Although the protein products of most of these genes have not yet been identified, functional analysis through mutation of the gene, or gene silencing, and the resultant phenotype give a clear indication of their function in PTGS and development. The involvement of these genes

in both PTGS and development further strengthen the idea that PTGS is an anti-viral and developmental mechanism that stretches across kingdoms.

2.6.2. Dicer: The initiator of RNA silencing

The Dicer (*Dcr*) enzyme was first discovered in *Drosophila* (Bernstein *et al.*, 2001), and is accepted as the name of a family of evolutionary conserved eukaryotic nucleases (Hannon, 2002). Dicer is a member of the RNase III family of dsRNA specific ribonucleases and functions in an ATP dependent manner (Bernstein *et al.*, 2001; Zamore *et al.*, 2000). Dicer binds to and cleaves dsRNA to form short interfering RNAs (siRNA) (Bernstein *et al.*, 2001; Zamore *et al.*, 2000). Short interfering RNAs were first observed, and associated with PTGS, by Hamilton and Baulcombe (1999). The length of the siRNAs produced by Dicer is species specific but range from 21-23 nt (~22 nt) (Hannon, 2002; Hutvágner and Zamore, 2002). The most effective siRNAs seem to be 19 bp long with 3' di-nucleotide overhangs containing 5'-phosphate and 3'-hydroxyl termini (Elbashir *et al.*, 2001). These siRNA features are important for the functioning of the silencing pathway. Short interfering RNAs that are blunt ended or contain no 5'-phosphates are inefficient catalysts of RNAi, both *in vivo* and *in vitro* (Nykänen *et al.*, 2001). Since the discovery of Dicer, endonucleolytic activity of its homologues has been demonstrated in all the organisms in which RNAi has been studied.

Dicer contains an ATP-dependent HEL domain at the amino terminus. It was initially speculated that the HEL domain of Dicer functions to unwind guide siRNAs bound to Dicer (Bernstein *et al.*, 2001). The presence of a HEL domain in the RNA-induced silencing complex (RISC) and the finding that the guide siRNAs are unwound by the inactive RISC to form an active RISC (Nykänen *et al.*, 2001, Hammond *et al.*, 2000), led to the conclusion that the Dicer HEL domain may function as an ATP powered translocase required to move Dicer along the dsRNA. No other RNase III nuclease is known to contain a HEL domain (Hutvágner and Zamore, 2002).

The *H. sapiens* homologue of Dicer has recently been cloned, expressed and characterised. This has resulted in new insight into Dicer's function and conserved nature. The *H. sapiens* Dicer is a 218 kDa multidomain enzyme including a putative DExH/DEAH RNA HEL, an ATPase domain, a PAZ signature, two neighbouring RNase III-like domains and a dsRNA-binding domain (RBD) (Provost *et al.*, 2002; Zhang *et al.*, 2002). A sequence alignment of Dicer proteins from *H. sapiens*, *Mus musculus*, *C. elegans, Drosophila melanogaster* and *A. thaliana* revealed a relatively high sequence similarity and conservation of these putative domains. *Schizosaccharomyces pombe* was included in the alignment studies but did not appear to have a PAZ domain (Provost *et al.*, 2002). Dicer produced ~22 nt siRNAs from the

dsRNA substrate. It was shown that Mg^{2+} ions are necessary for dsRNA cleavage but not dsRNA binding, and that ATP was not necessary for the cleavage of dsRNA, unlike the *Drosophila* and *C. elegans* Dicer. It was subsequently reasoned that in *H. sapiens* the ATP might be required for the release of the siRNA products resulting in the observed multiple turnover of the enzyme (Provost *et al.*, 2002; Zhang *et al.*, 2002).

Two potential functions have been ascribed to Dicer in the proposed mechanism of RNA silencing. The first is as the initiator of RNA silencing through the production of guide siRNAs from a dsRNA substrate. A second possible function is the degradation of dsRNAs produced by an RdRp from an mRNA template during the amplification of the guide siRNAs. This would make Dicer the initiator and the effector of RNAi (Hutvágner and Zamore, 2002).

Other endonucleolytic functions related to RNA silencing have been ascribed to Dicer. Disruption of Dicer activity can lead to dramatic developmental defects. This is believed to be due to Dicer's role in the generation of small temporal RNAs (stRNAs); ~22 nt ssRNAs thought to act in a sequence specific manner in a process known as translational repression (**Figure 2.9**) (Hutvágner and Zamore, 2002).

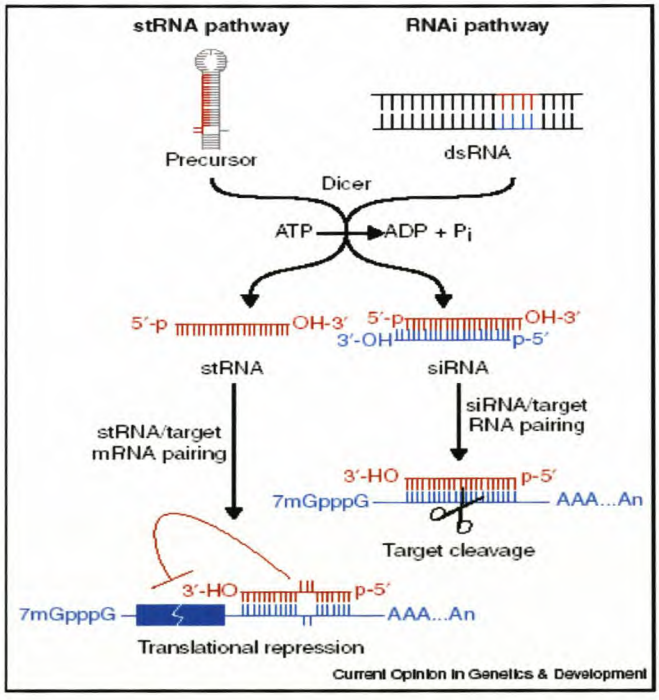

Figure 2.9. Dicer is an integral part of RNA silencing and developmental control. The RNAi and stRNA pathways intersect at Dicer, which generates the small RNA regulators. Short interfering RNAs (siRNAs) are generated from the dsRNA template for RNA silencing and bind with near perfect complementarity to the homologous RNA. Small temporal RNAs (stRNAs) are generated from ~70 nt stemloop precursors and bind in an imperfect fashion to the translationally repressed mRNA (Hutvágner and Zamore, 2002).

It has been shown in worms, flies and humans that Dicer processes ~70 nt stem-loop precursors into stRNAs that exhibit imperfect complementarity to regulatory sequences, thus regulating expression by blocking translation. The known stRNAs, *lin-4* and *let-7*, have been well characterised and seem to occur in all bilaterally symmetrical animals (Hutvágner *et al.*, 2001). Dicer has also been implicated in the generation of certain micro RNAs (miRNAs), similar to stRNAs, which seem to occur in all eukaryotes and also have a role in the regulation of expression. It is probable that stRNAs are a representative of the miRNA class (Hutvágner *et al.*, 2000). Advantages of these miRNAs are their small size, which can lead to rapid transcription, and that they do not need to be translated, resulting in a fast and efficient way of gene regulation.

2.6.3. Effectors of RNA silencing

2.6.3.1. RNA-induced silencing complex

The RISC is thought to be a multi-subunit nuclease; potentially containing HEL-, exonuclease- and endonuclease-subunits and a subunit that searches for homology between the guide sequence and the target mRNA (Hammond *et al.*, 2000; Elbashir *et al.*, 2001).

In vitro biochemical analysis during RNAi has shown that the active RISC contains a single siRNA molecule and a ribonuclease (Elbashir *et al.*, 2001; Nykänen *et al.*, 2001; Zamore *et al.*, 2000). Although this ribonuclease has not yet been identified, the RISC activity has been chromatographically shown as separate from that of Dicer, suggesting they are distinct (Bernstein *et al.*, 2001). It has also been shown in *Drosophila* that the active RISC can mediate sequence specific cleavage in the absence of ATP, whereas Dicer can not function without ATP (Nykänen *et al.*, 2001).

Both strands of the guide siRNAs are not equally eligible for assembly into the RISC. The 5'-end base pairs of the two complementary siRNAs determines their participation in RNAi, potentially resulting in only one of the siRNAs initiating RNAi. In the case of miRNAs, it is believed that the structure of single stranded miRNAs, initially generated as stem-loop duplexes, determine which strand enters the RISC. The other strand is degraded (Schwarz *et al.*, 2003).

The RISC has an inactive steady state and can not mediate RNAi before it binds to siRNAs. Once the siRNA has been incorporated and unwound in an ATP-dependent reaction facilitated by the HEL subunit, the RISC becomes active. The active RISC targets mRNA with sequence homology to the guide siRNAs (Hammond *et al.*, 2000). Mapping of the

cleavage sites triggered by siRNAs has shown that the target mRNA is endonucleolytically cleaved opposite the centre of the guide siRNA, and not the ends (Elbashir *et al.*, 2001; Zamore *et al.*, 2000). It is believed that the active RISC makes a single endonucleolytic cut resulting in two unstable products that are degraded by exonucleases (Hammond *et al.*, 2000), or can serve as a template for unprimed RdRp amplification of the dsRNA silencing trigger (Matzke *et al.*, 2001; Kooter *et al.*, 1999). Purified RISC preparations reveal no cleavage products suggesting that the RISC contains an exonuclease subunit (Hannon, 2002).

Initial studies showed that the RISC has a size of ~500 kDa (Hammond *et al.*, 2001a), but varying sizes have been observed since. Nykänen *et al.* (2001) have shown that the inactive RISC bound to double stranded guide siRNAs is ~360 kDa in size, and that the active core functional RISC bound to the single stranded siRNAs is ~232 kDa in size. The difference in size could possibly be due to the chromatographic procedure used, which might have caused a separation of the active complex from a larger ~500 kDa precursor (Nykänen *et al.*, 2001).

2.6.3.2. RNA-dependent RNA polymerase

Sequence analysis of a family of RNA silencing-associated proteins, found in a variety of species, suggests that these proteins are RdRps. Although no RdRps have been detected in flies and humans, they have been discovered in all the other organisms that have been found to exhibit RNA silencing. In *A. thaliana* it was found that an RdRp (SDE1) is required for PTGS induced by a transgene (Dalmay *et al.*, 2000). The discovery of RdRp activity during RNA silencing provides a possible explanation for the efficiency of dsRNA as an initiator and propagator of the silencing pathway (Hutvágner and Zamore, 2002; Lipardi *et al.*, 2001). An RdRp might amplify the dsRNA template or even the guide siRNAs, thus enforcing the silencing signal. The dsRNA amplification could occur through one, or both, of two methods. The guide siRNA could act as a primer to bind to the target mRNA, thus providing a template for the amplification of the dsRNA by the RdRp, or it might amplify RISC-cleaved aberrant mRNA in an unprimed dsRNA synthesis (many RNA polymerases, including RdRps, can synthesise RNA without an oligonucleotide primer) (Hutvágner and Zamore, 2002).

2.6.4. The proposed mechanisms of RNA silencing

The RNA silencing mechanism is activated through the presence of a dsRNA trigger, which can be introduced in several ways, depending on the organism. The dsRNA is recognised within the cytoplasm by Dicer, which binds to and cleaves it to form ~22 nt siRNAs (Bernstein *et al.*, 2001). The siRNAs are bound and then unwound by the HEL activity of RISC. This ATP dependent unwinding of the siRNAs is essential to activate the RISC

(Hammond *et al.*, 2000). The production of siRNAs by Dicer and their subsequent binding by and activation of the RISC is what will be referred to as the "core process" of RNA silencing.

Two mechanisms have been proposed to explain the production of siRNAs to fuel RNA silencing. It is believed that either or both of these occur during RNA silencing. They will be referred to as the "random degradative PCR"- and the "RISC mediated cleavage"-mechanisms. A model has been proposed that integrates these two mechanisms into the "core process" of RNA silencing (**Figure 2.10**) (Hutvágner and Zamore, 2002).

2.6.4.1. Random degradative PCR

In the "random degradative PCR"-mechanism, proposed by Lipardi *et al.* (2001), Dicer cleaves any dsRNA template present in the cytoplasm. The resulting siRNAs are unwound by the RISC during the "core process" to form guide siRNAs (Hammond *et al.*, 2000). In *Drosophila* the siRNAs may potentially be unwound by the HEL activity of Dicer (Bernstein *et al.*, 2001). The guide siRNA functions as a primer, binding to the mRNA target for the RdRp mediated synthesis of new dsRNA, which in turn again functions as a template for Dicer. This positive feedback mechanism results in mobilisation against any dsRNA template present in the cytoplasm. The reaction will continue until the entire dsRNA template has been destroyed. In this mechanism Dicer functions as the initiator of RNAi through siRNA production, but also as the target mRNA destroyer (Lipardi *et al.*, 2001).

2.6.4.2. RISC mediated cleavage

The "RISC mediated cleavage"-mechanism is the name that will be used in this study for the "It dices… it slices…"-mechanism proposed by Hutvágner and Zamore (2002), due to it being a more descriptive name. The activated RISC is believed to be a multiple-turnover ribonuclease with the bound siRNA acting as an RNA-encoded specificity determinant. Due to this characteristic a single RISC can target a complementary sequence to the guide siRNA and can degrade more than 30 mRNA substrates. Along with the production of multiple siRNAs from a single long dsRNA by Dicer, this mechanism results in targeted mobilisation against an mRNA species. This provides amplification of the silencing mechanism without the need for the continuous synthesis of new dsRNA molecules (Hutvágner and Zamore, 2002).

A single endonucleolytic cleavage of the target mRNA by the activated RISC would most likely create two unstable *in vivo* products, which might in itself be enough to eliminate a target mRNA molecule (Hutvágner and Zamore, 2002). In addition, the 5' endonucleolytic

cleavage product could function as the template for unprimed RdRp activity, resulting in more dsRNA template for Dicer cleavage. This would also be an amplification of the "RISC mediated cleavage"-mechanism. One of the two mRNA cleavage products generated by the RISC would correspond to the aberrant RNA believed to trigger silencing in plants and co-suppression in animals (Matzke *et al.*, 2001; Kooter *et al.*, 1999; Voinnet *et al.*, 1998).

2.6.4.3. Random degradative PCR vs. RISC mediated cleavage

Analysis of the RNA silencing initiators and effectors suggest that the cleavage of dsRNA into guide siRNAs and the degradation of the target mRNA occurs by two distinct mechanisms, catalyzed by Dicer and the RISC ribonuclease respectively, which can be integrated into a single model (**Figure 2.10**) (Hutvágner and Zamore, 2002).

In *H. sapiens* and *Drosophila* cells the siRNA 5' phosphate is required for RNAi, but the 3' hydroxyl group does not appear to have any role. The siRNA guides endonucleolytic cleavage at a single site in the target mRNA, and does not act as a primer. It is believed that RNAi is conserved between flies and humans and that RdRps play no role in these organisms (*Schwarz et al.*, 2002). These findings suggest that the "RISC mediated cleavage"-mechanism is more likely than the "random degradative PCR"-mechanism; however, this does not conclusively exclude a model where Dicer contains two distinct nuclease activities (Hutvágner and Zamore, 2002).

Studies of the different genes and proteins involved in silencing suggest that RNA silencing is not only the result of Dicer/RISC mRNA degradation, but is strongly associated with other processes including interactions with chromosomal DNA during TGS, a possible link to the translation process and the involvement of an RdRp (Waterhouse et al., 2001).

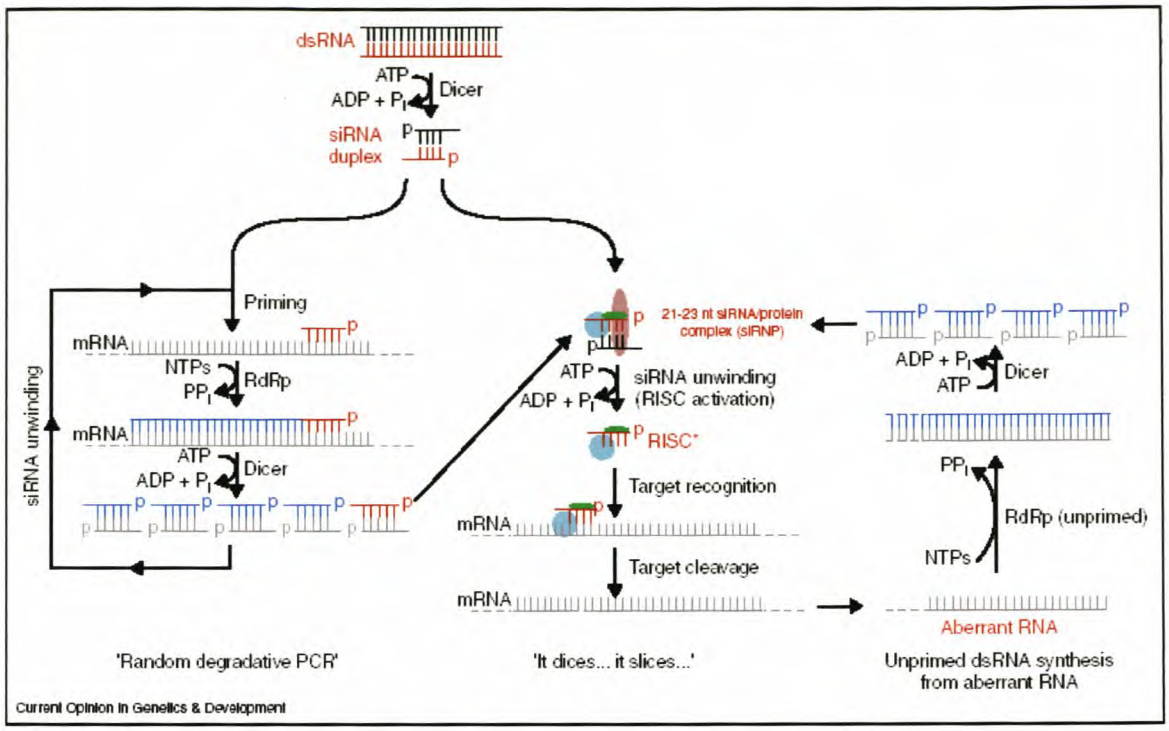

Figure 2.10. An integrated model for RNA silencing. The production of siRNAs by Dicer from the dsRNA template, and the binding of siRNAs by RISC and the subsequent activation of the RISC are referred to in the text as the "core process" of RNA silencing. In the "Random degradative PCR"-mechanism the guide siRNAs produced by Dicer act as primers for an RdRp mediated dsRNA amplification of the target mRNA. The resultant dsRNA is cleaved by Dicer to form siRNAs that can again act as primers for dsRNA amplification, or can be bound by the RISC for homologous mRNA recognition and subsequent mRNA destruction. In the "RISC mediated cleavage"-mechanism ("It dices… it slices…") the siRNAs produced during the "core process" and the "Random degradative PCR" are unwound in an ATP dependent reaction by the RISC helicase, thus activating the RISC. The activated RISC binds mRNA homologous to the guide siRNA, and proceeds to cleave the mRNA approximately in the center of the guide siRNA. The cleaved mRNA products are unstable. One of these cleaved mRNA molecules could act as template for unprimed RdRp mediated amplification resulting in more dsRNA template for Dicer cleavage and consequently more siRNAs (Hutvágner and Zamore, 2002).

2.6.5. Characteristics of PTGS and RNAi

2.6.5.1. Natural virus resistance

The natural ability of plants to defend themselves against challenging viruses manifests itself as the phenomenon known as cross-protection. During cross-protection infection with a mild virus strain confers resistance to closely related, more severe strains of the virus. The predominant mechanism of cross-protection is an RNA-mediated post-transcriptional process that targets a secondary-challenging virus in a sequence specific manner, known as PTGS

(Ratcliff *et al.*, 1999; Ruiz *et al.*, 1998). This defence system is remarkable in its ability to adapt to potentially any virus. Although PTGS is usually targeted against cytoplasmic RNA viruses, evidence is emerging that DNA viruses, like the ssDNA geminiviruses and the reverse transcribing pararetroviruses, can interact with the silencing mechanism on a transcriptional and post-transcriptional level (Covey and Al-Kaff, 2000). Along with the finding that, like RNA viruses, DNA viruses can encode suppressors of PTGS, it is becoming clear that the overall silencing mechanism can also target DNA viruses (Voinnet *et al.*, 1999). Thus, the beauty of PTGS is that the host does not genetically predetermine its specificity. Instead, it is dictated by the genome sequence of the challenging virus (Ruiz *et al.*, 1998).

Several studies have shown that PTGS is a general plant response to virus infections (Ruiz *et al.*, 1998). Virus resistance can be induced by pre-infection of non-transgenic plants with the same virus. Post-transcriptional gene silencing is induced in *Nicotiana* species by nepoviruses and caulimoviruses that contain no homologous sequences to the plant. Leaves emerging after the initial systemic infection are symptom free and contain reduced levels of the virus (Covey *et al.*, 1997; Ratcliff *et al.*, 1997), in what is known as a recovery phenotype (Ratcliff *et al.*, 1999). In nepovirus-infections the recovered plants are resistant to a secondary infection (Ratcliff *et al.*, 1997). *N. benthamiana* plants infected with tobraviruses exhibit RNA-mediated cross-protection and the recovery phenotype, whereas plants infected with potexviruses only exhibit RNA-mediated cross-protection (Ratcliff *et al.*, 1999).

Together with the discovery that certain viruses express proteins that suppress gene silencing (Li and Ding, 2001) these findings suggest that PTGS in plants evolved as an anti-viral defence mechanism.

2.6.5.2. Suppressor of transposon mobilisation

Transposable elements (TEs), or transposons, can be divided into two classes. Class I TEs are retroelements that amplify their copy number through reverse transcription and are particularly abundant in eukaryotes, especially in plants. Class II TEs contain terminal inverted repeats (TIRs), and one or more of the TEs encodes a transposase that interacts with the TIRs to excise and integrate the TEs in another region of the genome. They are found in all organisms, particularly in prokaryotes. Transposon activity needs to be kept in check to ensure survival of the host organism (Waterhouse *et al.*, 2001).

DNA methylation, or the accompanying changes in chromatin structure, is an epigenetic method to suppress transposon mobilisation. Insertion of a transposon in or close to a heterochromatic area often leads to silencing of the TE. However, TEs integrating into a

euchromatic area are probably controlled through PTGS. There are several methods through which TEs could form dsRNA intermediates, or RNA/DNA hybrids, that would lead to RNA silencing through the degradation of their transposase mRNA (Hammond *et al.*, 2001b; Waterhouse *et al.*, 2001).

Although it has not yet been conclusively shown for plants, mutations of certain RNAi effector elements lead to increased levels of transposon activity (Hammond *et al.*, 2001a). In *C. elegans* mutation of the *mut-7* RNAi effector gene leads to transposon mobilisation. Of 30 mutants that activate transposon mobilisation in nematodes, 22 also cause defects in RNAi (Ketting *et al.*, 1999). Similar conclusions have been drawn in *D. melanogaster* and in *Chlamydomonas reinhardtii* (Hammond *et al.*, 2001b). It has also been shown that extracts of the ~22 nt siRNA products of RNA silencing can contain TE sequences (Elabshir *et al.*, 2001; Waterhouse *et al.*, 2001). The relationship between RNAi and transposon silencing suggests that RNAi is involved in genome defence and maintenance of stability.

2.6.5.3. Transitivity

RNA silencing is amplified through a process known as transitivity or transitive RNAi (Himber *et al.*, 2003; Hannon, 2002). During transitivity new siRNAs that correspond to sequences located outside the primary siRNA-targeted region of a transcript are produced. This process increases the initial pool of siRNAs (Vaistij *et al.*, 2002; Sijen *et al.*, 2001).

Transitivity has been shown to occur in *N. benthamiana*, *A. thaliana* and *C. elegans* (Vaistij *et al.*, 2002; Sijen *et al.*, 2001). In both plants and *C. elegans* transitivity requires an endogenous RdRp (Himber *et al.*, 2003). Transitivity in *C. elegans* moves from 3' to 5' in the target transcript. Targeting of the 3' area of a transcript results in suppression of the mRNA and the production of siRNAs homologous to that region. Short interfering RNAs complementary to the region upstream of the targeted area (transitive siRNAs) also appear and accumulate. If homologous transcripts to these transitive siRNAs are present they are also targeted and silenced (Sijen *et al.*, 2001). In plants transitivity is bidirectional and seems to be transcript dependent i.e. some mRNAs induce transitivity while others do not (Vaistij *et al.*, 2002).

2.6.5.4. Systemic signaling

An important characteristic of PTGS is the ability of the silencing signal to spread systemically. The signal molecule is believed to be siRNA, due to the sequence specificity of the systemic silencing. It is thought that the siRNA is bound to a helper molecule and is

transported from cell-to-cell through the plasmodesmata and over long distances through the phloem channels from the bottom of the plant to the apical regions (Himber *et al.*, 2003; Voinnet *et al.*, 1998; Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997). Although the PTGS signal is bidirectional, transmission of silencing from silenced rootstock to un-silenced scion is more efficient (Sonoda and Nishiguchi, 2000).

Gene silencing through the systemic propagation of the silencing signal has been demonstrated in several different studies. Voinnet and Baulcombe (1997) have shown that the transient expression of GFP by agro-infiltration of a specific leaf can systemically silence the constitutive expression of an endogenous GFP transgene in *N. benthamiana*. It has also been shown that co-suppression induced against the endogenous Nitrate-reductase gene, and a Glucuronidase producing transgene, in a tobacco rootstock plant spreads systemically to an unsilenced scion. This systemic silencing occurs even when up to 30 cm of the wild-type plant is grafted in between the rootstock and scion (Palauqui *et al.*, 1997). Once targeted silencing is established in a scion, re-grafting of the silenced scion onto a silenced rootstock with different target mRNA specificity does not superimpose the new target specificity onto the target specificity of the silenced scion (Sonoda and Nishiguchi, 2000).

Another seemingly unique facet of PTGS in plants is the involvement of two functionally distinct dsRNAs: the ~22 nt siRNAs for RISC-mediated mRNA cleavage, and a new class of ~25 nt long siRNAs that correlates positively with long distance systemic silencing and DNA methylation (Hamilton *et al.*, 2002).

A recent study presented an alternative model for the PTGS systemic signaling phenomenon. Himber *et al.* (2003) characterised the silencing signal observed in plants. The existence of a short-distance signal movement process was revealed. This process is initiated from a small group of cells that produce the ~22 nt siRNAs and spreads to ~10-15 adjacent cells, without the amplification of the silencing signal. The ~25 nt siRNAs appear to be unnecessary for short-distance signal movement. The long-distance signaling mechanism requires SDE1 (an RdRp) and SDE3 (an RNA HEL). It was found that the long-distance movement was also associated with ~22 nt siRNAs (Himber *et al.*, 2003), contrary to the findings of Hamilton *et al.* (2002). Both the short- and long-distance cell-to-cell silencing signals occur within the same tissues, are triggered by the same molecules and may make use of the same plasmodesmata channels. This model of the silencing signal proposes that the long-distance movement occurs through multiple short-distance signaling events (Himber *et al.*, 2003).

2.6.6. Applications of RNA silencing

2.6.6.1. Functional genomics

Potentially the most powerful application of RNA silencing is its use in the determination of gene function i.e. functional genomics or reverse genetics. RNA silencing has been used in several plants species, *C. elegans* and *Drosophila* to determine gene function. With the discovery that RNAi can be induced in mammals, RNA silencing is quickly becoming the major functional genomics tool in the study of all model organisms (Hammond *et al.*, 2001b).

Introduction of gene specific dsRNAs into nematodes typically results in the same phenotype as the null mutant for the gene. RNA interference has been used to systematically and individually silence each gene on entire *C. elegans* chromosomes (Fraser *et al.*, 2000; Gönczy *et al.*, 2000). The initiation of RNAi through the introduction of gene specific dsRNAs in *Drosophila* has been used successfully to study embryonic phenotypes but dsRNA interferes with gene expression transiently and is not stably inherited, thus limiting studies of gene function in the late stages of development. The use of a stably inherited transgene from which hairpin dsRNAs (hpRNAs) are expressed, results in gene specific interference of expression in embryonic tissues, as well as adult tissues (Kennerdell and Carthew, 2000).

Introduction of gene specific silencing for functional studies in plants can be achieved in two ways. In the first a plant transformation vector is transformed with an expression construct that produces hpRNAs homologous to the target gene. The expression of this construct results in silencing of the target gene (Wesley *et al.*, 2001; Smith *et al.*, 2000; Waterhouse *et al.*, 1998). The second method is the phenomenon known as virus-induced gene silencing (VIGS). In VIGS an infecting DNA or RNA virus is introduced that contains homologous sequences to the target endogenous gene or transgene. The dsRNA replicative form of the virus serves as the template for Dicer, which initiates RNA silencing (Baulcombe, 1999; Ruiz *et al.*, 1998). Ultimately the function of the target gene can be deduced through the effect that its silencing has on the plant's phenotype (Ruiz *et al.*, 1998; Lindbo *et al.*, 1993). Several virus vectors have been produced to determine plant gene function through VIGS. A powerful example is the *tobacco rattle virus* (TRV) infectious cDNA clone. The TRV vector can accommodate any non-viral plant sequence, can mediate VIGS of endogenous genes without displaying virus-induced symptoms and, unlike other RNA virus vectors, can induce silencing in the growing points of plants (Ratcliff *et al.*, 2000). Virus induced gene silencing is a transient, versatile, fast and efficient method of silencing endogenous genes, and has the added advantage of not having to stably integrate transgenic copies of the target gene into the plant.

2.6.6.2. Transgenic virus resistance

Post-transcriptional gene silencing can be manipulated to induce sequence specific virus resistance in plants. The plant can be transformed with a transgene construct, containing complementary sense and antisense sequences of a virus gene coupled by a hairpin forming sequence. Once expressed, the mRNA complementary areas bind together to form an hpRNA. This hpRNA is transported to the cytoplasm where it is targeted by Dicer, leading to the production of virus specific guide siRNAs. The siRNAs are mobilised to degrade homologous virus mRNAs by the RISC (Smith *et al.*, 2000; Waterhouse *et al.*, 1998). Related virus sequences with 10% to 20% dissimilarity can be silenced through PTGS (Baulcombe, 1999).

Through the use of the above mentioned construct, derived from the pHannibal vector (**Figure 2.11**), that encodes a self-complementary hpRNA with a sense-intron as the hairpin forming sequence, Wesley *et al.* (2001) have shown highly efficient gene silencing of 90%-100% in independent transgenic plants. The efficiency of using this sense-intron containing construct to induce virus resistance has also been demonstrated. Of 23 tobacco plants containing a *potato virus Y* (PVY) derived transgenic construct, 22 (96%) were completely immune to PVY. Significantly less efficient silencing of PVY was obtained with control experiments where the same complementary hpRNA-forming construct was used, but with different sequences for the hairpin. A GUS derived hairpin induced 58% silencing, an antisense-intron hairpin induced 65% silencing, and transformation constructs expressing only the sense or antisense PVY gene, and not a complementary hpRNA, induced 7% and 4% silencing respectively (Smith *et al.*, 2000). The use of virus-derived self-complementary hpRNA expression to induce virus resistance in plants has proved very reliable, efficient and can target any plant virus, due to the specificity determinant being the virus's own genome.

The strategy used by the Waterhouse research group (Wesley *et al.*, 2001; Smith *et al.*, 2000; Waterhouse *et al.*, 1998), has been successfully applied to induce virus resistance against *plum pox virus* (PPV) (**Figure 2.12**) (Pandolfini *et al.*, 2003). A transformation vector was produced containing two homologous 197 bp fragments of the PPV genome in an inverted repeat conformation, with the *rolA* intron from *Agrobacterium rhizogenes* between the complementary sequences that would function as the intron-hairpin in transcribed mRNA. The construct was under the control of the *rolC* promoter (a phloem specific promoter, not active within epidermal and mesophyll cells) from *A. rhizogenes*. Transformation of *N. benthamiana* with this construct resulted in a high percentage (80%) of transformants exhibiting systemic resistance to PPV, but no resistance to local infection. In the plants

exhibiting infection in the apical leaves due to systemic infection (20%), symptoms were mild.

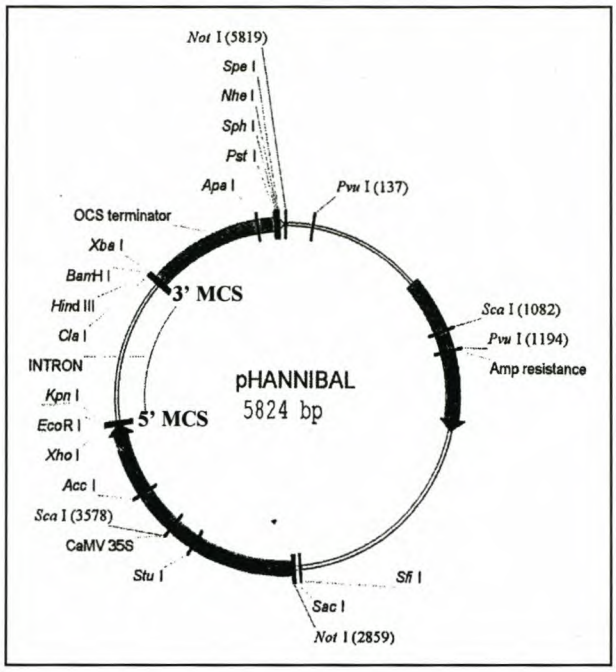

Figure 2.11. pHannibal cloning vector of the pHannibal/pART27 binary vector system, for the induction of gene silencing or virus resistance. The sense conformation of the target sequence is cloned into the 5' multiple cloning site (MCS). The antisense conformation of the target sequence is cloned into the 3' MCS. Transcription results in an mRNA with two complementary arms that fold around the intron to form target sequence specific dsRNA (Wesley *et al.*, 2001).

A similar strategy has been used to confer resistance to the fanleaf disease causing viruses: GFLV, *arabis mosaic virus* (ArMV) and *raspberry ringspot virus* (RRV). Self-complementary GFLV/ArMV/RRV constructs were made and used to transform *N. benthamiana*. Successful transformants challenged with the relevant viruses exhibited one of three phenotypes: susceptibility, recovery or resistance. The same constructs were used to successfully transform grapevine plants. The transformants have been screened and selected for low copy number insertion of the transgenes, but the results of the siRNA analysis are still pending (Reustle *et al.*, 2003). An analogous study attempting to induce GFLV resistance in tobacco plants through transformation with GFLV self-complementary constructs have yielded successful transformants, but the tobacco plants have not yet been challenged with GFLV or tested for the presence of siRNAs, which would indicate successful GFLV-specific PTGS (Bruno *et al.*, 2003).

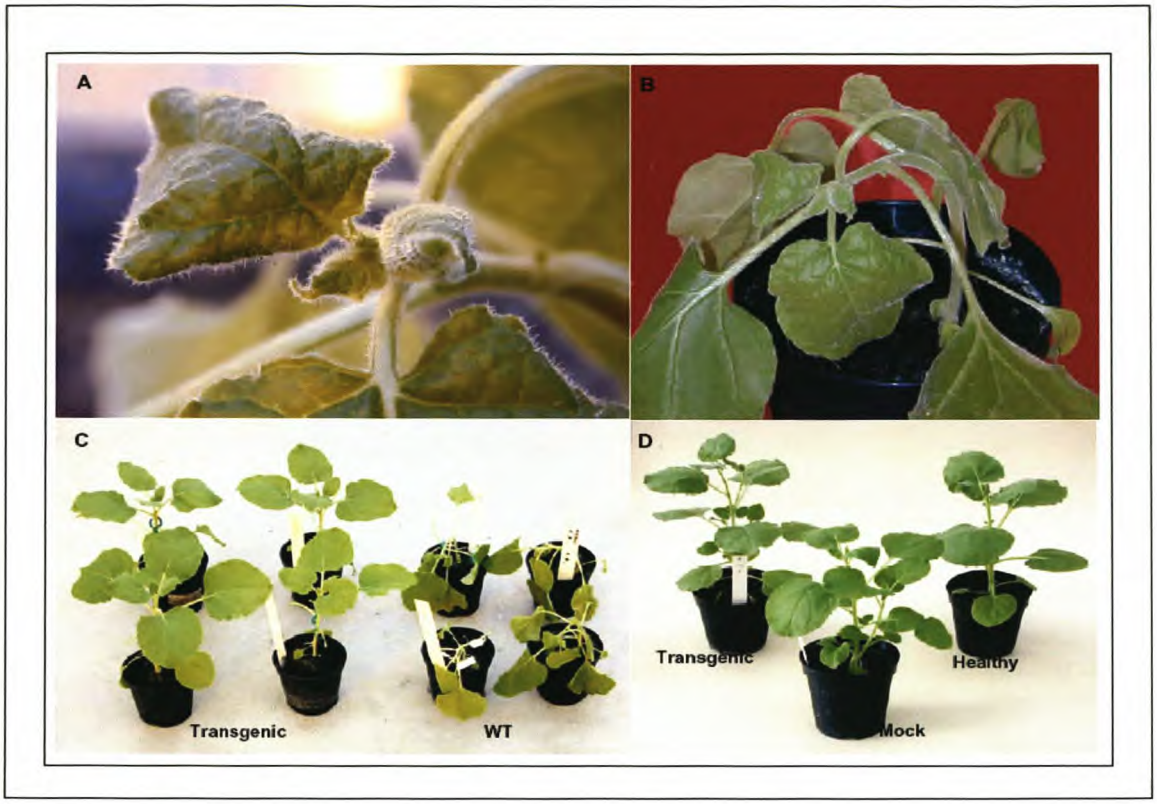

Figure 2.12. *Plum pox virus* inoculated transgenic and untransformed tobacco control plants. **(A)** Infected, untransformed plants 7 days post-infection, exhibiting curling of apical leaves. **(B)** Infected, untransformed plants 7 days post-infection, exhibiting severe wilting. **(C)** Infected transgenic and untransformed plants 7 days post-infection. The 4 transgenic plants on the left do not exhibit any virus-induced symptoms. The 4 untransformed control plants on the right exhibit severe virus induced symptoms. **(D)** Plants 7 days post-infection: transgenic inoculated plants (Transgenic), mock-inoculated transgenic plants (Mock) and healthy wild-type plants (Healthy) (Pandolfini *et al.*, 2003).

Cucumber mosaic virus (CMV) is a widespread virus with a host range of more than a 1000 species and can naturally suppress PTGS through the protein Cmv2b. Tobacco plants have been transformed with a CMV specific inverted-repeat construct that produces an RNA transcript with the ability to form dsRNA. Plants transformed with this CMV specific construct exhibited three phenotypes on challenging with CMV: susceptible plants with milder symptoms than the wild-type control; a recovery phenotype where newly emerging leaves were free of CMV symptoms and plants that exhibited complete CMV resistance. Northern analysis of CMV resistant lines showed the presence of the siRNAs associated with PTGS (Kalantidis *et al.*, 2002). The ability of transgenic dsRNA-induced virus specific PTGS to confer resistance to a virus with the ability to suppress PTGS is indicative of the potential of this resistance mechanism.

RNA mediated virus resistance has been conclusively demonstrated for several dicotyledonous plant species (Waterhouse *et al.*, 1999), but is also found in monocotyledonous plant species (Ding, 2000). In conjunction, resistance has been induced against most of the major plant viruses using a PTGS based protection mechanism, irrespective of their genome (RNA or DNA) (Goldbach *et al.*, 2003). This clearly demonstrates the power of applying PTGS to induce targeted virus resistance.

2.7. VIRAL SUPPRESSION OF RNA SILENCING

In contrast to plants' ability to silence viral RNAs, certain plant viruses have evolved genes that code for proteins that are suppressors of the silencing mechanisms (Li and Ding, 2001). It has been shown that diverse RNA and DNA plant viruses encode proteins that suppress PTGS (Voinnet *et al.*, 1999). This counter defence phenomenon was first described in 1998 (Anandalakshmi *et al.*, 1998; Béclin *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998) and provided the strongest evidence that plants evolved RNA silencing as a natural defence mechanism targeted against viruses. This discovery also resulted in a valuable tool, in the form of silencing suppression assays (SSAs), for the elucidation of the silencing mechanism (Li and Ding, 2001).

2.7.1. Silencing suppression assays

Silencing suppression assays are an effective technique to determine if a candidate viral protein has suppression activity of a specific stage of the RNA silencing mechanism. In SSAs a reporter transgene (e.g. GFP or GUS) is stably integrated into a plant. This reporter transgene is then either constitutively silenced through transformation or genetic crosses, or transiently through agro-infiltration (Voinnet *et al.*, 1998; Voinnet and Baulcombe, 1997).

To determine if a candidate protein exhibits silencing suppression activity, it can be introduced into silenced plants by genetic crosses, agro-infiltration or by a replicating virus vector. If the reporter gene is silenced constitutively, the candidate viral suppressor protein is introduced through genetic crosses with a plant containing the stably integrated viral suppressor transgene (Anandalakshmi *et al.*, 1998; Kasschau and Carrington, 1998). When the candidate suppressor is introduced through infection with a virus vector or through agro-infiltration, both the constitutive and transiently silenced reporters can be used to assay for suppression activity (Béclin *et al.*, 1998; Brigneti *et al.*, 1998).

Although the SSA is in essence a powerful technique, important considerations need to be taken into account before it is performed. It is critical to consider the potential influence of the silencing stage targeted by the suppressor on the current status of the reporter, i.e. a candidate suppressor that targets the production of the silencing signal will most likely be inactive in a silencing reversal assay when RNA silencing is complete. Due to different silencing pathways, suppressors are not always active when using a specific delivery mechanism, thus the activity of the suppressor needs to be compared when using different delivery systems. Finally, there are alternative reporters that can be used to induce silencing suppression (e.g. virus induced gene silencing) (Li and Ding, 2001). Because SSAs are not the focus of this project, and for the sake of brevity, these implications are not further discussed.

2.7.2. Suppressors of RNA silencing

To date, three types of viral suppressors have been identified through the use of several SSAs. The first two viral suppressor types are able to partially or completely reverse pre-existing RNA silencing and the third type, although not able to reverse RNA silencing, is able to prevent the systemic silencing signal. The general SSA used, involved transgenic *N. benthamiana* expressing GFP, which was silenced with agro-infiltration containing 35S-GFP before the plants were infected with viruses carrying the potential suppressor gene (Li and Ding, 2001).

HC-Pro (suppressor type 1), is encoded by Potyviruses and can completely suppress RNA silencing in all tissues, irrespective of the silencing stage (Li and Ding, 2001; Voinnet *et al.*, 1999; Brigneti *et al.*, 1998). Other proteins that fall within this category are P1 (*rice yellow mottle virus*) and AC2 (*african cassava mosaic virus*) (Voinnet *et al.*, 1999; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). Cmv2b (suppressor type 2), is encoded by CMV. Cmv2b suppresses silencing in the new leaves emerging from the growing points of plants, but can not suppress silencing in older tissue where silencing has been established (Brigneti *et al.*, 1998). Cmv2b can not completely reverse silencing once established (Brigneti *et al.*, 1998). Other proteins that fall within this category are Tav2b (*tomato aspermy virus*) and p19 (*tomato bushy stunt virus*) (Li *et al.*, 1999; Voinnet *et al.*, 1999). p25 (suppressor type 3), is encoded by PVX and does not cause suppression in an SSA. However, p25 can suppress localised and systemic PTGS through inhibition of the silencing signal, when simultaneously transfected with a silencing inducing construct, if silencing has not yet been induced (Voinnet *et al.*, 2000).

2.7.3. Targeting silencing suppressors for virus resistance

Many of the viral suppressors of gene silencing that have so far been found, have previously been shown to be required for virulence determination (Li and Ding, 2001). A further development in the study of the host/virus interaction was the discovery of a counter counter-defence mechanism. Li *et al.* (1999) found that Tav2b elicits a strong host resistance response (hypersensitive cell death and virus resistance) in tobacco when expressed from the unrelated TMV RNA genome. In the tobacco related species, *N. benthamiana*, Tav2b acts as a virulence determinant and suppresses PTGS. Thus the suppressor of the host gene silencing defence mechanism activates an independent host resistance mechanism, further illustrating the complexity of host/virus interaction (Li *et al.*, 1999). It has been shown that a strong PTGS inducer (*potato virus A* (PVA)) can induce PTGS in a transgenic plant expressing a silencing suppressor (HC-Pro) derived from PVA's genome. Transgenic plants expressing HC-Pro were initially susceptible to PVA and were systemically infected by 14 days post-inoculation, but showed a characteristic recovery phenotype by 28 days. It was also shown that the silencing is dependent on sequence similarity between the virus and the silencing suppressor transgene (Savenkov and Valkonen, 2002).

Silencing suppression appears to be a general viral counter-defensive strategy targeted against the virus specific PTGS mechanism of plants. This, along with the effectiveness of the previously mentioned Tav2b-targeted counter counter-defence response, hints at the potential effectiveness of targeting viral suppressors of gene silencing for virus control (Li and Ding, 2001; Li *et al.*, 1999).

2.8. CONCLUSION

Internationally, plant viruses and the diseases they cause are an extensive problem within the various agricultural industries. This is also reflected within the South African viticulture industry. Grapevine virus diseases cause extensive damage to vineyards, which translates into significant economic losses. Grapevine leafroll disease, with its main component being GLRaV-3, causes a continuous reduction in fruit yield and quality, a decline in grapevine growth and ultimately a reduction in the grapevine lifespan. Grapevine rupestris stem pitting, consistently associated with GRSPaV, causes a slow decline in the growth of *V. vinifera*, and a reduction in fruit yield and quality similar to those of GLR. Over the past century various approaches have been used to curb grapevine virus infections. In the last two decades several strategies, e.g. CP-MR, PDR and PTGS, have resulted in the improved plant protection against viruses.

It has been conclusively shown that PTGS is not just a naturally occurring defence mechanism of plants against their viral pathogens but is the fundamental mechanism behind most examples of induced plant virus resistance. The specific mechanism behind PTGS is only now being unraveled, even though it has been "applied" as cross-protection since the 1920s, and more recently in RNA-based PDR strategies. The power of PTGS as a defence mechanism lies therein that the specificity determinant of PTGS is the genome of the challenging virus. Better understanding of the nature of PTGS will potentially result in an improved ability to choose virus-derived sequences that elicit strong PTGS reactions. This will result in improved virus resistance. Thus, through the transformation of a plant with a construct containing complementary virus derived sequences that result in virus specific dsRNA when transcribed, targeted virus resistance can and has been successfully induced. The manipulation of these constructs to contain complementary sequences from more than one virus would almost certainly result in multiple-virus resistance.

Previous methods used for the introduction of plant virus resistance have mostly resulted in inconsistent and partial resistance. Through the application of PTGS, targeted resistance has been introduced with consistent results against many diverse plant viruses with diverse hosts. Although the resistance afforded through the expression of virus specific dsRNA is not broad spectrum, the relative ease with which it can be implemented, the lack of functional foreign proteins within the transformed plant and the degree of resistance that it imparts to the plant makes it the ideal and perhaps the only realistic choice for the introduction of effective and sustained plant virus resistance.

Chapter 3: Isolation, sequencing and expression of the GRSPaV coat protein gene of a South African isolate

3.1. INTRODUCTION

Due to the economic importance of both GLR and RSP in the South African viticulture industry, the main causative viruses associated with these two diseases were selected for the construction of a vector that would ultimately be used to impart multiple-virus resistance to grapevines. The vector system that was selected as the basis of the construct has been successfully used for plant transformation and the induction of virus resistance (Wesley *et al.*, 2001; Smith *et al.*, 2000). The selected system consists of the primary cloning vector pHannibal, based on pART7, and the binary vector pART27 (Gleave, 1992). It was decided to clone sense and antisense segments of the CP genes of both GLRaV-3 and GRSPaV in a complementary fashion, into a single pHannibal vector. The resultant vector could potentially be used to induce multiple-virus resistance against both GLRaV-3 and GRSPaV.

To amplify the CP gene segments necessary for the vector construction, clones of the CP genes of GLRaV-3 and GRSPaV were required. A GLRaV-3 CP gene clone was acquired (courtesy of H. Gardner), but no such clone was available for the GRSPaV CP gene. Thus the gene had to be isolated from GRSPaV infected grapevines. Grapevine rupestris stem pitting-associated virus is a phloem restricted ssRNA positive sense virus, with a dsRNA replicative phase. Consequently, dsRNA could be isolated from the petioles, leaf veins and bark scrapings of infected plants, and an RT-PCR performed using virus CP gene specific primers to isolate the GRSPaV CP gene.

For the isolation of the GRSPaV CP gene, three different methods were attempted. The first two methods were variants of dsRNA isolation protocols, each followed by an RT step using the isolated dsRNA as template, and subsequently a PCR step using the reverse transcribed cDNA as template. The advantage of this method was that once significant quantities of pure dsRNA were isolated, the RT-PCR worked well with little or no background amplification. The disadvantages were that the isolation of large quantities of high quality dsRNA from grapevine material was not always easily achieved. The third method was the rapid direct-one-tube-RT-PCR procedure which combines the RT and PCR steps into a single reaction, using grapevine petiole and leaf vein material that was ground in Grape-ELISA grinding buffer as template (La Notte *et al.*, 1997). This third procedure has definite advantages over the first two. It is faster, less laborious and almost always resulted in the amplification of the

required area. However, the disadvantage was that background amplification often occurred, due to the template being the phloem containing tissue, and not only dsRNA.

As a secondary aim of this project, it was decided to express the GRSPaV CP gene with the purpose of isolating the protein for the production of antibodies. These antibodies could be used by various institutions that supply certified grapevine propagation material as an additional tool for the large scale screening of grapevine plants for the presence of GRSPaV. There is a definite interest and need within the viticulture industry for this additional screening method, as the propagation of infected grapevine material is believed to be the main method of GRSPaV spread. Antibodies have been generated using recombinant GRSPaV CP in the USA, and have been effectively used for the detection of GRSPaV using an ELISA and/or a Western Blot approach (Meng et al., 2003; Minafra *et al.*, 2000).

Although molecular methods are potentially the only way to completely control RSP, detection of GRSPaV within rootstocks used for propagation purposes is an integral part in the management of the disease. If rootstocks are not effectively screened for the presence of GRSPaV, infected planting material will be distributed to grape growers. Currently biological indexing of candidate rootstocks on *V. rupestris* St. George and GRSPaV-specific RT-PCR is used to detect GRSPaV within rootstocks. Indexing, although reliable when performed correctly, is slow and laborious. Sequence variants of GRSPaV have a sequence homology of 75% to 93% (Meng *et al.*, 1999b). Due to the high sequence variability of GRSPaV, RT-PCR can result in false negative results, leading to the spread of infection through the propagation of infected material. It has been found that primers designed on different genomic areas of GRSPaV can have a varied detection rate of 58% to 90% (Zhang *et al.*, 1998b). Meng *et al.* (1999b) found that they detected GRSPaV in 85% of plants that had previously tested positive with indexing, when using an RT-PCR approach. However, when they designed primers from a highly conserved genomic area this detection rate increased to 99%. The use of degenerate primers based on various sequence variants could also increase the efficacy of RT-PCR based screening. Thus, RT-PCR can be used as a very effective method for the detection of plant viruses, if degenerate primers are designed from highly homologous areas of the genomes of different strains or sequence variants of the target virus. A third potential screening method is the ELISA technique, which is currently a preferred method of plant virus detection due to its simplicity, effectiveness and the ability to screen large quantities of plants simultaneously and hence, its cost effectiveness. The coat proteins from different GRSPaV sequence variants exhibit highly similar antigenic indices, suggesting serological relatedness (Meng *et al.*, 1999b). This makes ELISA an ideal method of screening for the GRSPaV in rootstocks and propagation material. In conjunction with this, ELISA has been found to be comparable to biological indexing and RT-PCR in its effectiveness as a

detection method for GRSPaV and the grapevine-leafroll associated viruses (Meng *et al.*, 2003; Ling *et al.*, 2001). It is recommended that the three above mentioned methods be used in conjunction to facilitate effective detection of GRSPaV within grapevine rootstocks, resulting in more effective disease management before plant propagation. An improvement in sampling strategies would also result in more effective detection when using RT-PCR and ELISA (Ling *et al.*, 2001).

For the expression of the GRSPaV CP, necessary for the production of antibodies, a single method of protein expression was attempted. The expression of virus proteins with the pET14b bacterial expression system has proven viable and efficient. The pET vectors were acquired from Novagen, but were originally constructed by Studier and colleagues (pET system manual, 2000; Studier *et al.*, 1990; Rosenberg *et al.*, 1987; Studier and Moffatt, 1986). Advantages of the system include the availability of a Histidine-tag for the detection and purification of expressed proteins, and the T7 promoter driven expression for high levels of protein expression. An advantage applicable to the expression of the GRSPaV CP gene of the SA isolate, which starts with the sequence ATGG, is the presence of the start codon of the His-tag within an *Nco*I restriction site, with the recognition sequence C/CATGG. This facilitated the cloning of the CP gene in frame and exactly 8 bp downstream of the ribosomal binding site (RBS), without the need to induce any mutations, while removing the His-tag from the vector.

The *E. coli* cell line BL21(DE3)pLysS was selected for expression (pET system manual, 2000). This cell line contains the *lacUV5* promoter that drives the *T7 RNA polymerase* gene. This cell line contains an additional plasmid, pLysS, that downregulates the *T7 RNA polymerase* and causes the cells to lyse after freezing, thus releasing the expressed protein. Within *E. coli* BL21(DE3)pLysS cells small amounts of T7 lysozyme (*T7 lysozyme* is situated on the pLysS plasmid) are also produced, which is a natural inhibitor of T7 RNA polymerase. T7 lysozyme induces stricter expression, i.e. less basal expression. Induction with Isopropyl-β-D-thiogalactopyranoside (IPTG) leads to the production of T7 RNA polymerase which, in turn, transcribes the gene of interest cloned into the pET14b plasmid, ultimately resulting in translated protein.

3.2. EXPERIMENTAL PROCEDURES

3.2.1. Primer design and calculation of T_m

All primers were designed using the Microsoft DOS-based program, Primer (Primer version 1.01. Serial number 50132, Copyright 1990, scientific & educational software). All melting temperatures were calculated using the Oligonucleotide Analyzer tool, which uses nearest neighbour parameters, on the website http://www.rnature.com.

The primers relevant to **Chapter 3** are given in **Table 3.1** below.

Table 3.1. Relevant primers used for the isolation, cloning, sequencing and expression of the GRSPaV CP gene. Underlined sequences represent the appropriate RE present within the 5' extension. When present within a primer, the start codon of the GRSPaV CP gene is indicated in bold font.

Primer	Sequence (5' - 3')	T_A (°C)	RE sites
StemCP2-For	ACT TTC AAA GAC GGT GGA CAT GAG	59	
StemCP2-Rev	AGC CAT AGC TTG TCT GAG CAC TTG	60	
RSP-RevTransc	GGA ATT ATT GGA CTG CGG	51	
RSP-CP-Forw	GCG TCT AGA GAA TTC **ATG** GCA AGT CAA ATT GGG	66	*Xba*I & *Eco*RI
RSP-CP-Rev	GGG TCT AGA GAA TTC CGG AAT TGT CGC TGA CTT	68	*Xba*I & *Eco*RI
RSP-CP-FlankForw	GGA GGT TTG AAG GCT TTA GGG	58	
RSP-CP-FlankRev	GTA CGG TAT TCC AGC GAA CAG G	59	
StempitCP2-For	CTG AAA TCA CTG GAG TTG GC	55	
RSP-CP-NewRev	CTT AAC CCA GCC TTG AAA TCG G	58	
RSP-CP Forw	CGT CTC GAG GGT TAA **ATG** GCA AGT CAG	64	*Xho*I
RSP-CP Rev	GCG CTC GAG TCA TCC TCC TTA TTC ATG	63	*Xho*I
T7-Prom	TAA TAC GAC TCA CTA TAG GG	47	
T7-Term	GCT AGT TAT TGC TCA GCG G	53	
RSPCPexprFnew	GTA CCA **TGG** CAA GTC AGA TTG G	57	*Nco*I

3.2.2. Double stranded RNA extraction: standard CTAB method

Petioles and veins were excised from grapevine leaves and bark scrapings prepared for dsRNA extraction. Grapevine material was frozen and stored at -80°C until processed.

Five hundred microlitres of N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) extraction buffer (0.5 M Tris-Cl pH 8.0, 1.4 M NaCl, 2% CTAB, 0.02 M EDTA) was used per 100 mg of plant material. β-Mercaptoethanol (β-ME) was added just prior to the RNA extraction to a final concentration of 0.5%. Plant material was frozen with liquid nitrogen in sterile mortars and ground to a fine powder. CTAB extraction buffer was added and the material further ground to a smooth paste. The sample was incubated at 65°C for 15 min, centrifuged at 13.2k rpm for 10 min and 500 µl of supernatant drawn off without disturbing the interface. Five hundred microlitres of chloroform/iso-amyl alcohol (24:1) was added to the supernatant and vortexed for 1 min, centrifuged at 13k rpm and 4°C for 15 min. The supernatant was drawn off without disturbing the interface. Eight molar LiCl was added to a third of the supernatant volume, and incubated on ice (4°C) overnight. The following day the extraction was centrifuged at 13k rpm and 4°C for 20 min. The supernatant was discarded and the pellet washed twice with cold 70% EtOH with microfuge tube re-orientation (14k rpm, 5 min, 4°C). Clean dry pellets were re-suspended in 20 µl sterile water and left for 1 h at room temperature in the dark. RNase A (50 µg/ml) was added and the extracted dsRNA was incubated (30 min, 37°C). RNase A was heat inactivated (15 min, 65°C).

3.2.3. Double stranded RNA extraction: CF11 cellulose method

The CF11 cellulose dsRNA extraction was essentially performed as per Rezaian and Krake (1987). The petioles and veins were excised from grapevine leaves and bark scrapings from canes prepared for dsRNA extraction. Grapevine material was frozen and kept at -80°C.

Fifteen grams of bark scrapings and petiole/vein material were finely cut, flash frozen in liquid nitrogen and ground to a fine powder in a sterile mortar. The resultant ground material was re-suspended in four times the volume (60 ml) of extraction buffer (400 mM Tris-HCl pH 8.0, 1.4 M NaCl, 3% CTAB, 20 mM EDTA, 15 mM β-ME). The mixture was incubated (10 min, 60°C) and subsequently stirred for 10 min. Half the total volume (30 ml) of chloroform was added to the mixture, which was stirred for 30 min at room temperature. The mixture was subjected to centrifugation (10 000 ×g, 10 min). The supernatant was retained, EtOH was added to 17% (v/v) and 2.5 g sterile CF11 cellulose powder (Whatman) was added. The mixture was stirred for 30 min, loaded onto a chromatography column (Promega) and allowed to run through. The cellulose was washed with three volumes (90 ml) of STE/EtOH (100 mM NaCl, 50 mM Tris-HCl, pH 7, 1 mM EDTA, 17% ethanol v/v). The dsRNA was eluted with one volume (30 ml) of STE (100 mM NaCl, 50 mM Tris-HCl pH 7.0, 1 mM EDTA), and the resultant volume measured. The dsRNA was further purified by adding ethanol to 17% (v/v) and 0.3 g sterile CF11 cellulose powder to the eluted dsRNA solution. This mixture was stirred for 30 min, loaded onto a smaller chromatography column (Promega), washed with

three volumes of STE/EtOH and the dsRNA was eluted with STE. dsRNA was precipitated overnight with sodium acetate, centrifuged and the pellet re-suspended in 50 µl double distilled H_2O (ddH_2O). RNase A (50 µg/ml) was added and the extracted dsRNA was incubated (30 min, 37°C). RNase A was heat inactivated (15 min, 65°C).

3.2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

3.2.4.1 Primer annealing

For the primer annealing step, "cocktail A" was prepared by adding together 2.5 µl of dsRNA extraction product, 0.25 µl (0.5 µM) reverse primer and 3.625 µl ddH_2O. Cocktail A was incubated for 10 min at 75°C when using dsRNA as template, and at 65°C for ssRNA template. After the incubation the reaction was flash cooled on ice.

3.2.4.2. cDNA synthesis

Reverse transcription was performed by means of the Superscript^{TM}II Reverse Transcriptase (Invitrogen) essentially according to the manufactures instruction.

"Cocktail B" was prepared by adding together 1× First Strand Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 0.25 mM dNTP's, 0.25 U RNasin, 10 mM dithiothreitol (DTT) and 0.3125 U Superscript^{TM}II reverse transcriptase.

"Cocktail A" was added to "cocktail B", for a total reaction volume of 10 µl, and incubated at 42°C for 1 h.

3.2.4.3. PCR amplification

One microlitre of the synthesized cDNA template was added to the PCR cocktail (1× Bioline PCR NH_4 reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8 at 25°C, 0.01% Tween-20], 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.5 U Bioline BIOTAQ^{TM} DNA polymerase).

The 10 µl PCR reaction was subjected to the following program in a GeneAmp PCR System 9700 thermal cycler (Perkin & Elmer/Applied Biosystems): 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; 72°C for 7 min; 4°C. The general primer annealing temperature used was 55°C, but this varied depending on the primer. Primer annealing temperatures are indicated in **Table 3.1**.

3.2.5. Polymerase chain reaction (PCR)

Polymerase chain reactions were generally performed as stated in **Section 3.2.4.3**.

3.2.6. Agarose gel electrophoresis

The following procedure was essentially performed as per Sambrook *et al.* (1989).

Agarose gel electrophoresis was generally performed using 1% (w/v) agarose/TAE (Tris-acetate/EDTA) gels. TAE was prepared as a 50× stock solution (2 M Tris, 5.71% (v/v) glacial acetic acid, 0.05 M EDTA pH 8.0), and used as a 1× working solution (0.04 M Tris-acetate, 0.001 M EDTA). Visualisation of smaller DNA fragments (<500 kb) was performed on 2% (w/v) agarose/TAE gels for improved DNA separation. Ethidium bromide (0.5 µg/ml) was added to the agarose gel for the visualisation of DNA through ultra-violet (UV) absorbance and transmission at 509 nm. Agarose gels were electrophoresed at a voltage of 75-120 mV.

To facilitate loading of DNA into the wells of agarose gels, 1× loading buffer (0.042% bromophenol blue, 0.042% xylene cyanol FF, 6.67% (w/v) sucrose) was used). The 1kb+ marker (Invitrogen) was generally used as a size standard during agarose gel electrophoresis of important results. For routine gel electrophoresis λ *Pst*I (30 µg of λ DNA digested with 100 U *Pst*I, in a total volume of 500 µl) and λ *Sty*I (30 µg of λ DNA digested with 100 U *Sty*I, in a total volume of 500 µl) were used as size markers.

3.2.7. Rapid direct-one-tube-RT-PCR procedure

The following procedure was adapted from La Notte *et al.* (1997), and performed on ice.

Cambium and petiole samples were ground in Grape-ELISA grinding buffer (carbonate ELISA coating buffer pH 9.6, 2% PVP40, 0.2% BSA, 0.05% Tween 20, 1% Sodium Metabisulfite). A recommended dilution factor for the grape extract of 1:20 for cambium and 1:40 for petiole was used. Four microlitres of the 1:20 diluted grape extract was pipetted in a microfuge tube containing 25 µl of sterile 1× GES (0.1 M glycine-NaOH pH 9.0, 50 mM NaCl, 1 mM EDTA + 0.5% Triton X-100). The sample was heated in a thermal cycler for 10 mins at 95°C. After heating, the microfuge tubes containing the sample were chilled in an ice bath for a minimum of 5 min. 2 µl of the sample was pipetted into 23.0 µl of the One-Tube-RT-PCR mix (1× Bioline PCR NH_4 buffer, 1× Sucrose/cresol red dye solution (10×: 20% (w/v) sucrose, 1 mM cresol red), 0.252 µM of each primer, 0.1 M DTT, 1.5 mM $MgCl_2$, 200

µM dNTP's, 25 U Superscript II (200 U/µl Life technologies), 1.25 U *Taq* DNA polymerase (5 U/µl Bioline Biotaq)). The sample was then subjected to the following thermal cycler program: 48°C for 30 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, 72°C for 7 min, 4°C.

3.2.8. Purification of DNA excised from agarose/TAE gels

For DNA purification, the DNA was excised from the agarose/TAE gels and purified by means of the QIAquick® Gel Extraction Kit (QIAgen). The PCR product purification protocol was essentially performed according to the manufacturer's instructions.

DNA was excised from an agarose/TAE gel and weighed. Ten microlitres of capture buffer (buffered solution containing acetate and chaotrope) was added for every 10 mg of agarose gel slice and vortexed vigorously. The microfuge tubes were incubated at 60°C until the agarose was completely dissolved (5-15 min). The sample was transferred to a QIAquick® Spin Column placed within a collection tube, incubated (room temperature, 1-2 min) and centrifuged (13.2k rpm, 30 sec - 1 min). The flow through was discarded and 500 µl of wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 80% EtOH) was added to the column, and centrifuged (13.2k rpm, 30 sec - 1 min). The flow through was discarded and the column centrifuged (13.2k rpm, 30 sec - 1 min). The column was transferred to a clean 1.5 ml microfuge tube; 30 µl to 50 µl of ddH_2O was added to the column and the sample incubated (room temperature, 1-5 min). The column was centrifuged (13.2k rpm, 2 min), and the flow through retained for further use.

3.2.9. High fidelity PCR

High fidelity PCR was performed by means of the Expand High Fidelity PCR System (Roche), essentially according to the manufacturer's instructions.

Approximately 0.1-0.5 ng of DNA was added to the high fidelity PCR cocktail (200 µM dNTPs, 300 nm of each primer, 1× Expand High Fidelity buffer (1.5 mM $MgCl_2$), 2.6 U of Expand High Fidelity PCR System enzyme mix). The 50 µl high fidelity PCR reaction was subjected to the following program in a GeneAmp PCR System 9700 thermal cycler (Perkin & Elmer/Applied Biosystems): 1 cycle of 94°C for 2 min; 10 cycles of 94°C for 15 sec, 55°C for 30 sec and 68°C for 45 sec; 30 cycles with the same steps, with an increase in the elongation step of 5 sec per cycle; 68°C for 7 min; 4°C. The general primer annealing temperature used was 55°C, but this varied depending on the primer. Primer annealing temperatures are indicated in **Table 3.1**.

3.2.10. Cloning of PCR products into pGem®-T Easy

Cloning of PCR products using the pGem®-T Easy Vector System (Promega) was performed essentially according to the manufacturer's instructions.
Ligations of PCR products to pGem®-T Easy was performed in 10 µl reactions (1× Rapid Ligation Buffer (30 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM adenosine triphosphate (ATP), 1% Polyethylene glycol (PEG)) for T4 DNA Ligase, 50 ng of pGem®-T Easy vector, 3 Weiss units T4 DNA Ligase (3 Weiss units/µl)). The concentration of PCR product used per ligation was calculated according to the manufacturer's instructions, which is essentially the same as the example in **Addendum (A)**. pGem®-T Easy is selected for with ampicilin (amp) (100 µg/ml) in the growth media, and successful ligation can be visually assessed through blue/white selection, by the addition of IPTG (0.16 mM) and 5-bromo-4-chloro-3-indocyl-β-D-galactoside (X-gal) (0.016 mg/ml) in the growth media.

3.2.11. Transformation of plasmids into competent cells

The following procedure was essentially performed as per Sambrook *et al.* (1989). All plasmids were transformed into competent *E. coli* DH5α, unless specified otherwise. *E. coli* DH5α was made competent with the rubidium-chloride method (**Addendum, B.1**).

One to 10 µl of a ligation reaction was added to 100 µl of competent cells. For ligations with low concentrations of vector and insert, the entire ligation was used for the transformation. If extracted plasmid was being re-transformed into competent cells, the plasmid was diluted 1000×, and 1-5 µl used for the transformation. The transformation reaction was tapped lightly to mix, and incubated on ice (20 min, 4°C). The transformation was heat shocked (45 sec, 42°C) and subsequently incubated on ice (2 min, 4°C). Nine hundred and fifty microlitres of Luria-Bertani (LB) (**Addendum, C.1**) medium was added to the transformation and shaken (150 rpm, 90 min, 37°C). A 100 µl volume of the resultant transformation was plated on LB/agar plates with the appropriate antibiotics (**Section 3.2.12**). During transformations where very few colonies were expected the transformed cells were pelleted (8 000 rpm, 5 min) and resuspended in 100 µl of LB medium before being plated on LB/agar/antibiotic plates.

3.2.12. Bacterial cell culture

Transformed bacteria were plated on LB/agar plates containing the relevant antibiotics. The plasmids used and the selectable antibiotic markers, including concentrations used, are given in **Addendum (D)**. Bacteria from freezer cultures, or that were re-streaked from a single

colony were also grown on LB/agar/antibiotic plates. Plates containing transformed *E. coli* bacteria were incubated for ~16 h at 37°C.

For plasmid extraction, transformed bacteria were grown in 5 ml LB medium containing the relevant antibiotic for the specific plasmids (**Addendum, D**).

3.2.13. Plasmid extraction – Kit

Plasmid extractions were adapted from the Wizard® *Plus* SV Minipreps DNA Purification System (Promega) centrifugation protocol. Plasmids were generally extracted using the kit for the purpose of sequencing.

The 5 ml overnight culture was pelleted (13.2k rpm, 30 sec), and the pellet was suspended in 250 µl resuspension solution (50 mM Tris-Cl pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). Two hundred and fifty microlitres cell lysis solution (0.2 M NaOH, 1% SDS) was added and the sample inverted 4 times. Ten microlitres alkaline protease solution was added to the sample, which was inverted 4 times and incubated (5 min, room temperature). Three hundred and fifty microlitres of neutralization solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) was added, inverted 4 times and centrifuged (13.2k rpm, 10 min). The cleared lysate was decanted into a spin column within a collection tube and centrifuged (13.2k rpm, 1 min). The flowthrough was discarded; 750 µl wash solution (60 mM potassium acetate, 8.3 mM Tris-Cl pH 7.5, 40 µM EDTA, 60% EtOH) was added to the spin column and centrifuged (13.2k rpm, 1 min). The flowthrough was discarded; 250 µl wash solution was added to the spin column and centrifuged (13.2k rpm, 1 min). The flowthrough was discarded and the spin column within the collection tube centrifuged (13.2k rpm, 2 min). The spin column was transferred to a sterile 1.5 ml microfuge tube, 50-100 µl ddH_2O added, incubated for 5 min and centrifuged (13.2k rpm, 2 min). Plasmid DNA was stored at -20°C.

3.2.14. Sequencing

Sequencing was either performed in the laboratory using a cycle sequencing method, which is essentially a combination of the dideoxy chain termination sequencing method (Sanger *et al.*, 1977) and thermal cycling methodology, or at the Core DNA Sequencing Facility, Stellenbosch University. All sequencing in the laboratory was performed using a Perkin & Elmer Applied Biosystems GeneAmp PCR System 9700 thermal cycler and the BigDye™ Terminator v3.0/v3.1 Cycle Sequencing DNA sequencing kit (Applied Biosystems) according

to the manufacturer's instructions. Sequencing products were run on an ABI PRISM^R 3100 Genetic Analyzer automated sequencer (Applied Biosystems).

3.2.15. Restriction digestion and ligation of inserts and vectors

Restriction digestions were performed according to the manufacturer's instructions, in the appropriate buffer. Restriction digestions were generally incubated for 1 h 30 min to 3 h at 37°C. Sticky ended ligations of RE digested inserts to vectors were performed using the NEB T4 DNA Ligase (New England Biolabs), essentially according to the manufacturer's instructions.

One cohesive end ligation unit is equal to ~0.015 Weiss units. The NEB T4 DNA ligase was supplied at 400 cohesive end ligation units per microlitre. Standard ligations were performed with 0.2 µl NEB T4 DNA ligase (80 U or 1.19 Weiss units) and 1× T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml bovine serum albumin (BSA), pH 7.5 at 25°C) in a total reaction volume of 10 µl or 20 µl. A total of 5 pmol vector and insert was used per ligation where possible, with an insert to vector ratio of 3:1. An example of the calculations used to determine vector and insert amounts for efficient ligation is given in **Addendum (A)**. Ligations were performed overnight at 16°C, or for 4 h at 20°C.

For difficult ligations or ligations where the amount of vector and/or insert available was less than the calculated necessary value, the following changes were made. A total of 160 U NEB T4 DNA ligase (2.38 Weiss units) were used. Prior to ligation the vector, insert and ddH_2O was added together and incubated for 10 min at 65°C, and kept on ice until the remaining components were added polyethylene glycol (PEG 8000) was added to a concentration of 5%, after all the other components were added and mixed. Ligations were performed overnight at 20°C.

3.2.16. Expression of GRSPaV coat protein in *Escherichia coli*

The following procedure was adapted from the pET System Manual (2000) (Novagen).

Competent BL21(DE3)pLysS cells were transformed with the pET14b-RSP-CP, pET14b-HSP70-3' (positive control) and the pET14b (negative control) expression vectors. The cells were made competent with the RbCl method (Hanahan, 1985).

Single colonies of BL21(DE3)pLysS::pET14bΔGRSPaVCP, BL21(DE3)pLysS::pET14b ΔHSP70-3' and BL21(DE3)pLysS::pET14b were inoculated into 5 ml LB medium containing

100 µg/ml amp, 34 µg/ml chloramphenicol (chlor) and 0.5% glucose. These were grown overnight (225 rpm, 37°C) until saturation was achieved. Two hundred and fifty microlitres of each culture was inoculated into 25 ml Terrific Broth (TB) (**Addendum, C.2**) and 0.5% glucose added.

The TB expression cultures were grown to an OD_{600} of approximately 0.6 (225 rpm, 37°C). One millilitre of each uninduced culture was harvested prior to induction. The cultures were induced with 0.4 mM IPTG for 3-5 h. If the OD_{600} was more than 0.6 after 2 h, a 200 µl sample of the culture was taken every 20 min and diluted to 1 ml with TB (0.5% glucose) until the OD_{600} was measured at 0.6. This was to ensure that the total cell proteins of the induced and uninduced samples were of a similar quantity for SDS-PAGE analysis. After induction, 1 ml of each induced culture was harvested.

3.2.17. Purification of the soluble- and insoluble whole cell protein fraction

The following procedure was adapted from the Novagen pET System Manual (2000).

One millilitre of the induced culture was taken as a total cell protein sample. The remainder of the culture was pelleted (10 000 ×g, 15 min, 4°C). The supernatant was discarded and the pellets were resuspended in 1/10× culture volume 1× STE (0.1 M NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0). The sample was sonicated in 1.5 ml aliquots with a microtip for 90 sec on ice, to prevent heat denaturation of proteins, until it was no longer viscous. The sonicated sample was centrifuged (10 000 ×g, 10 min, 4°C) to separate the soluble- and insoluble fractions. The supernatant (soluble fraction) was drawn off and kept at 4°C. The pellet (insoluble fraction) was washed three times with vortexing between washes (13.2k rpm, 5 min), with a wash buffer (1× STE, 1% Triton X-100). The supernatant was discarded and the clean dry pellet was resuspended in 1/10× culture volume 1% SDS. The insoluble fraction was kept at 4°C. The total cell protein sample was pelleted (13.2k rpm, 10 min), the supernatant removed and the pellet resuspended in 100 µl ddH_20.

A 100 µl volume of the total cell protein sample, the soluble fraction and the insoluble fraction was drawn off and 100 µl of 2× loading buffer (0.124 mM Tris-Cl pH 6.8, 4% SDS, 20% Glycerol, 10% β-ME, 0.25% Cresol red) was added. The samples were denatured (5 min, 95°C) for use in SDS-PAGE.

3.2.18. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE procedure was performed as per Doyle (1996).

The 1 ml induced and uninduced protein expression cultures were prepared for crude protein extractions. Each culture was centrifuged (8 000 rpm, 5 min), resuspended in 100 µl ddH_2O and 100 µl 2× loading buffer (0.124 mM Tris-Cl pH 6.8, 4% SDS, 20% Glycerol, 10% β-ME, 0.25% Cresol red) and stored at 4°C until denaturation (5 min, 95°C).

Denatured proteins were separated on a discontinous polyacrylamide gel (PAG) (3.2% stacking gel and 12% resolving gel) in electrode buffer (192 mM Glycine, 0.1% SDS, 250 mM Tris-Cl pH 8.3) on a Hoefer miniVE Vertical Electrophoresis System. The separated proteins were stained with Coomasie Brilliant Blue R250 for 30 min and the gel destained overnight with destaining solution (5% acetic acid, 10% methanol).

3.3. RESULTS AND DISCUSSION

3.3.1. Cloning and sequencing of GRSPaV CP gene

Grapevine rupestris stem pitting-associated virus infected grapevine material was received from the KWV germplasm collection. Prior to receiving the material it had tested positive for GRSPaV infection (Oosthuizen, T., Pers. comm., KWV Vititec). Double stranded RNA extractions were performed on petiole and leaf vein material from infected and control plants using two protocols: the CTAB based extraction protocol, and the CF11 cellulose based extraction protocol. Control plants were subjected to the same dsRNA extraction protocols (**Figure 3.1**). It was found that the CF11 protocol was superior to the CTAB protocol (results not shown), and was subsequently used for all dsRNA extractions. The resultant extracts were screened for infection using RT-PCR with the GRSPaV CP gene diagnostic primers, StemCP2-For and StemCP2-Rev. This diagnostic primer set had been used to amplify a 523 bp fragment of the GRSPaV CP gene. A 523 bp fragment was amplified using the dsRNA from the positive plants as template (results not shown). No amplification product was observed when the extraction product from control plants was used. This result indicated that the infected material contained GRSPaV and that the control material either did not contain GRSPaV, or did not contain GRSPaV at detectable levels when tested with the RT-PCR procedure.

The available GRSPaV CP gene sequences (Genbank: accession number NC001948, accession number AF026278) were used to design primers to amplify the complete CP gene of 780 bp. Three primers were designed: a reverse primer for reverse transcription (RSP-RevTransc), and a forward primer and reverse primer for PCR amplification (RSP-CP-Forw and RSP-CP-Rev). The PCR primers each contained a 15 nt extension consisting of an *Xba*I

and *Eco*RI restriction site and three extra nucleotides for efficient digestion. *Xba*I and *Eco*RI restriction sites were selected to facilitate the cloning of the complete CP gene into the pART7/pART27 binary vector system (Gleave, 1992). Initially this product would have been used to transform grapevines for use in a CP-MR strategy targeted against GRSPaV.

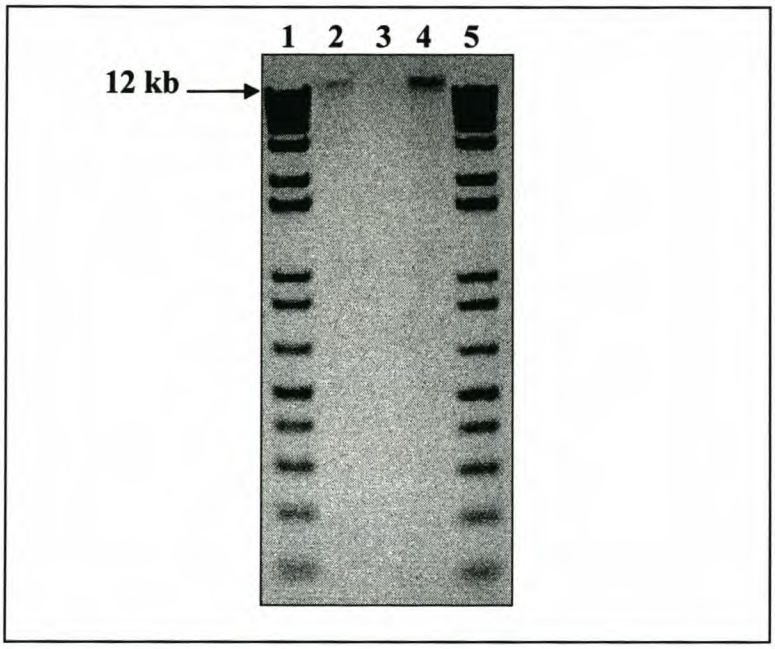

Figure 3.1. dsRNA extraction using the CF11 cellulose method. Lanes 1 and 5: 1 kb+ marker. Lane 2: extraction product from a grapevine potentially infected with GRSPaV. Lane 3: extraction product from a control plant. Lane 4: extraction product from a grapevine that had previously tested positive for GRSPaV. Subsequent RT-PCR confirmed that the dsRNA from lane 4 was GRSPaV. No product was amplified using the extraction products from lanes 2 and 3 as template. The band that can be seen is the full length dsRNA replicative form, and no subgenomic RNA bands are visible. The 1 kb+ marker is a DNA size marker, which separates faster than dsRNA during electrophoresis, and was used to give a rough indication if dsRNA was successfully isolated.

The RT-PCR amplification of the GRSPaV CP gene was unsuccessful with the primer set designed to amplify the complete CP gene, even when different annealing temperatures (T_A) and number of PCR cycles were used. Several RT-PCR variations were used in an attempt to amplify the complete GRSPaV CP gene. "Touch-up"- and "soft anneal"-RT-PCRs were performed. Combinations of the GRSPaV CP gene-specific primers (RSP-RevTransc, RSP-CP-Forw, RSP-CP-Rev, StemCP2-For, StemCP2-Rev) were used in an attempt to amplify the CP gene in segments, and RT-PCRs were performed using both forward and reverse primers simultaneously during the RT step. None of these methods proved successful in amplifying the entire CP gene or segments there of. Due to the successful amplification of the 523 bp GRSPaV CP gene fragment it was believed that the problem was not the RT-PCR or the

the new GRSPaV CP primers and the CP gene of the South African isolate for successful amplification.

New GRSPaV specific primers flanking the CP gene (RSP-CP-FlankForw and RSP-CP-FlankRev) were designed. Initial RT-PCRs using these primers were also unsuccessful, however when combining the primers StemCP2-Rev (used for RT and PCR) and RSP-CP-FlankForw, a product of the expected size (756 bp) was obtained. This amplified area contained 687 bp of the 5' region of the GRSPaV CP gene. To amplify the remaining 93 bp of the 3' region of the GRSPaV CP gene the same method was attempted, using the StemCP2-For (used for the RT and PCR) and RSP-CP-FlankRev primers. However, no amplification was observed. It was concluded that there were to many polymorphisms between the sequence of the CP gene and the RSP-CP-FlankRev primer for successful amplification.

The 756 bp RT-PCR product was cloned into pGEM®-T Easy and sequenced using the T7 promoter (T7-Prom) and SP6 primers. The sequencing results confirmed that a segment of the GRSPaV CP gene was amplified. Analysis of the sequence revealed that the sequence of the South African isolate was significantly different from the USA isolates. This explained the failure of many of the primers to amplify the GRSPaV CP gene, or parts there of, using RT-PCR. The start codon and a primer present in this sequenced area (RSP-CP-Forw: 5'-GCG TCT AGA GAA TTC ATG GCA AGT CAA ATT GGG-3') are indicated in blue in **Figure 3.2**. Polymorphisms between the primer and the CP gene are indicated in red. At this stage the infected grapevine material had been used up.

TGATTATCGTTGTTGAATTGGTTAAATGGCAAGTCAGATTGGTAAGCTCCCCGGCGAATCA
AATGAGGCATTTGAAGCCCGGCTGAAATCACTGGAGTTGGCTAGAACTCAAAAGCAGCCA
GAAGGCTCAAACACACCGCCTACTCTCAGTGGTGTGCTGGCCAAACGTAAGAGGGTTATT
GAGAATGCACTCTCAAAGACAGTGGACATGAGGGAGGTGTTGAAACACGAAACGGTTGTA
ATCTCTCCAAATGTCATGGATGAGGGTGCAATAGATGAGCTGATTCGTGCATTTGGAGAAT
CAGGCATAGCTGAGAGCGCACAATTTGATGTGGCTATAGATATAGCACGTCACTGCTCTGA
TGTTGGAAGCTCCCAAAGGTCAACCTTGATTGGCAAAAGTCCATTCTGTGATCTGAATAGA
TCAGAAATTGCCGGGATTATAAGGGAAGTAACCACATTGCGCAGATTTTGCATGTACTATG
CAAAAATCGTGTGGAATATCCATCTGGAGACGGGGATACCACCAGCTAATTGGGCCAAGA
AAGGGTTTAATGAGAATGAGAAATTTGCAGCCTTTGACTTCTTTCTGGGAGTCACAGATGA
GAGTGCGCTTGAACCAAAGGGTGGAATCAAAAGAGCTCCAACAAAAGCTGAGATGGTCGC
TAATATTGCC

Figure 3.2. The partial sequence of the GRSPaV CP gene from the first isolate of the virus. The first forward primer designed to amplify the GRSPaV CP gene (RSP-CP-Forw) is indicated in blue with the start codon of the gene underlined. Polymorphisms between the primer and the gene sequence are indicated in red, clearly showing the inability of this primer to amplify the CP gene. The primary stop codon (TGA) and secondary stop codon (TAA) of the gene preceding the CP gene, the triple gene block, is indicated in magenta.

New infected grapevine material was received from the KWV germplasm collection. A different approach was attempted to amplify the GRSPaV CP gene. The new material was subjected to a Rapid direct-one-tube-RT-PCR procedure that combines RT and PCR into a single step. Rapid direct-one-tube-RT-PCR was performed using the sap extract (cambium and petiole samples ground in Grape-ELISA grinding buffer) of infected grapevine material as template. A GRSPaV CP gene specific forward primer (StempitCP2-For) (courtesy of M. Engelbrecht), based on the initial 687 bp of GRSPaV CP gene sequence, was used along with a new reverse primer (RSP-CP-NewRev). An expected product of 786 bp was amplified, gel purified and a concentration gel was performed using Lambda DNA standards. The concentration of the PCR product was too low to see on an agarose gel and thus too low to clone efficiently. The CP gene was re-amplified using the Expand High Fidelity PCR system (Roche). The PCR product was gel purified, cloned into pGem®-T Easy and the clone sequenced using the T7-Prom and SP6 primers. The sequence confirmed that the remainder of the GRSPaV CP gene had been amplified.

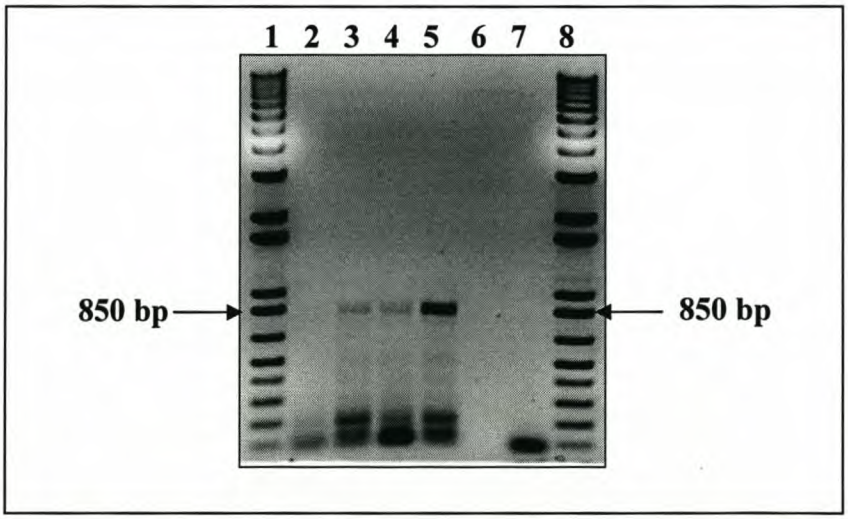

Figure 3.3. First amplification of the complete GRSPaV CP gene by means of Rapid direct-one-tube-RT-PCR. Lanes 1 and 8: 1 kb+ marker. Lanes 3 to 5: GRSPaV CP gene at 803 bp (780 bp plus the primer extensions). The products seem slightly larger than they are due to the gel having run slower in the centre lanes. Lane 2: it appears that amplification was to low to see the CP gene PCR product. Lane 6: empty. Lane 7: negative control RT-PCR, with water as the template.

Analysis of the two CP gene sequences that had been obtained showed that there were differences in the overlapping areas between the sequences of the first and second isolate. These polymorphisms indicated different sequence variants, as was found to be the case for GRSPaV by Meng *et al.* (1999b). The two sequences were combined to form a chimeric CP gene consisting of as-much-as-possible-of the sequence from the second isolate. The chimeric sequence was exactly 780 bp with no frameshifts, and was subsequently used as the

basis to design two new primers: RSP-CP Forw and RSP-CP Rev, which contained 5' extensions with *Xho*I restriction sites to facilitate cloning into the pET14b bacterial expression vector. These primers were used in a Rapid direct-one-tube-RT-PCR to amplify the entire gene in a single reaction (**Figure 3.3**). The amplified gene was gel purified and cloned into the pGEM®-T Easy. Clones were screened for the presence of the CP gene using the T7-Prom and the RSP-CP Rev primer. The plasmids from four positive clones were extracted, and a 100 ng/µl of each plasmid was used for sequencing with the T7-Prom primer and the SP6 primer. The sequencing results contained the complete GRSPaV CP gene of the second virus isolate. The sequence (SA-CPgene) is represented in **Figure 3.4** as an alignment with the first GRSPaV CP gene sequenced by Zhang *et al.* (1998).

3.3.2. Pairwise alignment of available GRSPaV CP gene sequences

A pairwise alignment was performed on the two previously sequenced GRSPaV CP sequences (Genbank: accession number NC001948 (New York); accession number AF026278 (California)) and our complete and incomplete South African GRSPaV CP gene sequences, using the web-based program, ClustalW (http://clustalw.genome.ad.jp/) (Thompson *et al.*, 1994; Higgins *et al.*, 1992).

Due to the first GRSPaV CP gene segment sequenced being incomplete, the other three CP sequences were truncated to make all four sequences the same length. The results of the pairwise alignment can be seen in **Table 3.2**.

Table 3.2. The pairwise alignment between CP gene sequences of the two USA isolates and the two SA isolates.

	SAcomplete	SAincompl	New York
SAcomplete	-	-	-
SAincomplete	96%	-	-
New York	89%	90%	-
California	89%	90%	99%

These results show a clear grouping of the two sequence variants from the USA, and the two sequence variants from South Africa. A pairwise alignment between the three untruncated complete sequences provides the same result except that homology between the SA strain and the USA strains is slightly more, but at 90% it still shows a clear distinction.

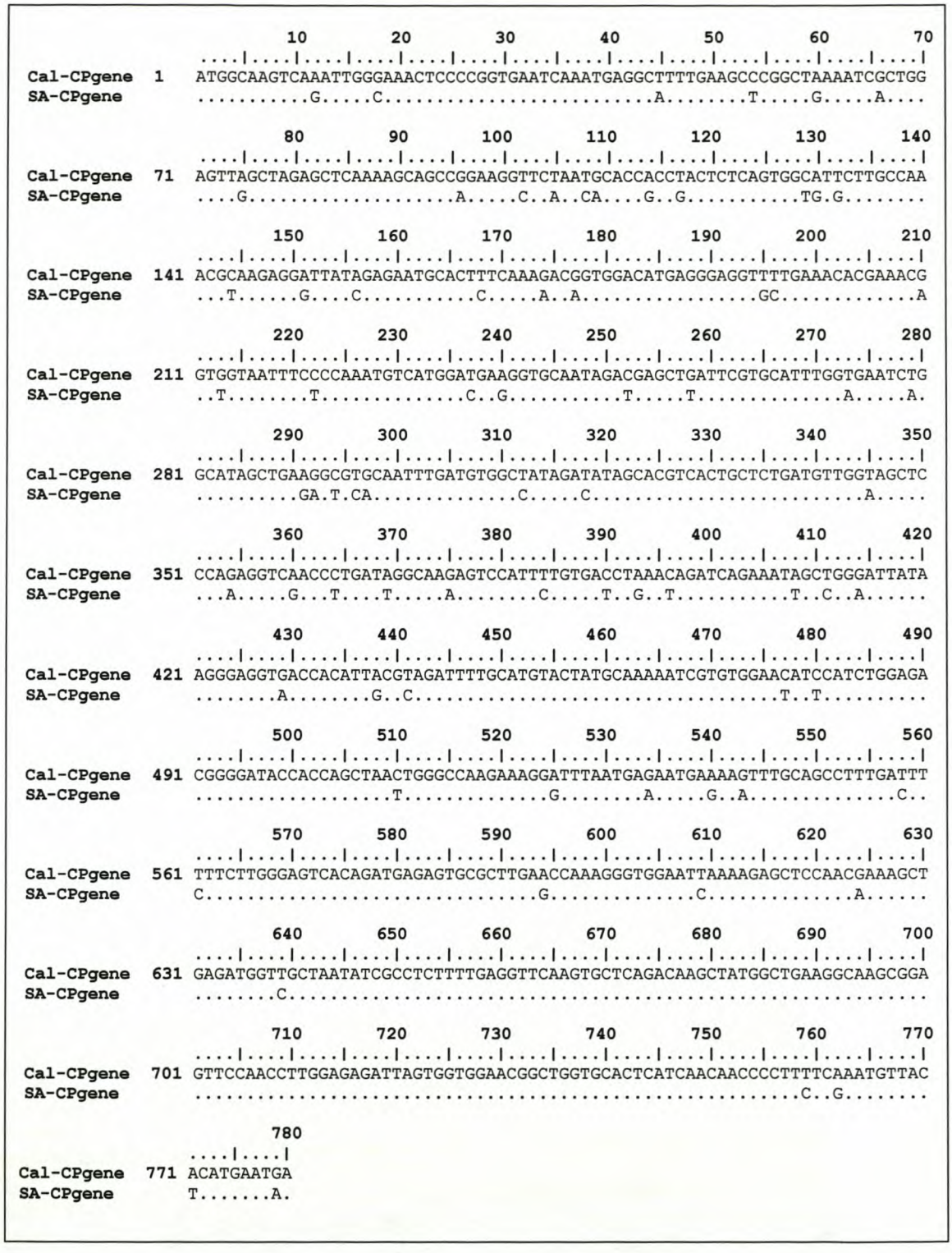

Figure 3.4. Sequence alignment of the first isolated GRSPaV CP gene (Cal-CPgene), and the GRSPaV CP gene of the second South African isolate (SA-CPgene). Polymorphisms are indicated at the relevant positions below the full sequence of the Californian isolate (Zhang *et al.*, 1998).

3.3.3. Phylogenetic analysis

A multiple sequence alignment of the available CP gene sequences of all the foveaviruses was performed (CP gene sequences available from Genbank). Coat protein gene sequences were used for the alignment. This is the only sequence available for the SA isolates of GRSPaV, and is of most interest to this project. Both the complete CP gene sequence from the second SA isolate and the incomplete CP gene sequence from the first SA isolate were used. The initial multiple alignment was performed using ClustalW (http://clustalw.genome.ad.jp/) (Thompson *et al.*, 1994; Higgins *et al.*, 1992). The alignment data was imported into BioEdit (Version 5.0.9) (Hall, 1999), where it was further manually aligned. The final alignment data was imported into MEGA (Molecular Evolutionary Genetics Analysis, Version 2.1) (Kumar *et al.*, 2001) and used to generate a phylogenetic tree. A neighbour-joining (NJ) tree was generated using pairwise deletions and bootstrap analysis (**Figure 3.5**). The phylogenetic tree shows the position of the SA isolates of GRSPaV within the *Foveavirus* family. This tree also shows the relationship between the GRSPaV isolates from the USA and SA. These isolates form two distinct branches within the same clade. Although the branch value of 72 is not above the threshold of 75 it does not mean that the viruses are unrelated. The complete genome sequence was not used for this phylogenetic analysis and genome organisations were not taken into account. For a review of phylogentic analysis see Baldauf (2003).

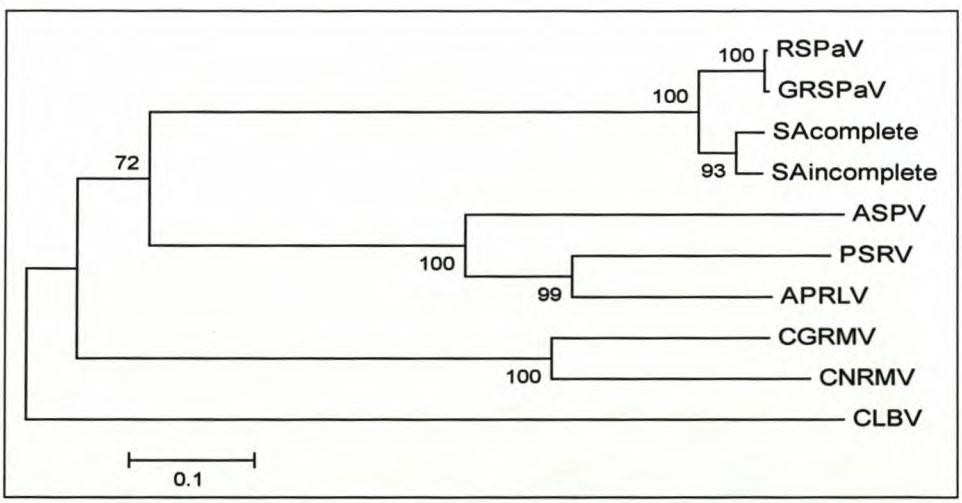

Figure 3.5. Neigbour-joining phylogram of a multiple alignment of the available *Foveavirus* CP gene sequences. (RSPaV: *rupestris stem pitting-associated* virus; GRSPaV: *grapevine rupestris stem pitting-associated* virus; SAcomplete: GRSPaV, completely sequenced CP gene from second SA isolate; SAincomplete: GRSPaV, partially sequenced CP gene from first SA isolate; ASPV: *apple stem pitting virus*; PSRV: *peach sooty ringspot virus*; APRLV: a*pricot latent virus* (not currently ICTV approved); CGRMV: cherry green ring mottle virus; CNRMV: cherry necrotic rusty mottle virus; CLBV: *citrus leaf blotch virus*).

3.3.4. Expression of the GRSPaV coat protein

The CP gene was excised from pGEM®-T Easy (**Section 3.3.1**) using *Xho*I and cloned into the *Xho*I RE site of pET14b (**Figure 3.6**). Clones were screened for the presence of the CP

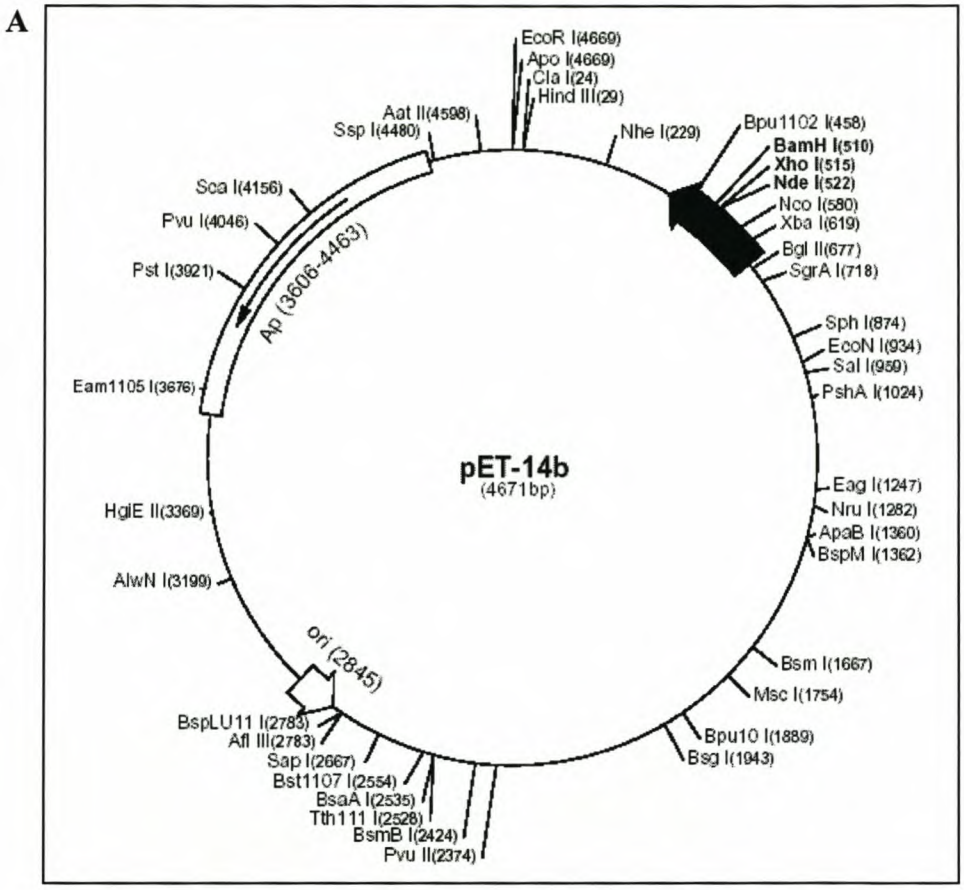

Figure 3.6. pET14b bacterial expression vector. **(A)** Restriction map of pET14b, with the cloning and expression region indicated with the black arrow. **(B)** Sequence, RE sites and details of the cloning and expression region.

gene and orientation using the T7-Prom and the RSP-CP Rev primer. Positive clones were grown up under selection (amp) and the pET14b-RSP-CP plasmid extracted for sequencing.

A 100 ng/µl plasmid was used for sequencing with the T7-Prom primer and the T7 terminator primer (T7-Term). The sequencing results confirmed that the GRSPaV CP gene was successfully cloned and in the correct orientation with no mutations, insertions or deletions. pET14b-RSP-CP was transformed into competent *E.coli* BL21(DE3)pLysS, and grown under selection (amp/chlor).

Initial expression was performed using BL21(DE3)pLysS::pET14bΔGRSPaV-CP, a positive control, BL21(DE3)pLysS::pET14bΔHSP70-3', and a negative control, BL21(DE3)pLysS:: pET14b. The expression was performed as stated in the experimental procedures. The total cell protein samples were visualized through SDS-PAGE. The positive control (courtesy of M.-J. Freeborough) worked, resulting in a protein fragment of ca. size 23.63 kDa. The banding pattern of the negative control and GRPSaV CP expression was the same, thus no expression occurred. The expression was attempted several times to ensure human error was not the cause of the failure of expression. No expression of the CP was observed at the expected size of 28 kDa.

Analysis of the GRSPaV sequencing results (sequenced from the pET14b expression vector) revealed an in-frame stop codon directly before the GRSPaV CP start codon. Sequence analysis of the results of the first SA isolate sequenced revealed that the triple gene block, preceding the CP gene, contained two stop codons, a primary stop codon and a secondary stop codon three codons downstream. This was confirmed through analysis of the available GRSPaV sequences (Genbank: accession number NC001948, accession number AF026278), which each contained an extra stop codon at the 3' end of the triple gene block. The extra stop codon is probably present to ensure readthrough does not occur during protein translation. During the design of the GRSPaV CP primers, the primers were designed to make them optimal for PCR amplification without including the primary stop codon of the triple gene block. The secondary stop codon was not detected and inadvertently include in the primer. Both stop codons are indicated in magenta in **Figure 3.2**.

It was decided to re-clone the GRSPaV CP gene into pET14b, but without the Histidine tag. The *Nco*I restriction enzyme (RE) recognition sequence is CCATGG, with the ATG being the start codon for the Histidine tag in pET14b, exactly 8 nt downstream of the RBS (the optimal distance for translation). Due to the sequence of the GRSPaV CP gene starting with ATGG, a primer was designed to clone the GRSPaV CP gene in frame into the *Nco*I site. The new GRSPaV CP forward primer (RSPCPexprFnew) contained a 5' extension with an *Nco*I site, facilitating the directional cloning of the GRSPaV CP gene into the *Nco*I/*Xho*I sites of pET14b when used together with the RSP-CP Rev primer to amplify the CP gene.

The GRSPaV CP gene was amplified using the Expand High Fidelity polymerase, gel purified and cloned into pGEM®-T Easy. By means of an *Nco*I/*Xho*I double-digest, the GRSPaV CP gene was excised from pGEM®-T Easy and gel purified. An *Nco*I/*Xho*I double-digest was performed on purified pET14b, resulting in the loss of the Histidine tag. The digested GRSPaV CP gene was ligated to the digested pET14b expression vector and transformed. Colonies were PCR screened for the presence of the insert using the T7-Prom and the GRSPaV CP rev primer. No colonies contained the insert. The double digestion of the vector, the ligation and transformation was repeated, and the resultant colonies were again PCR screened. No colonies contained the insert. To test that *Nco*I and *Xho*I were capable of digesting the vector, Lambda DNA and pET14b was digested with both *Nco*I and *Xho*I. Both enzymes digested the Lambda DNA but, while *Xho*I linearised pET14b, *Nco*I failed to digest the expression vector. Thus, it appears that the *Nco*I restriction site within pET14b had been altered.

Two *E. coli* DH5α freezer cultures containing pET14b were grown up under selection (amp) and the plasmid isolated. The extracted plasmids were linearised with *Nco*I and *Xho*I, which both digested the two plasmid isolates. The newly isolated pET14b was subjected to an *Nco*I/*Xho*I double digested, separated through agarose gel electrophoresis and gel purified. The double digested pET14b and GRSPaV CP gene was ligated and transformed into competent *E. coli* DH5 (**Figure 3.8**). Resultant colonies were PCR screened using the T7-Prom primer and RSP-CP Rev primer. Eleven of the 12 PCR screened colonies contained the GRSPaV CP gene. Four of the positive colonies were grown up under selection (amp) and the plasmid (pET14b-NewGRSPaV-CP) isolated. pET14b-NewGRSPaV-CP had a total size of 5397 bp. The isolated plasmid was sequenced using the T7-Prom and T7-Term primers.

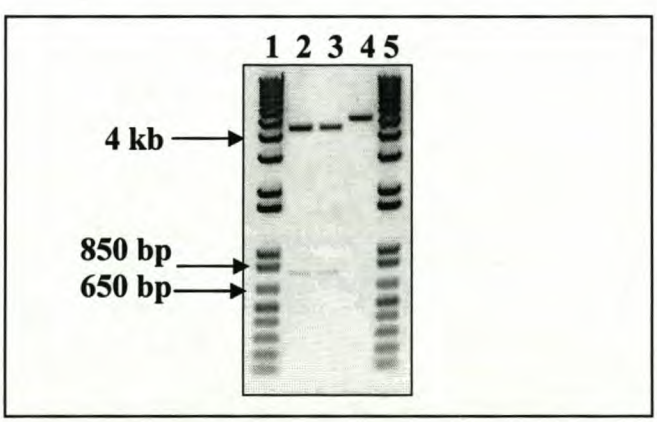

Figure 3.8. pET14b-NewRSP-CP. Lanes 1 and 5: 1 kb+ marker. Lane 2 and 3: *Nco*I/*Xho*I digested pET14b-NewRSP-CP, with the GRSPaV CP gene (~780 bp). Lane 4: *Bam*HI linearised pET14b-NewRSP-CP (~5397 bp).

The sequencing results revealed that the GRSPaV CP gene was directionally cloned into the pET14b expression vector. No mutations or frameshifts were present in the sequence preceding the cloned gene, including the RBS. Of the four sequenced clones, one was selected for the expression study. The selected clone contained a single nucleotide polymorphism in the GRSPaV CP gene, which did not result in a change in amino acid sequence. No frameshift causing mutations were present.

The pET14b-NewRSP-CP vector was transformed into BL21(DE3)pLysS and grown under selection (amp/chlor). Protein expression was performed as stated in the experimental procedures. A faint novel band of approximately 28 kDa was present in the induced pET14b-NewRSP-CP sample, but was also present in the uninduced samples. The protein expression was repeated with the following changes: 1% Glucose was added to the 5 ml overnight LB culture and to the 25 ml TB culture; the samples were induced for 5 h; 1 ml of the induced cultures were taken every hour. The total cell protein samples were visualized through SDS-PAGE. Although a faint 28 kDa was still present in the uninduced GRSPaV CP sample, the expression of the 28 kDa CP could clearly be seen in the 5 induced samples (1-5 h) (**Figure 3.9**).

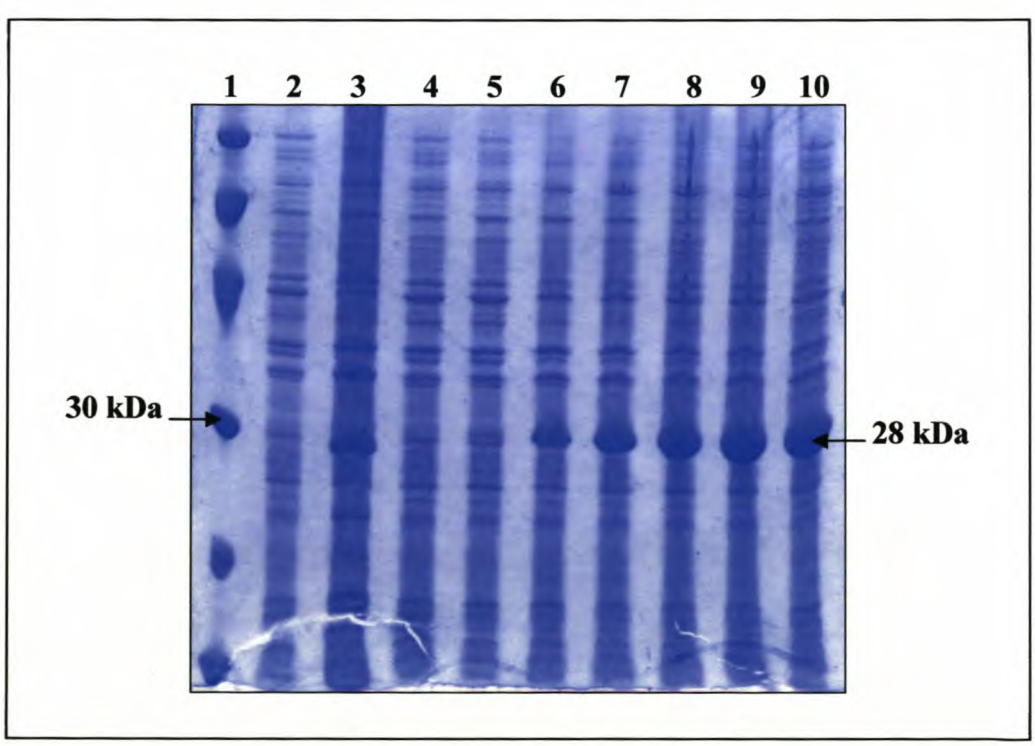

Figure 3.9. Coomasie stained SDS-PAG of total cell protein extracts from *E. coli* BL21(DE3)pLysS, containing pET14b-HSP70-3' (positive control), pET14b (negative control) and pET14b-NewRSP-CP. Lane 1: molecular weight protein marker (Amersham). Lanes 2 and 3: uninduced and induced pET14b-HSP70-3'. Lane 4: induced pET14b. Lane 5: uninduced pET14b-NewRSP-CP. Lanes 6-10: induced pET14b-NewRSP-CP (1-5 h).

To determine if the expressed protein was present in the soluble phase or in the insoluble phase as inclusion bodies, an expression was initiated with the following change: 2% Glucose was added to the 5 ml overnight LB culture and to the 25 ml TB culture to prevent basal expression of the gene by completely switching off the T7 promoter. One millilitre of each uninduced culture was taken and 4 h after induction 1 ml of the induced GRSPaV CP sample was taken as a total cell protein sample. The remainder of the induced culture was subjected to soluble and insoluble protein purification. The soluble and insoluble cytoplasmic fractions were isolated as stated in the experimental procedures, and visualized through SDS-PAGE, along with the positive- and negative control total cell protein samples and the uninduced and induced total cell protein samples. It could clearly be seen that the recombinant GRSPaV CP product was present almost exclusively in the soluble fraction (**Figure 3.10**).

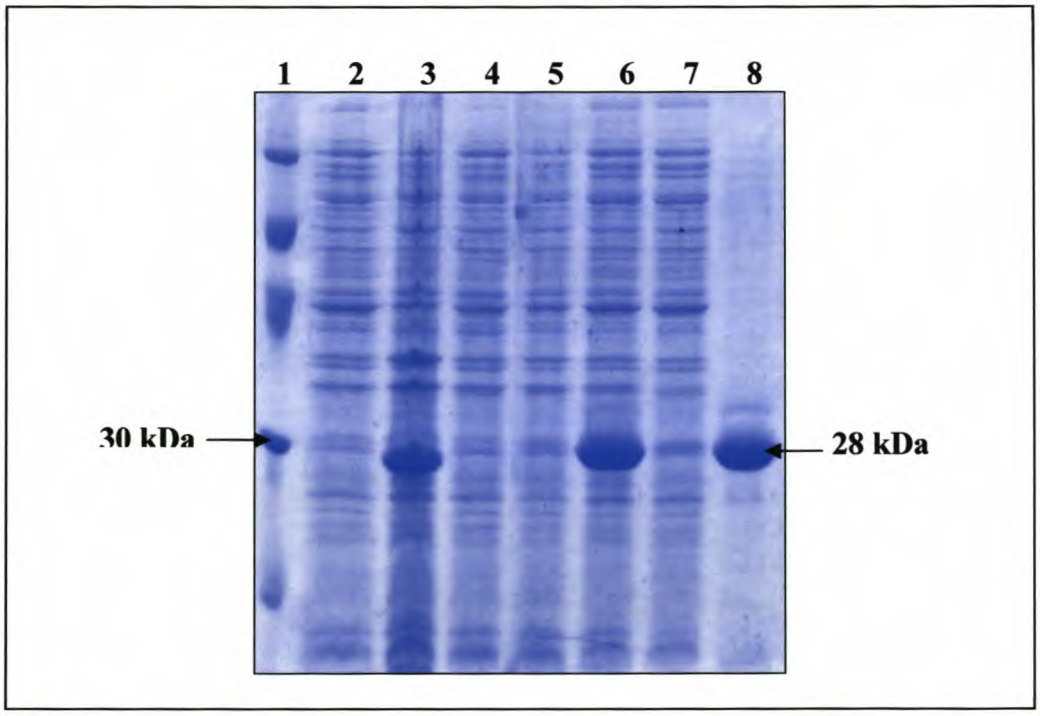

Figure 3.10. Coomasie stained SDS-PAG of total cell protein extracts and soluble and insoluble fractions from *E. coli* BL21(DE3)pLysS, containing pET14b-HSP70-3' (positive control), pET14b (negative control) and pET14b-NewRSP-CP. Lane 1: molecular weight protein marker (Amersham). Lanes 2 and 3: uninduced and induced pET14b-HSP70-3'. Lane 4: induced pET14b. Lane 5: uninduced pET14b-NewRSP-CP. Lane 6: induced pET14b-NewRSP-CP (total cell proteins). Lane 7: induced pET14b-NewRSP-CP (soluble fraction). Lane 8: induced pET14b-NewRSP-CP (insoluble fraction).

To initiate the production of GRSPaV CP specific antibodies, a large-scale protein expression will be peformed, the protein containing inclusion bodies isolated and denatured and thoroughly washed before antibodies will be generated in rabbits. The efficacy of the generated antibodies will be tested in enzyme linked immuno sorbent assays (ELISA) and

western blots on GRSPaV infected and uninfected grapevine material that has tested positive and negative respectively, using GRSPaV specific RT-PCR.

Chapter 4: Cloning of GRSPaV and GLRaV-3/2 specific plant transformation vectors and tobacco transformation

4.1. INTRODUCTION

Several methods have been applied to introduce plant virus resistance through the transformation of virus derived genes or sequences. Most of these methods have achieved limited success. It is known today that many of these methods were unknowingly utilising PTGS to induce virus resistance.

Natural resistance genes to plant viruses have been applied with success, but no such *R* genes have been found for grapevine viruses (Goldbach *et al.*, 2003). Methods such as CP-MR and other forms of PDR have been applied with varying degrees of success. These methods often produced inconsistent results, with few transformants exhibiting resistance (Bendahmane *et al.*, 1997; Baulcombe, 1996; Lomonossoff, 1995; Lindbo and Dougherty, 1992; Powell-Abel *et al.*, 1986; Sanford and Johnston, 1985). Potentially the biggest problem with PDR-based resistance methods is that most of these methods rely on the expression of a functional viral protein to induce resistance. This is especially relevant today with the negative sentiment experienced globally toward genetically modified organisms (GMOs). Several unrelated methods have achieved similar inconsistent levels of resistance. These include the expression of virus-specific antibodies (plantibodies), ribosome-inactivating proteins (RIPs) and the expression of the mammalian 2',5'-oligoadenylate synthetase (Goldbach *et al.*, 2003; Tavladoraki *et al.*, 1993). Compared to these methods, the application of PTGS to induce virus resistance has achieved tremendous success.

Post-transcriptional gene silencing has several major advantages over the methods used in the past. It is believed to have evolved as a plant defence mechanism for protection against viruses, which makes it an ideal candidate for the introduction of transgenic virus resistance. No functional virus-derived protein needs to be expressed to convey resistance. The PTGS signal spreads systemically through the plant, resulting in virus-targeted PTGS having been activated in the tissues away from the point of infection before the virus has had enough time to infect systemically (Voinnet and Baulcombe, 1997). Resistance can potentially be induced against closely related viruses with genome areas containing high degrees of homology (80-90%). With careful planning resistance could be induced against several viruses, e.g. the closely related grapevine leafroll-associated viruses (Baulcombe, 1999). Grafting of a scion onto a transgenic virus resistant rootstock might impart the virus resistance to the scion through the systemic signal without having to transform the scion. Thus, the scion would

gain the benefits of PTGS induced virus resistance without being genetically modified itself. This would be a tremendous advantage in a consumer market dominated by anti-GMO sentiment. However, the silencing signal production and propagation has to be better understood before this can be confirmed. Finally, the beauty and power of PTGS lies therein that the genome of the target virus is the specificity determinant for resistance. Consequently, PTGS induced virus resistance is mobile, specific and adaptable.

Post-transcriptional gene silencing has been successfully applied to induce virus resistance against most of the major DNA and RNA plant viruses, in both monocotyledonous and dicotyledonous plants (Goldbach *et al.*, 2003; Ding, 2000; Waterhouse *et al.*, 1999). The Waterhouse research group has developed a system for the introduction of efficient gene silencing or virus resistance in plants (Wesley *et al.*, 2001; Smith *et al.*, 2000; Waterhouse *et al.*, 1998). Their system has been effectively used to introduce virus resistance against various viruses. The Waterhouse group have achieved 96% resistance against PVY in transformed plants (Smith *et al.*, 2000). Pandolfini *et al.* (2003) achieved 80% resistance against PPV in transformed plants, using the Waterhouse system. Several other studies are currently utilising the same strategy (Bruno *et al.*, 2003; Reustle *et al.*, 2003).

The Waterhouse system consist of two primary transformation plasmids (pHannibal and pKannibal) based on pART7, and the binary plant transformation vector pART27 (Wesley *et al.*, 2001; Gleave, 1992). pHannibal and pKannibal differ only in their selectable markers, which confer amp and kanamycin (kan) resistance, respectively. The pHannibal/pKannibal vectors each contain two MCSs that are situated 5' and 3' to an intron sequence. These MCSs facilitate the cloning of sense and antisense virus derived sequences in a complementary conformation. The expression cassette-construct, containing these complementary sequences upstream and downstream of the intron, is excised from the pHannibal backbone with a *Not*I digestion and cloned into the *Not*I site in pART27. The *Not*I site within pART27 is situated within a *lac* operon (*lacZ'* gene), which facilitates blue/white (β-galactosidase) selection of successful transformants. The *lacZ'* and chimeric *nptII* (kan resistance) genes are situated between the right border (RB) and left border (LB), which are necessary for the 5'-3' transfer of the transfer-DNA (T-DNA) from *A. tumefaciens* to the plant genome. pART27 contains the necessary elements for maintenance in *A. tumefaciens* and high-copy maintenance in *E. coli*, and can be selected for with kan, spectinomycin and streptomycin (Gleave, 1992). The pART27 vector containing the T-DNA with the cloned cassette is used to transform the relevant plant through *A. tumefaciens* mediated transformation (Wesley *et al.*, 2001).

The pHannibal/pART27 binary vector system would facilitate the construction of three virus-specific plant transformation vectors for the introduction of multiple-virus and single-virus

resistance. The primary vector, to be used for the introduction of multiple-virus resistance, would ultimately contain complementary CP gene sequences of both GRSPaV and GLRaV-3. It was decided to construct two additional vectors; a GLRaV-3 specific vector, due to a demand identified within the viticulture industry, and a GLRaV-2 specific vector, due to the need for a system that could be applied for proof of concept. *N. benthamiana* is a host of GLRaV-2. Subsequent to the transformation of *N. benthamiana* with the GLRaV-2 specific vector, the transformed plant can be infected with GLRaV-2 to test for resistance. Instead, due to the importance of the multiple-virus (GLRaV-3 and GRSPaV) construct for the viticulture industry, it was ultimately decided to use this construct to transform *N. tabacum* for proof of concept. Through infection of transformed plants with a recombinant virus vector (PVX) (containing the GFP gene as a screenable marker, and the GRSPaV and GLRaV-3 CP gene sequences used for the construction of the vector), and subsequent molecular tests, it can be determined if multiple-virus resistance has been induced.

Tobacco was chosen for plant tranformation instead of grapevine because of the relative ease of transformation and the short regeneration time of tobacco when compared to grapevine (2-3 months vs. ~1 year). Grapevine transformation will be attempted once successful transformation of tobacco is achieved, and the RNA silencing of a challenging virus, containing homologous sequences to the transformed sequences, is demonstrated. The tobacco species *N. tabacum* cv. Petit Havana (SR1) was chosen for plant transformation.

A. tumefaciens mediated plant transformation was chosen for the transformation of tobacco. It is the most commonly used method of tobacco transformation, generally has a high success rate and does not require the level of skill and resources necessary for other transformation techniques such as particle (biolistic) bombardment. *A. tumefaciens* is a soil-borne bacterium that possesses the ability to transfer genetic material to the chromosomes of a host plant, resulting in crown gall disease. Infection is directed by the tumor inducing (Ti) plasmid that facilitates the insertion of the T-DNA into the genome of infected plant cells. The genes located within the T-DNA encode plant phytohormones, resulting in gall formation, and opines, which are used as carbon and nitrogen sources by *A. tumefaciens*. This mechanism of natural gene transfer has been adapted for the transfer of a gene of interest (GOI) into a target plant. To create a non-oncogenic (disarmed) *A. tumefaciens* strain, the T-DNA region of the Ti-plasmid is removed. A binary vector carrying the RB and LB elements of the T-DNA, an expression cassette containing the GOI, and a selectable marker gene is transformed into *A. tumefaciens*, either directly or through tri-parental mating. The *vir* (virulence) genes on the resident disarmed Ti-plasmid catalises the 5'-3' T-DNA transfer into a plant chromosome, ultimately resulting in a transgenic plant that expresses the GOI (Armitage *et al.*, 1988).

4.2. EXPERIMENTAL PROCEDURES

4.2.1. Primer design and calculation of T_m

Primers were designed and their T_ms calculated as described in **Section 3.2.1**.

The primers relevant to **Chapter 4** are given in T**able 4.1** below.

Table 4.1. Relevant primers used for the cloning of the pHannibal-based transformation vectors. Underlined sequences represent the appropriate RE present within the 5' extension.

Primer	Sequence (5' - 3')	T_A (°C)	RE sites
RSP-Sense-Forw	GCT CTC GAG GCG AAT CAA ATG AGG CAT	65	*Xho*I
RSP-Sense-Rev	GAC GAA TTC AGG TTG ACC TTT GGG AGC	65	*Eco*RI
RSP-A.Sense-Forw	GCT GGA TCC GCG AAT CAA ATG AGG CAT	65	*Bam*HI
RSP-A.Sense-Rev	GAC AAG CTT AGG TTG ACC TTT GGG AGC	64	*Hin*dIII
LR3-Sense-Forw	ACG GAA TTC GAT GCG GCA CAA GGA AA	65	*Eco*RI
LR3-Sense-Rev	ATC GGT ACC CAA AGC TAT TCC CTT GCC	65	*Kpn*I
LR3-A.Sense-Forw	TCG AAG CTT GAT GCG GCA CAA GGA AA	65	*Hin*dIII
LR3-A.Sense-Rev	CGC ATC GAT CAA AGC TAT TCC CTT GCC	64	*Cla*I
pHannIntr-Forw	CTT CGC AAG ACC CTT CCT CTA	57	
pHannIntr-Rev	CAT AGG CGT CTC GCA TAT CTC	55	
T7-Prom	TAA TAC GAC TCA CTA TAG GG	47	
SP6	TAC GAT TTA GGT GAC ACT ATA G	49	
LR3CP-S-Fdir	GCT CTC GAG AAG TAC GTT AAG GAC GGG	65	*Xho*I
LR3CP-S-Rdir	ATC GGT ACC ACT CTT TGA ACT CCG TCG	65	*Kpn*I
LR3CP-AS-Fdir	GTC TCT AGA AAG TAC GTT AAG GAC GGG	61	*Xba*I
LR3CP-AS-Rdir	GAC AAG CTT ACT CTT TGA ACT CCG TCG	62	*Hin*dIII
RSPasconFN	CCG ATC GAT GCG AAT CAA ATG AGG CAT	68	*Cla*I
LR2CP-S-Forw	GTA CTC GAG CAA CCT GGT GAT AAC CGA	64	*Xho*I
LR2CPsRN	GTA CTC GAG CGT ACA TAC TCG CGA ACA	63	*Xho*I
LR2CPasFN	CGC ATC GAT CAA CCT GGT GAT AAC CGA	65	*Cla*I
LR2CP-AS-Rev	GCC ATC GAT CGT ACA TAC TCG CGA ACA	65	*Cla*I
LR3CPsRN	ATC CTC GAG ACT CTT TGA ACT CCG TCG	65	*Xho*I
LR3CPasFN	GTC AAG CTT AAG TAC GTT AAG GAC GGG	61	*Hin*dIII

4.2.2. High fidelity PCR

High fidelity PCRs were performed as described in **Section 3.2.9**.

4.2.3. Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described in **Section 3.2.6**.

4.2.4. Purification of DNA excised from agarose/TAE gels

Purification of DNA was performed as described in **Section 3.2.8**.

4.2.5. Plasmid extraction – Alkaline lysis

The following procedure was performed essentially according to Sambrook et al. (1989). Alkaline lysis plasmid extractions were performed for routine extraction of plasmids.

Five millilitres of LB medium, containing the appropriate antibiotics (**Addendum, D**), was inoculated with a single colony from an LB/Agar/antibiotic plate and shaken overnight at 37°C and 225 rpm. Three millilitres of overnight culture was pelleted by centrifugation (13.2k rpm, 30 sec). The supernatant was poured off and excess media drawn off. The pellet was resuspended in 200 µl of solution 1 (50 mM Glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0) and incubated (5 min, room temperature). Four hundred microlitres of fresh solution 2 (0.2 M NaOH, 1% SDS) was added, mixed by inversion and incubated (5 min, room temperature). Three hundred microlitres of cold KOAc (solution 1) was added, mixed by inversion and incubated on ice (5 min). The extraction was then centrifuged for 10-15 min at 4°C and 14k rpm. Approximately 800 µl of the supernatant was removed without drawing off any of the white precipitate. The supernatant was roughly measured and 0.8 volumes of isopropanol was added, mixed by inversion and incubated (10 min, room temperature). The extraction was centrifuged for 10-15 min at 14k rpm. The supernatant was poured off and the excess removed with a pipette. Five hundred microlitres of cold 70% ethanol was added and centrifuged (14k rpm, 5 min, 4°C). The wash step was repeated after microfuge tube re-orientation. The 70% EtOH was poured off and the excess EtOH drawn off. The extracted plasmid was dried on a heating block (5 min, 65°C). Sterile water was added to a total of 30 µl to 100 µl, depending on the anticipated yield. RNase A (50 µg/ml) was added and incubated (30 min, 37°C). The isolated plasmid was subjected to a phenol/chloroform DNA clean-up (**Section 4.2.6**) in the event of an impure plasmid DNA preparation.

4.2.6. Phenol/Chloroform DNA clean-up

The following procedure was adapted from Sambrook et al. (1989).

The volume of the plasmid DNA solution was adapted to 180 µl with ddH_2O. Twenty microlitres of phenol was added and the mixture was emulsified by vigorous shaking. One volume of chloroform/isoamyl alcohol (24:1) was then added to the mixture and again emulsified by vigorous shaking. The mixture was then centrifuged (13.2k rpm, 5 min) and the top aqueous phase was recovered. The previous steps were repeated until no protein was visible at the interface between the organic and aqueous phases. One tenth the recovered volume 3 M sodium acetate and an equal volume isopropanol was added. This was then mixed and kept on ice for 5 min, after which it was centrifuged (13.2k rpm, 10 min). The supernatant was discarded and 200 µl 70% EtOH was added to wash the pellet by centrifugation (13.2k rpm, 2 min) with microfuge tube re-orientation. The 70% EtOH was poured off and the excess EtOH drawn off. The pellets were dried on a heating block (5 min, 60-65°C) and resuspended in 50 µl ddH_2O. RNase A was added to a final concentration of 50 µg/ml.

4.2.7. Restriction digestion and ligation of inserts and vectors

Restriction digestions and ligations were performed as described in **Section 3.2.15**.

4.2.8. Transformation of plasmids into competent cells

Transformation of plasmids into competent cells was performed as described in **Section 3.2.11**.

4.2.9. Bacterial cell culture

Bacterial cell culture was performed as described in **Section 3.2.12.**

4.2.10. Polymerase chain reaction (PCR)

Polymerase chain reactions were performed as stated in **Section 3.2.4.3**.

4.2.11. Cloning of PCR products into pGem®-T Easy

Cloning of PCR products into pGem®-T Easy was performed as described in **Section 3.2.10**.

4.2.13. Plasmid extraction – Kit

Plasmid extractions for sequencing were performed as described in **Section 3.2.13**.

4.2.12. Sequencing

DNA Sequencing was performed as described in **Section 3.2.14**.

4.2.14. Direct transformation of *Agrobacterium tumefaciens*

The following procedure was adapted from Tzfira *et al.* (1997).

Competent *A. tumefaciens* (GV3101) was obtained (courtesy of H. Gardner). These cells were rendered competent using the $CaCl_2$-based protocol described by Tzfira *et al.* (1997). A minimum of 50 ng of plasmid DNA should be used for transformation

Hundred nanograms of plasmid DNA (pART27-HSAScon) was added to 100 µl of competent *A. tumefaciens*. The cells were frozen in liquid nitrogen. Frozen cells were thawed through incubation at 37°C for 5 min. One millilitre of LB medium, without antibiotics, was added to the thawed cells, and incubated with gentle shaking (150 rpm, 3 h, 28°C). The transformation was centrifuged (13.2k rpm, 30 s). One hundred microlitres of media was drawn off (for resuspension of the cells), and the excess discarded. The cell pellet was resuspended in 100 µl LB medium, and plated on LB/agar plates with kan (20 µg/ml), rifampicin (rif) (50 µg/ml) and tetracycline (tet) (5 µg/ml). Plates were incubated for 2-3 days at 28°C. Single colonies obtained on the LB/agar/antibiotic plates were re-streaked on LB/Agar/kan/rif/tet plates to confirm successful transformation through antibiotic selection.

4.2.15. *Agrobacterium tumefaciens* mediated transformation of *Nicotiana tabacum*

Plant transformations were essentially performed as described in Section 4 of Methods in Plant Molecular Biology (Svab *et al.*, 1995).

The tobacco leaf disks used for transformation were from two sources: *in vitro* tobacco plants and hardened off tobacco plants from a greenhouse. The tobacco plants from both sources were *Nicotiana tabacum* cv. Petit Havana (SR1). Tobacco leaves were first surface sterilised by emersion in 70% EtOH (30 sec), followed by emersion in 0.5% sodium hypochlorite with vigorous shaking (5 min). The leaves were rinsed five times in ddH_2O. Leaves were again immersed in 0.5% sodium hypochlorite with vigorous shaking (5 min), and rinsed five times

in ddH_2O. Leaf disks were prepared from leaf material by dissecting the leaves into disks of approximately 1 cm^2.

A. tumefaciens GV3101 containing pART27-HSAScon was grown to an OD_{600} of approximately 1 (150 rpm, 48-60 h, 28°C) in LB with antibiotic selection (kan [20 µg/ml], rif [50 µg/ml], tet [5 µg/ml]). The cells were pelleted and resuspended in LB (with no antibiotics). Leaf disks were incubated in the resuspended *A. tumefaciens* cells (30 min, room temperature) and blotted dry on sterile filter paper. The leaf disks were subsequently placed on co-cultivation MS media for 2 days in the dark at 24°C (**Addendum, C.3**) (approximately 10 disks per Petri dish). The plant cytokinin 6-benzylaminopurine (BAP) (shoot generation) and the plant auxin 1-napthaleneacetic acid (NAA) (root generation) were used for plant regeneration (**Addendum, C.3**). Leaf disks were incubated with a 16 h "day light" and 8 h dark cycle, at 24°C. The leaf disks were co-cultivated for 2 days, washed vigorously in water containing cefotaxime (cef) (400 µg/ml) to kill the *A. tumefaciens*, and placed on MS regeneration medium (MS shooting medium) (**Addendum, C.3**) containing the antibiotics kan (50 µg/ml) and cef (400 µg/ml). Leaf discs were replanted on fresh media, with callus tissue touching the media, every two weeks until shoot development. Shoots were removed from regeneration media and placed on MS rooting media (**Addendum, C.3**) containing kan (50 µg/ml) and cef (400 µg/ml). Due to the failure of the plants to root on the MS rooting media, plants could not be hardened off for further analysis.

4.3. RESULTS AND DISCUSSION

4.3.1. Initial attempt at cloning the virus specific transformation vectors

As the main focus of this study, a vector based on pHannibal (Wesley *et al.*, 2001), had to be constructed that contained virus specific CP gene segments of two grapevine viruses, GLRaV-3 and GRSPaV. pHannibal (**Figure 2.11**) (Wesley *et al.*, 2001), which is itself based on pART7 (Gleave, 1992), contains two multiple cloning sites (MCS) situated 5' and 3' to the pyruvate orthophosphate dikinase (pdk) intron. These facilitate the cloning of the sense and antisense conformations of a specific sequence. The 5' MCS contains 3 restriction enzyme sites (5' to 3'): *Xho*I, *Eco*RI and *Kpn*I. The 3' MCS contains 4 restriction enzyme sites (5' to 3'): *Cla*I, *Hin*dIII, *Bam*HI and *Xba*I. The pdk intron functions as a hairpin forming sequence, facilitating the formation of dsRNA when the complementary sequences in the transcribed mRNA hybridise.

Initial primers were designed to mediate the directional cloning of the CP gene segments of both GRSPaV and GLRaV-3 within the same construct. Sense and antisense conformations of the CP genes were to be cloned within the pHannibal vector to induce the formation of multiple-virus specific dsRNA once expressed within transformed plants (Wesley *et al.*, 2001). It is generally accepted that the 5' region of genes are more conserved, thus primers were designed to amplify segments from the 5' regions of the GRSPaV and GLRaV-3 CP genes. It has been shown that dsRNA templates of between 98 bp and 853 bp are sufficient for efficient RNA silencing of a homologous RNA or mRNA (Wesley *et al.*, 2001). *Grapevine rupestris stem pitting-associated virus* specific primers (RSP-Sense-Forw, RSP-Sense-Rev, RSP-A.Sense-Forw, RSP-A.Sense-Rev) were based on the incomplete CP gene sequence of the first SA GRSPaV isolate that consisted of most of the CP gene from the 5' region (**Figure 3.2**). The GRSPaV primers were designed to amplify a 336 bp region of the CP gene. *Grapevine leafroll-associated virus 3* specific primers (LR3-Sense-Forw, LR3-Sense-Rev, LR3-A.Sense-Forw, LR3-A.Sense-Rev) were based on the GLRaV-3 CP gene sequence (Genbank: accession number AF037268). The GLRaV-3 primers were designed to amplify a 291 bp region of the CP gene. Both primer sets were designed with restriction enzyme recognition site-containing extensions that would facilitate cloning in pHannibal in both the sense and antisense conformation (**Table 4.1**). The LR3-A.Sense-Rev primer contains a *Cla*I RE site. The *Cla*I RE is *dam*-methylation sensitive, and will not digest *Cla*I RE site containing plasmids extracted from methylating bacteria. Cells from a *dam*-methylation negative (*dam*-) *E. coli* GM41, were rendered competent using the calcium-chloride method (**Addendum, B.2**). When necessary to digest plasmids with *Cla*I, the plasmid in question was first transformed into competent *E. coli* GM41 cells.

The pHannibal/pART27 binary vector system was obtained from P. Waterhouse. The GRSPaV and GLRaV-3 sense and antisense CP gene segments were amplified using the above mentioned primers in high fidelity PCR reactions. The GRSPaV CP gene segments were amplified using a pGem®-T Easy clone of the incomplete CP gene of the first SA GRSPaV isolate as template (**Section 3.3.1**). The GLRaV-3 CP gene segments were amplified using a pGem®-T Easy clone of the CP gene of a SA GLRaV-3 isolate (courtesy of H. Gardner) as template. Polymerase chain reaction products were separated by means of agarose gel electrophoresis, excised and gel purified.

The primers used to amplify the CP gene segments contained 3 extra bps on the 5' ends to facilitate restriction digestion. Thus, PCR products were digested directly. Each CP gene segment was directionally cloned into pHannibal, or pHannibal already containing the complementary CP gene segment, through double digestion of vector and insert, ligation and transformation. Resultant colonies were screened using insert specific PCR. Using this

method a GRSPaV specific vector (pHann-RSPCPs/as) and a GLRaV-3 specific vector (pHann-LR3CPs/as) was constructed. The GLRaV-3 sense and antisense CP segments were also cloned into the pHann-RSPCPs/as vector, resulting in the primary vector containing both GRSPaV and GLRaV-3 complementary CP gene segments (pHann-S/AScon).

For final confirmation of the presence of the CP segments within pHann-RSPCPs/as, pHann-LR3CPs/as and pHann-S/Ascon, multiple digests using the same enzymes used to clone the segments were performed. These multiple digests revealed that none of the final vectors seemed to contain the relevant inserts. To confirm this, digests were performed using the *Not*I RE. *Not*I cleaves a 2960 bp segment out of pHannibal for cloning into pART27. If the CP segments were present the size of this *Not*I digest product would change from e.g. 2960 bp to 4214 bp if both the GRSPaV/GLRaV-3 sense and antisense constructs were cloned. The *Not*I digestions confirmed that the three plasmids did not contain the cloned CP segments.

During the process of understanding why the cloning did not work, it was realised that REs need a certain minimum amount of extra base pairs on *both* ends of a restriction site for it to digest optimally. Although it was known that a RE needs extra bps adjacent to the recognition sequence for efficient digestion, the effect of not having any or having too few bps was underestimated. The optimal number of extra bps necessary for efficient digestion of RE sites close to the ends of PCR products or oligonucleotides, or RE sites close to the ends of a digested vector, can be found in New England Biolabs (2001). Also, Sambrook and Russel (2001) recommend that when RE sites present within a MCS are chosen for directional cloning, they should not be within 12 bp of each other. Thus it was virtually impossible to achieve ligation of digested PCR products and pHannibal digested with enzymes that digest immediately adjacent RE sites. To remedy this error it was decided to change the RE sites present in the PCR primer extensions in such a way that the new RE sites chosen for cloning, present within the MCS, were separated by another RE site. This created a 6 bp gap between the chosen RE sites for directional cloning, which was thought to be enough to facilitate efficient digestion. It was also decided to clone all PCR products with RE site containing extensions into the pGem®-T Easy vector before digestion with the relevant REs. This resulted in the ability to determine if both RE sites present in the cloned segment were efficiently digested, through the presence of a band at the expected size of the cloned PCR product and a band at the size of the pGem®-T Easy vector (± 3 kb) after digestion and gel electrophoresis. Two pHannibal specific primers (pHannIntr-Forw, pHannIntr-Rev) were designed for sequencing of inserts and determination of insert orientation in pHannibal through PCR with a vector specific and insert specific primer. pHannIntr-Forw is situated upstream of the 5' MCS of pHannibal, and pHannIntr-Rev is situated downstream of the 3'

MCS of pHannibal., thus making it possible to sequence inwards (toward the intron and the two MCSs) from these primers.

Two potential explanations exist for the false positive PCR results indicating successful cloning. In the first possibility the use of insert specific primers for colony PCR screening, and not one vector and one insert specific primer, would have produced the false positive results if contaminating DNA was present. However, when a water negative control was performed there was no amplification present (results not shown). In the second, due to inefficient digestion of the vector and insert, a ligation might have resulted in a very large number of religated vector and a very small number of ligated vector and insert. Subsequent transformation might have resulted in transformation of many copies of the religated vector and a few copies of the vector/insert. In such a case PCR screening would still have had yielded a positive result, but digestion of extracted plasmids would show that the transformed *E. coli* DH5α contained only re-ligated vector.

4.3.2. "Ligation-PCR" for the amplification of the multiple-virus constructs

A strategy was devised to create the pHannibal construct containing sense and antisense CP segments of both GRSPaV and GLRaV-3, without having to order new primers. During this strategy, named "ligation-PCR", the PCR amplified GRSPaV sense CP segment (RSP-Sense-Forw and RSP-Sense-Rev) and GLRaV-3 sense CP segment (LR3-Sense-Forw and LR3-Sense-Rev) were individually digested with *Eco*RI. Although the 3 extra bps on the 5' extension were not enough for optimal digestion, it was hoped that partial digestion might still take place. The digested CP segments were gel purified after agarose gel electrophoresis. A ligation was performed using both *Eco*RI digested CP segments. The resultant ligation product was used as the template for a high fidelity PCR reaction, using the RSP-Sense-Forw and LR3-Sense-Rev primers to amplify the sense "construct". A similar PCR was performed using the RSP-A.Sense-Forw and LR3-A.Sense-Rev primers to amplify the antisense "construct". The "ligation-PCR" proved successful (**Figure 4.1**), and the 651 bp PCR products were gel purified and cloned into the pGem®-T Easy vector (pGem-Scon, pGem-AScon), pGem-Scon and pGem-AScon was sequenced using the T7-Prom and SP6 primers, confirming that the cloned inserts were indeed the sense- and antisense "constructs". The sense and antisense constructs would be cut out of pGem-Scon and pGem-AScon, and subsequently directionally cloned into digested pHannibal.

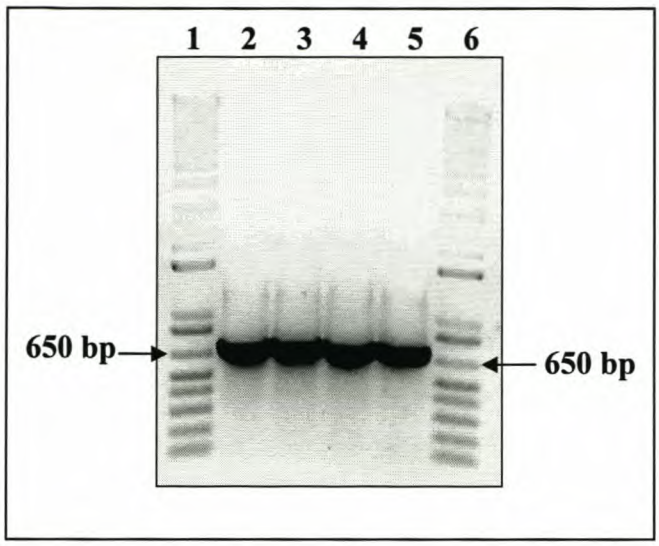

Figure 4.1. "Ligation-PCR" of the sense "construct" and antisense "construct". Lanes 1 and 6: 1kb+ marker. Lanes 2 and 3: PCR amplified GRSPaV/GLRaV-3 sense "construct" (651 bp). Lanes 4 and 5: PCR amplified GRSPaV/GLRaV-3 antisense "construct" (651 bp).

4.3.3. Cloning of the multiple-virus pHannibal vector containing the complementary GRSPaV and GLRaV-3 CP gene segments

pGem-SCon and pHannibal were sequentially digested with *Kpn*I (*Kpn*I requires the addition of BSA (100µg/ml) for digestion) and *Xho*I. The digested sense "construct" was gel purified and ligated to the digested gel purified pHannibal, which was subsequently transformed and the resultant colonies screened for the presence of the insert. Digestion of the vector with the REs used for the cloning of the insert, and a vector/insert specific PCR using the pHannIntr-Forw and LR3-Sense-Rev primers, confirmed that the sense "construct" was cloned. The plasmid was named pHann-SCon.

pGem-ASCon and pHann-SCon were extracted from the methylation negative *E. coli* GM41 cells in which they were cloned. Both plasmids were double digested with *Cla*I and *Bam*HI, and the digested antisense "construct" and pHann-SCon gel extracted and ligated. The resultant clones were screened using digestion and PCR, but none contained the insert. The process was repeated several times, however the antisense "construct" could not be cloned into pHann-SCon. To induce efficient digestion of the vector, pHann-SCon was sequentially digested by first digesting with *Cla*I, followed by gel purification and digestion with *Bam*HI. The process was repeated with the initial digest being performed with *Bam*HI and the second digest with *Cla*I. Ligations using both sequentially digested vectors and the digested insert proved unsuccessful. It was reasoned that, although it is not mentioned within the literature (Wesley *et al.*, 2001), the pHannibal vector was not designed to routinely clone more than one

insert within each MCS, and that for efficient digestion and ligation the RE sites on the ends of each MCS should be used. For the 5' MCS *Xho*I and *Kpn*I, and for the 3' MCS *Cla*I and *Xba*I, should be used. This would appear to make sense as both *Cla*I and *Xba*I are *dam*-methylation sensitive REs.

It was decided to clone the antisense "construct" non-directionally, and to determine orientation with PCR and sequencing after the antisense "construct" was cloned. A new forward primer (RSPasconFN), containing a *Cla*I RE site, was designed. The antisense "construct" was amplified from pGem-ASCon using the RSPasconFN and LR3-A.Sense-Rev primers and cloned into the pGem®-T Easy vector (pGem-ASConNew). The pGem-ASConNew and pHann-SCon plasmids were extracted from *E. coli* GM41 and digested with *Cla*I. Initial ligations and transformations resulted in very few colonies, which tested negative for the presence of the antisense "construct" using vector/insert specific PCRs. Through the isolation, digestion and gel purification of large amounts of vector and insert, and the optimisation of ligations, conditions were created for efficient ligations. The inclusion of PEG 8000 and the incubation of the insert, vector, and ddH_2O at 65°C for 10 min resulted in much more efficient ligations. The ligation efficiency was increased to such an extent that the amount of colonies per ligation/transformation increased from ±20 colonies to >1000. Polyethylene glycol absorbs H_2O molecules, thus increasing the vector and insert concentration. The incubation (10 min, 65°C) causes the plasmid DNA to de-clump, increasing the chances of digested vector and insert ends coming into proximity. Subsequently the extracted plasmids were screened through digestion to determine if the insert was present. Plasmids containing the insert were screened with a vector/insert specific PCR to determine the orientation of the insert. The plasmids containing the antisense "construct" in the complementary orientation to the sense "construct" were sequenced using the pHannIntr-Forw and pHannIntr-Rev primers, which confirmed that they contained both the sense "construct" and antisense "construct" in the required complementary conformation. The final vector was named pHann-SAScon (**Figure 4.2**).

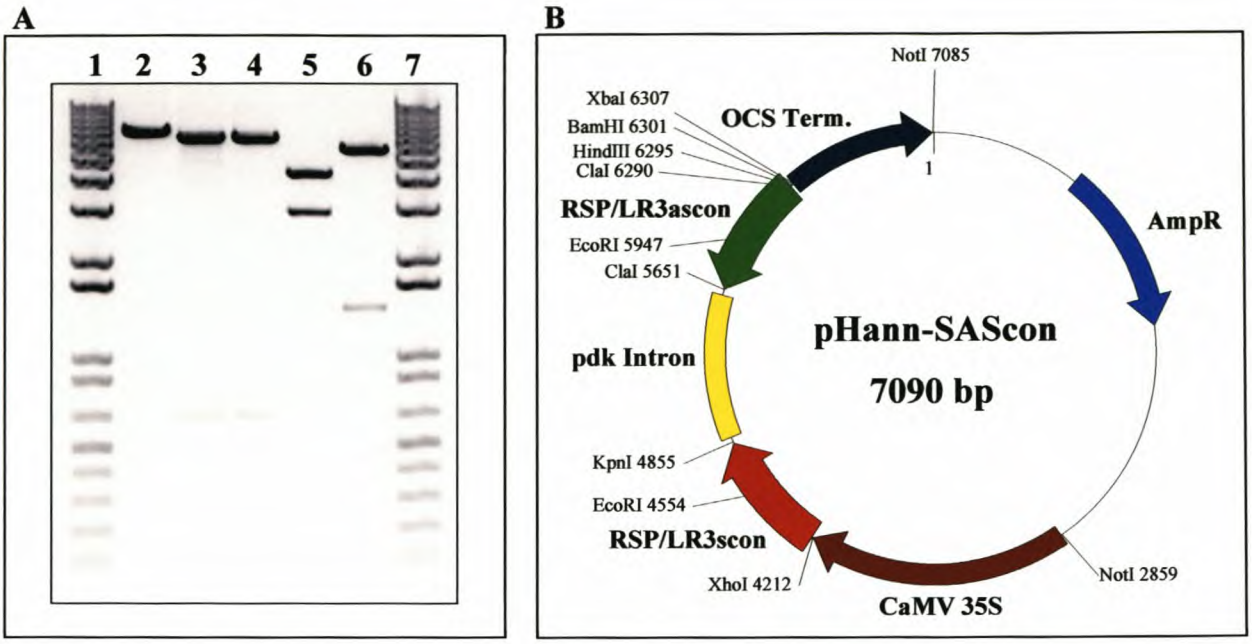

Figure 4.2. pHann-SAScon **(A)** Multiple digests of pHann-SAScon. Lane 1 and 7: 1kb+ marker. Lane 2: *Bam*HI linearised vector (7090 bp). Lane 3: *Kpn*I/*Xho*I excised sense "construct" (~651 bp). Lane 4: *Cla*I excised antisense "construct" (~651 bp). Lane 5: *Not*I digested vector. The top band (~4226 bp) containing the complementary CP gene segments is cloned into pART27. Lane 6: *Eco*RI digested vector. *Eco*RI digests the *Eco*RI restriction sites incorporated within the sense and antisense "constructs" during the "ligation-PCR" **(Section 4.3.2)**, resulting in a ~1393 bp band. **(B)** Restriction map of pHann-SAScon.

4.3.4. Cloning of the GLRaV-3 pHannibal vector

During the creation of the pHann-SAScon vector, GLRaV-3 CP specific primers were designed. These primers would have facilitated the directional cloning of the GLRaV-3 sense CP gene segment into the *Xho*I and *Kpn*I sites of the 5' MCS (LR3CP-S-Fdir, LR3CP-S-Rdir), and the antisense segment into the *Xba*I and *Hin*dIII sites of the 3' MCS (LR3CP-AS-Fdir, LR3CP-AS-Rdir). These primers were designed to amplify a 463 bp segment of the 5' region of the GLRaV-3 CP gene. After the completion of the pHann-SAScon vector it was decided to redesign the LR3CP-S-Rdir and LR3CP-AS-Fdir primers to contain *Xho*I and *Hin*dIII RE sites respectively. The new primers were named LR3CPsRN and LR3CPasFN. The strategy was adapted from directional cloning to non-directional cloning due to the difficulty experienced with directional cloning into the limited MCSs of pHannibal. Due to the non-directional nature of this cloning, the cloned segments could insert in either the forward of reverse orientation, and the vector could also potentially re-ligate. Thus the presence of the insert and its orientation had to be determined using other methods. Colonies resulting from ligation/transformations could be the screened for the presence of the insert through plasmid extraction and restriction digestion. If the segment was present its

orientation could be determined with restriction digestions of novel RE sites that lie close the ends of the segment, or with a vector/insert specific PCR. For final confirmation of both presence of the insert and conformation, the inserts could be sequenced within pHannibal using the pHannibal specific primers, pHannIntr-Forw and pHannIntr-Rev.

The GLRaV-3 CP gene segments were PCR amplified using a pGem®-T Easy clone of the CP gene of a SA GLRaV-3 isolate (courtesy of H. Gardner) as template. The LR3CP-S-Fdir and LR3CPsRN primers were used to amplify the sense segment, and the LR3CPasFN and LR3CP-AS-Rdir primers were used to amplify the antisense segment. The sense and antisense GLRaV-3 CP segment PCR products were cloned into the pGem®-T Easy. These plasmids were named pGem-LR3CPs and pGem-LR3CPas respectively. pHannibal and pGem-LR3CPs were digested with *Xho*I and the digested pHannibal and GLRaV-3 sense CP segment gel purified, ligated and transformed into competent *E. coli* DH5α. Plasmid was extracted from the resultant colonies and screened with *Xho*I digestion for the presence of the insert, and a vector/insert specific PCR was performed on the plasmids containing the insert to determine the insert orientation. This plasmid was named pHann-LR3CPs and was transformed into competent *E. coli* DH5α. pHann-LR3CPs and pGem-LR3CPas were digested with *Hin*dIII and the digested pHann-LR3CPs and GLRaV-3 antisense CP segment gel purified, ligated and transformed into competent *E. coli* DH5α. Plasmid was extracted from the resultant colonies and screened with *Hin*dIII digestion for the presence of the insert, and a vector/insert specific PCR was performed on the plasmids containing the insert to determine the insert orientation. The plasmids containing the GLRaV-3 antisense CP segment in the complementary orientation to the GLRaV-3 sense CP segment were sequenced using the pHannIntr-Forw and pHannIntr-Rev primers, which confirmed that they contained both the sense and antisense segments in the required complementary conformation. The final vector was named pHann-LR3CPsas (**Figure 4.3**).

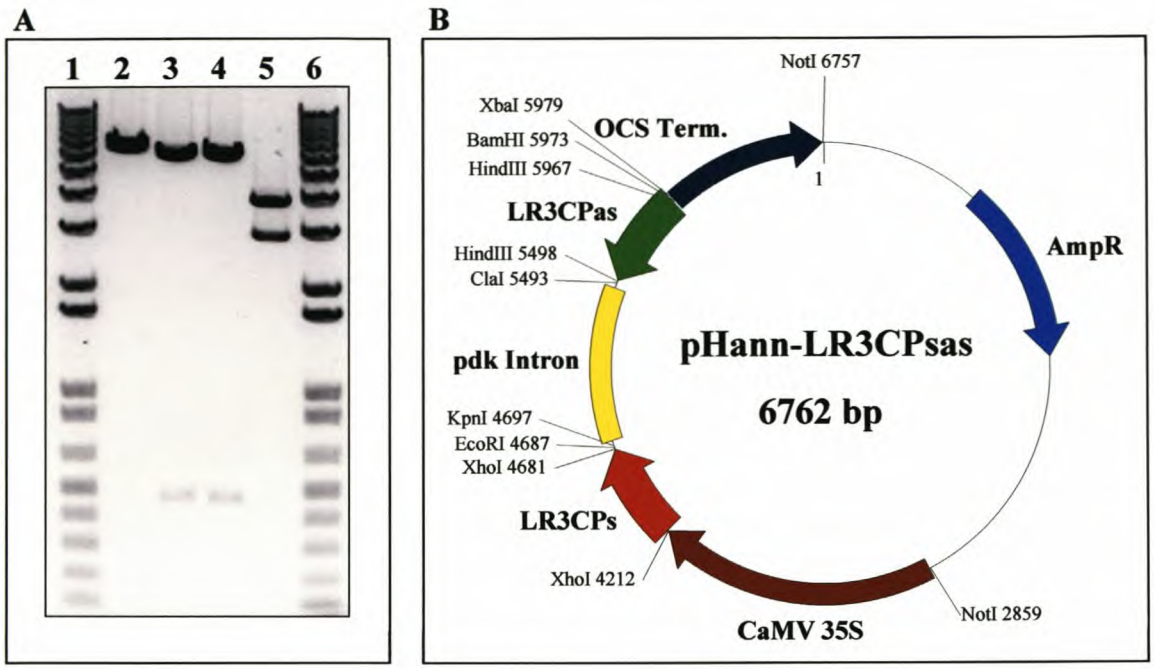

Figure 4.3. pHann-LR3CPsas **(A)** Multiple digests of pHann-LR3CPsas. Lane 1 and 6: 1kb+ marker. Lane 2: EcoRI linearised vector (6762 bp). Lane 3: *Xho*I excised sense GLRaV-3 CP gene segment (~463 bp). Lane 4: HindIII excised antisense GLRaV-3 CP gene segment (~463 bp). Lane 5: *Not*I digested vector. The top band (~3885 bp), containing the complementary CP gene segments, is cloned into pART27. **(B)** Restriction map of pHann-LR3CPsas.

4.3.5. Cloning of the GLRaV-2 pHannibal vector

During the course of this study an attempt was made by another student in the laboratory to clone the sense and antisense CP segments of GLRaV-2 into pHannibal. Although the cloning was unsuccessful, the GLRaV-2 sense and antisense CP segments had been cloned into the pGem®-T Easy vector. These clones were obtained (courtesy of L. Wentzel), and the plasmids extracted. New primers were designed: LR2CPsRN containing a *Xho*I RE site in the 5' extension, and LR2CPasFN containing a *Cla*I RE site in the 5' extension. These primers facilitated the non-directional cloning of the sense and antisense CP segments when used in conjunction with the existing complementary GLRaV-2 sense and antisense CP segment specific primers containing the same RE sites in their extensions. The primers were designed to amplify a 491 bp segment of the 5' region of the GLRaV-2 CP gene. Due to the non-directional cloning strategy employed, the same strategies as described in **Section 4.3.4** were used to determine if the segment was cloned, and to determine the orientation of the cloned segment.

These new GLRaV-2 CP gene specific primers were used along with the existing complementary primers (courtesy L. Wentzel) to amplify the GLRaV-2 sense CP segment

(LR2CP-S-Forw and LR2CPsRN) and the antisense CP segment (LR2CPasFN and LR2CP-AS-Rev) in a PCR, using the extracted plasmid clones of the sense and antisense CP segments as template. The resultant sense CP segment contained *Xho*I RE sites on both ends, and the antisense CP segment contained *Cla*I RE sites on both ends. The PCR products were gel purified, cloned into the the pGem®-T Easy vector, and named pGem-LR2CPs and pGem-LR2CPas respectively. pHannibal and pGem-LR2CPs were digested with *Xho*I, and the digested pHannibal and GLRaV-2 sense CP segment gel purified, ligated and transformed into competent *E. coli* DH5α. Plasmid was extracted from the resultant colonies and screened with *Xho*I digestion for the presence of the insert, and a vector/insert specific PCR was performed on the plasmids containing the insert to determine the insert orientation. This plasmid was named pHann-LR2CPs and was re-transformed into competent *E. coli* DH5α. pHann-LR2CPs and pGem-LR2CPas was digested with *Cla*I and the digested pHann-LR2CPs and GLRaV-2 antisense CP segment gel purified, ligated and transformed into competent *E. coli* GM41. Plasmid was extracted from the resultant colonies and screened with *Cla*I digestion for the presence of the insert, and a vector/insert specific PCR was performed on the plasmids containing the insert to determine the insert orientation. The plasmids containing the GLRaV-2 antisense CP segment in the complementary orientation to the GLRaV-2 sense CP segment were sequenced using the pHannIntr-Forw and pHannIntr-Rev primers, which confirmed that they contained both the sense and antisense segments in the required complementary conformation. This final vector was named pHann-LR2CPsas (**Figure 4.4**).

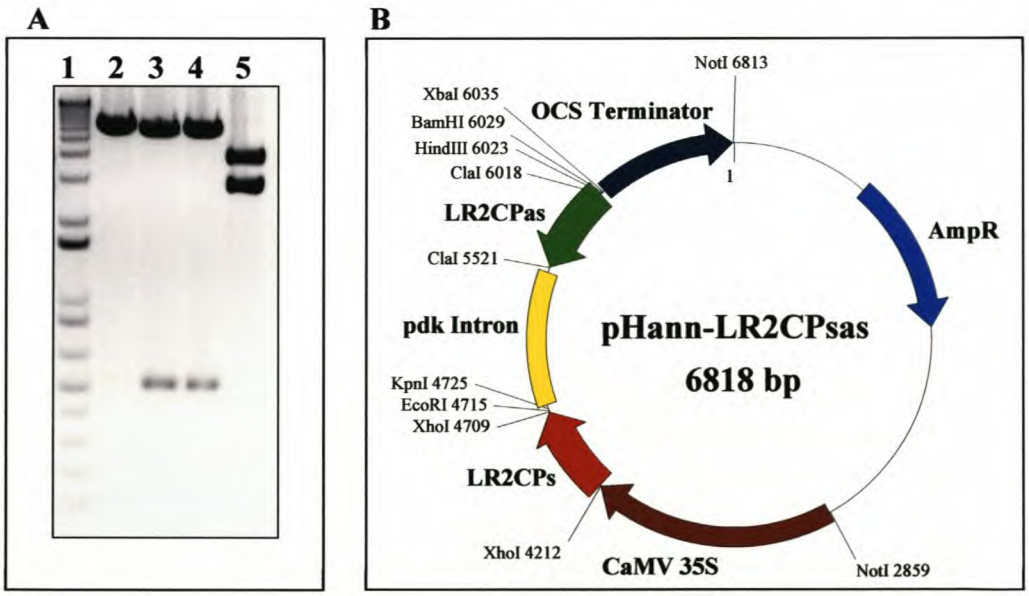

Figure 4.4. pHann-LR2CPsas **(A)** Lane 1: 1kb+ marker. Lane 2: EcoRI linearised vector (6762 bp). Lane 3: *Xho*I excised sense GLRaV-2 CP gene segment (~491 bp). Lane 4: *Cla*I excised antisense GLRaV-2 CP gene segment (~491 bp). Lane 5: *Not*I digested vector. The top band (~3941 bp) containing the complementary CP gene segments is cloned into pART27. **(B)** Restriction map of pHann-LR2CPsas.

4.3.6. Cloning of the Sense/Antisense construct from pHann-SAScon into pART27

pART27 is the binary vector of the pART7 primary cloning vector (Gleave, 1992). pHannibal is based on the pART7 primary cloning vector, which facilitates the excision and transfer of the cloned complementary inserts from pHannibal to pART27 (Wesley *et al.*, 2001). pART27 and pHann-SAScon were digested with *Not*I. The *Not*I digestion of pHann-SAScon resulted in the pHannibal backbone (~2864 bp) and the "HSAScon" cassette (~4226 bp) containing the CaMV 35S promoter, the sense "construct", the pdk intron, the antisense "construct" and the octopine sythase (ocs) terminator. The linearised pART27 (11 667 bp) and the "HSAScon" cassette was gel purified and ligated. The transformation was plated on LB/Agar plates containing kan (20 μg/ml), IPTG (0.16 M) and X-gal (0.016 mg/ml), for blue/white selection of *E. coli* DH5α containing ligated pART27 and the "HSAScon" cassette. The total vector would be 15 893 bp.

Successful cloning of an insert into the *Not*I site of pART27 was first visually assessed. White colonies potentially contained pART27-HSAScon, due to the disruption of the *lac* operon. Several white colonies were chosen and their plasmids extracted. The extracted plasmids were screened by RE digestion. Two suitable enzymes, *Not*I and *Eco*RI, were selected for digestion. If the ligations were successful, the *Not*I digestion would result in the 11 667 bp pART27, and the ~4226 bp "HSAScon" cassette. Due to the presence of *Eco*RI sites between the GRSPaV CP segment and the GLRaV-3 CP segment of both the sense "construct" and the antisense "construct" (**Section 4.3.2**), digestion with *Eco*RI would result in a ~14 500 bp backbone, and ~1393 bp insert, containing the sense GLRaV-3 CP segment, the pdk intron and the antisense GLRaV-3 CP segment. Approximately 87% (20 of 23) of the screened colonies contained the insert. Three of the extracted plasmids containing the insert were chosen for the transformation of *A. tumefaciens*. The final plasmid was named pART27-HSAScon (**Figure 4.5**).

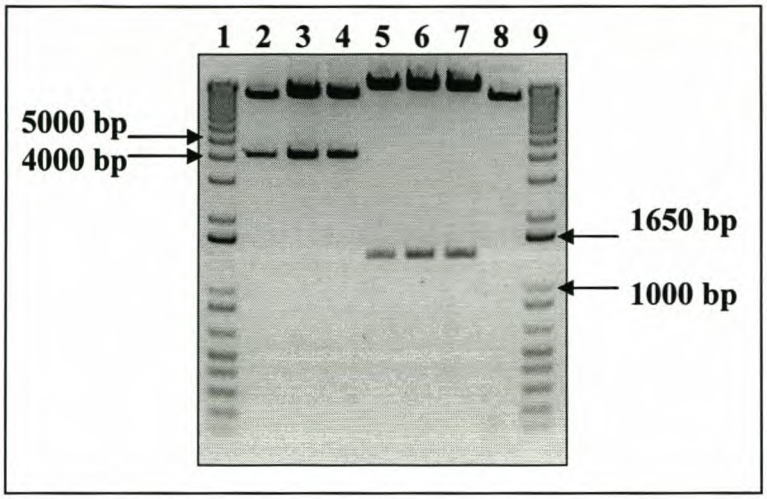

Figure 4.5. pART27-HSAScon digested with *Not*I and *Eco*RI for confirmation of tranformation. Lanes 1 and 9: 1kb+ marker. Lanes 2-4: *Not*I digested pART27-HSAScon isolated from three different single colonies that contained ligated pART-27 and the "HSAScon" cassette from pHann-SAScon. *Not*I excises the ~4226 bp cassette cloned into pART27. Lanes 5-7: *Eco*RI digestion of the same three colonies in the same order. *Eco*RI digests the *Eco*RI restriction sites incorporated within the sense and antisense "constructs" during the "ligation-PCR" (**Section 4.3.2**), resulting in a characteristic ~1393 bp band. Lane 8: *Eco*RI linearised untransformed pART27 (11 667 bp). Note, the vector backbone in lanes 2-4 is the same size as the linearised untransformed pART27. The vector backbone in lane 5-7 is bigger than 12 kb (the top band of 1kb+), after the ~1393 bp segment was excised by *Eco*RI digestion.

4.3.7. Transformation of *Agrobacterium tumefaciens* with pART27-HSAScon

Direct transformation of the pART27-HSAScon vector into *A. tumefaciens* was chosen above tri-parental mating due to it being a simpler and less time consuming method, and because of the availability of competent *A. tumefaciens* cells. *A. tumefaciens* strain GV3101 was chosen for plant transformation. *A. tumefaciens* GV3101 contains the helper plasmid pSoup (formerly known as pJic_Sa rep) that has tet resistance, and can be used with the binary vector pGreen (Hellens *et al.*, 2000).

pART27-HSAScon from three single colonies was directly transformed into competent *A. tumefaciens* GV3101, and plated on LB/agar plates. Transformants containing the correct plasmids were selected on antibiotics (kan [20 µg/ml], rif [50 µg/ml], tet [5 µg/ml]). Resultant single colonies were re-streaked out on LB/agar/kan/rif/tet plates to confirm successful transformation through antibiotic selection and to ensure that selected single colonies were not contaminating *E. coli*. Selected single colonies were screened for the

presence of the sense "construct" with PCR. The colonies that tested positive for the construct were used to grow up *A. tumefaciens* GV3101 for plant transformation (**Figure 4.6**).

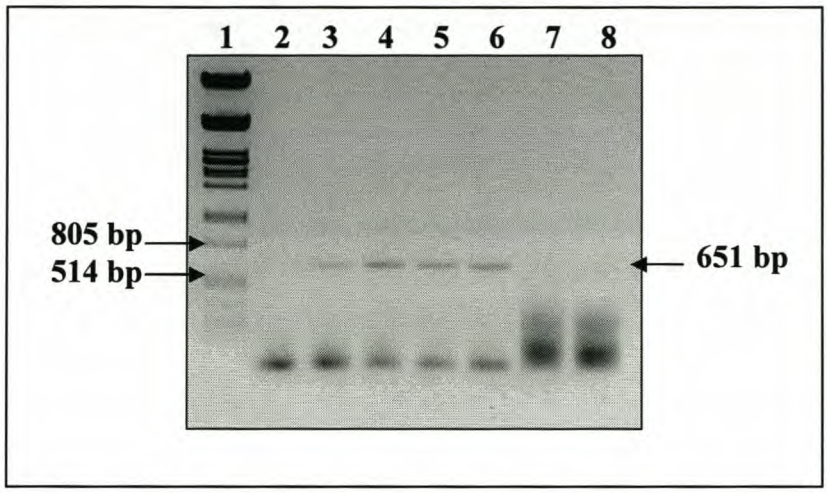

Figure 4.6. PCR to confirm the presence of pART27-HSAScon within transformed *A. tumefaciens* GV3101. Lane 1: λ *Pst*I marker. Lanes 2-7: two colonies from each of 3 *A. tumefaciens* GV3101::pART27ΔHSAScon plates, screened for the presence of the sense "construct" with colony PCR. The lack of a visible band in lane 2, and the complete lack of amplification in lane 7 were probably due to the very low copy number of pART27 in *A. tumefaciens*. Lane 8: PCR negative control with ddH_2O as template.

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

N. tabacum cv. Petit Havana (SR1) leaves from two sources (an *in vitro* plant and hardened off greenhouse plants) were used for the preparation of leaf disks for transformation. Ten leaves from the *in vitro* plant were surface sterilised. The surface sterilization was too severe for the leaves, which were partially burned and bleached. Approximately 40 leaf disks were prepared from the damaged leaves, but these were also partially damaged. Due to the loss of most of the *in vitro* plant leaves, several large leaves were harvested from hardened off greenhouse plants and surface sterilised. The surface sterilization did not have a negative effect on these leaves. Approximately 250 leaf disks were prepared from these leaves for transformation. Two sets of ~100 leaf disks were prepared for the transformation controls. The first set was used for the generation of untransformed negative control plants, without antibiotics in the media. The second set was used for untransformed negative controls with antibiotics in the media, to determine the efficacy of the antibiotics to kill off untransformed leaf disks. This antibiotic control was also used to determine the incidence of "escapes", which are untransformed plants that grow on selection. In conjunction with the transformation of the 250 leaf disks, as mentioned above, 100 *N. tabacum* cv. Petit Havana (SR1) leaf disks from *in vitro* plant material were also transformed by ARC-

Infruitec/Nietvoorbij with the *A. tumefaciens* GV3101 containing the pART27-HSAScon vector (*A. tumefaciens* GV3101::pART27ΔHSAScon).

The damaged leaf disks from the *in vitro* plants and the leaf disks from the greenhouse plants were incubated in the *A. tumefaciens* GV3101::pART27ΔHSAScon cell suspension for 30 min at room temperature, blotted dry and kept on co-cultivation media for 2 days. Almost no *A. tumefaciens* could be seen after 2 days. The leaf disks of the two control sets were also incubated on co-cultivation media for 2 days. All the leaf disks were transferred to regeneration media with antibiotics (kan (50 μg/ml) and cef (400 μg/ml)), except for the untransformed negative control leaf disks for the generation of negative control plants, which were transferred to MS regeneration media without antibiotics.

Callus developed on the transformed leaf disks between 2 and 4 weeks after infection with *A. tumefaciens* GV3101::pART27ΔHSAScon. The shoots did not develop at similar times after infection and callus development, but developed sporadically between 4 and 8 weeks after infection. Approximately 20 shoots developed on the MS regeneration media. Of these, 4 shoots developed quickly. All 20 shoots were transferred to the MS rooting media. The four well developed shoots continued to develop for ~2 weeks after being transferred. No further shoot development was observed after ~2 weeks and no root development was observed. None of the remaining transferred shoots developed any further or developed roots on the MS rooting media. The leaf disk transformation performed by ARC-Infruitec/Nietvoorbij resulted in 20 well developed shoots. These were transferred to MS rooting media and five developed rudimentary roots. However, plants did not continue to develop further.

Callus started to develop on the negative control antibiotic free leaf disks after ~2 weeks. Shoots started to develop within 4 weeks. After 6 weeks, shoots were excised from the callus and transplanted to MS rooting media. Roots started to develop after ~2 weeks on MS rooting media, but most root development was initially above the media and not within it. Root development within the media was observed ~2 weeks after initial root development. Negative control plants were not hardened off due to the lack of rooted transformed plants. The negative control leaf disks on the antibiotic containing MS regeneration media were transferred to new media every two weeks for the duration of the plant transformation experiment. These leaf disks gradually died, and no shoots developed during the duration of the experiment.

The 20 plants at ARC-Infruitec/Nietvoorbij did not appear to have been transformed (**Figure 4.7**). Five of the plants developed roots. The leaves of these plants were a dark green colour, indicating that the plants were resistant to the selective antibiotic kan, and thus contained the

"HSAScon" cassette from pART27. The failure of the plants to develop further implies that they were not transformed and that the plants were all escapes. This could possibility be due to the transgene e.g. homology between sequences within the transgene and areas within the plant genome resulting in homologous recombination, which would disrupt the expression of the construct. Zubco *et al.* (2000) observed the spontaneous loss through homologous recombination of a transgenic *nptII* gene, and the adjacent transgene sequences, in 3 out of 23 transgenic tobacco shoots. This is several orders of magnitude higher than has previously been observed. The disruption of endogenous genes through homologous recombination has also been demonstrated in *Arabidopsis* (Kempin *et al.*, 1997; Miao and Lam, 1995). Molecular analysis was not performed to determine if transformation was successful, due to the obvious inability of the plants to regenerate and develop completely on selective media.

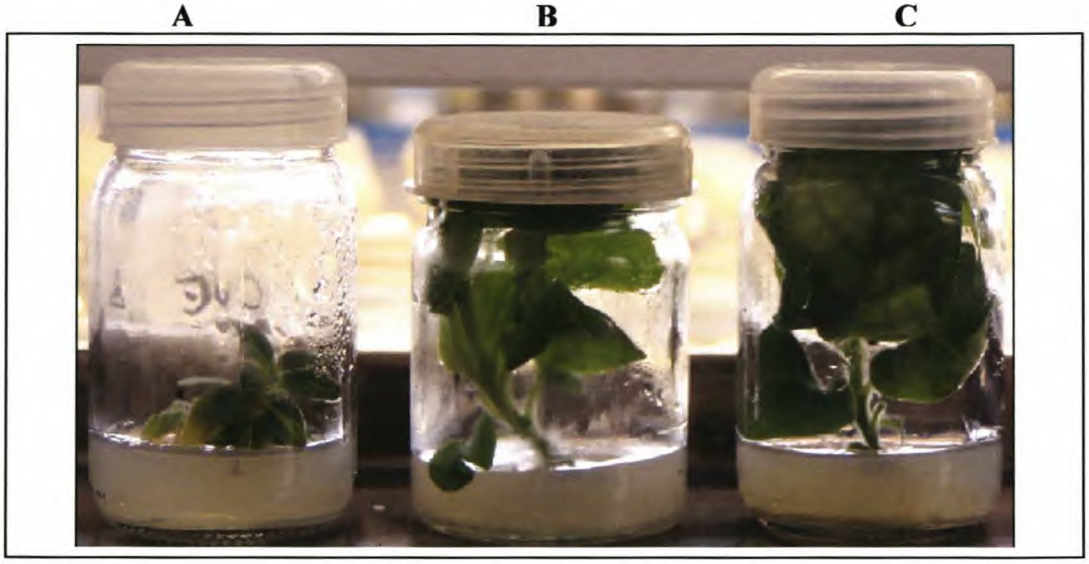

Figure 4.7. Transformation of *N. tabacum* cv. Petit Havana (SR1) with *A. tumefaciens* GV3101::pART27ΔHSAScon. **(A)** *A. tumefaciens* GV3101::pART27ΔHSAScon transformed tobacco plant on rooting media with selection. **(B)** Untransformed negative control tobacco on rooting media, regenerated at the same time as plant A. **(C)** Unrelated transformed tobacco on rooting media with selection, regenerated at the same time as plant B (this plant was included in the photo to depict what the plant on the left should look like). Although roots can't bee seen on this photo, rudimentary roots are present on plant A. Plant B and C have extensive roots. All three plants were transferred to new rooting media 2 months prior to this photo. Plant A had not developed any further since the transfer, and yellowing of the leaves can be seen. The "transformed" plant was clearly not successfully transformed, due to the lack of further shoot and root development and the yellowing of the leaves. Other potential transformants looked similar and also did not develop further.

Although it isn't clear why the transformation failed, there were several steps where the transformation might have gone awry. Certain plant transformation protocols indicate that the leaf disks should be co-cultivated for 4 days until extensive *A. tumefaciens* growth has taken

place. The co-cultivation for 2 days might have resulted in inefficient infection and thus transformation. The leaf disks from the *in vitro* plant might have been extensively damaged by the surface sterilisation, which may have inhibited transformation. The hardened off greenhouse plants were very big when compared to the *in vitro* plant used. The possibility that flower buds were beginning to develop in the greenhouse material can not be conclusively excluded, which would significantly influence the probability of successfully transformation of the plants. Also, the co-cultivation media and MS regeneration media did not contain NAA as recommended by Svab *et al.* (1995). Although plant transformation/regeneration is routinely performed in this way by ARC-Infruitec/Nietvoorbij, the absence of auxins within the shooting media may have had a long term detrimental effect on the ability of the plants to properly develop roots due to hormonal imbalance and an excess of BAP. Another possibility is that significant sequence homology might exist between the genome of *N. tabacum* and the GRSPaV and GLRaV-3 CP gene segments used for the construction of the vector. Previous attempts to transform the complete CP gene of GLRaV-3 into *N. tabacum* in a CP-MR strategy repeatedly resulted in failure, which may indicate that there is some post-transcriptional interaction resulting in the failure of tobacco plants to regenerate. A BLAST (http://www.ncbi.nlm.nih.gov/) search between the CP gene sequences used and the genbank nucleotide database did not reveal any homology with known *N. tabacum* sequences, however, the genome of *N. tabacum* has not been fully sequenced. Several near-perfect homologous sequences (~15-30 bp in length) with the genome of the model plant *A. thaliana* were found. A BLAST search between the sequence of the pdk intron within pHannibal and the genbank nucleotide database did not reveal any significant homology with *N. tabacum* or any other related or model plants (Altschul *et al.*, 1990).

To eliminate possible oversights or mistakes during the experimental procedures of the tobacco transformation, the constructs used for the transformation (pART27-HSAScon) have been re-transformed into *E. coli* DH5α at very low concentrations to ensure that each resultant single colony contained only plasmids from a single transformation event. These plasmids were isolated and subjected to the *Not*I and *Eco*RI digestions, and the *Kpn*I/*Xho*I and *Cla*I digestions, to confirm the presence of the "HSAScon" cassette containing the sense and antisense "constructs". Sequencing will still be performed using the pHannIntr-Forw and pHannIntr-Rev primers as a final check. Subsequent to confirmation, these constructs will again be used for direct transformation of *A. tumefaciens*, which will be used in another attempt to transform *N. tabacum* cv. Petit Havana (SR1). For this transformation plant material will be used that has never been subjected to regeneration through plant tissue culture, and selection for potential transformants will be performed on 100-120 μg/ml kan (as recommended by ARC-Infruitec/Nietvoorbij) to reduce the amount of escapes that develop on shooting media.

Chapter 5: Conclusion

The primary aim of this study was the construction of three plant transformation vectors that would potentially facilitate specific multiple-virus and single-virus resistance to economically important grapevine viruses in the South African viticulture industry. Also, the multiple-virus construct would be used to transform *N. tabacum* cv. Petit Havana (SR1). Subsequent to this study, the efficiency of the multiple-virus construct as an inducer of multiple-virus resistance will be tested in a virus-induced gene silencing (VIGS) based analysis. A secondary aim was the expression of the GRSPaV CP in bacteria to be used for the production of GRSPaV specific antibodies. The antibodies will be used by parties within the viticulture industry for the detection of GRSPaV in potential propagation material.

The three plant transformation vectors were constructed. Each virus-specific vector contains complementary grapevine virus CP gene segments cloned upstream and downstream of an intronic sequence in such a way as to induce the formation of hairpin-dsRNA (hpRNA) upon transcription. Transgenic plants expressing the construct, containing the complementary segments of both GRSPaV and GLRaV-3, should result in expression and formation of multiple-virus specific hpRNA. This will be tested by infecting the transgenic tobacco with a recombinant PVX virus incorporating sequences homologous to the GRSPaV and GLRaV-3 sequences within the hpRNA, which will induce PTGS. The presence of sequences from two different grapevine virus pathogens within the dsRNA-producing construct should induce targeted gene silencing of both viruses. Because GLRaV-3 is economically the most important grapevine virus pathogen in South Africa, a GLRaV-3 specific vector was also constructed. If the multiple-virus vector fails to introduce resistance against one or both viruses, the GLRaV-3 vector could still be used to introduce resistance to GLRaV-3. In conjunction, the GLRaV-3 vector facilitates the introduction of GLRaV-3 resistance without the expression of extraneous sequences. To demonstrate the proof of concept of induced virus resistance through PTGS, a GLRaV-2 specific vector was constructed. The GLRaV-2 construct can be used for the transformation of *N. benthamiana*, which is a host of GLRaV-2. The transformed plants can be inoculated with a pure source of GLRaV-2. Resistance to GLRaV-2, exhibited by the transformed *N. benthamiana* plants, would indicate successful gene silencing of the targeted virus. Both GRSPaV and GLRaV-3 infect only grapevine. Therefore, due to the difficulty in transforming grapevine and the long regeneration time of grapevine, it is more feasible to test the system by transforming tobacco with a self complementary GLRaV-2 construct and infecting with GLRaV-2. The limited MCSs of pHannibal, and the difficulty of digesting immediately adjacent or very close RE sites, makes the routine directional cloning of more than one gene segment into the MCSs of pHannibal

very difficult. For the construction of a pHannibal-based vector containing more than one gene segment per MCS, it is recommended that the sense and antisense "construct" first be assembled in an intermediate vector with a more versatile MCS before cloning into pHannibal.

The multiple-virus cassette was transferred from pHannibal to pART27, which was subsequently used in *A. tumefaciens* mediated transformation of *N. tabacum* cv. Petit Havana (SR1). Unfortunately transformation was unsuccessful. Following this study transformation will once again be attempted, and successful integration of the construct within the plant genome confirmed with Southern analysis. Due to the importance of the primary construct for imparting multiple-virus resistance, an alternative VIGS-based strategy was devised to demonstrate the proof of concept of multiple-virus resistance, while at the same time testing the efficiency of the GRSPaV/GLRaV-3 construct to introduce resistance in transgenic plants. For this strategy a recombinant replicating virus vector was constructed, consisting of *potato virus X*, *green fluorescent protein* (GFP) as a screenable marker, and the GRSPaV/GLRaV-3 sense construct used to design the primary vector (courtesy M.-J. Freeborough). *Potato virus X* can infect both *N. benthamiana* and *N. tabacum*, which makes it an ideal vector to test PTGS-based resistance in tobacco before applying the system to grapevine. This recombinant virus will be used to infect plants transformed with the *A. tumefaciens* (GV3101)::pART27ΔHSAScon, and gene silencing of the transformed sequences tested with northern analysis. Resistance to the recombinant virus and the lack of virus specific RNA, transcribed from the transformed construct and the replicating virus, will indicate successful gene silencing. This will also be the proof of concept that multiple-virus resistance can be induced through the use of more than one virus-derived gene or sequence in a single self complementary construct. It has been shown that infection of a plant with PVX carrying a transgene will silence a homologous endogenous plant gene or transgene in *N. benthamiana* and *N. tabacum* while at the same time inducing targeted resistance against PVX through a PTGS-based mechanism (Ruiz *et al.*, 1998; English et al., 1996).

The GRSPaV CP gene of a South African isolate of the virus was isolated. Sequence variability of GRSPaV caused difficulty with the isolation of the gene. It is recommended that when isolating genes of viruses that potentially exhibit a high degree of sequence variability, primers with degenerate 3'-nts are used. The isolated GRSPaV CP gene was used for the expression of the CP. Subsequent to this study, large scale expression of this protein will be performed, the protein isolated and purified from the insoluble fraction and used for the generation of GRSPaV CP specific polyclonal antibodies. The South African viticulture industry relies on biological indexing and serological screening of propagation material for virus infection, but do not currently have antibodies available for the detection of GRSPaV.

We hope that the production of such antibodies will aid in the control of GRSPaV, which is believed to be primarily spread through infected propagation material.

To the best of our knowledge this is the first attempt at the introduction of multiple-virus resistance utilising the principles and processes of PTGS. Multiple-virus resistance has previously been induced through the transformation of plants with constructs containing complete multiple virus CP genes that each contain a promoter and terminator, and is completely transcribed and translated as in CP-MR (Gonsalves *et al.*, 2000). Gonsalves *et al.* (2000) successfully introduced multiple-virus resistance and showed that the resistance was induced through PTGS. However, they followed a traditional PDR based approach where they transformed *N. benthamiana* with a construct containing the complete CP gene of *turnip mosaic virus* (TuMV) and a 218 bp N gene segment of *tomato spotted wilt virus* (TSWV). It was reasoned that the TuMV CP gene was an inducer of PTGS, and that multiple-virus resistance could be induced through the use of transformation constructs containing such an PTGS inducing sequence linked to other non-inducing virus derived sequences (Gonsalves *et al.*, 2000) Although no Southern analysis results were presented it is probable that multiple transformation events of the TuMV/TSWV construct, in complementary orientations, led to the formation of virus specific dsRNA, which induced PTGS.

The use of PTGS for the induction of virus resistance has several advantages over previously used methods. Post-transcriptional gene silencing is a naturally occurring anti-viral plant mechanism. The specificity of PTGS is determined by the genome of the virus, the signal that activates PTGS spreads systemically through the plant ahead of the infecting virus and the signal is amplified through a process named transitivity, which increases the effectiveness of gene silencing. Thus, PTGS is adaptive, mobile and specific. A major advantage of PTGS-based resistance over most PDR based methods is that PTGS results in degraded RNA. No functional virus genes or proteins are produced, and no transgenic RNA is present that could become engaged in RNA events. Virus resistance based on PTGS seems to fulfill the current high demands concerning biosafety.

The application of plant virus resistance mechanisms based on PTGS is ideal for the introduction of targeted plant virus resistance. Because the specificity determinant of PTGS is the virus' genome, the mechanism could be adapted for the introduction of multiple-virus resistance. We believe that the transformation of *N. tabacum* with our GRSPaV/GLRaV-3 specific transformation vector, pART27-HSAScon, will result in efficient gene silencing of a recombinant virus vector containing homologous sequences, resulting in targeted resistance. Thus, the first steps have been taken to ultimately introduce multiple-virus resistance in grapevines. Through the introduction of PTGS-based multiple-virus resistance to grapevine

viruses and more efficient detection of GRSPaV infection within grapevine propagation material, South African grapevines will be improved and the viticulture industry significantly enhanced.

Addendum

A. CALCULATION OF MOLAR RATIOS FOR LIGATION

This is an example of the calculation of molar ratios for efficient ligation. For this example the pHannibal plasmid (5.824 kb) and a fictitious insert (0.6 kb) will be used to illustrate the theory of a ligation. The relationship between the molecular weight (M_r) and size (kb) of DNA is such that 1 kb of DNA has an approximate M_r of 0.662 Mega Daltons (MD).

Thus: 1 mol pHannibal = 0.662 MD × kb (pHannibal)
= 662 000 D × 5.824 kb
= $\underline{3.855 \times 10^6 \text{ g}}$

1 pmol pHannibal = 1 mol pHannibal × 10^{-12}
= $(3.855 \times 10^6 \text{ g}) \times 10^{-12}$
= 3.855×10^{-6} g
= $\underline{3.855 \text{ µg}}$

The following simplified equation gives the same result:

1 pmol pHannibal = (0.662 × kb (pHannibal)) µg
= (0.662 × 5.824 kb) µg
= $\underline{3.855 \text{ µg}}$

Thus: 1 pmol Insert = (0.662 × kb (Insert)) µg
= (0.662 × 0.6 kb) µg
= $\underline{0.397 \text{ µg}}$

For a 1 ml ligation with a total of 5 pmol DNA:

5 pmol pHannibal = 5 × 3.855 µg
= $\underline{19.275 \text{ µg}}$

5 pmol Insert = 5 × 0.397 µg
= $\underline{1.985 \text{ µg}}$

In a 1 ml ligation with 5 pmol total DNA, and a standard insert to vector ratio of 3:1, there should be a total of 4 molecules per ligation event (1 molecule vector and 3 molecules insert), thus ¼ vector (pHannibal) and ¾ Insert.

Thus: Amount of pHannibal = ¼ × 5 pmol pHannibal
= ¼ × 19.275 µg
= 4.819 µg

Amount of Insert = ¾ × 5 pmol Insert
= ¾ × 1.985 µg
= 1.489 µg

These vector and insert values are for a 1 ml ligation. To adapt these values for a 10 µl ligation they are divided by 100 (1000 µl / 10 µl = 100).

B. COMPETENT CELL PREPARATION PROTOCOLS

B.1. Competent cell preparation - rubidium chloride protocol

The following procedure was adapted from Hanahan (1985).

An *E. coli* BL21(DE3)pLysS single colony was inoculated in 2.5 ml of LB medium with chloramphenicol (chlor) (34 µg/ml). The culture was incubated overnight (225 rpm, 37°C). After approximately 16 h the overnight culture was used to inoculate 250 ml LB medium containing 20 mM $MgSO_4$ and chlor. The cells were grown in a one-litre flask, for proper aeration (225 rpm, 37°C). The OD_{600} was checked after 2 h and every 30 min thereafter, until the OD_{600} reached 0.4-0.6. The cells were pelleted by centrifugation (7 000 rpm, 5 min, 4°C). The cell pellets were gently resuspended in 0.4× original culture volume of ice-cold TFB1 (30 mM KaOc, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15% glycerol. pH adjusted to 5.8 with 1 M acetic acid, filter sterilised (0.2 µm)). The resuspended cells were combined and incubated on ice for 5 min at 4°C (for the remainder of the procedure the cells were kept on ice and all pipettes, tips, microfuge tubes and flasks were also chilled). The cells were pelleted by centrifugation (7 000 rpm, 5 min, 4°C), and gently resuspended in 1/25 original culture volume of ice-cold TFB2 (10 mM MOPS or PIPES pH 6.5, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol. pH adjusted to 6.5 with 1 M KOH. Filter sterilised (0.2 µm) and stored at room temperature). The competent cells were treated very gently due to their high sensitivity to handling and elevated temperature. The competent cells were incubated on ice

for 15-60 min and then 100 µl/tube was aliquoted for storage at –80°C. The cells were quick frozen in a dry ice/isopropanol bath.

B.2. Competent cell preparation - calcium chloride protocol

The following procedure was adapted from Hanahan (1983).

A single colony *E. coli* GM41 was inoculated in 5 ml of LB medium, and incubated overnight (225 rpm, 37°C). After approximately 16 h the overnight culture was used to inoculate 500 ml LB medium (1:100 dilution). The cells were grown in a one litre flask, for proper aeration, at 37°C and 225 rpm. The OD_{600} was determined after 1.5 h and then every 30 min until the OD_{600} reached 0.4-0.6. The cells were pelleted by centrifugation (5 000 rpm, 10 min, 4°C) and kept on ice. The cell pellets were gently resuspended in 100 ml of ice-cold buffer 1 (100 mM $MgCl_2$) by pipetting. The resuspended cells were incubated on ice (20-30 min, 4°C) (for the remainder of the procedure the cells were kept on ice and all pipettes, tips, microfuge tubes and flasks were also chilled). The cells were pelleted by centrifugation (4 000 rpm, 10 min, 4°C) and gently resuspended in 10 ml ice-cold buffer 2 (100 mM $CaCl_2$, 15% glycerol). The competent cells were treated gently due to their high sensitivity to handling and elevated temperature. The competent cells were aliquoted at 1 ml/pre-chilled microfuge tube for storage at –80°C. The competent cells were later divided into 100 µl and 200 µl aliquots for easy use.

C. BACTERIAL GROWTH AND PLANT REGENERATION MEDIA

C.1. Luria-Bertani (LB) medium for bacterial growth

The protocol was adapted from Sambrook *et al.* (1989).

Per litre:	950 ml	ddH_2O
	10 g	bacto-tryptone
	5 g	bacto-yeast extract
	5 g	NaCl

pH was adjusted to 7.5 with 10 M NaOH, and the volume adjusted to one litre with ddH_2O. The solution was sterilised by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle.

C.2. Terrific broth (TB) medium for bacterial growth

The protocol was essentially performed as per Sambrook *et al.* (1989).

Per litre:	900 ml	ddH_2O
	12 g	bacto-tryptone
	24 g	bacto-yeast extract
	4 ml	glycerol

The solution was sterilised by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle, and allowed to cool to approximately 60°C. A 100 ml volume of sterile phosphate buffer (0.17 M KH_2PO_4, 0.72 M K_2HPO_4) was added to the autoclaved solution.

C.3. Plant tissue culture media for the regeneration of *N. tabacum* from leaf disks

Table C.3.1. Table indicating the components of media used for the regeneration of *N. tabacum* from leafdisks. MS salts and vitamins prepared according to Murashige and Skoog (1962). Where applicable kan (50 µg/µl) was used as a selectable marker, and cef (400 µg/ml) was used as a broad-spectrum antibiotic.

Plant transformation media for *N. tabacum* leaf disks	
Co-cultivation MS medium	MS salts and vitamins 10.5 g/l Phyto agar 1.0 mg/l BAP 30 g/l Sucrose pH 5.7 to 5.8 with 1 M KOH
Regenerating MS medium	MS salts and vitamins 10.5 g/l Phyto agar 1.0 mg/l BAP 30 g/l Sucrose pH 5.7 to 5.8 with 1 M KOH
Rooting MS medium	MS salts and vitamins 10.5 g/l Phyto agar 0.1 mg/l BAP 1.0 mg/l NAA 30 g/l Sucrose pH 5.7 to 5.8 with 1 M KOH

D. ANTIBIOTIC SELECTION OF PLASMIDS

Table D.1. Relevant antibiotic selectable markers and their concentrations used for the selection of specific plasmids used during the course of this study.

Plasmid	Antibiotic	Concentration (μg/μl)
pGem-T Easy	Ampicilin (amp)	100
pET-14b	Ampicilin (amp)	100
pLysS	Chloramphenicol (chlor)	34
pHannibal	Ampicilin (amp)	100
pART27 (bacterial selection)	Kanamycin (kan)	20
pSoup	Tetracycline (tet)	5
pART27 (plant selection)	Kanamycin (kan)	50

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