THE DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF THE SOUTH AFRICAN STRAIN(S) OF GRAPEVINE FANLEAF NEPOVIRUS

by

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DECLARATION

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ABSTRACT

South Africa is one of the top ten wine producing countries in the world. The South African wine industry contributes approximately R16.3 billion to South Africa's annual gross domestic product with 42.8% of wine being exported. To compete with the top wine producing countries and to ensure a viable export market, South Africa needs to ensure that healthy, virus free propagation material is produced and sold. One of the viruses that need to be tested for is *Grapevine fanleaf virus* (GFLV). *Grapevine fanleaf virus* causes degeneration and malformation of berries, leaves and canes and is responsible for significant economic losses by reducing crop yields by as much as 80%, reducing the longevity of the vines and affecting fruit quality. It is widespread in the Breede River Valley of the Western Cape where the nematode vector, *Xiphinema index,* is prevalent. The Breede River Valley contributes approximately 30% of the total production of the local wine industry, and severe losses in this region could threaten the viticulture. The Plant Improvement Act states that all propagation material sold must be tested for GFLV by a reputable scientific technique. The technique commonly used in South Africa is the Double Antibody Sandwich - Enzyme-linked Immunosorbent Assay (DAS-ELISA) and the kits are imported from Europe at a significant cost to the South African viticulture industry.

The objective of this study was to produce a reliable and sensitive diagnostic assay specific for the South African strains of GFLV. This project aimed to develop and optimize a DAS-ELISA, by using recombinant DNA technology to produce antibodies against bacterially expressed viral coat protein. Total RNA was extracted from GFLV infected grapevine material and the viral coat protein (CP) amplified. The CP was cloned into the pGex-6P-2 expression vector, fusing a Glutathione S-Transferase (GST) partner to the viral coat protein enhancing solubility and protein purification. Insufficient amounts of the soluble protein were expressed and purified, preventing the production of antibodies and thus the development of the DAS-ELISA.

An alternative diagnostic rapid-direct-one-tube-RT-PCR assay was developed. This rapid-direct-one-tube-RT-PCR assay was compared to commercially available DAS-ELISA and ImmunoStrip tests (Agdia) to assess the reliability, sensitivity and specificity of the rapid-direct-one-tube-RT-PCR assay. Twelve GFLV isolates from South Africa were sequenced to investigate the variability between the isolates as well as the variability between the South African isolates and GFLV sequences available in Genbank. Sequence identities between clones from different GFLV isolates from South Africa were between 86-99% and 94-99% at the nucleotide and amino acid levels, respectively. Phylogenetic analysis based on the coat protein gene sequences showed that the South

African isolates form two distinct clades or sub-populations. No significant correlation was found between geographical origin and symptoms, nor between geographical origin and sequence variability or between grapevine cultivar and symptom expression. Of the 23 samples tested with all three tests, 21 tested positive with rapid-direct-one-tube-RT-PCR, 19 with the ImmunoStrips and 17 with an imported DAS-ELISA kit (Agdia). Rapid-direct-one-tube-RT-PCR was found to be the most reliable technique for GFLV detection.

Although the establishment of a DAS-ELISA directed to the South African strain(s) of GFLV was not successful, an alternative PCR based diagnostic system was developed, and proved to be sensitive and reliable. RT-PCR based diagnostic assays are generally accepted to be more sensitive than DAS-ELISA, but the latter is still used as the diagnostic assay of choice for routine testing due to ease of use. This rapid-direct-one-tube-RT-PCR assay is a rapid, sensitive and reliable diagnostic test, reducing the prevalence of false negatives, contributing to a virus free viticulture industry. The rapid-direct-one-tube-RT-PCR assay is as easy to use as DAS-ELISA, faster and can be performed by semi skilled workers, thus providing all the advantages associated with DAS-ELISA.

OPSOMMING

Suid-Afrika is een van die top tien wyn produserende lande in die wêreld. Die Suid-Afrikaanse wynbedryf dra ongeveer R16.3 biljoen by tot Suid-Afrika se jaarlikse bruto binnelandse produk waarvan 42% van die wyn uitgevoer word. Om mee te ding met die top wyn produserende lande asook die uitvoer mark te verseker, is dit nodig vir Suid-Afrika om gesonde, virus vrye voortplantings materiaal te produseer en te verkoop. Een van hierdie virusse waarvoor getoets moet word is Wingerd netelblaar virus (GFLV). Wingerd netelblaar virus veroorsaak degenerasie en misvorming van korrels, blare en stokke en is verantwoordelik vir ernstige ekonomiese verliese deur die oes opbrengs te verlaag met tot 80%, die leeftyd van die wingerd te verminder en die kwaliteit van die vrug te affekteer. Die virus is wyd verspreid in die Breede Rivier Vallei van die Wes Kaap waar die nematode vektor, *Xiphinema index* algemeen voorkom. Die Breede Rivier Vallei dra tot 30% van die totale produksie van die plaaslike wyn bedryf by en ernstige verliese in die omgewing kan die wingerdkunde ekonomie bedreig. Die plantverbeterings wet bepaal dat alle voortplantings materiaal getoets moet word vir GFLV deur ’n betroubare wetenskaplike tegniek. “Double Antibody Sandwich - Enzyme-linked Immunosorbent Assay” (DAS-ELISA) word ingevoer vanuit Europa teen uitermatige kostes aan die Suid Afrikaanse wingerdkunde industrie.

Die objektief van hierdie studie was om ’n betroubare sensitiewe diagnostiese toets spesifiek vir die Suid Afrikaanse isolate van GFLV te produseer. Ons het gepoog om ’n DAS-ELISA te ontwikkel en te optimiseer deur gebruik te maak van rekombinante DNA tegnologie om antiliggame teen die bakterieel-uitgedrukte virus mantelproteïen te produseer. Totale RNA is geïsoleer vanuit GFLV geïnfekteerde wingerdmateriaal en die virus mantelproteïengeen is geamplifiseer. Die mantelproteïengeen is gekloneer in die pGex-6P-2 uitdrukkingsvektor, wat ’n Glutatioon S-Transferase (GST) vennoot aan die virus mantelproteïen geen heg. Hierdie GST vennoot verbeter oplosbaarheid van die mantelproteïen en vergemaklik die suiwering daarvan. Onvoldoende hoeveelhede van die oplosbare proteïen is egter uitgedruk en gesuiwer, wat die produksie van antiliggame verhinder het en dus die ontwikkeling van die DAS-ELISA onmoontlik gemaak het..

’n Alternatiewe diagnostiese “rapid-direct-one-tube-RT-PCR” toets is ontwikkel. Hierdie “rapid-direct-one-tube-RT-PCR” toets is vergelyk met kommersieël beskikbare DAS-ELISA en ImmunoStrip toetse (Agdia) om die betroubaarheid, sensitiwiteit en spesifisiteit van die ‘rapid-direct-one-tube-RT-PCR” toets te evalueer. Twaalf Suid-Afrikaanse GFLV isolate se nukleotiedvolgorde is bepaal om die genetiese variasie tussen die isolate te ondersoek, asook tussen die Suid-Afrikaanse isolate en isolate beskikbaar op GenBank. Volgorde ooreenkomste tussen klone

van verskillende isolate van Suid-Afrika was tussen 86-99% en 94-99% op nukleotied en aminosuur vlak respektiewelik. Filogenetiese analise gebaseer op die mantelproteïengeen volgordes toon dat die Suid-Afrikaanse isolate in twee groeperings of sub-populasies verdeel. Geen betekenisvolle korrelasie is gevind tussen die sub-populasies en simptoomuitdrukking of tussen die geografiese oorsprong of die wingerdkultivar en die simptoom uitdrukking nie. Van die 23 monsters wat getoets is vir GFLV het 21 positief getoets met die “rapid-direct-one-tube-RT-PCR”, 19 met die ImmunoStrip toets (Agdia) en 17 met die ingevoerde DAS-ELISA (Agdia). “Rapid-direct-one-tube-RT-PCR” is bevind as die mees betroubaarste en sensitiefste GFLV diagnostiese toets.

Alhoewel die ontwikkeling van ’n DAS-ELISA gerig tot die Suid Afrikaanse GFLV variante nie suksesvol was nie, is ’n alternatiewe betroubare en sensitiewe RT-PCR gebaseerde toets daargestel. RT-PCR gebaseerde toetse word algemeen aanvaar om meer sensitief as DAS-ELISA toetse te wees, maar laasgenoemde is steeds die voorkeur diagnostiese toets vir roetine toetsing, moontlik as gevolg van die eenvoud van die toets. Die “rapid-direct-one-tube-RT-PCR” is ’n vinnige, sensitiewe en betroubare toets, wat die voorkoms van vals negatiewe resultate verminder en so dus bydrae tot ’n virus vrye wingerdkunde industrie. Die “rapid-direct-one-tube-RT-PCR” is net so eenvoudig om uit te voer soos die DAS-ELISA, vinniger, kan deur semi-opgeleide werkers uitgevoer word en lewer dus al die voordele geassosieer met die DAS-ELISA.

ABBREVIATIONS

µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
µm	Micrometer
1A	RNA1 gene coding for a putative proteinase cofactor
$1B^{Hel}$	RNA1 gene coding for a putative helicase and NTP-binding domain
$1C^{VPg}$	RNA1 gene coding for a virus genome linked protein
$1D^{Pro}$	RNA1 gene coding for a chymotrypsin-like cysteine proteinase
$1E^{pol}$	RNA1 gene coding for a putative RNA-dependant RNA polymerase
$2A^{HP}$	RNA2 gene coding for a homing protein
$2B^{MP}$	RNA2 gene coding for a movement protein
$2C^{CP}$	RNA2 gene coding for a single coat protein
3'NC	3' Non-coding
Amp	Ampicillin
ArMV	*Arabis mosaic virus*
bp	base pairs
CP	Coat protein
dH2O	Distilled water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphate(s)
DTT	1,4-Dithiothreitol
DAS-ELISA	Double Antibody Sandwich - Enzyme-Linked Immunosorbent Assay
EDTA	Ethylene Diamine Tetra-acetic Acid di-sodium Salt
GFLV	*Grapevine fanleaf virus*
GST	Glutathione S-Transferase
IPTG	Isopropyl-β-D-thiogalactoside
kb	kilobase
kDa	kiloDalton
LB	Luria Bertani broth
M	Molar
mA	milliAmpere
MBP	Maltose Binding Protein
mM	Millimolar
MP	Movement protein
Mr	Molecular weight
ng	Nanograms
nm	Nanometer(s)
nt	Nucleotides
OD_{600}	Absorpsion value at 600 nm
ORF	Open reading frame
P1	RNA1 polyprotein
P2	RNA2 polyprotein
P3	Protein that is encoded for by RNA3

PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNA1	First single stranded positive sense genomic Ribonucleic acids
RNA2	Second single stranded positive sense genomic Ribonucleic acids
RNA3	Satellite RNA
RT-PCR	Reverse Transcription - Polymerase Chain Reaction
SAPO	South African Plant Improvement Organisation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
TB	Terrific Broth
TEMED	Tetramethylethylenediamine
U	Units
V	Volt
v/v	Volume per volume
VPg	Viral Protein genome-linked
w/v	weight per volume
β-ME	β-mercaptoethanol

CHEMICAL COMPOUNDS

Chemical Name	Chemical Abbreviation
Acetic Acid	HOAc
Ammonium	NH_4
Calcium Cloride	$CaCl_2$
Disodium Hydrogen Phosphate	Na_2HPO_4
Hydrogen Cloride	HCl
Lithium Cloride	LiCl
Magnesium Cloride	$MgCl_2$
Potasium Orthophosphate Dihydrogen	KH_2PO_4
Potassium Acetate	KOAc
Potassium Cloride	KCl
Potassium Phosphate	K_3PO_4
Reduced Glutathione	$C_{10}H_{17}N_3O_6S$
Sodium Acetate	NaOAc
Sodium Hydrogen Carbonate	$NaHCO_3$
Sodium Carbonate	Na_2CO_3
Sodium Cloride	NaCl
Sodium Deoxycholate	$C_{24}H_{39}NaO_4$
Sodium Hydroxide	NaOH
Sodium Metabisulfite	$Na_2S_2O_5$
Sodium Dihydrogen Orthophosphate	NaH_2PO_4
Tris	$(HOCH_2)_3CNH_2$

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"Protein expression is an art, rather than a science"

http://www.exptec.com/Stategies/Strategies.htm

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1 INTRODUCTION

1.1 General introduction

South Africa is one of the ten largest wine producing country's in the world and the South African wine industry contributes 1.5% to the countries total annual gross domestic product (2003, SAWIS). South Africa exports 42.8% of the produced wine, of which the United Kingdom, Germany, the Netherlands, Sweden and Denmark are the greatest importers (SAWIS).

Grapevine is the most important fruit species worldwide and is under constant threat of viral infections that could cause severe economic losses if the infections are not properly controlled. One such virus is *Grapevine fanleaf virus* (GFLV). It was discovered in the 1950's and causes one of the oldest known viral diseases of grapevines. It is widespread in all major wine growing regions where the parasitic nematode *Xiphinema index* is present. *Grapevine fanleaf virus*, a member of the genus *Nepovirus* in the family *Comoviridae*, causes severe damage to leaves, canes and berries. Infection can cause a yield loss of up to 80%, depending on the strain and susceptibility of the host grapevine plant. It is an economically important virus that could decrease the longevity of the vine by 50% (Andret-Link et al., 2004; Martelli et al., 2001). Three leaf symptom types are associated with the disease: fanleaf, yellow mosaic and vein banding. No association between these symptoms and virus strains have been observed, but multiple infections have been associated with increased severity in symptoms (Bashir et al., 2007c). Studies on the variability of the virus has been done in France (Vigne et al., 2004a, 2005), Iran (Bashir et al., 2007a, 2007b, 2007c), Slovenia (Pompe-Novak et al., 2007), Tunisia (Fattouch et al., 2005a, 2005b) and the USA (Naraghi-Arani et al., 2001). Up to 13% variability on nucleotide level and 9% on amino acid level have been observed in the coat protein gene, 9% and 7% respectively for the movement protein (MP) gene and 6.7% and 2.5% respectively for the complete RNA2 open reading frame (ORF) (Bashir et al., 2007a, 2007b, 2007c; Fattouch et al., 2005a, 2005b; Naraghi-Arani et al., 2001; Pompe-Novak et al., 2007; Vigne et al., 2004a, 2005).

In South Africa GFLV is widespread in the Breede River valley of the Western Cape (Malan and Hugo, 2003). The Breede river valley contributes approximately 33% to the wine economy (SAWIS) and uncontrolled spread of the virus could have devastating affects on the wine industry as well as on the South African economy. The control of GFLV is thus essential for the sustainability and growth of the wine industry. The South African Plant Improvement Act, 1979, states that all vine canes sold must be tested for GFLV infection. Two diagnostic assays that are widely used for routine testing are Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

and Double Antibody Sandwich - Enzyme-Linked Immunosorbent Assay (DAS-ELISA). Although RT-PCR is proven to be more sensitive than ELISA, ELISA is still the method of choice because of its simplicity in operation, minimum laboratory equipment required and low cost associated with the assay. Currently, DAS-ELISA tests are imported from Europe and pose two immediate threats to the industry, the possible non-specificity of the imported test to the South African strain(s) and the great cost to the industry. To overcome these problems, a sensitive and reliable diagnostic assay directed to the South African strain(s) of the virus was viewed to be essential. No research has been done previously on the variability of the South African strain(s) of the virus, these studies are essential to develop a sensitive diagnostic assay that would detect all variants of the virus.

1.2 Project proposal

The aim of this study was to develop a diagnostic assay directed to the South African strain(s) of the GFLV. This project aimed to develop and optimise a diagnostic DAS-ELISA by using recombinant DNA technology to express the virus coat protein and produce antibodies for use in this assay. The GFLV coat protein gene will be amplified and cloned into the pGex-6P-2 expression vector, and the expression of soluble GFLV coat protein will be attempted in two *Escherichia coli* strains: BL21(DE3)pLysS and Rosetta2(DE3)pLysS.

We also aimed to develop an RT-PCR diagnostic assay that detects all GFLV strain(s) present in South Africa. To do so, information was required on the variability of the South African stain(s). *Grapevine fanleaf virus* samples were collected from different areas in the Western Cape of South Africa displaying fanleaf, vein banding and yellow mosaic symptoms. Different cultivars of the host plant were also selected.

The developed RT-PCR diagnostic assay will be compared to an available DAS-ELISA and ImmunoStrip tests to asses the reliability and sensitivity thereof.

2 LITERATURE REVIEW

2.1 Introduction

Grapevine fanleaf virus causes a devastating disease and affects all winegrowing regions where the nematode vector *Xiphinema index* is prevalent, including the Breede River valley in the Western Cape of South Africa. In this review, GFLV and *X. index* distribution in South Africa is discussed to highlight the effect of what a GFLV epidemic could have on the economic status of the wine industry as well as on the South African economy. *Grapevine fanleaf virus* is described in detail to emphasise the genome variability, functioning, spreading, control strategies and the devastating effect of GFLV infection on grapevine. Diagnostic assays are discussed and compared to give insight in the available techniques for virus identification and control.

2.2 South African Grapevine Industry

2.2.1 Wine regions of South Africa

The South African wine industry dates back to approximately 350 years ago when the Dutch Governor, Jan van Riebeeck planted the first vineyard in 1655 from which the first wine was produced from these Cape grapes in 1659. He encouraged the farmers to plant vineyards all around the Cape area, but with little viticulture experience the industry did not flourish. His successor, Simon van der Stel, brought winemaking immigrants from France in 1667 and introduced the knowledge, skills and secrets of viticulture and winemaking to South Africa (http://www.vineyardvarieties.com/history.php).

The Wine of Origin System was introduced in 1973 and the South African winelands were divided into official regions and districts. The wine growing regions are mostly situated in the Western Cape (fig. 2.1) near the coast with Overberg, Stellenbosch, Paarl, Swartland, Robertson, Worcester and Swellendam as the most prominent areas. Wine is also produced in the Klein Karoo, Oliphant's River, Douglas and lower Orange River regions with much warmer and drier climates (http://www.vineyardvarieties.com/regions.php).

Today South Africa is one of the top 10 wine producing countries in the world and according to the South African Wine Industry and Systems (SAWIS) website (http://www.sawis.co.za) contributes approximately R16.3 billion (excluding tourism) to SA annual gross domestic product in 2003. 101 957 hectares of land is planted to vineyards (wine grapes) producing 730.4 million litres of wine and exporting 312.6 million litres (42.8%) in 2007 (SAWIS).

Figure 2.1: South African wine regions (http://www.wineanorak.com/safricamap.htm)

2.2.2 *Xiphinema index* and GFLV distribution

Grapevine fanleaf virus is spread by the ectoparasitic nematode *Xiphinema index*. Although not all vineyards infected with *X. index* are infected with GFLV, it is a clear indicator of where the virus is prevalent.

X. index was first reported in the Western Cape Province in the Robertson, Bonnievale and Swellendam regions in 1971 (Heyns, 1971). It was also reported in the Worcester area (Barbercheck and Heyns, 1986) as well as in Calitzdorp and Ladysmith in the Klein Karoo region (Malan and Meyer, 1994). Barbercheck et al. (1985) reported that *X. index* was spread from Rawsonville all along the Breede River to Bonnievale including Worcester, Robertson, Ashton, McGregor and Montagu. This indicate that *X. index* was spread by means of irrigation water from the Breede River used in the vineyards. Isolated cases of *X. index* were also detected in the Paarl region (Malan and Meyer, 1994) and the Franshoek region (Van Reenen and Heyns, 1986).

Grapevine fanleaf virus is widely distributed in the Breede River Valley where *X. index* is prevalent (Malan and Hugo, 2003). Isolated cases of the virus have also been detected in the Paarl-Wellington and Stellenbosch regions of the Western Cape (this report).

2.2.3 South African quarantine and certification regulations

The Agricultural Pest Act, 1983 (Act No. 36 of 1983) provides measures to prevent and combat agricultural pests including plant pathogens such as viruses. The Directorate of Plant Health regulates the importation and exportation of plants and plant products. This important material may be infected with quarantine pathogens which may endanger the South African agriculture and the agriculture of countries to which plant material is exported to. This could result in the banning of South African exports of plant material to these countries. The Plant Improvement Act, 1976, (Act No. 53 of 1976) for the phytosanitary requirements for plants and shoots states that all certified plants and plant materials of rootstocks varieties and all certified plants and plant materials of scion varieties need to be tested for GFLV.

2.2.4 Impact on Agriculture

Inconsistant results from locally produced GFLV antiserum forced the wine industry to import DAS-ELISA kits from Europe which pose two immediate problems: first, by the significant cost to the viticulture industry and second, by the potential of non-specificity of these kits to South African strain(s) of the virus. No research has been done on the variability of South African GFLV strain(s). This data is required to predict the potential non-specificity of these kits and to ensure the development of a DAS-ELISA diagnostic test directed to the South African strain(s) of the virus. Without proper diagnostic systems in place, infected material may be falsely diagnosed as GFLV free certified material and could cause a GFLV epidemic in South African vineyards.

The Breede River Valley, where *X. index* is prevalent, contributes to approximately 33% of the South African wine industry and is under threat by infection with GFLV (SAWIS). Without the availability of accurate diagnostic assays, infection could spread to surrounding vine growing regions causing significant economic losses to the South African wine industry.

2.3 Grapevine fanleaf virus (GFLV)

2.3.1 Genus nepovirus

Grapevine fanleaf virus belongs to the genus *Nepovirus*, one of three genera belonging to the family *Comoviridae* (Wellink et al., 2000). The genus consists of 28 species and 8 tentative species including *Arabis mosaic virus* (ArMV) to which GFLV is related – both serologically and on genome sequence level (Martelli et al., 2001). *Nepoviruses* consist of two single stranded positive

sense genomic ribonucleic acids (RNAs), each containing a single ORF encoding a polyprotein which is proteolytically processed by the RNA1- encoded protease into functional proteins (Margis and Pinck, 1992; Ritzinthaler et al., 1991; Sergheni et al., 1990).

2.3.2 Morphology

The icosahedral virus particles have a 28 nm diameter with an angular outline and contain three density components with different sedimentation coefficients. These particles are serologically identical (fig. 2.2). The top component (T) consists of an empty shell, the middle component (M) consists of the capsid structure containing the RNA2 species and the bottom component (B) consists of the capsid structure containing both the RNA1 and 2 species (Quacquarelli et al., 1976). The ratio (T:M:B) of these components varies seasonally, from 1.0:0.5:3.5 in summer to 1.0:0.5:0.75 in winter (Martelli et al., 2001). The reason for this variance is unknown at present.

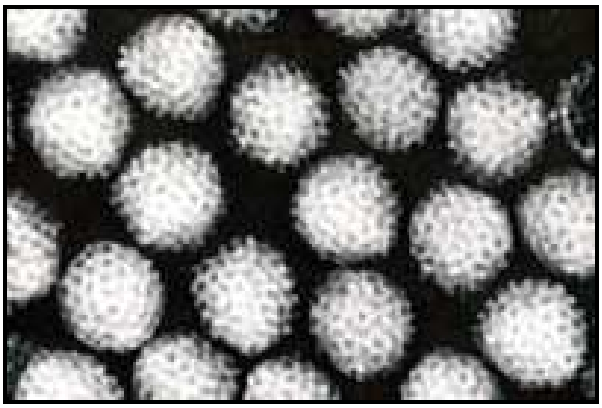

Figure 2.2. Electron micrograph of purified icosahedral virus particles (Gergerich and Dolja, 2006)

2.3.3 Genome organisation

Grapevine fanleaf virus has two single stranded positive sense RNA genomes, RNA1, with a molecular weight (Mr) of 2.4 x 10^6 and RNA2 with an Mr of 1.4 x 10^6 (Quacquarelli et al., 1976). RNA1 is 7342 nucleotides (nt) in length with a single ORF extending from nt 243 to 7097 and RNA2, 3774 nt in length with an ORF extending from nt 233 to 3562. Both RNA species are monocistronic and carry a small covalently linked viral protein (VPg) at their 5'ends and a poly adenosine (poly(A)) tail at their 3'ends (Pinck et al., 1988) (fig. 2.3).

The RNA1 polyprotein (P1) is proteolytically processed into 5 mature products (fig. 2.3); a putative proteinase cofactor (1A), a putative helicase and NTP-binding domain ($1B^{Hel}$), a virus genome linked protein ($1C^{VPg}$), a chymotrypsin-like cysteine proteinase ($1D^{Pro}$) and a putative RNA-dependant RNA polymerase (RdRp, $1E^{pol}$) (Margis et al., 1991, 1994; Margis and Pinck, 1992; Pinck et al., 1991; Ritzenthaler et al., 1991). The RNA2 polyprotein is processed into three mature

products; a homing protein ($2A^{HP}$) also required for RNA2 replication, a movement protein ($2B^{MP}$), and a single coat protein ($2C^{CP}$) that encapsidate the viral RNA for the virion (Gaire et al., 1999; Margis et al., 1993; Ritzenthaler et al., 1995a; 1995b; Serghini et al., 1990) (fig. 2.3). The viral capsid is composed of 60 subunits of the $2C^{CP}$ protein and has a Mr of 56 019 as predicted from the nucleotide sequence (Serghini et al., 1990). Seventy percent of the weight of the M component and 58% of the weight of the B component consists of the $2C^{CP}$ protein (Quacquarelli et al., 1976).

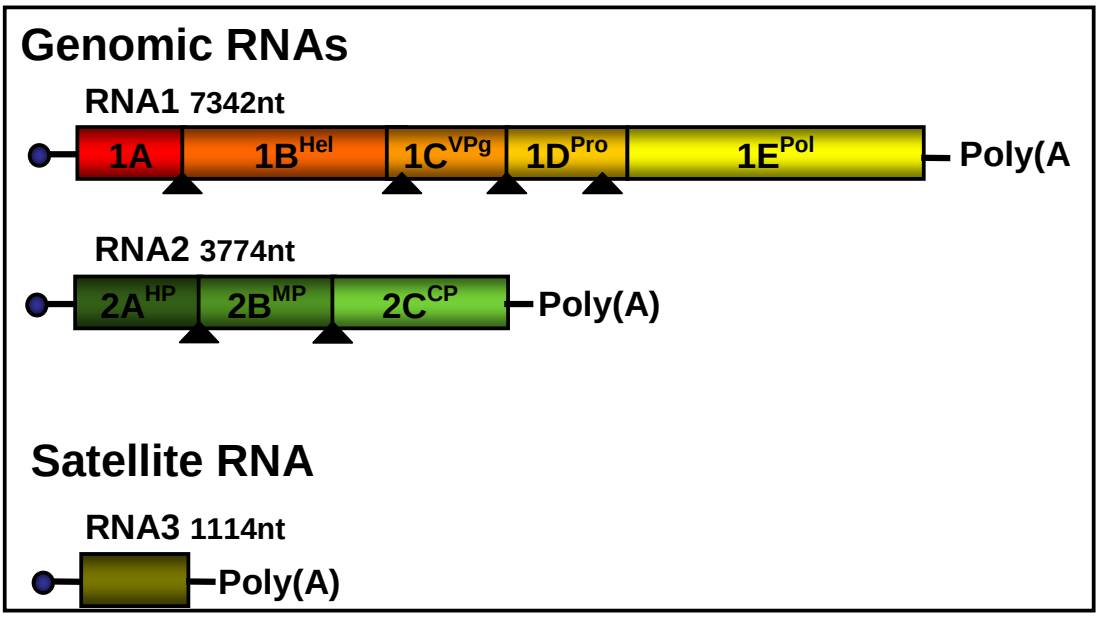

Figure 2.3. Genome organisation of *Grapevine fanleaf virus* genomic and satellite RNA. The boxes represent the ORF of each RNA species. The VPg is represented by the blue circles. The triangles represent the cleaving sites of each polyprotein (Andret-Link et al., 2004; Martelli et al., 2001). Elements in figure are not drawn to scale.

Satellite RNA was discovered in association with some GFLV isolates and is refered to as RNA3 (Pinck et al., 1988; Saldarelli et al., 1993). The RNA3 is 1114 nt in length and carries a small covalently linked viral protein (VPg) at its 5' terminus and a poly(A) tail at its 3' terminus (Fuchs et al., 1989; Pinck et al., 1988) (fig. 2.3). The satellite RNA contains a single ORF extending from nt 14 to 1040 and encodes a highly hydrophilic and extremely basic (pH 11) protein (P3) (Fuchs et al., 1989). The P3 protein is unable to replicate on its own and requires RNA1 and RNA2 for its encapsidation and replication, and appears to be necessary for the replication of RNA3 (Hans et al., 1992, 1993; Pinck et al., 1988).

2.3.4 Replication and cell to cell movement

The infection of a plant cell with GFLV causes uncontrolled production of membranes that accumulate within the nuclear periphery and forms the viral compartment (fig. 2.4). Endoplasmic reticulum-derived membranous vesicles form within the viral compartment (Andret-Link et al., 2004; Ritzenthaler et al., 2002). The viral particles enter the viral compartment, are decapsidated

and the two genomic RNA's replicate (Ritzenthaler et al., 2002) and are translated into polyproteins that are proteolytically spliced by the $1D^{pro}$ protease into the mature products (Margis et al., 1991). The mature products accumulate within the compartment except the MP, which is rapidly transported to the cell periphery (Gaire et al., 1999).

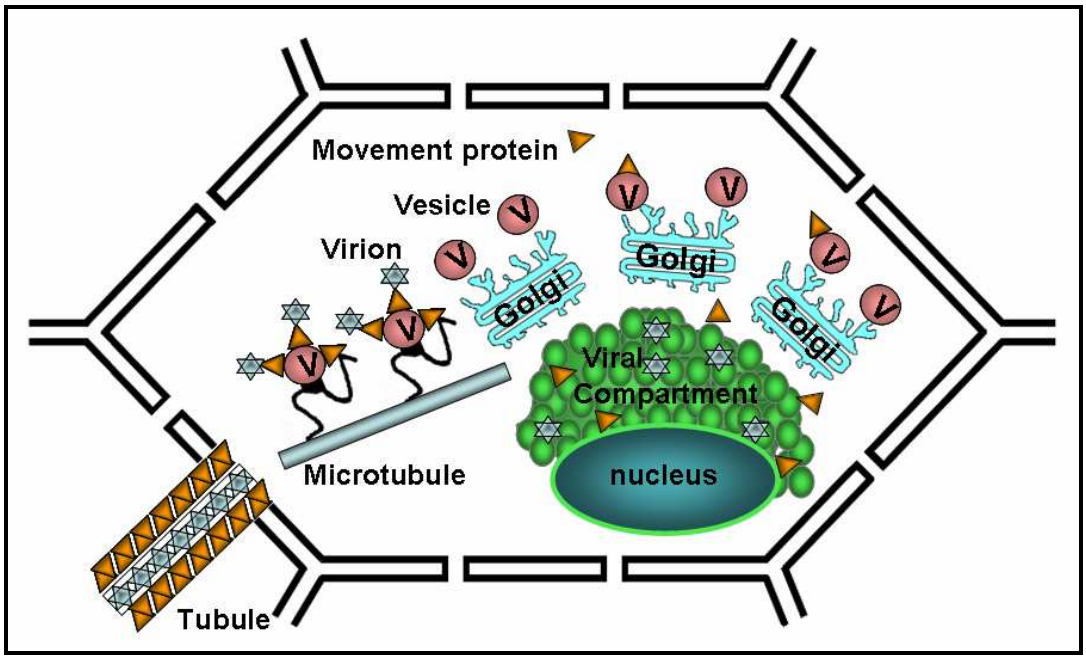

Figure 2.4. Schematic representation of the replication, intracellular trafficking, and cell-to-cell movement steps of *Grapevine fanleaf virus* (Andret-Link et al., 2004). Elements in figure are not drawn to scale.

A functional secretory pathway and intact microtubules are essential for intracellular MP transport across cell walls through plasmodesmata (Laporte et al., 2003). It is suggested that MP could be transported intracellulary together with the CP or viral particles on Golgi-derived secretory vesicles along microtubules (fig. 2.4) (Andret-Link et al., 2004; Laporte et al., 2003). From the viral compartment the GFLV particles move to the cell periphery via the microtubules followed by movement through the plasmodesmata to infect adjacent cells (Andret-Link et al., 2004). The MP self-assembles into unidirectional membranous tubules with their base embedded in the cross-wall and their tip in the cytoplasm of the adjacent cell to form a pathway for the viral particles to move from cell to cell (fig. 2.4) through MP-CP or MP-virion interactions (Ritzenthaler et al., 1995a, b, 2002; Belin et al., 1999; Carvalho et al., 2003). This is the first step in the systemic infection of the entire plant.

2.3.5 Geographical distribution and transmission

Grapevine fanleaf virus causes fanleaf degeneration in almost all temperate regions where *Vitis vinifera* is cultivated, including the Breede River Valley of South Africa (Andret-Link et al., 2004; Barbercheck et al., 1985). *Grapevine fanleaf virus* has been reported in North and South America,

Asia, Africa, Europe, New Zealand, and South Australia (Bovey et al., 1990; Martelli and Savino, 1990). *Grapevine fanleaf virus* is native to *V. vinifera* and probably originated in the Caucasus area between the Black and Caspian seas and was transferred from there to Europe and the rest of the world through exchange of *V. vinifera* material (Raski et al., 1983). *Grapevine fanleaf virus* can be distributed through grapevine seeds, *X. index,* and human actions like grafting, soil transfer, mechanical inoculation and exchange of propagation material (Martelli et al., 2001). The ectoparasitic nematode *X. index* from the family *Longidoridae* is responsible for transmission of GFLV from grapevine to grapevine (Brown et al., 1995; Hewitt et al., 1958). *Xiphinema index* feeds on the root tips of grapevine and ingests the GFLV from infected vines during feeding. The virus is then retained in the odontophore, oesophagus and oesophageal pump and released into adjacent vines (Raski et al., 1983; Taylor and Robertson, 1970). *Grapevine fanleaf virus* can be transmitted by both juvenile and adult nematodes but the virus is not transmitted to their progeny (McFarlane et al., 2002; Taylor and Raski, 1964). Virus transmission also occur through grafting (Martelli and Holland, 1987; Martelli et al., 2001) As a result of the limited movement of the GFLV nematode vector, *X. index* in soil, GFLV infected grapevines often show an erratic distribution in vineyards.

2.3.6 Fanleaf disease

Grapevine fanleaf virus causes a variety of degenerating symptoms that affect the leaves, canes and berries of vines. These symptoms vary in severity (Martelli, 1993). Irregular vein formation occurs, leaves develop open marginal and petiolar sinuses, and become asymmetrical and distorted with prominent marginal teeth (Andret-Link et al., 2004; Martelli et al., 2001). These symptoms cause the leaves to resemble a fan-like structure, hence the name “fanleaf”. Other foliar symptoms include chlorotic mottling, yellow mosaic, ringspot, and light green to chrome yellow chlorotic bands along the veins (Andret-Link et al., 2004; Martelli et al., 2001). The virus also causes cane malformations that include uneven internode spacing, fasciations, zigzag growth, double nodes and flattening of the canes (Raski et al., 1983). The berry clusters are reduced in size and number and berry ripening is irregular and some berries do not reach maturity (Martelli and Savino, 1990). Crop losses range from moderate (10%) to high (80%) and in extreme cases, the total crop can be lost depending on the susceptibility of the cultivar and the virulence of the virus strain. The fruit quality is affected by a reduction in sugar content and vine longevity could decrease by 50% in GFLV infected grapevines (Andret-Link et al., 2004; Martelli et al., 2001). Symptoms develop in early spring and fade away throughout the vegetative season (Martelli, 1993). *Grapevine fanleaf virus* also causes the formation of abnormal ribbon shaped bodies that cross the lumen of infected vines (Graniti and Russo, 1965). These endocellular tubular structures consist mainly of cellulose sheaths coated with

lignin, suberin or cutin depending on the tissue, and are visible in the lignified shoots and basal internodes (Martelli and Savino, 1990).

2.3.7 Host Range

Vitis species especially, *V. vinifera* and *V. rupestris* are the major natural hosts of GFLV, but the virus can occasionally infect weeds (Horvath et al., 1994). Diagnostically susceptible host species are limited to species from the families *Amaranthaceae, Chenopodiaeae, Cucurbitaceae, Leguminoseae, Solanaceae* and *Fabaceae* (Andret-Link et al., 2004; Martelli and Holland, 1987). These species can be infected by GFLV through the inoculation of infected grapevine sap.

2.3.8 Molecular diversity

In a single host plant, RNA viruses show a heterogeneous population structure, called "quasispecies" (Kissi et al., 1999; Schneider and Roossinck, 2000). *Grapevine fanleaf virus* is no different. Quasispecies are mainly caused by to two factors: firstly, the RNA virus infects the host plant for long periods of time and the genomes are subjected to a continuous process of competition and selection (Holland and Domingo, 1998; Roossinck, 1997; Sevilla et al., 1998). Secondly, there is no proofreading mechanism associated with the RNA1-encoded RNA-dependant RNA polymerase causing an error prone replication process that generates mutant genomes at a high rate (Gracia-Arenal et al., 2001). The high mutation rate allows several master sequences and related variants, with different biological properties to form, causing the population to become more stable rather than genetically more diverse or variable (Dockter et al., 1996; Gracia-Arenal et al., 2001).

Numerous molecular studies have been done in several countries to determine the diversity of GFLV isolates. Naraghi-Arani et al. (2001) investigated the quasispecies nature of 14 isolates of GLFV in California. A 1557 bp fragment spanning a part of the CP gene and part of the 3' untranslated region was analysed through sequencing and RFLP analysis. Sequence identities of 87% were found on nucleotide level and 91% on amino acid level. The data suggested quasispecies populations within the GFLV genome. Vigne et al. (2004a) characterised 347 GFLV isolates from the Champagne region in France and found that 55% of the population had one predominant restrictotype (clones that have the same RFLP profiles) and 33% of sequenced isolates had a population structure with 2 distinct haplotypes. A strong genetic stability was found in the CP gene, with sequence identities of between 86.2 to 99.5% at nucleotide level and 93.1 to 99.8% at amino acid level. Fattouch et al. (2005b) characterised a 605 bp fragment from the CP gene from 20 GFLV isolates from naturally infected grapevine from north Tunisia and found that the GFLV population consisted of two restrictotypes corresponding to 2 distinct sub-populations (Sp1 and Sp2). The sequences from these two sub-populations vary as much as 11% on nucleotide level. Based on the

severity of symptoms, GFLV isolates were previously separated into two forms, chromogenous and deforming strains (Hewitt, 1971; Martelli, 1993). No association was found between yellow mosaic and leaf malformation symptoms and single Sp1 and Sp2 infected plants; however increased severity of symptoms was observed in grapevine containing both Sp1 and Sp2 strains. Bashir et al. (2007c) analysed a 1620 bp fragment, corresponding to the CP gene and the 3'non-coding (3'NC) region, of 8 GFLV isolates from Iran and found two distinct isolates. Identities between the two isolates were between 83 to 94% on nucleotide level.

Wetzel et al. (2001) analysed the complete nucleotide sequence of the RNAs 2 of GFLV and ArMV from isolates of south-west Germany. Their study revealed nucleotide homology levels of up to 72% between isolates. The MP gene of ArMV and GFLV was found to be conserved with homologies ranging from 76-78% on nucleotide level and 86-88% on amino acid level. The coat protein gene of these isolates was found to be very similar with homologies ranging from 67-68% on nucleotide level and 68-69% on amino acid level. Most variability was found in the 2A genes with homologies ranging from 68-78% on nucleotide level and 59-75% on amino acid level (Wetzel et al. 2001). ArMV infected vines show symptoms similar to GFLV infected vines and mixed infections can occur (Weber et al., 2002).

2.3.9 Serology

Grapevine fanleaf virus is serologically homogeneous virus species and natural variants are rare. In 1985, Savino et al. identified one such variant in Tunisia by using a gel double diffusion test. Huss et al. (1987) distinguished between different GFLV variants from five countries with the use of monoclonal antibodies. These variants could not be identified by polyclonal antibodies.

Grapevine fanleaf virus and ArMV are from the same *nepovirus* sub-group and are serologically related (Dias and Harrison, 1963), this is supported by similarities found between the amino acid sequences of their CPs (Wetzel et al., 2001). The low nucleotide and amino acid sequence variability between the CPs of different GFLV strains enhance serology based diagnostic techniques and permits cross reaction in these techniques.

2.3.10 Cytopathology

Grapevine fanleaf virus particles have been observed in the roots, mesophyll cells and vascular parenchyma and cytoplasm of grapevines; here the particles are rare and aligned in short rows (Gergola et al., 1969; Kalasjan et al., 1979). Empty particle rows in the nucleoplasm were observed (Savino et al., 1985) and virus particles within the membranous tubules connected to the plasmodesmata (Gerola et al., 1969; Savino et al., 1985). Protein P3 of the satellite particle is

detected in subcellular membrane fractions and nucleus-enriched fractions but not in cytoplasmic or cell wall fractions (Moser et al., 1992).

2.3.11 GFLV control

Grapevine fanleaf virus could have a dramatic economic impact on the grape industry. The prevention of the spread of the virus is therefore essential for the industry. The availability of virus-free propagation material has greatly increased in most viticultural regions because of the implementation of grapevine certification and the establishment of quarantine facilities (Andret-Link et al., 2004). For these strategies to be successful, sensitive and reliable molecular and serological tests are essential. Although the long distance spread of the virus is somewhat restricting through these certification strategies, GFLV control is still a major problem in diseased vineyards. To win this fight, the natural cycle of the nematode-virus complex must be broken (Andret-Link et al., 2004). This could be done through cultural practices and soil disinfestations.

To eliminate GFLV from vineyards where *X. index* is absent, infected grapevines need to be replaced by new, virus-free grapevine material. Where both *X. index* and GFLV are present, the main control strategy is to disinfect the soil to reduce *X. index* populations (Raski et al., 1983; Taylor and Brown, 1997). However, because this strategy is limited to upper soil, and the nematicides is often very toxic and prohibited in certain countries and the efficacy is low (Abawi and Widmer, 2000; Burrows et al., 1998). The most effective strategy is crop rotation and the removal of vineyards and root debris from the infected areas for a period of 10 years (Vuittenez et al., 1969). Since only grapevines and figs are natural host plants for *X. index*, the nematode cannot reproduce and survive when other crop are planted on *X. index* infested soils (Siddiqi, 1974; Wyss, 2000). However, this is not a viable strategy for premium commercial vineyards where fallow periods are limited to 2 years (Andret-Link et al., 2004).

The conventional breeding for resistance to GFLV is an ideal strategy to control the virus, but no useful source of resistance to GFLV has so far been identified in wild or cultivated grapevine (Lahogue and Boulard, 1996; Raski et al., 1983). Breeding of grapevine that is resistant to *X. index* is another control strategy. Currently varieties that are tolerant to *X. index* have been identified in Muscadine grapes, (Bouquet, 1981; Harris, 1983; Malan and Meyer, 1993; McKenry et al., 2001; Meredith et al., 1982; Raski et al., 1983; Walker et al, 1985) but they do not completely exclude GFLV (Staudt and Weischner, 1992).

With the progress in determining the genomic structure, protein function and sequence diversity of GFLV, genetic engineering became a viable and attractive strategy to engineer GFLV resistant

rootstocks and thus control GFLV (Andret-Link et al., 2004, Fuchs, 2003). Transgenic *Nicotiana benthamiana* expressing the CP gene from GFLV has shown high levels of resistance against the virus (Bardonnet et al., 1994). Through the use of complete, truncated, sense or antisense, translatable or untranslatable gene constructs, GFLV $2C^{CP}$, $2B^{MP}$and $1E^{Pol}$ genes have been transferred to grapevine successfully (Krastanova et al., 1995; Mauro et al., 1995; Xue et al., 1999). Vigne et al. (2004b) recently reported resistance to GFLV in transgenic rootstocks after a three year trial in naturally infected vineyards in France; this study indicates that genetic engineering could be a successful strategy to control GFLV infections.

2.4 Plant virus diagnostic testing

2.4.1 Diagnostic systems used in grapevine virus detection and identification

The sensitive, reliable and rapid identification of plant viruses is essential for effective disease control. Virus detection and identification is important for preventing the spread of the viral infection and is needed to implement quarantine regulations. It is also important in disease epidemiology research and for designing new control strategies.

Before the development of laboratory testing, field and biological indexing was used to detect and identify viral pathogens using morphological criteria. However, this method is time consuming and requires an extensive knowledge in taxonomy and disease symptomology. Most grapevine virus diseases only show diagnostic symptoms during certain times of the year and it is virtually impossible to diagnose grapevine diseases on morphology alone in dormant seasons. It is also difficult to discriminate between closely related viruses and mixed infections, and some viruses may never show obvious symptoms. The concentration of the viral agents could also be too low for the plant to show any disease symptoms, and the vine could be perceived as virus free. More reliable, sensitive, rapid and cost effective diagnostic assays that can be taught quickly and easily to semi-skilled staff were required to detect and identify plant viruses. This led to the development of laboratory diagnostic techniques relying on serological and molecular properties of the viral agents.

2.4.2 Serological testing

The first breakthrough in the development of a laboratory diagnostic testing technique came in 1977 with the development of the ELISA (Clark and Adams, 1977). This technique is based on the production of antibodies in an animal host against the virus of interest. Different variations of the ELISA have been developed. Indirect DAS-ELISA is the most commonly used variation (fig. 2.5). Sap extract samples are added to a microtitre plate coated with capture antibodies raised against the virus of interest (in example a rabbit). If the virus is present in the sap extract it will bind to the antibodies fixed on the microtitre plate. After adding the sap-extracts a detecting antibody (also

raised against the viral agent) is added. A third antibody or enzyme-linked secondary antibody (example a goat anti-rabbit antibody) is then added and binds to the detecting antibody. This secondary antibody antibody is coupled to a reporter molecule that allows for indirect detection of the virus. The reporter molecule, usually an enzyme, acts on a substrate causing it to change colour, which can be measured by a spectrophotometer (O'Donnell, 1999; Ward et al., 2004). Antibodies against viruses have also been used in other serological diagnostic techniques including western blots, dot-blot immunobinding assay, immunodiffusion assays, immunostrip tests and serologically specific electron microscopy (SSEM) (Schaad et al., 2003).

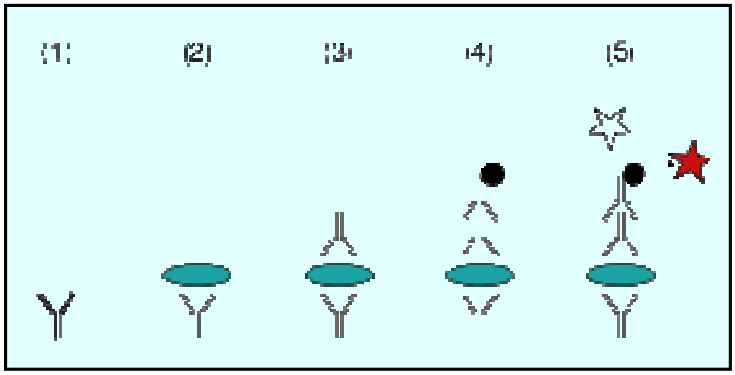

Figure 2.5: Schematic presentation of an indirect Double Antibody Sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (1) the microtitre plate is coated with a capture antibody; (2) sap extract containing the target virus is added and binds to capture antibody; (3) the detecting antibody is added, and binds to the viral agent (4) the secondary antibody coupled to an enzyme is added and binds to the detecting antibody; (5) substrate is added, and is converted by the enzyme to detectable form.
(http://en.wikipedia.org/wiki/ELISA#.22Indirect.22_ELISA)

2.4.2.1 Antibody production

There are two main routes for the production of antibodies for use in diagnostic assays, polyclonal antibodies and monoclonal antibodies. Polyclonal antibodies are prepared by injecting purified virus particles into a host animal. The serum is collected after a period of time and the polyclonal antibodies purified from the serum. The recognition of multiple epitopes of the virus particles makes the polyclonal antibodies tolerant to small changes in the nature of the antigen or virus. Polyclonal antibodies are heterogeneous and the serum contains a complex mixture of antibodies with different affinities. They are rapid and cost effective to produce and can be generated in a variety of animals.

The disadvantages of polyclonal antibodies are that they are generated in limited amounts and the specificity varies from batch to batch, the antibodies recognize multiple specificities and could react to similar viruses and plant protein extracts, leading to false positives. The development of monoclonal antibodies against pathogens increased the specificity of these serological tests (Köhler

and Milstein 1975). Monoclonal antibodies are prepared by injecting mice with the purified virus. Single antibody forming cells, developed in the spleen of the mouse, are collected and fused *in vitro* with immortal myeloma cells to form hybridomas. Each hybridoma is cloned and will produce identical monoclonal antibodies. In contrast to polyclonal antibodies, monoclonal antibodies recognize a single epitope, are highly specific and are available in unlimited supply making it ideal for the detection and identification of specific viral strains. However, the rapid mutation rate of especially RNA viruses could cause small changes in the epitope, leading to false negatives. The production of monoclonal antibodies are also time consuming and expensive.

Traditionally, virus particles were used as viral antigens for polyclonal and monoclonal antibody production. However, virus purification is a labour-intensive process, with varying and sometimes substandard results concerning the purity and concentration of the purified virus (Fajardo et al., 2007). The purified virus preparation could contain host plant proteins as well as other viruses present in the host plant that could lead to non-specific reactions (Ling et al., 2007). Other difficulties in the purification of virus particles is low virus titre in woody plants, the lack of sufficient herbaceous hosts and the presence of inhibitor compounds such as polyphenols, tannins and polysaccharides (Ling et al., 2000; Ling et al., 2007). Antibodies produced against different batches of purified viruses may cause varying specificities and titres and could lead to inconsistent results (Barbieri et al., 2004).

With the development of molecular techniques and the increasing number of available virus sequences, the genes of structural proteins such as viral CPs could be amplified, cloned, expressed, purified and used as a viral antigen for polyclonal (Vaira et al., 1996) and monoclonal antibody production. Recombinant DNA technology has become widely acceptable technique for the production of high amounts of stable antibodies with uniform concentrations (Targon et al., 1997; Barbieri et al., 2004; Nickel et al., 2004).

2.4.2.2 Host cells for Recombinant protein expression

Different organisms are available for antibody expression, each with its advantages and disadvantages (table 2.1). When selecting the appropriate expression system, important factors such as protein solubility, functionality, speed, and yield need to be considered. The low cost, high protein yield, short growth time, convenient expression control, well-known genetics, large selection of available plasmids and strains makes *Escherichia. coli* the most commonly used recombinant protein expression host cell in research and diagnostics (http://www.exptec.com/Stategies/Strategies.htm). The selection of an appropriate *E. coli* strain for recombinant expression is extremely important. The selected *E. coli* strain should be able to

maintain the expression plasmid stably, posses the genetic elements necessary for the expression system and be deficient in the major natural harmful proteases encoded by the *lon* gene (Phillips et al., 1984; Sorensen and Mortenson, 2005).

The *E. coli* strain BL21 is a *Ion* and *ompT* protease deficient strain (http://www.nextgensciences.com/pdf/documentation/comp_cells/BL21%20DE3%20pLysS%20PI.pdf) and is recommended for use with Glutathione S-Transferase (GST) gene fusion system for optimal expression of the fusion protein (Harper and Speicher, 2001; GST gene fusion system handbook, 2002). BL21(DE3)pLysS is a chloramphenicol resistant derivative of *E. coli* B strain that encodes for T7 phage lysozyme. T7 Lysozyme is a natural inhibitor of T7 RNA polymerase and suppresses basal expression from the T7 promoter (http://openwetware.org/wiki/E._coli_genotypes#BL21.28AI.29). Rosetta(DE3)pLysS is a chloramphenicol resistant derivative of *E. coli* B strain that also encodes for T7 phage lysozyme. It contains the tRNA genes argU, argW, ileX, glyT, leuW, proL, metT, thrT, tyrU and thrU that supplements the rare codons AGG, AGA, AUA, CUA, CCC and GGA. (http://openwetware.org/wiki/E._coli_genotypes#BL21.28AI.29).

2.4.2.3 Protein expression and purification strategies

The choice of vector or plasmid is important for the expression strategy as well as for the purification of the expressed protein. To enhance the solubility of the expressed protein and simplify the purification, a wide range of fusion partners has been developed. Most fusion partners have their own specific affinity purification method. A fusion partner or affinity tag is linked to the target protein by a recognition site that is specific for a given protease.

Fusion partners offer several advantages, such as the prevention of fusion body formation, the improvement of protein folding characteristics, protection of the target protein from intracellular proteolysis, improved solubility of the target protein, facilitation of one-step adsorption purification, easy to remove from the target protein and it has a minimal effect on the tertiary structure and biological activity of the target protein (Baneyx, 1992; Hanning and Makrides, 1998; Sorensen et al., 2005; Terpe, 2003). Several different fusion partners or affinity tags have been developed and are commercially available, of which the histidine fusion partner (His-tag), maltose binding protein (MBP), NusA and GST are some examples. The choice of fusion partner depends on the target protein expressed, for example the hydrophobicity and stability of the target protein.

Table 2.1: Comparison of commonly used host cells for protein expression

Host cell	Advantages	Disadvantages
Cell free	Rapid expression directly from the plasmid. Open system, easy to incorporate components to enhance expression.	Low amounts of expressed protein.
E. coli	High amounts of expressed protein, up to 80% of total cellular proteins. Inclusion bodies can facilitate purification. High range of fusion systems and vectors available. Rapid growth rate. Low costs. Uncomplicated culture conditions. Convenient expression control systems.	Over-expression may cause insoluble expressed protein. Lack of post-translational modification. Endotoxins. Solubility (can be enhanced with vector selection). Growth conditions may require optimization.
Yeast	Eukaryotic protein expression and processing. No endotoxins. Uncomplicated media requirements. Secretion facilitates purification.	Yeast cell walls are difficult to break and limit protein purification. Limited vectors available. More difficult expression control than *E. coli*. Fermentation required for large scale protein expression. Growth conditions may require optimization.
Insect (*Baculovirus*)	Post-translational modifications similar to mammalian host cells. High yields facilitate protein purifications. Greater yield than mammalian host cells.	Difficult culture conditions. Expressed protein activity not 100%. Over-expression may cause insoluble expressed protein.
Mammalian	Expressed protein activity equal to natural protein. Highest level of post-translational modifications.	Large scale protein expression only possible in suspension cultures. Difficult culture conditions, requires expensive labour, facilities and consumables. Low cell growth rate. High costs.

(http://www.exptec.com/Stategies/Host%20cells.htm) (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Protein-Expression.reg.za.htmlwww.invitrogen.com)

The GST gene fusion system is one of the most widely used expression systems. This pGex vector fuses a *Schistosoma japonicum* 26 kDa GST protein to the target protein (GST gene fusion system handbook, 2002). One of the advantages of GST as fusion partner is that the protein is not folded into inclusion bodies and thus helps to increase the solubility of the fusion protein (Einarson et al., 2005). The GST fusion partner also improves expression levels and eases the purification of the fusion protein. A specific protease cleavage site is located between the GST fusion partner and target protein and is easily cleaved to remove the GST fusion partner (Harper and Speicher, 2001).

Table 2.2: Comparison of ELISA and PCR techniques for the diagnosis and detection of plant viruses.

Characteristics	ELISA techniques	PCR techniques
Ease of development	Time consuming and costly to develop sensitive antibodies	Rapid if sequence information is available
Sample preparation	Minimal	Minimal if crude plant extracts are used, but for larger fragment amplifications, DNA or RNA extraction is necessary.
Procedure	Generally simple, no skilled staff necessary.	Generally complex, technically skilled staff required.
Portability	Assays can be adapted for field testing	Assays can only be done in a laboratory
Specificity	Depends on antibody quality	Excellent, can be adapted to be genus, species or isolate specific
Sensitivity	Generally less sensitive than PCR	Excellent, can detect a few copies of the target virus
Quantification	Directly quantitative	Laborious in standard PCR, but simple and rapid quantification is possible using real-time PCR.
Detection of multiple pathogens	Possible, depends largely on antibody quality	Easy with multiplex PCR
Cost	Cost effective, especially for high throughput applications	Expensive, especially real-time PCR, but costs are decreasing with the increased acceptance of this technique

(Ward et al., 2004)

2.4.3 Nucleic acid testing

Molecular techniques specifically target the genetic material of plant viruses. The availability of genome sequences of more plant viruses allows molecular techniques to be more sensitive and specific than serologically based techniques. Polymerase chain reaction (PCR) was developed in the early 1990's and has the ability to selectively amplify a part of the target deoxyribonucleic acid (DNA).

In contrast with serological based techniques, molecular techniques, could target the entire viral genome not just the CP as with ELISA diagnostic tests. For the amplification of RNA, PCR was adapted to include a reverse transcription (RT) step to convert the RNA to DNA before amplification, making it more viable for virus diagnostics, because most viruses have an RNA genome (Waterhouse and Chu, 1995). PCR was also adapted to test for multiple viruses by adding extra sets of primers or with the use of a single pair of degenerate primers.

The sensitivity and specificity of PCR could be enhanced by using nested PCR. This technique involves two PCR reactions, in the second reaction primers are used to recognise and amplify a region within the PCR product of the first reaction (Schesser et al., 1991; Mutasa et al., 1995; Foster et al., 2002). Although ELISA is not as sensitive as molecular techniques, it is still widely used because of low technical skill requirements, cost effectiveness and portability, making it ideal for large scale field testing (table 2.2).

3 DAS-ELISA DEVELOPMENT

3.1 Introduction

Grapevine fanleaf virus causes one of the most devastating diseases in grapevine. To successfully control the virus a sensitive and reliable diagnostic assay is necessary. In this study an attempt was made to design a reliable and sensitive diagnostic DAS-ELISA specific to the South African strain(s) of GFLV. The use of recombinant DNA technology to express the protein was selected as the method of choice for the production of GFLV specific antibodies. This method eliminates the possibility of false positive results by producing antibodies against the purified virus coat protein and not to the purified virus particles that could contain plant proteins and inhibitory compounds (Ling et al., 2007). The method is reproducible, and a constant reservoir for the development of antibodies, with the same sensitivity and specificity eliminating inconsistent results (Barbieri et al., 2004).

The synthesis of recombinant coat proteins could be performed with or without a fusion protein. The pGex-6P-2 expression system was selected as a vector, this system creates a fusion protein by attaching a GST fusion partner to the target virus coat protein. The GST fusion partner enhances the solubility of the target protein by improving the folding characteristics and facilitates a single-step adsorption purification (Einarson et al., 2005). The *Escherichia coli* strain BL21 is the recommended bacterial strain for the GST gene fusion system and promotes optimal expression of the fusion protein (Harper and Speicher, 2001; GST gene fusion system handbook, 2002).

In this study, the GFLV CP gene was cloned into the pDrive cloning vector and subcloned into the pGex-6P-2 expression vector. The plasmid containing the GFLV CP gene was transformed into BL21(DE3)pLysS and Rosetta(DE3)pLysS for expression. The expressed protein was purified using GST-bind chromatography.

3.2 Materials and methods

3.2.1 Plant material

Grapevine fanleaf virus-infected grapevine leaf material was obtained from the South African Plant Improvement Organisation (SAPO), from Vititec, Paarl, South Africa, as well as from the South African Agricultural, Food, Quarantine & Inspection Services, Stellenbosch, South Africa.

3.2.2 Total RNA extraction

Total RNA extraction was performed according to a modified method of Davies and Robinson (1996). Two and a half grams of leaf material was ground to a fine powder with liquid nitrogen using a mortar and pestle. The ground leaf material was resuspended in 25 ml of extraction buffer (3 M $NaClO_4$, 0.2 M Tris-HCl, pH 8.3, 5% (w/v) Sodium Dodecyl Sulphate (SDS), 8.5% (w/v) Polyvinyl Polypyrrolidone (PVPP), 2% (w/v) Polyethylene Glycol (PEG) 6000, 1% (v/v) β-mercaptoethanol (β-ME)), stirred (30 min, room temperature) and centrifuged (10 000 x g, 30 min, 4°C). The supernatant was removed, placed into a new polypropylene tube and precipitated with 2.5 volumes of 99% (v/v) ethanol (2 h, -20°C) followed by centrifugation (8 300 x g, 15 min, 4°C). The pellet was washed (70% (v/v) ethanol, 16 000 x g, 10 min, 4°C) and resuspended in 2 ml TE buffer (10 mM Tris-HCl, pH 8, 0.1 mM Ethylene diamine tetra-acetic acid di-sodium salt (EDTA), 0.2% (v/v) β-ME). The sample was divided into four 1.5 ml Eppendorf tubes and six wash steps were performed with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1, phenol pH 4.3,) followed by a single wash step in an equal volume of chloroform:isoamylalcohol (24:1) (16 000 x g, 10 min, 4°C). The supernatant was precipitated with 2.5 volumes of 99% (v/v) ethanol and 0.1 volumes of 3 M NaOAc and incubated (2 h, -20°C), followed by centrifugation (16 000 x g, 20 min, 4°C). The pellet was washed with 300 µl of 70% (v/v) ethanol (16 000 x g, 10 min, 4°C), dried on ice and resuspended in 100 µl of dH_2O. The RNA was precipitated by adding 0.3 volumes of 8 M LiCl and incubated overnight (4°C). The pellet was washed with 300 µl of 70% (v/v) ethanol (16 000 x g, 10 min, 4°C), dried on ice and resuspended in 50 µl of dH_2O, aliquoted and stored at -80°C. The RNA quality was analysed by gel electrophoresis (90 min, 100 V) and the concentration determined using the NanoDrop®ND-1000 Spectrophotometer according to the manufacturer's instructions.

3.2.3 GFLV coat protein amplification

3.2.3.1 Primer design

Primers were designed to conserved regions of available GFLV sequences from the GENBANK database of the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) using

PRIMER DESIGNER (Version 1.01). The primers were synthesised by Inqaba Biotech (Pretoria, South Africa) and Integrated DNA Technologies, Inc (Coralville, IA, USA).

Table 3.1: Primers used in this study

Primer name	Primer sequence (5' - 3')	Region	Annealing temperature
GFLV-500-F	AGTGAGTGGAACGGGACCACTATGG	Coat protein	62.0°C
GFLV-500-R	CACCAGCTTCGTGATGGTAACGCT	Coat protein	61.4°C
GFLV-MP-F	ACCTTCTCTATCAGRAGYCG	Movement protein	50.8–55.8°C
GFLV-NC-R	ACAAACAACACACTGTCGCC	Non-coding region	56.3°C
GFLV-INV-F	CACATACACCCCGGGATACT	Coat protein	56.2°C
GFLV-CP(*Sal*I)-F	CGGTCGAC**T**GGATTAGCTGGTAGAGG	Coat protein	61.8°C
GFLV-CP(*Sal*I)-R	AGTCGACCTAGACTGGGAAGCTGG	Coat protein	61.8°C
T7	TAATACGACTCACTATAGGG	pDrive	47.5°C
SP6	TACGATTTAGGTGACACTATAG	pDrive	48.8°C
pGex-5'	GGGCTGGCAAGCCACGTTTGGTG	pGex-6P-2	65.7°C
pGex-3'	CCGGGAGCTGCATGTGTCAGAGG	pGex-6P-2	64.0°C

Underlined nucleotides represent the *Sal*I recognition site. The Bold nucleotide in primer GFLV-CP-(*SalI*)-F represents the inserted nucleotide to ensure that the CP sequence remains in frame.

3.2.3.2 Reverse transcription polymerase chain reaction (RT-PCR)

The rapid-direct-one-tube-RT-PCR was performed according to the modified method of Osman et al. (2007). The GFLV-500-F and GFLV-500-R primers were used for testing the grapevine material received for GFLV infection. The rapid-direct-one-tube-RT-PCR method was used to amplify small fragments (≤500 bp). Four leaf disks or 60 µg of infected leaf material was ground in 600 µl (1:20) grinding buffer (, 1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$ (pH 9.6), 2% (w/v) Polyvinyl Pyrrolidone (PVP) 40, 0.2% (w/v) Bovine Serum Albumin (BSA), 0.05% (v/v) Tween 20, 1% (w/v) $Na_2S_2O_5$). Four microlitres of the extract was then pipetted into a tube containing 1 x GES buffer (100 mM glycine-NaOH pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X100) to give a final volume of 50 µl. The sample was denatured for 10 min in a waterbath at 95°C followed by direct cooling on ice for 5 min. Two microlitres of the denatured RNA was directly pipetted into the one-step-RT-PCR mix (1 x BIOLINE PCR NH_4 reaction buffer, 1.5 mM $MgCl_2$, 1 x cresol, 0.2 mM dNTPs, 0.4 µM GFLV-500-F primer, 0.4 µM GFLV-500-R primer (table 3.1), 5 mM 1,4-Dithiothreitol (DTT), 1 U AMV reverse transcriptase (Promega), and 1 U BioTaq DNA polymerase (BIOLINE)) to a final volume of 25 µl per reaction. The RT-PCR cycle conditions were: 1 cycle of 45 min at 45°C, 1 cycle of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C respectively, 1 cycle of 7 min at 72°C.

RNA was extracted from the grapevine samples that tested positive for GFLV through the rapid-direct-one-tube-RT-PCR. These RNA samples were used to amplify a 1765 bp fragment extending the CP, from within the MP to the 3'NC region of the virus, through one-step-RT-PCR and the primers GFLV-MP-F and GFLV-NC-R (table 3.1).

The one-step-RT-PCR method was used to amplify large fragments (≥ 1 kb). Two microlitres of the extracted RNA was directly pipetted into the one-step-RT-PCR mix (1 x BIOLINE PCR NH_4 reaction buffer, 1.5 mM $MgCl_2$, 1 x cresol, 0.2 mM dNTPs, 0.4 µM GFLV-MP-F primer, 0.4 µM GFLV-NC-R primer (table 3.1), 5 mM DTT, 1 U AMV reverse transcriptase (Promega), and 1 U BioTaq DNA polymerase (BIOLINE)) to a final volume of 25 µl per reaction. The RT-PCR cycle conditions were: 1 cycle of 45 min at 42°C, 1 cycle of 3 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 53.5°C and 2 min at 72°C respectively, 1 cycle of 7 min at 72°C.

PCR re-amplification was performed using Ex Taq (Takara), an enzyme with proofreading capabilities, to reduce the chance of incorporating mutations. The PCR was performed according to the manufacturer's instructions.

Agarose gel electrophoresis was performed according to Sambrook et al. (1989). DNA fragment separation was performed on a 1% (w/v) agarose gel in 1 x TAE (40 mM Tris, 0.114% (v\v) HOAc, 1 mM EDTA pH 8.0) at 100 V for 60 min. Ethidium bromide (0.5 µg/ml) was added to the agarose gel to a final concentration of 0.01% (v/v) for ultra violet visualisation (gel documentation system, SynGene). Six times (6x) Loading Dye (Fermentas) was used to assist in DNA loading. Gene Ruler 1 kb DNA ladder (Fermentas) were used to determine the molecular size of the DNA fragments, together with GeneTools and GeneSnap (SynGene) gel analysis tools.

3.2.4 Cloning and transformation of the 1765 bp GFLV fragment into pDrive cloning vector

The Wizard® SV Gel and PCR cleanup system (Promega) were used to purify the PCR fragments from the agarose gels. The purified GFLV CP gene was ligated into pDrive cloning vector using the Qiagen PCR cloning kit. The purification and ligation was done according to the manufacturer's specifications.

Chemically competent cells were prepared according to a modified method of Sambrook et al. (1989). A single colony of *E. coli* DH5α was inoculated into 5 ml of Luria Bertani (LB) broth and incubated (225 rpm, overnight, 37ºC). The overnight culture was used to inoculate 500 ml (1:100 dilution) LB broth and incubated (225 rpm, 37ºC) until an optical density, at an absorption value of 600 (OD_{600}) was between 0.5 and 0.6. The culture was centrifuged (5 000 x g, 10 min, 4°C), the pelleted cells were resuspended in 100 ml ice cold $MgCl_2$ (100 mM) and incubated on ice (30 min). The cells were pelleted (4 000 x g, 10 min, 4°C), and resuspended in 10 ml filter sterilised (0.22 µm) $CaCl_2$ (100 mM, with 15% glycerol,). One hundred microlitres of cells were aliquoted into prechilled 1.5 ml tubes, flash frozen in (ice-cold 96% (v/v) ethanol and stored at -80°C until use.

All transformations were performed using the protocol specified by Sambrook et al. (1989). One hundred microlitres of the chemically competent dH5α cells was added to the ligation reaction, mixed gently and incubation on ice for 10 min. The cells were heat shocked (45 sec, 42°C waterbath) and directly incubated on ice for 5 min. Nine hundred microlitres of LB broth (Merck) was added to the transformation reaction and incubated (155 rpm, 60 min, 37°C). One hundred microlitres was plated out onto LB bacteriological agar (Merck) plates containing 100 µg/ml Ampicillin (Amp), for pDrive selection and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, Fermentas) and 0.2 mM Isopropyl-β-D-thiogalactoside (IPTG, Fermentas), for blue-white colony selection. The remaining 900 µl of the transformation reaction was centrifuged (2 000 x g, 60 sec), the cells resuspended in 100 µl of LB broth and plated out, when low transformation efficiencies were expected. The plates were incubated overnight at 37°C.

3.2.5 pDrive screening for the 1765 bp GFLV insert

White colonies were screened for the correct insert size using PCR with insert specific primers. The white colonies were picked with a sterile toothpick and the tip inserted and briefly swirled in a tube containing 25 µl PCR reaction mixture (1 x BIOLINE PCR NH_4 reaction buffer, 1.5 mM $MgCl_2$, 1 x cresol, 0.2 mM dNTPs, 0.4 µM GFLV-500-F primer, 0.4 µM GFLV-500-R primer (table 3.1), and 1 U BioTaq DNA polymerase (BIOLINE)). The PCR cycle conditions were: 1 cycle of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 53.5°C and 2 min at 72°C respectively, 1 cycle of 7 min at 72°C. The confirmed positive white colonies were inoculated in 5 ml LB broth containing 100 µg/ml Amp and incubated (225 rpm, overnight, 37°C).

A GeneJet Plasmid Miniprep kit (Fermentas) was used according to the manufacturer's specifications to purify the plasmid DNA. When large quantities of plasmid DNA was purified for restriction enzyme analysis the plasmid alkaline lysis mini-prep method was used (Sambrook et al., 1989). Three millilitres of the overnight culture was centrifuged (16 000 x g, 1 min) and the supernatant discarded. The pellet was resuspended in 100 µl of solution 1 (25 mM Tris-HCl pH 8, 500 mM Glucose, 100 mM EDTA) and incubated (5 min, room temperature). Two hundred microlitres of solution 2 (200 mM NaOH, 1% (w/v) SDS) was added, inverted 4 times and incubated on ice for 5 min. Thereafter 150 µl of ice-cold solution 3 (3 M KOAc, 2 M HOAc, pH 4.8) was added, inverted 4 times, incubated (5 min, on ice) and centrifuged (16 000 x g, 10 min, 4°C). The supernatant was recovered, transferred to a new tube and precipitated with 2.5 volumes of ice cold 99% (v/v) ethanol. The solution was inverted, incubated (5 min, -20°C) and centrifuged (16 000 x g, 10 min, 4°C). The supernatant was discarded and the pellet washed with 70% (v/v) ethanol (16 000 x g, 5 min, 4°C). The pellet was dried (10 min, room temperature) and resuspended

in 20-50 µl of dH_2O depending on the pellet size. DNA concentrations were determined using the NanoDrop® ND-1000 Spectrophotometer according to the manufacturer's instructions.

Freezer cultures of positive clones were made by adding 420 µl 50% glycerol to 980 µl of overnight culture and stored at -80°C.

All plasmid DNA samples were screened with restriction enzyme digestion for the appropriate size insert, before sequencing. The plasmids were digested with *Eco*RI (Fermentas) according to the manufacturer's specifications.

3.2.6 Sequencing and sequence analysis

Plasmid DNA templates were sequenced with the Applied Biosystems ABI PRISM BigDye_ Terminator v3.0 Ready Reaction Cycle Sequencing Kit according to the manufacturer's instructions. The T7 and SP6 primers (table 3.1) were used as well as the GFLV-INV-F primer to sequence the full length of the fragment extending from the MP to the 3'NC region of the RNA2 genome. Sequencing was performed by the Core DNA Sequencing Unit, Department of Genetics, Stellenbosch University.

Chromas (version 1.45) (www.technelysium.com.au/chromas.html) and BioEdit (version v7.0.4, Hall, 1999) were used to perform sequence editing. Sequence comparisons were performed using the BLAST algorithm (Altschul et al., 1990) against the GENBANK database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and BioEdit (version v7.0.4, Hall, 1999). Restriction sites within the CP were determined with NEBcutter, New England BioLabs inc., (www.tools.neb.com/NEBcutter2). The GFLV CP gene was analysed with NEBcutter, New England BioLabs inc., (www.tools.neb.com/NEBcutter2) to select a restriction enzyme that did not cut within the CP. *Sal*I sites were subsequently introduced at the 5' and 3' ends of the CP fragment (1515 bp) after amplification with primers containing these sites (table 3.1, GFLV-CP(*Sal*I)-F and GFLV-CP(*Sal*I)-R). One extra nucleotide was also incorporated in the forward primer to maintain the CP ORF (appendix 1).

To amplify the CP, pDrive containing the extended fragment was re-amplified with the CP specific primers, GFLV-CP(*Sal*I)-F and GFLV-CP(*Sal*I)-R (fig. 3.1). Plasmid DNA dilutions were made and 100 ng of plasmid DNA was pipetted into the PCR reaction to a final volume of 25 µl (1 x Ex Taq reaction buffer containing 20 mM $MgCl_2$, 1 x cresol, 0.2 mM dNTPs (Takara), 0.5 µM GFLV-CP(*Sal*I)-F primer, 0.5 µM GFLV-CP(*Sal*I)-R primer (table 3.1), and 0.5 U Ex Taq (Takara). The PCR cycle conditions were: 1 cycle of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 55°C and 2 min at 72°C respectively, 1 cycle of 7 min at 72°C.

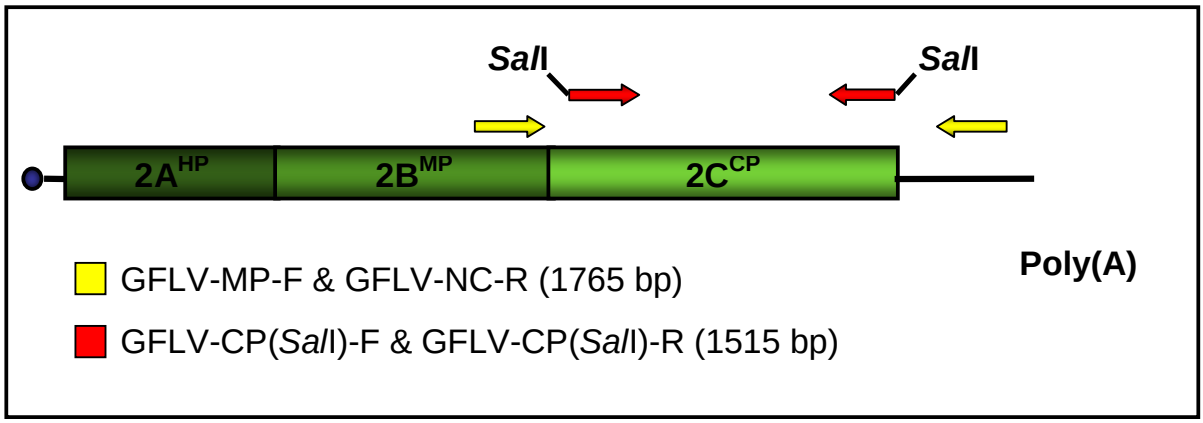

Figure 3.1: Primers positions on the RNA2 genome. The GFLV-CP(*Sal*I) primer set incorporates a *Sal*I restriction site into the *Grapevine fanleaf virus* coat protein fragment.

3.2.7 Cloning and transformation of the GFLV coat protein product into pGex-6P-2

The PCR product was analysed by gel electrophoresis (section 3.2.3.2), the fragment excised and gel purified (section 3.2.4). The fragment was ligated into the pDrive cloning vector (section 3.2.4) and transformed into chemically competent dH5α cells (section 3.2.4). Positive colonies were screened for using PCR amplification (section 3.2.5), picked and grown in LB broth and plasmid DNA extractions were performed (section 3.2.5). Freezer cultures were made from all positive clones (section 3.2.5).The coat protein was excised from the pDrive cloning vector by digestion with the *Sal*I restriction enzyme (Fermentas,) according to the manufacturer's specifications. Both the digestion products were analysed on an agarose gel (section 3.2.3.2). The coat protein fragment was excised from the gel and purified (section 3.2.4).

The pGex-6P-2 expression vector (Amersham Biosystems,) was prepared for ligation by digesting the vector with the *Sal*I restriction enzyme according to the manufacturer's specifications. The vector was treated with Shrimp Alkaline Phosphatase (SAP, Fermentas) as prescribed by the manufacturer. The pGex-6P-2 expression vector was transformed into chemically competent *E. coli* dH5α cells (section 3.2.4), and plated out. Different primer combinations (table 3.2) were used to screen the colonies through PCR amplification for the presence of the GFLV CP gene in the correct orientation (section 3.2.5). The positive colonies were picked and grown in liquid LB broth and plasmid DNA purified (section 3.2.5). Restriction enzymes that cut once in the vector and once in the CP insert were selected to confirm the PCR results (NEBcutter, New England BioLabs Inc., www.tools.neb.com/NEBcutter2). The plasmid DNA was digested with *Pst*I (Roche) according to the manufacturer's specifications, to confirm the PCR results (table 3.2). Positive clones were sequenced and analysed to confirm the orientation and the integrity of the ORF (section 3.2.6) Freezer cultures were made from all positive clones (section 3.2.5).

Table 3.2: Primer combinations and restriction enzyme digest product sizes for orientation screening of the pGex-6P-2 expression vector containing the *Grapevine fanleaf virus* coat protein fragment.

Primer set /Restriction enzyme	Product size	Orientation
pGex-5' and GFLV-500-R	1380 bp	Forward
pGex-5' and GFLV-500-F	831 bp	Reverse
*Pst*I	2360 bp and 4147 bp	Forward
*Pst*I	1100 bp and 5407 bp	Reverse

All transformations into BL21(DE3)pLysS and Rosetta2(DE3)pLysS cells were performed using the protocol specified by Sambrook et al. (1989) (section 3.2.4). One hundred microlitres of the transformation reaction in LB Broth was plated out onto LB agar (Merck) plates containing 100 µg/ml Amp, for pGex-6P-2 selection and 37 µg/ml Chbramphenicol (Roche). Freezer cultures were made from all positive clones (section 3.2.5).

3.2.8 Recombinant protein expression and purification

3.2.8.1 Recombinant protein expression

Ten microlitres of the freezer culture BL21(DE3)pLysS and Rosetta2(DE3)pLysS transformed with the pGex-6P-2 containing the GFLV CP gene insert) was inoculated in 5 ml of Terrific broth (TB, 12 g/l Bacto-tryptone, 24 g/l Bacto-yeast, 0.4% (v/v) glycerol, 1% (w/v) glucose, 1 x KPO_4 buffer (17 mM KH_2PO_4, 72 mM K_2HPO_4, pH 6.5)) and grown overnight. The culture was diluted 50 or 100 fold with TB containing 100 µg/ml Amp and 37 µg/ml Chloramphenicol and incubated (150-200 rpm, depending on the flask used, 37ºC) until the appropriate density was reached (OD_{600} of 0.5 or 1.0). The culture was induced with IPTG (0.01mM, 0.02mM, 0.05 mM 0.1 mM 0.2 mM or 1.0 mM) and incubated (150-200 rpm, 25ºC or 37ºC). After induction, samples were taken every hour for 6 hours, overnight or grown until an OD_{600} of 1.3 was reached. An uninduced control sample was subjected to the same conditions. The bacterial cells were collected (8 000 x g, 20 min, 4°C) and resuspended in 500 ml TEN50 lysis buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol, 0.2 mM DTT). To rupture the bacterial cell walls, the cell suspension was incubated at -80°C for 15 min and then at 37°C for 10 min, these freeze thaw steps were repeated three times. Fifty to 100 µg/ml DNase was added and incubated (37°C waterbath, 2 h) and sheared by passing through a 12 gauge syringe needle. One millilitre of the total cell protein was kept for SDS-PAGE analysis and the remainder of the sample centrifuged (8 000 x g, 20 min). The supernatant containing the soluble fraction of the GFLV CP was collected. The pellet containing the insoluble fraction of the GFLV CP was resuspended in lysis buffer. All samples were stored at -20°C.

A modified method of Chen et al. (2002) was also used for protein expression. In this method 3% of ethanol was added to the Terrific Broth. The cultures were incubated (150-200 rpm, 37°C) until the

correct optical density (OD_{600}) was reached (table 3.3). The cultures were heat shocked at 42°C (150 rpm), induced with 0.2 mM or 1 mM IPTG and incubated at (150-200 rpm, 37°C) until the appropriate OD_{600} was reached (table 3.3). After expression the samples were centrifuged (500 x g, 15 min and the pellet resuspended in 1/3 (of the original volume) Buffer A (25 mM Tris, 5 mM $MgCl_2$, 5 mM β-ME) or TEN150 lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 5 mM DTT, 0.2% sarkosyl, 0.4% Triton X100). Cell walls were ruptured as described above (section 3.2.8.1).

Table 3.3: Protein expression optimisation using a modified method of Chen et al. (2002)

Medium	**1st incubation at 37°C (OD_{600})**	**Heat shock at 42°C (OD_{600})**	**Expression temperature**	**Expression (OD_{600})**
Terrific broth	0.0-0.1	0.1-0.5	16°C	0.5-0.9
Terrific broth	0.0-0.1	0.1-0.5	30°C	0.5-0.9
Terrific broth	0.0-0.5	0.5-0.9	16°C	Overnight
Terrific broth	0.0-0.5	0.5-0.9	30°C	Overnight
Terrific broth with ethanol	0.0-0.1	0.1-0.5	16°C	0.5-0.9
Terrific broth with ethanol	0.0-0.1	0.1-0.5	30°C	0.5-0.9
Terrific broth with ethanol	0.0-0.5	0.5-0.9	16°C	Overnight
Terrific broth with ethanol	0.0-0.5	0.5-0.9	30°C	Overnight

3.2.8.2 Solubility optimisation

Soluble protein is needed for antibody production. The optimisation protocol described by Mercado-Pimentel et al. (2002) was used to optimise the expression of soluble protein. The transformed BL21(DE3)pLysS cells were grown overnight at 37°C in LB broth containing 100 µg/ml Amp. Twenty millilitres of the overnight culture was added to a 2 L Erlenmeyer flask containing 1 l Terrific broth, 100 µg/ml Amp and 37 µg/ml Chloramphenicol and incubated (150 rpm, 37°C), until an OD_{600} of 0.5 was reached. The culture was induced to 0.1 mM IPTG and incubated under the same conditions until an OD_{600} of approximately 1.3 was reached. The culture was centrifuged (7 000 x g, 7 min, 4°C) and the pellet washed (7 000 x g, 7 min, 4°C) with 60 ml of ice cold STE buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 100 µg/ml Phenylmethanesulphonylfluoride (PMSF)). The bacterial pellet was resuspended in STE, and treated with 100 µg/ml lysozyme (Boehringer Mannheim GmbH) for 15 min on ice, followed by the addition of DTT to a final concentration of 5 mM. The bacterial solution was then divided into 6 aliquots and different concentrations of sarkosyl were added to a final concentration of 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0% (w/v) respectively. Cell walls were ruptured as described above (section 3.2.8.1).

The soluble and insoluble fractions were analysed on a SDS-PAGE as described below and the lowest sarkosyl concentration able to solubilise the fusion protein was selected to continue with. The sample was divided into 5 aliquots and Triton X100 was added to a final concentration of 0.0,

0.5, 1.0, 2.0, and 4.0 % (v/v) respectively. The samples were briefly vortexed and 500 µl incubated with 200 µl GST-resin (Novagen, 50% slurry) for 60 min at 4°C. The resin was centrifuged (500 x g, 5 min) and washed six times with ice cold 500 µl PBS-1 (8.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) through resuspension and centrifugation (500 x g, 5 min). The pellet was resuspended in 50 µl elution buffer 2 (75 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT, 0.1% (w/v) SDS), vortexed and the supernatant collected. Samples were analysed on a SDS-PAGE gel and the lowest amount of Triton X100 able to bind to the GST resin was selected. The protocol was repeated with the selected amounts of sarkosyl and Triton X100 on a 10 ml column containing 5 ml resin (50% slurry).

3.2.8.3 Protein purification using GST-bind chromatography (Novagen)

Purification of the expressed protein was performed using affinity chromatography with GST-bind resin, adapted from Novagen's specifications. For GST:GFLV-CP fusion protein purification, soluble and solubilised fractions were loaded on a 10 ml polypropylene column containing 1 ml settled GST Bind™ resin (Novagen). Before loading, the resin was washed and equilibrated with 2 x 1 column volume of equilibration buffer (1 x PBS-2 (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) or 1 x TEN150 (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl). The protein solution was inverted for 30 min at 37°C or 16°C. The flow through was collected and stored for SDS-PAGE analysis. The column was washed with 3 volumes of wash buffer (1 x PBS-3 (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 300 mM NaCl, 2.7 mM KCl, pH 7.3) or 1 x PBS-2 containing 0.1% Triton X100). The fusion protein was eluted 3 x with 1 ml Glutathione reconstitution buffer (10, 25 or 50 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0). Each elution were collected and stored for SDS-PAGE analysis. The resin was washed with 3 column volumes PBS-2 or PBS-3 and the flow through collected. The GST-resin was stored in 5 ml 20% ethanol at 4°C.

3.2.8.4 Solubilisation of inclusion bodies

The solubilisation of inclusion bodies was performed according to Burgess (1996). The transformed BL21(DE3)pLysS cells were grown overnight at 37°C in LB broth containing 100 µg/ml Amp. Twenty millilitres of the overnight culture was added to a 2 l Erlenmyer flask containing 1 l Terrific broth, 100 µg/ml Amp and 37 µg/ml Chloramphenicol and incubated (150 rpm, 37°C) until an OD_{600} of 0.5 was reached. The culture was induced to 0.1 mM IPTG and incubated under the same conditions until an OD_{600} of approximately 1.3 was reached. The culture was centrifuged (7 000 x g, 7 min, 4°C) and the pellet washed (7 000 x g, 7 min, 4°C) with 60 ml of ice cold STE buffer. The bacterial pellet was resuspended in STE containing 5 mM DTT, Complete EDTA-free protease inhibitor (Roche) and 10 % glycerol. Cell walls were ruptured as described above (section 3.2.1.8).

The pellet containing the inclusion bodies of the GFLV CP was washed four times with STE containing 2% $C_{24}H_{39}NaO_4$ to remove cell debris, resuspended in STE containing 0.5% sarkosyl 5 mM DTT and 10% glycerol and incubated on ice for 30 min. The solution was centrifuged (7 000 x g, 7 min, 4°C), the insoluble pellet resuspended in STE and the supernatant containing the solubilised inclusion bodies stored for SDS-PAGE analysis.

3.2.9 Solubility screening through SDS-PAGE analysis

Soluble and insoluble fractions were analysed on a denaturing sodium dodecyl sulphate polyacrylamide gel. The resolving gel contained 1 x resolving buffer (375 mM Tris-HCl, pH 8.8, 0.1% SDS), 10% acrylamide solution (9.725% (w/v) acrylamide, 0.275% (w/v) bisacrylamide), 0.1% (w/v) ammonium persulphate (APS, freshly prepared), and 0.13% (v/v) Tetramethylethylenediamine (TEMED). The stacking buffer contained 1 x stacking buffer (125 mM Tris-HCl, pH 6.8, 0.1% SDS), 3.25% acrylamide solution (3.16% (w/v) acrylamide, 0.09% (w/v) bisacrylamide), 0.15% (w/v) APS, and 0.15% (v/v) TEMED. All SDS- polyacrylamide gels were electrophoresed in 1 x gel running buffer (25 mM Tris-HCl, pH 8.3, 200 mM glycine, 0.1% SDS). Protein loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) was added to the samples and denatured at 95°C for 10 min before loading on the gel. Molecular size markers used include Unstained Protein Molecular Weight Marker (Fermentas), PageRuler^{TM} Unstained Protein Ladder (Fermentas), Prestained Protein Molecular Weight Marker (Fermentas) and Rainbow Molecular Weight Marker (GE Amersham). All markers were used according to the manufacturer's specifications. Electrophoresis was performed at 30 mA per gel for 2-3 h. Protein bands were visualised by staining with 0.025% (w/v) Coomassie Blue R-250 dissolved in 40% (v/v) methanol for 1 h and destaining (30% (v/v) methanol, 10% (v/v) HOAc) overnight.

3.2.10 SDS-PAGE gel purification and electro-elution

The solubilised proteins were loaded on the SDS-PAGE gel (section 3.2.9) and electrophoresed at 30 mA per gel for 2-3 h. One lane of the gel containing the solubilised fusion protein was excised from the gel, stained and destained and aligned to the rest of the gel. The protein band containing the fusion protein was excised and placed in an electro-elution tray containing 1 x electro-elution buffer (25 mM Tris-HCl, pH 8.5, 200 mM glycine). The fusion protein was electro-eluted at 20 V overnight and the current reversed for 30 sec. The sample was removed and replaced with 700 µl of fresh electro-elution buffer and electro-eluted for another 30 sec. The eluted fusion protein was stored for SDS-PAGE analysis.

3.2.11 Protein processing

The GST:GFLV-CP fusion protein concentration was determined by Quick Start Bradford Protein Assay (Bio-Rad) according to the manufacturer's specifications. The purified fusion protein samples were dialysed against 500 ml PreScission Protease cleavage buffer for 8 h at 16ºC, the buffer was replaced and dialysed for another 8 h. The dialysed fusion protein was concentrated against PEG 20000 for approximately 4 h.

The GST fusion partner removal was performed using PreScission Protease (GE Healthcare) according to the manufacturer's specifications. One unit of PreScission Protease cleaves 100 µg of GST-fusion protein in cleavage buffer (50 mM Tris-HCl, pH 7, 1 mM EDTA, 150 mM NaCl).

3.2.12 Western Blot

The GFLV fusion protein specificity was analysed with western blot according to the specifications of GE Health care. Prestained Protein Molecular Weight Marker or Rainbow Molecular Weight Marker were used for protein size determination. The protein samples were separated using SDS-PAGE, the resolving gel was removed and soaked in protein transfer buffer (25 mM Tris, 192 mM glycine) for 20 min. A sheet of Amersham Hybond-P PVDF membrane (GE Healthcare) was prepared by cutting the membrane to the size of the resolving gel and soaking the membrane in 100% methanol for 10 sec followed by a wash step in distilled water for 5 min and equilibrating the membrane in transfer buffer for 10 min. The electro-blotting cassette was assembled and transferred at 20 V overnight. The membrane was removed, the orientation marked and briefly rinsed in TBS (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) and the non-specific binding sites blocked with TBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk powder for 1 h. The membrane was briefly rinsed and washed with TBS-T (1 x TBS, 0.1% (v/v) Tween 20) for 5 min. The membrane was incubated with primary antibody (GFLV specific antibody or GST specific antibody) diluted in TBS-T (1:40 000) for 1 h. The membrane was briefly rinsed and washed twice with TBS-T for 10 min. The membrane was incubated with secondary antibody (goat anti-rabbit), coupled to the enzyme alkaline phosphatase and diluted in TBS-T (1:20 000) for 1 h. The membrane was briefly rinsed and washed with TBS-T for 10 min (x 3). 5-Bromo-4-chloro-3-indolyl-phosphate/Nitro blue tetrazolium (BCIP/NBT) was used as detection system (0.615 mg/ml BCIP, 0.33 mg/ml NBT in substrate buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl, pH 9.5).

3.3 Results and discussion

3.3.1 Total RNA extraction

The GLFV titre in plants is temperature sensitive and drops drastically in the hot summer months. It is thus important to collect grapevine leaf material in early spring to ensure a high virus concentration in the grapevine leaves. Unfortunately, for reasons beyond our control, leaf material for this study was obtained in the summer, which hampered efforts to extract high concentrations of intact viral RNA and the amplification of the CP.

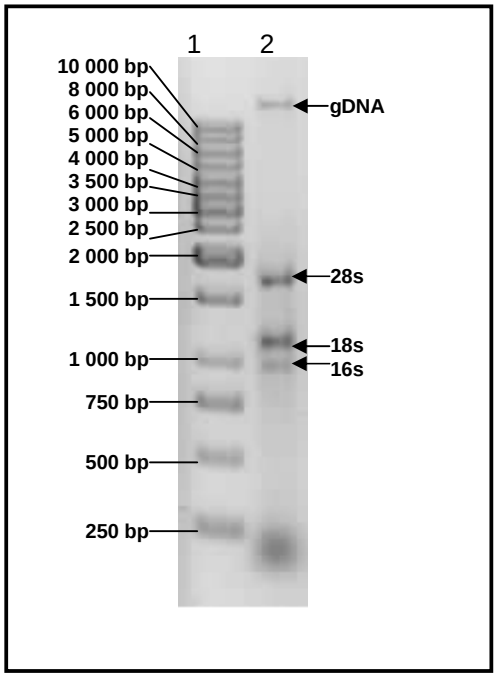

Figure 3.2: Agarose gel electrophoresis analysis of the total RNA extracted (Davies and Robbinson, 1996). Lane 1: Ruler 1 kb DNA ladder. Lane 2: Total RNA extracted (± 1.5 µg/lane).

Grapevine material that displayed distinct fanleaf symptoms were initially tested for GFLV infection using the rapid-direct-one-tube-RT-PCR amplification method, using the GFLV-500-F and GFLV-500-R primer set (table 3.1). However when primers GFLV-MP-F and GFLV-NC-R, for the amplification of the entire CP were used, amplification was unsuccessful. Therefore, total RNA extractions (Davies and Robinson, 1996) were performed on all positive plant material. A total RNA yield of approximately 900 ng was obtained from 2.5 g of leaf material. The quality of the RNA was assessed by agarose gel electrophoresis and ethidium bromide staining. Four bands were visible representing the genomic DNA, the ribosomal 28s, 18s and 16s RNA subunits (fig. 3.2).

3.3.2 GFLV coat protein gene amplification

A fragment of 1765 bp in size, comprising a 3' portion of the MP gene, the entire CP gene, and a portion of the 3'NC region of RNA2, was amplified by one-step-RT-PCR. Agarose gel analysis showed a faint amplification product. This could be due to the low viral RNA concentration. The PCR product was re-amplified to obtain enough of the PCR product for cloning (fig. 3.3).

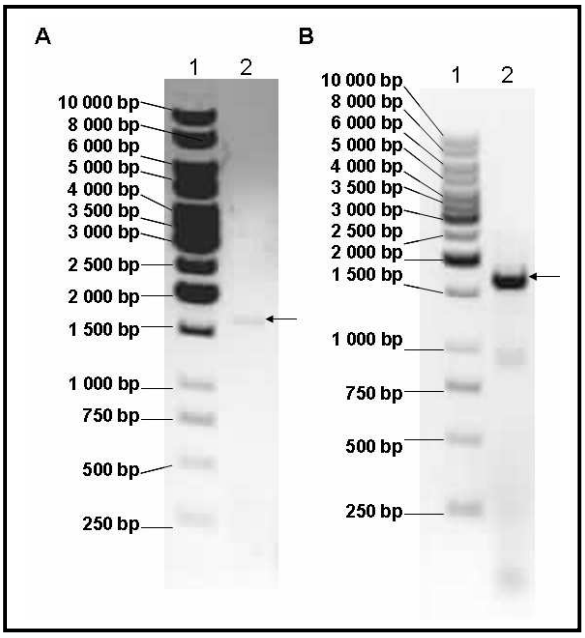

Figure 3.3: One-step-RT-PCR and re-amplification of the 1765 bp *Grapevine fanleaf virus* fragment comprising a 3' portion of the MP gene, the entire CP gene, and a portion of the 3'NC region of RNA2. **A**) Lane 1: Ruler 1 kb DNA ladder. Lane 2: One-step RT-PCR 1765 bp product. **B**) Lane 1: Ruler 1 kb DNA ladder. Lane 2: Re-amplification of the 1765 bp product.

3.3.3 Cloning and transformation of the PCR fragment.

The amplified product was excised from the gel, purified, ligated into the pDrive cloning vector (fig. 3.4) and transformed into chemically competent dH5α *E. coli* cells. The cloned fragments were analysed by PCR amplification and restriction enzyme digestion. Positive clones were selected for sequencing. Sequence analysis confirmed that the 1765 bp amplified product extends from the MP through the entire CP into the 3'NC region. No deletions or insertion were detected. Sequence data were analysed to select restriction enzymes for sub-cloning into the pGex-6P-2 expression vector. From the sequence analysis, *Sal*I was selected as the enzyme of choice to clone the CP into pGEX-6P-2. The coat protein sequence data was used to design primers to amplify the 1515 bp CP gene of the South African GFLV isolate. These primers included *Sal*I recognition sequences in their 5'-ends.

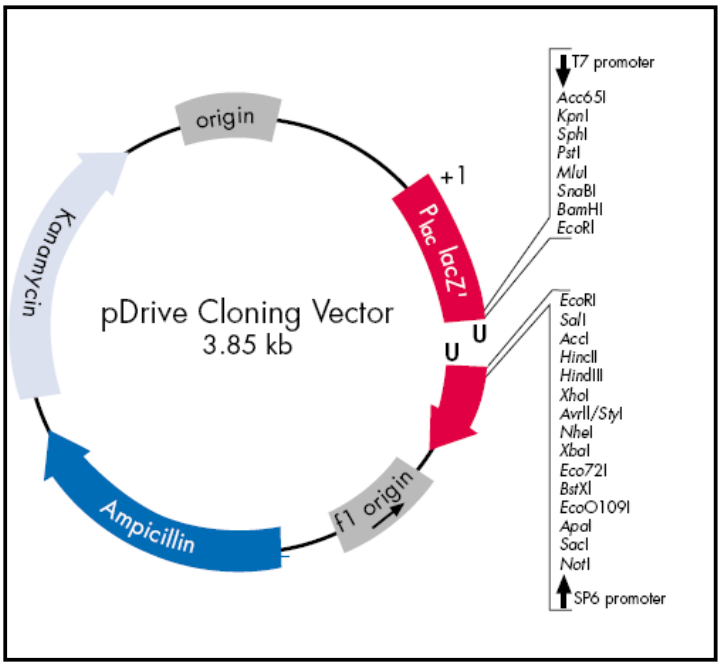

Figure 3.4: Restriction map of pDrive cloning vector.
(http://www1.qiagen.com/literature/pDrive/pcr_cloning21.pd)

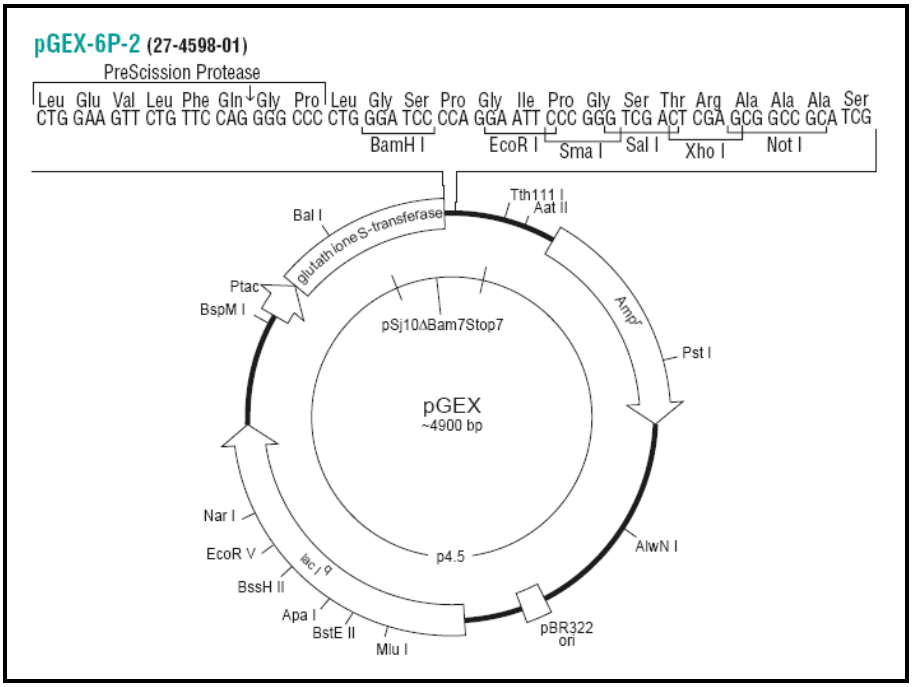

Figure 3.5: Restriction map of pGex-6P-2.
(http://www.sgc.utoronto.ca/SGC-WebPages/StructureDescription/M&M/Vectors/pGEX-6P-2.pdf)

The coat protein gene was amplified and cloned into pDrive with T/A-cloning. The CP gene was subcloned into the pGex-6P-2 by *Sal*I digestion. The clones were analysed with PCR and *Pst*I digestion to select clones with the correct orientation. Two of the ten clones digested with *Pst*I showed a 2360 bp and 4147 bp fragment and tested positive for the forward orientation (fig. 3.6).

Although the clones in lane 5 and 11 show the same digestion pattern, the fragments in lane 5 are slightly lower than those in lane 11, this could be due to the high amount of RNA present in the sample. The same could be seen for the slightly lower fragment of lane 6 when compared to lanes 7 and 8 (fig. 3.6). Clones 5 and 11 also tested positive for the forward orientation with PCR amplification and a 1380 bp fragment was amplified (fig. 3.6, lane 5 and 11). Both clones were sequenced and the ORF and orientation confirmed. The plasmids containing the GST:GFLV-CP fusion protein was transformed into BL21(DE3)pLysS and Rosetta2(DE3)pLysS cells for recombinant protein expression.

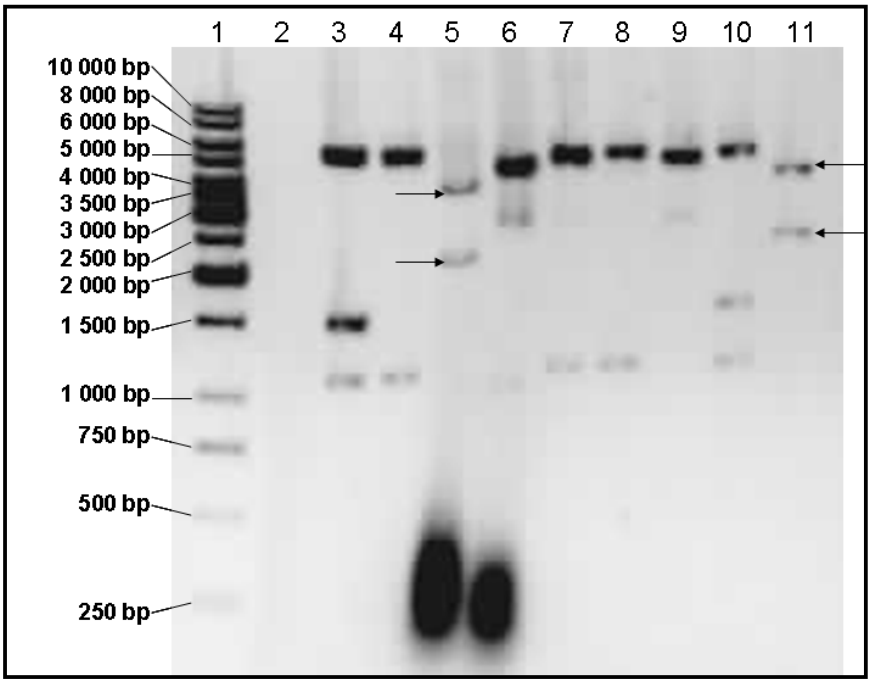

Figure 3.6: Orientation screening through restriction enzyme (*Pst*I) digestion of the pGex-6P-2 expression vector containing the 1515 bp GFLV-CP gene clones. Lane 1: Ruler 1 kb DNA ladder. Lane 5 and 11: Clones showing two fragments of approximately 2360 and 4147 bp indicating inserts in the correct orientation. Lane 3 and 10: Clones in the reverse orientation, showing two fragments of approximately 1100 and 5407 bp.

3.3.4 Recombinant protein expression and protein purification

The amino acid sequence of the CP was analysed to predict the size of the GST:GFLV-CP fusion protein (26 kDa + 57.41 kDa = 83.41 kDa). Initially the pGex-6P-2 vectors containing the GFLV-CP gene were transformed into both BL21(DE3)pLysS and Rosetta2(DE3)pLysS. Both these cell lines were used for protein expression with no apparent differences in the solubility of the proteins (fig. 3.7A). BL21(DE3)pLysS cells yielded a better growth curve compared to Rosetta2(DE3)pLysS cells and was selected for further analysis and optimisation.

For the first expression experiment, the following protocol was performed. Five millilitres of the overnight culture was inoculated in 500 ml TB and grown to an OD_{600} of 1.0 and induced with 0.1

mM IPTG. The samples was grown overnight at 37°C, collected and processed for SDS-PAGE analysis. The 83.4 kDa fusion protein was visible by SDS-PAGE, but very little of the fusion protein was soluble. Variable IPTG concentration (0.05, 0.075, 0.1, 0.15 and 0.2 mM) did not appear to lead to improved solubility. The expression time was changed, and samples were collected each hour for six hours after induction, but with no visible enhancement of the solubility. The first expression experiment was repeated with the following adaptations (fig. 3.7A). The inoculated cultures was grown to an OD_{600} of 0.9 at 25°C and induced with 0.2 mM IPTG. After induction the sample was incubated overnight at 25°C. The culture was centrifuged and the pellet resuspended in TEN50 Lysis buffer. The purification was performed as described by Novagen (fig. 3.7B). All washes were performed using PBS-3. The protein was eluted with 10 mM Glutathione reconstitution buffer.

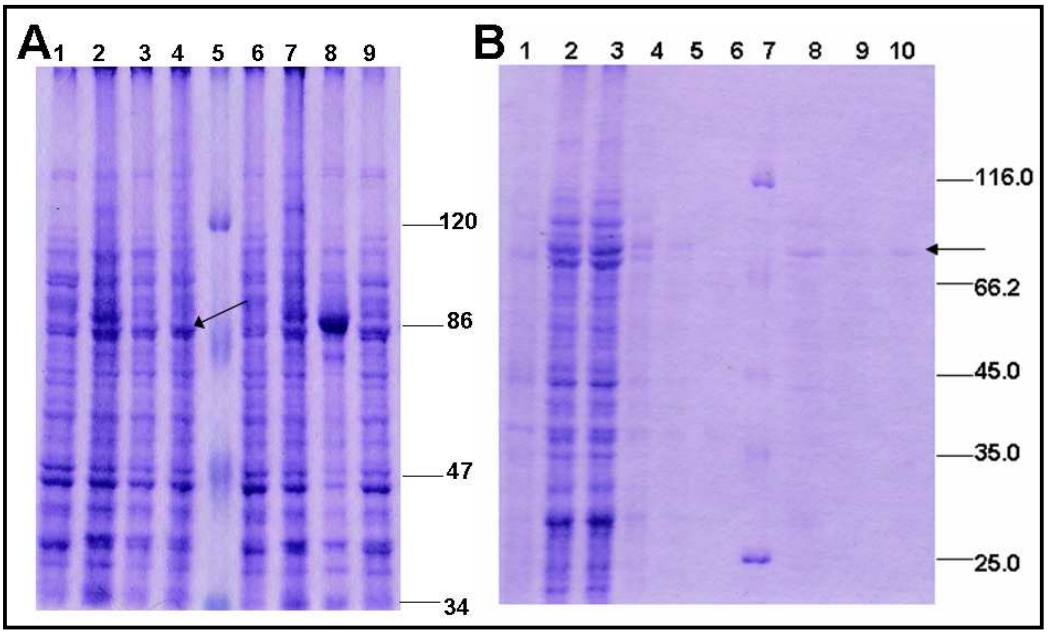

Figure 3.7: SDS-PAGE analysis of GST:GFLV-CP fusion protein expression and purification. **A**) SDS-PAGE analysis of GST:GFLV-CP fusion protein expression in BL21(DE3)pLysS (lane 1-4) and Rosetta2(DE3)pLysS (lane 6-9). Lane 1 and 6: Induced soluble fraction of pGex-6P-2 without the GFLV-CP insert. Lane 2 and 7: Total cell protein of the expressed GST:GFLV-CP. Lane 3 and 8: Insoluble fraction of the expressed GST:GFLV-CP. Lane 4 and 9: Soluble fraction of the expressed GST:GFLV-CP. Lane 5: Prestained protein molecular weight marker (kDa). **B**) SDS-PAGE analysis of the purification of GST:GFLV-CP fusion protein in (BL21(DE3)pLysS). Lane 1: Uninduced soluble fraction. Lane 2: Induced soluble protein fraction. Lane 3: Induced soluble protein flow through. Lane 4 – 6: PBS-2 washes. Lane 7: Unstained Protein Molecular Weight Marker. Lane 8-10: Reduced glutathione elution 1 to 3 (± 83.4 kDa).

A small amount of purified fusion protein was eluted from the column (fig. 3.7) decreasing with every elution step. A fairly high amount of purified fusion protein is required for GST removal with PreScission protease. The protocol was adjusted by changing the temperature and the induction time in order to slow the growth of the cultures, and thus produce more soluble fusion-protein. Three inoculated samples were grown for 1 hour at 37°C after which it was moved to room temperature

and grown until an OD_{600} of 0.5, 1.0 and 1.4 were reached respectively. Samples were collected at 3 hours and overnight, after induction with 0.05 mM IPTG. All samples yielded low amounts of soluble fusion protein.

A new protocol was followed based on the heat shock method of Chen et al. (2002). The growth medium, IPTG concentration, time of induction, expression time, and expression temperature were varied to enhance the solubility of the GST:GFLV-CP fusion protein (table 3.3). Best results were obtained when the sample was grown in TB (without 3% ethanol) at 37°C until an OD_{600} of 0.1 was reached, heat shocked at 42°C until an OD_{600} of 0.5, induced with 0.2 mM IPTG and grown at 16°C for 4 hours or until an OD_{600} of 0.9 (fig. 3.8, culture 3). Protein purification was performed with GST-bind chromatography as specified by Novagen. PBS-2 was used for the equilibration of the column and PBS-3 for the wash steps and the protein eluted with 25 mM reduced glutathione to further improve the yield of the eluted protein (GST gene fusion system handbook). No purified protein was visible on the SDS-PAGE gel (figure not shown).

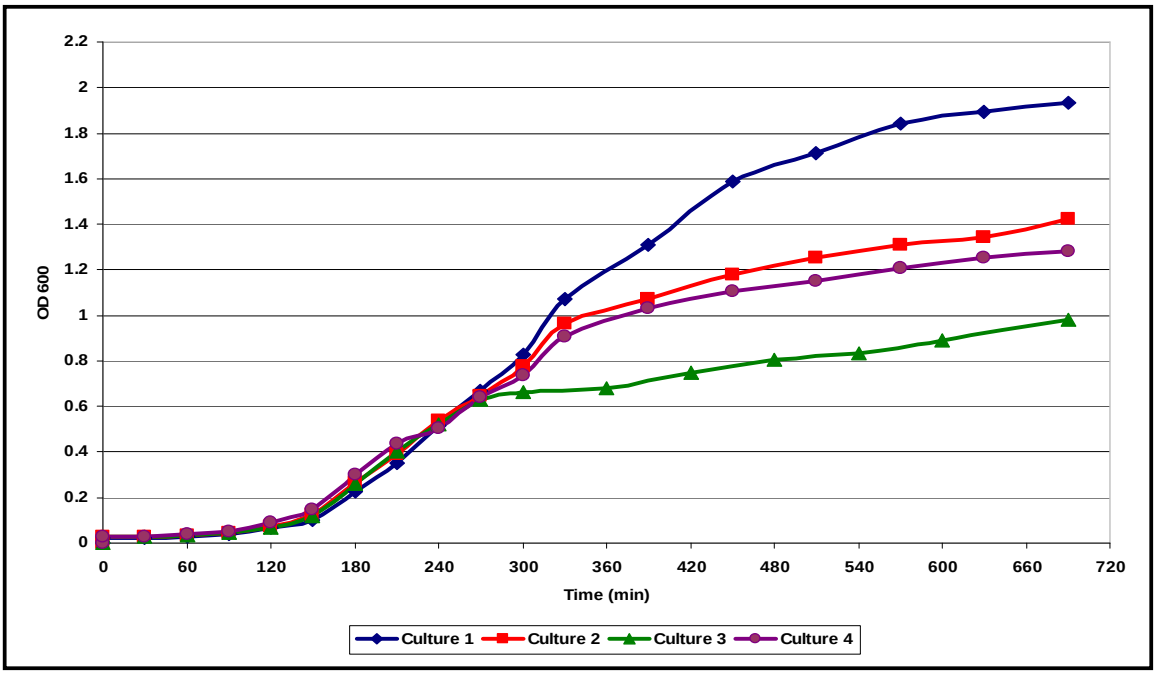

Figure 3.8: Culture growth curve. Culture 1: Grown at 37°C until an OD_{600} of 1.0, expressed at 37°C. Culture 2: Grown at 37°C until an OD_{600} of 1.0, expressed at 16°C. Culture 3: Grown at 37°C until an OD_{600} of 0.1, heat shocked at 42°C until an OD_{600} of 0.5, expressed at 16°C. Culture 4: Grown at 37°C until an OD_{600} of 0.5, heat shocked at 42°C until an OD_{600} of 0.9, expressed at 16°C.

Protein expression and purification was performed on pGex-6P-2, without the GFLV CP gene insert, to optimise the experimental procedures for the GST protein. The heat shock method from Chen et al. (2002) was performed with the TEN150 lysis buffer. The cultures were grown to an OD_{600} of 0.5 at 37°C, heat shocked to an OD_{600} of 1.0 at 42°C, induced with 0.1 mM IPTG and grown for 4 h at 16°C. Protein purification was performed with GST-bind chromatography

specified by Novagen. TEN150 was used for the equilibration of the column and PBS-2 containing 0.1% Triton X100 for the wash steps. The protein was eluted with 50 mM reduced glutathione (fig. 3.9). The exact protocol was repeated with pGex-6P-2 containing the GFLV-CP gene but failed to purify the fusion protein.

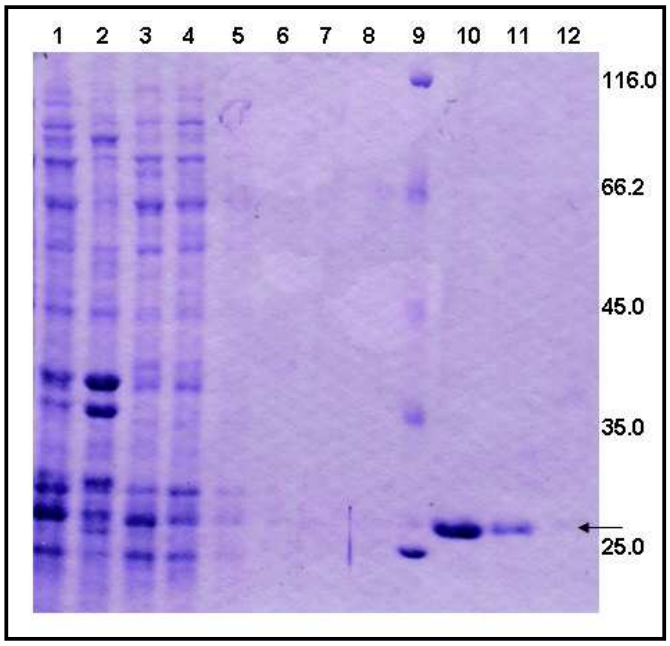

Figure 3.9: SDS-PAGE analysis of GST protein expression and purification. Lane 1: Total cell protein of the GST protein. Lane 2: Insoluble GST protein fraction. Lane 3: Soluble GST protein fraction. Lane 4: Soluble GST protein flow through. Lane 5-8: PBS-2 containing 0.1% Triton X100 washes. Lane 9: Unstained Protein Molecular Weight Marker (kDa). Lane 10 - 12: 50 mM Reduced glutathione elution 1 to 3 (± 26 kDa).

To express and purify sufficient amounts of the fusion protein in the soluble fraction, more optimisation was needed. Mercado-Pimentel et al. (2002) designed an optimisation protocol to enhance protein expression in the soluble fraction. Figure 3.10A shows the difference in solubility of the fusion protein after 0.25, 0.5, 1.0, and 2.0% of sarkosyl was added. The extra band between 85 and 70 kDa in the marker lane 6, is due to overflow from lane 5. The best results were obtained with 0.5 and 1.0% sarkosyl. These two samples were selected to continue the optimisation and different amounts of Triton X100 was added to a final concentration of 0.0, 0.5, 1.0, 2.0, 3.0, 4.0% respectively (fig. 3.10B). All samples were eluted with Buffer 2 (fig. 3.10B). The experiment was repeated and 0.7% sarkosyl and no Triton X100 was added (fig. 3.10C). The fusion protein was eluted with Buffer 2 (fig. 3.10C, lane 8 and 9) and reduced glutathione (lane 10 and 11) Low amounts of soluble fusion protein were eluted and non-specific proteins that bound to the resin were also eluted with the GST:GFLV-CP fusion protein.

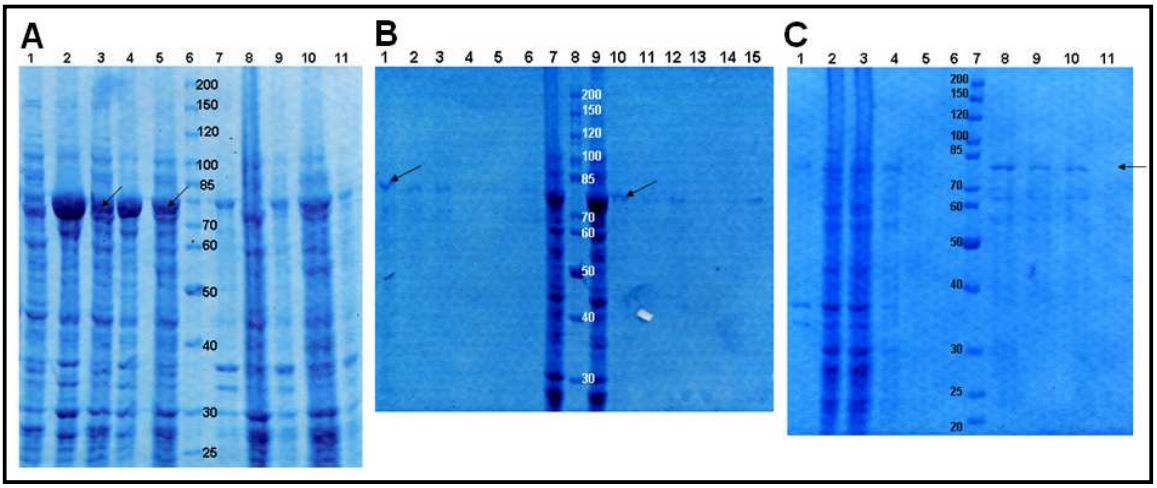

Figure 3.10: SDS-PAGE analysis of the GST:GFLV-CP fusion protein expression and purification optimisation using the protocol described by Mercado-Pimentel et al. (2002). **A**) Comparison of the soluble and insoluble fractions of the fusion protein samples treated with 0.25, 0.5, 1.0, 2.0, and 4.0 sarkosyl respectively. Lane 1: Soluble protein fraction containing 0.25% sarkosyl. Lane 2: Insoluble protein fraction 0.25% sarkosyl. Lane 3: Soluble protein fraction containing 0.5% sarkosyl. Lane 4: Insoluble protein fraction 0.5% sarkosyl. Lane 5: Soluble protein fraction containing 1.0% sarkosyl. Lane 6: PageRuler^{TM} Unstained protein Ladder (kDa). Lane 7: Insoluble protein fraction 1.0% sarkosyl. Lane 8: Soluble Protein fraction containing 2.0% sarkosyl. Lane 9: Insoluble protein fraction 2.0% sarkosyl. Lane 10: Soluble protein fraction containing 4.0% sarkosyl. Lane 11: Insoluble protein fraction 4.0% sarkosyl. **B**) Comparison of the protein samples treated with different amounts of Triton X100. Lane 1 -6: Samples treated with 0.5% sarkosyl and 0.0%, 0.5%, 1.0%, 2.0%, 3.0% and 4.0% Triton X100 respectively. Lane 7: Soluble protein fraction containing 0.5%. Lane 8: PageRuler^{TM} Unstained Protein Ladder (kDa). Lane 9: Soluble protein fraction containing 1.0% sarkosyl. Lane 10 – 15: Samples treated with 1.0% sarkosyl and 0.0%, 0.5%, 1.0%, 2.0%, 3.0% and 4.0% Triton X100 respectively. **C**) Protein expression and purification using 0.7% sarkosyl and 0.0% Triton X100. Lane 1: Insoluble fusion protein fraction. Lane 2: Soluble fusion protein fraction. Lane 3: Soluble fusion protein flow through. Lane 4-6: Washes 1-3. Lane 7: Unstained Protein Molecular Weight Marker (kDa). Lane 8 - 10: Elution 1 and 2 of Buffer 2. Lane 10-12: Elution 3 and 4 with 25 mM Reduced glutathione elution.

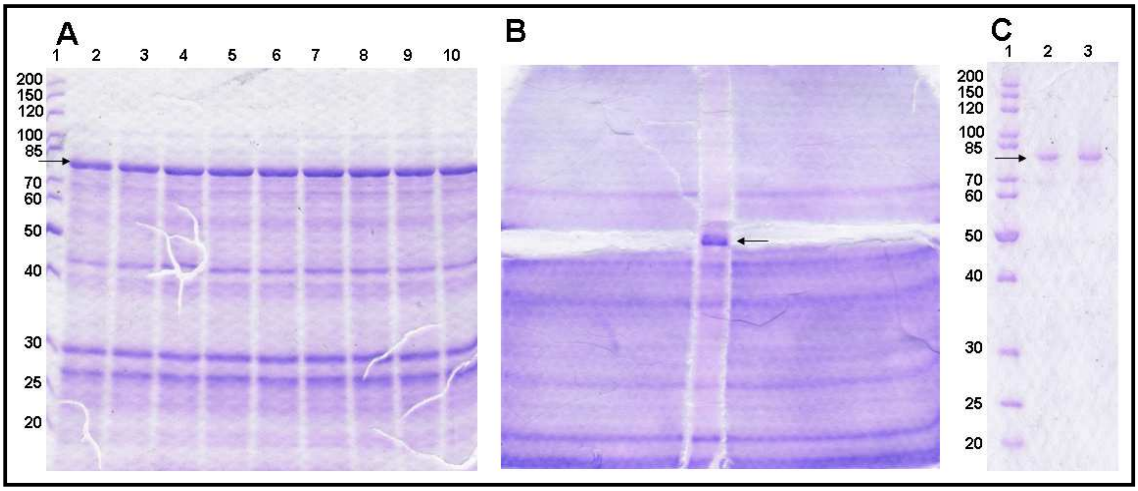

Figure 3.11: SDS-PAGE analysis and excision of the solubilised fusion protein. **A**) Lane 1: Unstained Protein Molecular Weight Marker (kDa). Lane 2-9: Solubilised fusion protein. **B**) SDS-PAGE gel with excised fusion protein. **C**) SDS-PAGE analysis of the electro-eluted fusion protein. Lane 1: Unstained Protein Molecular Weight Marker (kDa). Lane 2: first electro-elution protein sample. Lane 2: Second electro-elution sample.

To enhance the solubility of the fusion protein, protein solubilisation was performed (Burgess, 1996). The protein was solubilised, and electrophoresed on a SDS-PAGE gel. The protein band containing the fusion protein was excised and electro-eluted from the gel (fig. 3.11B and C).

3.3.5 Protein processing

After electro-elution the concentration of the GST:GFLV-CP fusion protein was determined with Quick Start Bradford Protein Assay and found to be 25 µg/ml, with a total amount of 200 µg. For antibody production 2.5-3 mg of protein is needed. The eluted samples were dialysed against PreScission Protease cleavage buffer, to prepare the GST:GFLV-CP fusion protein for GST removal and concentrated with PEG 20000 to increase the protein concentration. The concentration was again determined with Quick start Bradford protein assay and found to be insufficient for antibody production. The concentrated fusion proteins was analysed on a SDS-PAGE gel and showed degradation of the fusion protein.

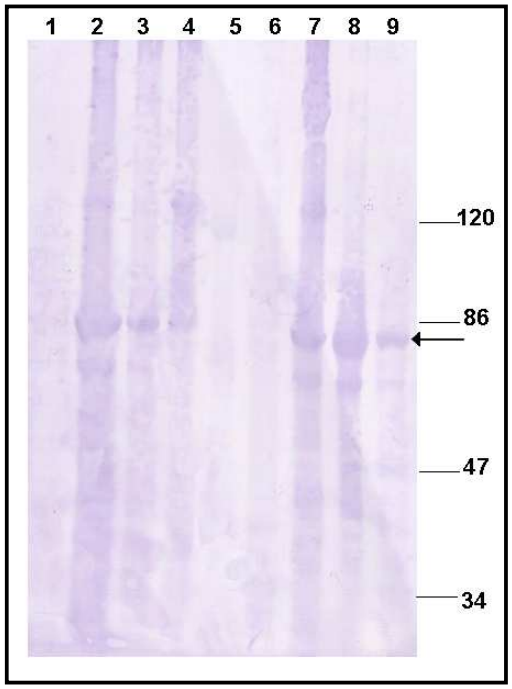

Figure 3.12: Western blot analysis of GST:GFLV-CP fusion protein expression in BL21(DE3)pLysS (lane 1-4) and Rosetta2(DE3)pLysS (lane 6-9), transferred to the membrane from figure 12.. Lane 1 and 6: Induced soluble fraction of pGex-6P-2 without the GFLV-CP insert. Lane 2 and 7: Total cell protein of the expressed GST:GFLV-CP. Lane 3 and 8: Insoluble fraction of the expressed GST:GFLV-CP. Lane 4 and 9: Soluble fraction of the expressed GST:GFLV-CP. Lane 5: Prestained protein molecular weight marker (kDa).

The PreScission Protease failed to remove the GST-partner from the GST:GFLV-CP fusion protein. The concentration of fusion protein and enzyme was varied with no effect on the cleavage of the fusion protein. This could be due to incorrect folding of the protein.

3.3.6 Western Blot

Western blot analysis was performed with the GFLV specific and GST antibodies. Both antibodies were non-specific and detected the fusion protein as well as the bacterial proteins. The best results were obtained using GFLV specific antibodies received from SAPO (fig. 3.12). The western blot was performed according to GE Healthcare's specifications (section 3.2.12). No proteins were detected in the induced control samples (without the GFLV-CP insert) (figure 17 lane 1 and 6). In the induced experimental samples from both the BL21(DE3)pLysS (Figure 17, lane 2-4) and Rosetta2(DE3)pLysS cell lines (Figure 17, lanes 7-9) containing pGex-6p-2:GFLV-CP, a band of the approximate size expected for the fusion protein was observed. However, many other bands were also observed indicating that the specificity of the antibody was insufficient, it could also be due to degradation of the GST:GFLV-CP fusion protein or that fusion protein products are present in the sample. The western blot was optimised by varying the antibody concentrations, with no real effect on the specificity and sensitivity to the assay (data not shown).

3.4 Conclusion

The use of recombinant DNA technology to express the target protein is an ideal strategy to develop antibodies against a target viral agent. It eliminates the possible development of antibodies to non-specific proteins such as plant proteins and provides a reservoir that can be used to produce antibodies of the same quality, sensitivity and specificity in each batch produced. Unfortunately, not all proteins are produced as soluble proteins and many accumulate in insoluble protein aggregates or inclusion bodies in the *E. coli* cells (Baneyx, 1992). The aim of this project was to develop a sensitive and reliable DAS-ELISA directed at the South African isolates of GFLV. Unfortunately the expression of insufficient concentrations of soluble protein prohibited the production of antibodies for the completion of the assay. High expression levels were achieved, but the fusion protein was expressed as insoluble aggregates. To enhance the solubility of the expressed protein, factors such as temperature, growth rate, induction time, growth media and *E. coli* cell lines were varied (Harper and Speicher, 2001). None of theses factors had any effect on the solubility of the protein. The expression of the GST fusion partner was optimised, and produced soluble protein proving that the system itself worked. When these conditions were used for the expression of the GST:GFLV-CP fusion protein, no soluble fraction were expressed.

The expression of this protein in inclusion bodies could be the result of one or more of the following factors: The overload of the folding pathway during over-expression of the target protein increases the probability of misfolding, leading to the formation of inclusion bodies (Baneyx and Mujacic, 2004). The expression of foreign proteins that do not fit well into the *E. coli* folding machinery elevates the problem of insolubility and the formation of inclusion bodies even further (Niiranen et al., 2007). Another inhibitory factor for the production of soluble proteins is the presence of disulphide bridges. A target protein with a structure, stabilized by disulphide bonds are difficult to express correctly, due to the non-specificity of the cysteine oxidation within the bacterial cytoplasm (Schrödel et al., 2005). The higher the percentage of cysteine residues the more the protein will aggregate to form inclusion bodies (Dyson et al., 2004). Terpe et al. (2003) suggested that the possible cause of the formation of inclusion bodies could be due to the presence of hydrophobic regions or highly charged residues within the target protein. The expression of rare codons, foreign to *E. coli*, could also cause the protein to be folded in inclusion bodies. Dyson et al. (2004) suggested that the following factors could help to predict if the expressed protein will be soluble: the percentage cysteine content, number of coiled coils, sub-cellular location, size of the expressed protein and the grand average of hydropathicity index (GRAVY).

We eliminated the effect of rare codons by using the *E. coli* strain Rosetta(DE3)pLysS that contains a pRARE plasmid that supplies an additional tRNA for the rare codon for arginine. This strain, however, had no effect on the solubility of the expressed protein. The GFLV CP (isolate A1) used in this study contained ten cysteine amino acids, 2% of the total amino acids in the fragment. Each subunit of the GST also contains four solvent-exposed cysteines and could lead to a high degree of oxidative aggregation (Kaplan et al., 1997). The high amount of cysteine amino acids present in the fusion protein could contribute to the incorrect folding of the protein by forming incorrect disulfide bridges, altering the structure of the protein and rendering it insoluble.

Fusion partners are proteins with the rare trait to enhance the solubility of the protein it is fused to, one such partner is GST. Studies done by Smith and Johnson (1988), Nygren et al. (1994), McTigue et al. (1995), Scheich et al. (2003), Rabhi-Essafi et al. (2007) suggest that the GST fusion partner enhances the solubility of the target proteins. Although these studies showed that GST enhances the solubility of these target proteins, the results of the expression and solubility studies is often only relevant within closely related species or protein families and cannot be directly transferred to unrelated proteins (Niiranen et al., 2007). Hammarstrom et al. (2002) and Dyson et al. (2004) concluded that GST is a poor enhancer. Kapust and Waugh, (1999) Fox et al. (2003), De Marco et al. (2004), Dyson et al. (2004) and Niiranen et al. (2007) compared the effect of GST, MBP and/or NusA fusion partners on the solubility of the target protein and found that MBP and NusA fusion partners enhance solubility much more effectively than GST.

For future expression studies it is necessary to compare different expression vector and fusion partners to select the vector that is most advantageous for each specific target protein. There are fusion partners, such as the MBP and NusA that solubilise proteins better than others on average, but there is no guarantee that the fusion partner will solubilse the selected target protein (Esposito and Chatterjee, 2006). Unlike GST, both these fusion partners are natural *E. coli* proteins and could be a contributing factor to the excellent solubility expression results obtained when expressing with these fusion partners. More research needs to be done on the soluble expression of large fragments, and the prediction of solubility expression and large scale comparisons of protein solubility with different fusion tags.

4 GENETIC VARIABILITY WITHIN THE COAT PROTEIN GENE OF GRAPEVINE FANLEAF VIRUS ISOLATES FROM SOUTH AFRICA AND THE EVALUATION OF RT-PCR, DAS-ELISA AND IMMUNOSTRIPS AS VIRUS DIAGNOSTIC ASSAYS

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Abstract

Grapevine fanleaf virus (GFLV) is responsible for severe fanleaf degeneration in grapevines of all major wine producing regions of the world, including South Africa. In order to successfully control the spread of the virus, specific and reliable diagnostic assays are necessary. The genetic variability of 12 GFLV isolates recovered from naturally infected grapevine plants in the Western Cape region of South Africa were characterised. These samples were subjected to RNA extraction, RT-PCR analysis and sequencing of the coat protein gene ($2C^{CP}$). Sequence identities between different GFLV isolates from South Africa were between 86-99% and 94-99% at the nucleotide and amino acid levels, respectively. Phylogenetic analysis based on the $2C^{CP}$ gene sequences showed that the South African isolates form two distinct clades or subpopulations. The specificity and sensitivity of three diagnostic techniques (rapid-direct-one-tube-RT-PCR, DAS-ELISA and ImmunoStrips) for the detection of GFLV were analysed to determine the appropriate diagnostic assay for virus infection. Rapid-direct-one-tube-RT-PCR was found to be the most reliable technique for detection. This is the first report on sequence analysis of full-length $2C^{CP}$ gene cDNA clones of GFLV isolates from South Africa.

1. Introduction

Grapevine fanleaf disease was discovered in the 1950's and is one of the oldest known viral diseases of grapevines. It occurs in all vine-growing regions of the world and has been reported in Asia, Africa, Europe, New Zealand, Australia, North and South America. The disease is caused by the *Grapevine fanleaf virus* (GFLV) a member of the genus *Nepovirus* in the family *Comoviridae* and is spread by the ectoparasitic nematode *Xiphinema index,* as well as by vegetative propagation and grafting. *Grapevine fanleaf virus* causes degeneration and malformation of berries, leaves and canes and is responsible for significant economic losses by reducing crop yields by as much as 80%, reducing the longevity of the vines and affecting fruit quality (Andret-Link et al., 2004; Martelli and Savino, 1990; Raski et al., 1983).

Grapevine fanleaf virus has two single stranded positive sense RNA genomes, RNA1 and RNA2 (Quacquarelli et al., 1976). Both RNA species are monocistronic and carry a small covalently

linked viral protein (VPg) at the 5' terminus and a poly(A) tail at the 3' terminus (Pinck et al., 1988). Each of the RNA genomes encodes a polyprotein that is proteolytically processed into functional proteins. The RNA1 polyprotein (P1) is cleaved into 5 mature products; a putative proteinase co-factor (1A), a putative helicase and NTP-binding domain ($1B^{Hel}$), a virus genome linked protein ($1C^{VPg}$), a chymotrypsin-like cysteine proteinase ($1D^{Pro}$) and a putative RNA-dependent RNA polymerase ($1E^{pol}$) (Margis and Pinck, 1992; Pinck et al., 1991; Ritzenthaler et al., 1991). The RNA2 polyprotein is cleaved into three mature products; a homing protein ($2A^{HP}$) also required for RNA2 replication, a movement protein ($2B^{MP}$) found in tubules observed in the plasmodesmata and a coat protein ($2C^{CP}$) that encapsidate the RNA molecules to form a virus particle (Gaire et al., 1999; Margis et al., 1993; Ritzenthaler et al., 1995a, 1995b; Serghini et al., 1990).

The diversity and the quasispecies nature of the GFLV genome have been assessed in several countries where this virus occurs naturally. These include France (Vigne et al., 2004a, 2005), Iran (Bashir et al., 2007a, 2007b, 2007c), Slovenia (Pompe-Novak et al., 2007), Tunisia (Fattouch et al., 2005a, 2005b) and the USA (Naraghi-Arani et al., 2001). Variability studies were performed using IC-RT-PCR-RFLP and sequencing of the complete (Bashir et al., 2007c; Naraghi-Arani et al., 2001; Vigne et al., 2004a, 2005) and partial $2C^{CP}$ gene (Bashir et al., 2007a; Fattouch et al., 2005a, 2005b), $2B^{MP}$ gene (Bashir et al., 2007b) as well as the complete RNA2 ORF (Pompe-Novak et al., 2007). In these studies nucleotide sequence similarities of 87% and amino acid sequence identities of 91% were observed for the $2C^{CP}$ gene, 91% and 93% respectively for the $2B^{MP}$ gene, and 93.3% and 97.5% for the RNA2 ORF, respectively. (Bashir et al., 2007a, 2007b, 2007c; Fattouch et al., 2005a, 2005b; Naraghi-Arani et al., 2001; Pompe-Novak et al., 2007; Vigne et al., 2004a, 2005).

The natural occurrence of recombination in the GFLV $2C^{CP}$ gene was first reported by Vigne et al. (2004a, 2004b, 2005). Five recombinant isolates were identified among the 347 GFLV isolates investigated, however, no differences were observed between recombinant and non-recombinant isolates regarding symptom expression, disease incidence or vigour (Vigne et al., 2004a, 2004b, 2005). Although the variability in the $2C^{CP}$ gene was high at nucleotide level (0.5-13.8%), less diversity was found at the amino acid level (0.2-6.9%) (Vigne et al., 2004a), indicating that there is strong genetic stability in the GFLV $2C^{CP}$ gene. Considering the putative roles in virus particle structure and stability, virus movement, as well as interactions with host and vector ascribed to the $2C^{CP}$ gene, limited levels of genetic variation can be tolerated in order to maintain viability (Andret-Link et al., 2004; Belin et al., 1999).

In South Africa, GFLV infections occur mostly in the Breede River Valley in the Western Cape, an area with high *X. index* infestation. Quarantine regulations in South Africa prescribe that vines (nuclear and mother block material) need to be tested for the presence of GFLV, as the planting of uninfected propagation material is one of the most effective ways to control grapevine fanleaf disease. The specific and rapid identification of GFLV is therefore essential for the effective prevention of disease spread. Different diagnostic methods are available for testing of viral infections; these include molecular techniques such as RT-PCR and immunological techniques such as DAS-ELISA and ImmunoStrips. Whilst the choice of the diagnostic assay depends on factors such as ease of assay development, specificity, sensitivity, skill levels of technicians, portability of the test (e.g. field testing) and cost (Ward et al., 2004); in the South African context it is important that any routine diagnostic assay is able to detect all genetic variants of GFLV prevalent in local vineyards.

In the present study the genetic variability of the GFLV $2C^{CP}$ gene of 12 isolates collected from the grapevine growing regions in the Western Cape province of South Africa were investigated. Variability studies were conducted to investigate a possible correlation between sequence variability in the $2C^{CP}$ gene and the sensitivity and/or specificity of diagnostic assays. Three diagnostic assays, rapid-direct-one-tube-RT-PCR, DAS-ELISA and ImmunoStrips were compared using naturally GFLV-infected material collected from vineyards in the Western Cape.

2. Materials and Methods

2.1 Plant material

Grapevine fanleaf virus infected grapevine leaf material was obtained from vineyards in the Breede River valley (Robertson, Bonnievale and Slanghoek) as well as from Stellenbosch and the Paarl-Wellington area in the Western Cape, and from Vititec, Paarl, South Africa (table 2). Not all collected samples displayed symptoms, some samples were collected from vines adjacent to symptomatic vines, and others from within the same vineyard but not adjacent or near to symptomatic vines.

2.2 RT-PCR, cloning and sequencing

Total RNA was isolated according to the method of Chang et al. (1993). A fragment of approximately 1760 bp in size, comprising a 3' portion of the $2B^{MP}$ gene, the entire $2C^{CP}$ gene, and a portion of the 3' non-coding region of RNA2, was amplified by RT-PCR. For primer annealing, 1 μM of primer GFLV-NC-R (5'-ACAAACAACACACTGTCGCC-3') was added to 0.45-1.0 μg of total RNA and incubated (5 min, 95°C) followed by 2 min on ice. cDNA synthesis was performed with AMV reverse transcriptase according to the instructions of the manufacturer (Fermentas). Five

microliters of the cDNA was added to an Ex Taq PCR amplification cocktail containing 1 x Ex Taq buffer (Takara), 0.2 mM dNTP's, 0.5 μM GFLV-MP-F (5'-ACCTTCTCTATCAGRAGYCG-'3) and 0.5 μM GFLV-NC-R, 1 mM cresol, 20% sucrose and 0.5 U Takara Ex Taq. The RT-PCR cycle conditions were: 1 cycle of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 53.5°C and 2 min at 72°C respectively, 1 cycle of 7 min at 72°C. The amplification products were gel purified using the ZymoClean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions. A PCR cloning kit (Qiagen) was used to clone the purified products into pDrive as per instructions and transformed into chemically competent *E. coli* dH5α cells (Invitrogen). The GeneJet Plasmid Miniprep kit (Fermentas) was used to purify the plasmid DNA for sequence analysis. The T7, SP6 and GFLV-348-Forw (5'-CGGCAGACTGGCAAGC-3') primers were used to sequence the entire length of the fragment extending from the movement protein to the noncoding region of the RNA2 genome. Sequencing was performed by the Core DNA Sequencing Unit at Stellenbosch University.

2.3 Sequence and Phylogenetic analysis

BioEdit (Ver. 7.0.4, Hall, 1999) was used to perform sequence editing and compilation. Sequence comparisons were performed using the BLAST algorithm (Altschul et al., 1990) against the GENBANK database of the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The generated GFLV nucleotide sequences from South Africa were compared to GFLV sequences downloaded from GenBank, using the ClustalW (Ver. 1.4) alignment function embedded in the BioEdit software. Although the sequences generated by RT-PCR included 100 bp of the $2B^{MP}$ gene, the complete $2C^{CP}$ gene and 141 bp or 147 bp of the 3' non-coding region, only the 1 515 bp $2C^{CP}$ gene sequence was used to perform sequence and phylogenetic analysis. The number of available $2C^{CP}$ gene sequences that include the partial $2B^{MP}$ gene and 3' non-coding region are limited, and a more complete phylogenetic tree was necessary for variability analysis. The sequences of the GFLV RNA2 fragments were submitted to the Genbank database and have been assigned accession numbers **EU70240** to **EU70251.**

Phylogenetic analysis of the aligned $2C^{CP}$ gene nucleotide sequences was performed using the parsimony option in PAUP (Ver. 4.0b10) (Swafford, 2002). Twelve $2C^{CP}$ gene nucleotide sequences from South Africa and 49 $2C^{CP}$ gene nucleotide sequences from GenBank were used in the phylogenetic analysis (table 1). Three *Nepovirus* outgroups were selected for the phylogenetic analysis, two *Arabis mosaic virus (*ArMV*)* isolates closely related to GFLV and *Tobacco ringspot virus* (TRSV) as the most distant outgroup. Gaps were introduced into the GFLV $2C^{CP}$ gene matrix to ensure sufficient alignment with the ArMV and TRSV out groups. To search for the shortest possible trees from the data matrix, a heuristic search of 1 000 replicates were performed using tree

bisection reconnection (TBR) branch swapping. All characters were weighted equally. Clade support was calculated with 1 000 bootstrap replicates using TBR branch swapping. Bootstrap percentages of ≥75% were considered as well supported, between 75% and 50% as moderately supported and values below 50% as weakly supported. Bootstrap percentages below 50% are not indicated on the phylograms.

2.4 Plant material preparation for diagnostic assays

All the grapevine leaf samples were ground in sample mesh bags containing 3 ml of grapevine sample extract buffer (Agdia). Leaf material (1:20 (w/v)) was inserted between the mesh linings of the bag and rubbed with a pestle to completely crush and mix the sample. The extracts were used in the rapid-direct-one-tube-RT-PCR as well as the GFLV ImmunoStrip test (Agdia) and GFLV indirect DAS-ELISA (Agdia), to standardise the technique comparison.

2.5 Rapid-direct-one-tube-RT-PCR

The rapid-direct-one-tube-RT-PCR was performed according to the modified method of Osman et al. (2007). This method was optimised using GFLV-348-Forw and GFLV-348-Rev (5'-TGGTCCCGTTCCACTCAC-3') primers to amplify a 348 bp fragment with low variability in the coat protein region. Four microliters of the mesh extract was pipetted into a microcentrifuge tube containing 1x GES buffer (100 mM glycine-NaOH pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X100) to give a final volume of 50 µl. The sample was denatured for 10 min in a waterbath at 95°C followed by direct cooling on ice for 5 min. Two microliters of the denatured extract was pipetted directly into the one-tube-RT-PCR mix (1 x KapaTaq buffer with Mg2+, 1 mM cresol, 20% sucrose, 0.2 mM dNTP's, 0.4 µM GFLV-348- Forw, 0.4 µM GFLV-348-Rev primers, 5 mM DTT, 1 U AMV reverse transcriptase (Fermentas), and 1 U KapaTaq (Kapa Biosystems)) to a final volume of 25 µl per reaction. The RT-PCR cycle conditions were: 1 cycle of 45 min at 45°C, 1 cycle of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 57°C and 30 sec at 72°C respectively, 1 cycle of 7 min at 72°C.

2.6 GFLV ImmunoStrip and DAS-ELISA assay

The ImmunoStrip test was performed according to the manufacturer's (Agdia) instructions. An ImmunoStrip was inserted 0.5 cm deep into each of the sample mesh bags, containing the macerated leaf material, held in a vertical position. The strips were incubated for 30 min at room temperature or until the control line was visible on the ImmunoStrip. If the control line was not present after 30 min, the test was regarded as invalid. ImmunoStrips were photographed to document results (data not shown). DAS-ELISA was performed using the GFLV DAS-ELISA reagent kit (Agdia), according to the manufacturer's instruction. The carbonate coating buffer, the

PBST wash buffer, p-nitrophenyl phosphate (PNP) substrate pellets and PNP substrate buffer from Agdia were used.

Table 1. South African and previously published $2C^{CP}$ gene sequences for *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* isolates (ArMV) and a *Tobacco ringspot virus* (TRSV) isolate used for phylogenetic analysis.

Accession	Virus	Isolate/Strain	Host cultivar	Country
AJ318415	GFLV	Hangzhou	Grapevine (no CV. specified)	China
AY017338	GFLV	NW	*V. vinifera*/Huxel	Germany
AY370941	GFLV	A2b	*V. vinifera*/Chardonnay	France
AY370960	GFLV	A18a	*V. vinifera*/Chardonnay	France
AY370962	GFLV	A19c	*V. vinifera*/Chardonnay	France
AY370967	GFLV	A23b	*V. vinifera*/Chardonnay	France
AY370968	GFLV	A24a	*V. vinifera*/Chardonnay	France
AY370969	GFLV	A26b	*V. vinifera*/Chardonnay	France
AY370975	GFLV	A30f	*V. vinifera*/Chardonnay	France
AY370977	GFLV	A30h	*V. vinifera*/Chardonnay	France
AY370985	GFLV	A34c	*V. vinifera*/Chardonnay	France
AY370992	GFLV	A40a	*V. vinifera*/Chardonnay	France
AY370994	GFLV	A42b	*V. vinifera*/Chardonnay	France
AY370998	GFLV	B10a	*V. vinifera*/Chardonnay	France
AY370999	GFLV	B11a	*V. vinifera*/Chardonnay	France
AY371002	GFLV	B12d	*V. vinifera*/Chardonnay	France
AY371007	GFLV	B19a	*V. vinifera*/Chardonnay	France
AY371012	GFLV	B3a	*V. vinifera*/Chardonnay	France
AY371013	GFLV	B3c	*V. vinifera*/Chardonnay	France
AY371016	GFLV	B4e	*V. vinifera*/Chardonnay	France
AY371017	GFLV	B5a	*V. vinifera*/Chardonnay	France
AY371025	GFLV	b844	*V. vinifera*/Cabernet franc	France
AY780899	GFLV	A17a	*V. vinifera*/Chardonnay	France
AY780901	GFLV	A17d	*V. vinifera*/Chardonnay	France
AY780902	GFLV	A10a	*V. vinifera*/Chardonnay	France
AY997695	GFLV	B5-7	Grapevine (no CV. specified)	Iran
AY997699	GFLV	SH3-3	Grapevine (no CV. specified)	Iran
DQ362923	GFLV	SG10	*V. vinifera*/Sangiovese	Italy
DQ362925	GFLV	SG12	*V. vinifera*/Sangiovese	Italy
DQ362927	GFLV	MS43	*V. rupestris*/St. George/Moscato	Italy
DQ362932	GFLV	FA31	*V. rupestris*/St. George/Favorita	Italy
DQ526452	GFLV	Ch-80	*V. vinifera*/Cabernet Sauvignon	Chile
DQ922653	GFLV	Vol471c	*V. vinifera*/Volovnik	Slovenia
DQ922660	GFLV	Vol50c1	*V. vinifera*/Volovnik	Slovenia
DQ922662	GFLV	Vol51c1	*V. vinifera*/Volovnik	Slovenia
DQ922665	GFLV	Vol51c4	*V. vinifera*/Volovnik	Slovenia
DQ922667	GFLV	Vol52c1	*V. vinifera*/Volovnik	Slovenia
DQ922669	GFLV	Vol54c2	*V. vinifera*/Volovnik	Slovenia
DQ922671	GFLV	Vol55c1	*V. vinifera*/Volovnik	Slovenia
DQ922675	GFLV	Vol57c2	*V. vinifera*/Volovnik	Slovenia
EU038294	GFLV	RS	*V. vinifera*/Prosecco Tondo	Brazil
EU258680	GFLV	RUP	IAC 514-6 grafted on Rupestris du Lot	Brazil
EU258681	GFLV	IAC	106-8 grafted on cv. IAC 766	Brazil
U11768	GFLV	GFLV-FC	*V. vinifera*/French Colombard	Austria
X16907	GFLV	F13	*V. vinifera*/Muscat	France
X60775	GFLV	Not specified	*V.rupestris*	USA
EU702440	GFLV	A1	*V. vinifera*/Cabernet Sauvignon	South Africa
EU702441	GFLV	S2	*V. vinifera*/Sauvignon blanc	South Africa
EU702442	GFLV	G2	*V. vinifera*/Cabernet Sauvignon	South Africa
EU702443	GFLV	W5	*V. vinifera*/Cabernet Sauvignon	South Africa
EU702444	GFLV	W1	*V. vinifera*/Cabernet Sauvignon	South Africa
EU702445	GFLV	D1	*V. vinifera*/Petit Verdot	South Africa
EU702446	GFLV	D12	*V. vinifera*/Chardonnay	South Africa
EU702447	GFLV	Du	*V. vinifera*/Chardonnay	South Africa

EU702448	GFLV	V2	*V. vinifera*/Pinotnoir	South Africa
EU702449	GFLV	V1	*V. vinifera*/Pinotnoir	South Africa
EU702450	GFLV	D7	*V. vinifera*/Chardonnay	South Africa
EU702451	GFLV	W8	*V. vinifera*/Cabernet Sauvignon	South Africa
AY363727	TRSV	Not specified	Not specified	USA
X81814	ArMV	P2-U	Not specified	France
X81815	ArMV	P2-L	Not specified	France

3. Results

3.1 GFLV symptoms

Three distinct GFLV-associated symptoms were observed in the leaf samples obtained from the vineyards in the Breede River Valley; fanleaf, yellow mosaic and vein banding (table 2, fig 1). Both fanleaf and yellowing mosaic were observed in the Robertson and Bonnievale vineyards, while only fanleaf symptoms were observed in the leaf material obtained from Wellington-Paarl. In contrast to the typical fanleaf and yellow mosaic symptoms observed in the Robertson area, one sample also showed leaf curling and malformation that is not characteristic of GFLV infection or of grapevine leafroll disease. Leaf material from this unknown (Du) vine was included in the analysis. Definite vein banding symptoms were only seen in leaf material collected from the Stellenbosch area.

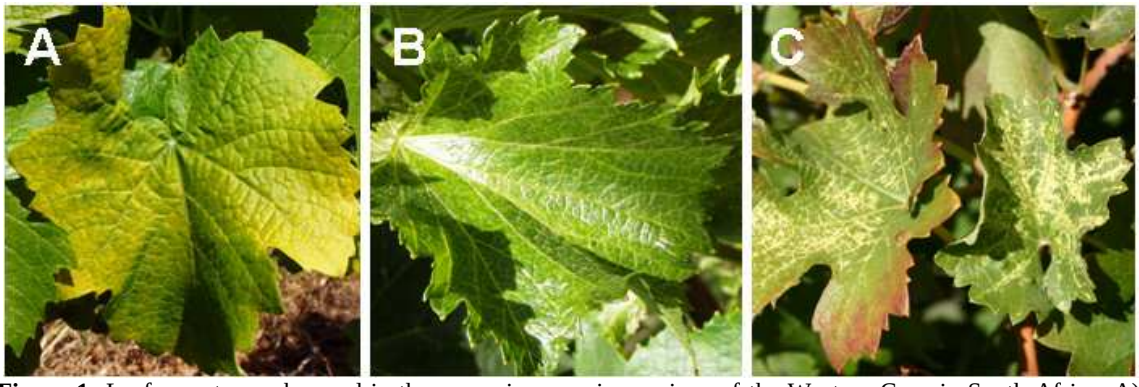

Figure 1: Leaf symptoms observed in the grapevine growing regions of the Western Cape in South Africa. A) Yellow mosaic, B) Fanleaf, C) Vein banding.

3.2 Variability analysis of the 2C^{CP} gene

From the 30 leaf samples that tested positive for GFLV with the rapid-direct-one-tube RTPCR, 12 samples were selected for variability analysis of the 1 515 bp 2C^{CP} gene. Sequence identities between clones from different GFLV isolates from South Africa were between 86- 99% and 94-99% at the nucleotide and amino acid levels, respectively (table 3). Nucleotide and amino acid sequence identities of 82-90% and 92-99%, respectively, in the GFLV 2C^{CP} gene were observed between South African isolates and previously published isolates (data not shown). Nucleotide variation was distributed throughout the 2C^{CP} gene rather then being conserved to specific regions or sites within the gene.

3.3 Phylogenetic analysis of the 2C^{CP} gene

The aligned GFLV 2C^{CP} gene nucleotide matrix had 390 (25.25%) constant characters, 765 (49.5%) parsimony informative characters (PICs) and 390 (25.25%) parsimony uninformative characters (PUCs). Four trees with a tree length of 4268 were retrieved with the heuristic search. Tree statistics revealed a consistency index (CI) of 0.408 and a retention index (RI) of 0.632. Bootstrap (BS) support of over 75% was found in 24 out of potentially 61 nodes (39.3%) and was used to compute a strict consensus tree. The monophyly of GFLV was strongly supported. The South African isolates formed two distinct clades. Clade A (containing isolates D1, A1, S2, G2, W1 and W5) and clade B (containing isolates D12, W8, V2, D7 and V1) (fig 2) were retrieved in the strict consensus analysis with a 100% bootstrap support. The South African isolate Du was retrieved sister to clade B.

3.4 Diagnostic assay comparison

Grapevine fanleaf virus was detected with rapid-direct-one-tube-RT-PCR in 30 of the 36 samples collected (table 2). Samples S1, S3, W2, W9, W10 and W12 showed no amplification of the expected 348 bp fragment. All the samples, except for the material obtained from Stellenbosch, were also tested for GFLV infections with the GFLV specific ImmunoStrips (Agdia) and DAS-ELISA (Agdia). Of the 23 leaf samples tested with all three diagnostic assays, 21 amplified the expected 348 bp fragment, 19 tested positive with the ImmunoStrip assay and 17 samples with the DAS-ELISA (table 2). Samples (D12 and V1) that showed a faint fragment with rapid-direct-one-tube-RT-PCR under the applied amplification conditions, tested negative with the ImmunoStrips and the samples that showed poor reactions with the rapid-direct-one-tube-RT-PCR (D12 and V1) and with ImmunoStrips (G3 and G4) tested negative with the DAS-ELISA. Samples W1-W12 were collected in a vineyard that showed severe grapevine leafroll disease symptoms. Some of these samples (W5, W9, W10 and W12) showed no clear vein banding, fanleaf or yellow mosaic symptoms. Of these, sample W5 tested positive for GFLV in rapid-direct-one-tube-RT-PCR. Samples W9, W10 and W12 did not amplify the expected 348 bp fragment.

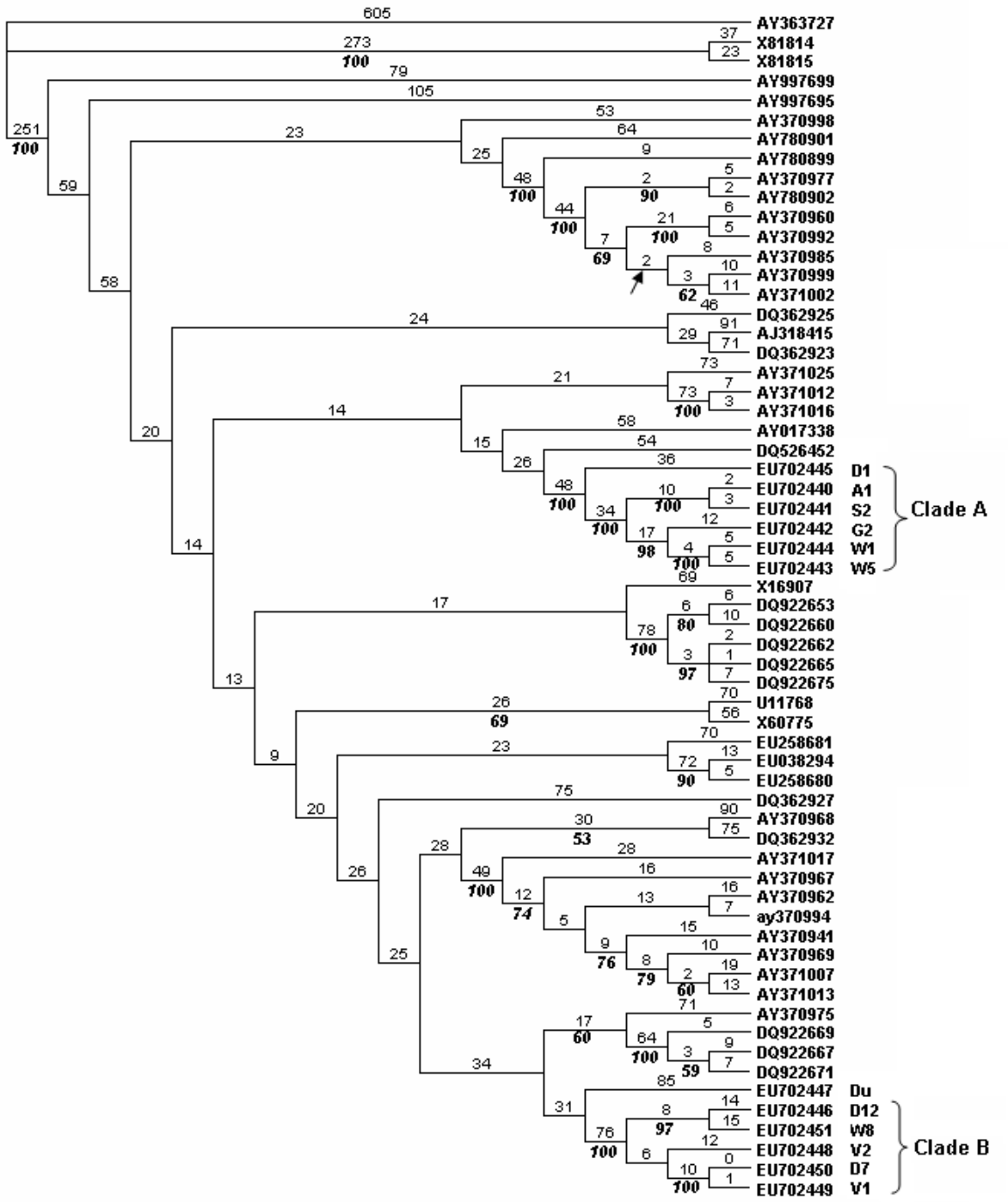

Figure 2: Parsimony phylograms based on the nucleotide sequences of GFLV $2C^{CP}$ genes generated by PAUP 4.0b10. Branch lengths are indicated above the nodes and the bootstrap support percentages are indicated below. Arrows indicate branches that collapse in the strict consensus. Bootstrap support percentages lower than 50% are not indicated.

Table 2. Origins of GFLV isolates from the wine growing regions of the Western Cape, South Africa, and symptoms observed on the leaves and methods of virus testing.

Sample name	District	Cultivar	Symptoms on leaves			RT-PCR[A]	Immuno-Strips[B]	DAS-ELISA[C]
			V	Y	F			
A1	Paarl	Cabernet Sauvignon	-	+	-	+	n.t.	n.t.
S1	Slanghoek	Sauvignon Blanc	-	-	-	-	-	-
S2	Slanghoek	Sauvignon Blanc	-	-	+	+	+	+
S3	Slanghoek	Sauvignon Blanc	-	-	-	-	-	-
D1	Robertson	Petit Verdot	-	-	+	+	+	+
D2	Robertson	Petit Verdot	-	+	+	+	+	+
D3	Robertson	Petit Verdot	-	+	-	+	+	+
D4	Robertson	Petit Verdot	-	-	+	+	+	+
D5	Robertson	Petit Verdot	-	+	-	+	+	+
D6	Robertson	Petit Verdot	-	-	+	+	+	+
D7	Robertson	Chardonnay	-	-	+	+	+	+
D8	Robertson	Chardonnay	-	+	-	+	+	+
D9	Robertson	Chardonnay	-	-	+	+	+	+
D10	Robertson	Chardonnay	-	+	-	+	+	+
D11	Robertson	Chardonnay	-	-	+	+	+	+
D12	Robertson	Chardonnay	-	+	+	+ (poor)	-	-
Du	Robertson	Chardonnay	und	und.	und.	+	+	+
V1	Bonnievale	Pinotnoir	-	+	+	+	+	+
V2	Bonnievale	Pinotnoir	-	+	-	+ (poor)	-	-
G1	Paarl-Wellington	Cabernet Sauvignon	-	-	+	+	+	+
G2	Paarl-Wellington	Cabernet Sauvignon	-	-	+	+	+	+
G3	Paarl-Wellington	Cabernet Sauvignon	-	-	+	+	+ (poor)	-
G4	Paarl-Wellington	Cabernet Sauvignon	-	-	+	+	+ (poor)	-
G5	Paarl-Wellington	Cabernet Sauvignon	-	-	+	+	+	+
W1	Stellenbosch	Cabernet Sauvignon	+	-	-	+	n.t.	n.t.
W2	Stellenbosch	Cabernet Sauvignon	+	-	-	-	n.t.	n.t.
W3	Stellenbosch	Cabernet Sauvignon	+	+	-	+	n.t.	n.t.
W4	Stellenbosch	Cabernet Sauvignon	+	+	-	+	n.t.	n.t.
W5	Stellenbosch	Cabernet Sauvignon	und.	und.	und.	+	n.t.	n.t.
W6	Stellenbosch	Cabernet Sauvignon	+	+	-	+	n.t.	n.t.
W7	Stellenbosch	Cabernet Sauvignon	+	+	-	+	n.t.	n.t.
W8	Stellenbosch	Cabernet Sauvignon	+	+	-	+	n.t.	n.t.
W9	Stellenbosch	Cabernet Sauvignon	und.	und.	und.	-	n.t.	n.t.
W10	Stellenbosch	Cabernet Sauvignon	und.	und.	und.	-	n.t.	n.t.
W11	Stellenbosch	Cabernet Sauvignon	+	-	-	+	n.t.	n.t.
W12	Stellenbosch	Cabernet Sauvignon	und.	und.	und.	-	n.t.	n.t.

[A] Amplification of 348 bp DNA fragment with rapid-direct-one-tube-RT-PCR. +, amplification of the expected fragment, -, did not amplify the expected fragment, n.t., not tested. [B] GFLV ImmunoStrip test. [C] GFLV direct double-antibody sandwich enzyme-linked immunosorbent assay. +, reacted, -, did not react, n.t., not tested, und, undefined symptoms. V, Vein banding. Y, Yellow mosaic. F, fanleaf. und., samples of undefined status.

4. Discussion

Three distinct types of grapevine leaf symptoms were observed in South African vineyards: vein banding, yellow mosaic and fanleaf, with vein banding symptoms predominant in the Stellenbosch area. The factors affecting symptom expression are still unknown. Martelli and Savino (1990) suggested that these factors could include virus strain, host genotype, multiple infections with different GFLV strains, the effect of other viruses and environmental conditions. No association between sequence variants and symptom expression were observed in the present study. Pompe-Novak et al. (2007) analysed the variability within the RNA2 of GFLV and found no association between the genetic variability of the complete RNA2 ORF and symptomology. They suggested

that the viral determinants for symptomology could be linked to the RNA2 non-coding region and/or RNA1. Our results confirm that the $2C^{CP}$ gene is not responsible for symptom expression. In a study on the diversity of GFLV isolates from Tunisia, two distinct sub-populations (Sp1 and Sp2) were observed (Fattouch et al., 2005a; 2005b). Increased severities of symptoms on grapevine plants containing both isolates were observed, suggesting that multiple infections with different GFLV strains could affect symptom expression. Testing for mixed infections of GFLV isolates and the effect of other viruses on symptom expression were not included in this study, but needs further investigation.

Although vein banding symptoms were only visible in Stellenbosch vineyards, no significant correlation was found between geographical origin and symptoms, nor between geographical origin and sequence variability or between grapevine cultivar and symptom expression. The genetic structure described here suggests a displacement of some GFLV isolates among geographically isolated populations, since isolates from various distinct regions had almost identical genetic structure. For example, identities of 99 % were obtained among variants from the Slanghoek region and Paarl (S2 and A1), as well as variants from Bonnievale and Robertson (V1 and D7). The current study could not correlate the observed sequence variability with specific geographical regions. It seems therefore more likely that variability is the result of different sources of grapevine propagation material that entered these regions.

Table 3. Percentage nucleotide (bottom) and amino acid (top) sequence identities between *Grapevine fanleaf virus* (GFLV) $2C^{CP}$ genes of isolates from South Africa as determined by NCBI Blast.

	A1	**S2**	**D1**	**D7**	**D12**	**Du**	**V1**	**V2**	**G2**	**W1**	**W5**	**W8**
A1		99	98	94	95	94	94	94	98	99	99	94
S2	99		97	94	95	94	94	94	98	98	99	94
D1	94	94		94	95	94	94	94	98	97	98	94
D7	88	88	87		98	95	99	99	94	94	95	98
D12	88	88	87	97		96	98	98	95	95	95	99
Du	86	86	86	88	88		95	95	94	94	94	95
V1	88	88	87	99	97	88		99	94	94	94	98
V2	88	88	87	98	97	88	98		94	94	94	98
G2	97	97	93	88	88	86	88	88		99	99	98
W1	97	97	93	88	88	86	88	88	98		99	88
W5	97	97	94	87	87	86	87	87	93	99		95
W8	88	88	87	97	98	88	97	97	88	94	88	

Phylogenetic analysis showed that the Du isolate was closely related to sequence variants in clade B (fig. 2). Pairwise alignments of Du with sequence variants from clade A showed sequence identities of 86% on nucleotide level and 94% on amino acid level and 88% and 95 to 96%, respectively with sequence variants from clade B (table 3). These values are significantly lower than the values for

intra-clade comparisons (clade A: 93-99% on nucleotide level and 98-99% on amino acid level and clade B: 97-99% on nucleotide level and 97-99% on amino acid level) (table 3).There is no significant difference between the interisolate variability of the South African isolates (86%) compared to France (86.2%) (Vigne et al., 2004a), Iran (84%) (Bashir et al., 2007c), Slovenia (13.2%) (Pompe-Novak et al., 2007) and the USA (87%) (Naraghi-Arani et al., 2001).

RT-PCR amplification of the 12 South African isolates resulted in 1 756 bp or 1 762 bp products, extending from the $2B^{MP}$ gene to the 3' non-coding region of the RNA2. Sequence analysis showed that all the sequence variants from clade B amplified a 1 762 bp fragment and have a six nucleotide insertion in the 3' non-coding region of the RNA2. All sequence variants from clade A amplified a 1 756 bp product, and had no insertion of these six nucleotides. Random mutations or nucleotide variations in the third base of codons were also noticed in the South African isolates. Most of these variations are silent mutations with little effect on the amino acid sequence. However, even if these silent mutations do not influence the amino acid sequence, they can potentially modulate RNA structure (Jończyk et al., 2004).

In a single host plant, RNA viruses often show a heterogeneous population structure, called "quasispecies" (Kissi et al., 1999; Schneider and Roossinck, 2000). *Grapevine fanleaf virus* is no different. A recent model of RNA population evolution suggests that viral RNA populations naturally evolve towards sub-population organisation; this prediction is confirmed with the appearance of sub-populations in GFLV quasispecies (Fattouch et al., 2005b; Huynen et al., 1996). Two sub-populations of the South African isolates were evident from the phylogenetic analysis. Clade A (D1, A1, S2, G2, W1 and W5) grouping with isolates from France, Germany and Chile, and clade B (D12, W8, V2, D7 and V1) grouping with isolates from France and Slovenia, perhaps indicating that these areas are the origins of these strains.

Rapid, sensitive and reliable diagnostic assays are necessary to effectively control the spread of GFLV. Three diagnostic techniques, rapid-direct-one-tube-RT-PCR, DAS-ELISA and ImmunoStrips were evaluated in this study to determine the sensitivity and specificity of each assay. No significant differences in specificity were observed among the three diagnostic assays. Isolates D12 and V1 (grouping with clade B, fig. 2) and G3 and G4 (grouping with clade A, data not shown) were not reliably detected with the DAS-ELISA and/or the ImmunoStrips. These results are probably due to low virus titres in the infected leaf material, rather than being attributed to sequence variability of these isolates. One of the samples (W2) that tested negative with the rapid-direct-one-tube-RT-PCR showed vein banding symptoms; this could suggest that these plants were infected with other viruses (*Yellow mosaic virus, Arabis mosaic virus*) or viriods (*Grapevine yellow*

speckle viriod) with similar symptomology. Samples S1 and S3 were collected from symptomless vines in a GFLV infected vineyard to screen for possible latent infection. These samples tested negative in all three assays, indicating no GFLV infection and thus validating the integrity of these assays.

The rapid-direct-one-tube-RT-PCR was found to be the most sensitive assay in this study. Although RT-PCR is generally accepted to be more sensitive than DAS-ELISA, the latter is still used as the diagnostic assay of choice for routine testing. DAS-ELISA is a high throughput, sensitive, cost effective assay that requires moderately skilled personnel and basic laboratory equipment. In our experience, the ImmunoStrip assay was more sensitive than the DAS-ELISA. Although the assay is more costly, it can be performed on site by less skilled personnel, and requires no specialised equipment. The ImmunoStrip assay is applicable to high throughput analysis, the results are obtained rapidly (30 min vs. 2 days) and easy to interpret, with minimal chance for contamination and false positives. This makes ImmunoStrips a viable alternative to DAS-ELISA for routine testing. We however recommend that rapid-direct-one-tube-RT-PCR be implemented and used for the most accurate, sensitive and reliable detection of GFLV.

This paper gives a general overview of the GFLV diversity within South African vineyards. Phylogenetic analysis of the $2C^{CP}$ gene revealed two distinct sub-populations within the South African GFLV population. There was no association between GFLV $2C^{CP}$ gene sequence variability and symptom expression or geographical origin of sub-populations. RT-PCR was found to be the most sensitive and reliable diagnostic technique to be used for GFLV detection. In future work, it would be important to investigate the effect of mixed GFLV infections and other viruses on the symptom expression of GFLV. For more reliable diversity analysis, the complete genome of GFLV should be compared. This could provide more insight in the viral component responsible for symptom expression as well as the geographical origin(s) of the South African isolates and sub-population evolution.

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5 FINAL CONCLUSION

Grapevine fanleaf virus is one of the most devastating grapevine viruses in the world and is a significant problem in the Breede River valley of South Africa. To control the spread of the virus in South Africa, all certified grapevine plants must be tested by a sensitive and reliable diagnostic assay. The aim of this study was to develop a sensitive and reliable diagnostic DAS-ELISA specific to the South African strain(s) of GFLV. To produce sensitive antibodies against the target GFLV CP, recombinant DNA technology was utilised to express the target GFLV CP, was. The CP was successfully cloned into the pGex-6P-2 vector and the GST:GFLV-CP fusion protein was expressed. High amounts of fusion protein were expressed, but the protein was localised in the insoluble inclusion bodies. Different strategies to improve soluble protein expression were performed but none had any significant effect on the GFLV CP solubility.

The best results were obtained when GST:GFLV-CP fusion protein expression was induced at OD_{600} 0.5 with 0.1 mM IPTG and grown to an OD_{600} of 1.3. After centrifugation the pellet was washed and resuspended in STE buffer. The sample was treated with 100 µg/ml lysozyme followed by the addition of 5 mM DTT. To solublise the inclusion bodies 0.5-1.0% sarkosyl was added to the STE bacterial solution resulting in approximately 50% soluble fusion protein expression without degradation of the protein (fig.3.10A). For protein purification elution buffer 2 was preferred over Glutathione reconstitution buffer and resulted in approximately 40% purification of the GST:GFLV-CP fusion protein (fig. 3.10B), resulting in low amounts of soluble protein being purified, which was insufficient for antibody production. Expression of the control sample, pGex-6P-2 without the GFLV-CP was optimised as a control experiment to prove the functioning of the system. High amounts of soluble GST were expressed and purified, but when the GST:GFLV-CP was expressed under the same conditions, no soluble fraction was visible when subjected to SDS-PAGE analysis.

To determine the probable cause for the expression of the protein as an inclusion body, the CP sequence fused to the GST partner was analysed. The incorporated thymine (T) kept the ORF in frame and no premature stop codons were expressed (appendix 1). Kaplan et al. (1997) suggested that the higher the percentage of cysteine residues present in the protein, the more likely the protein would be expressed in the insoluble inclusion bodies. Bioinformatics analysis showed that the GST:GFLV CP fusion protein had 14 cysteine residues, 1.9% of the total amino acids present that form disulfide bridges. These cysteine residues can form incorrect disulfide bridges when expressed, resulting in incorrect folding of the fusion protein. Christendat et al. (2000) and Bertone

et al. (2001) state that the following sequence based parameters could be helpful for solubility prediction: proteins with long hydrophobic stretches (> 20), low glutamine content (Q<4%), low negatively charged residue content (DE<17%), high content of aromatic residues (FYW> 7.5%) and low content of the amino acids aspartic acid, glutamic acid, asparagines and glutamine residues (DENQ< 16%) tend to be insoluble. Using this criteria, three of the five parameters predict that the GST:GFLV-CP fusion protein would be insoluble (fig 5.1). The fusion protein consists of 16.8% D, E, N, Q residues, 0.8% more than the predicted insoluble value. A stretch of only 7 hydrophobic amino acids are present in the GST:GFLV-CP fusion protein.

Table 5.1: Insolubility prediction of the GST;GFLV-CP fusion protein by calculating the amino acid percentages.

	Hydrophobic stretch V,I,L, M,F,W,A,P	Glutamine (Q)	Negatively charged (D,E)	Aromatic (F,Y,W)	D,E,N,Q
Insoluble	>20 aa	<4%	<17%	>7.5%	<16%
GST:GFLV-CP	7 aa	2.5%	11.4%	25.1%	16.8%

V, Valine; I, Isoleucine; L, Leucine; M, Methionine; F, Phynylalanine; W, Tryptophan; A, alanine; P, Proline; Q, Glutamine; Y, Tyrosine; D, Aspartic acid; E, Glutamic acid; N, Aspargine.

For future use of recombinant DNA technology to express fusion proteins, more research needs to be done on solubility prediction and different fusion proteins must be compared to select the best possible solubility enhancer for the target protein.

Although a sensitive and reliable DAS-ELISA could not be developed, an RT-PCR based diagnostic assay was developed and optimised. Twelve samples were collected from GFLV infected areas in the Western Cape and the CP sequenced (appendix 2) to analyse the variability of the South African isolates of the virus. The isolates grouped in to two clades based on sequence and phylogenetic analysis. Only isolate Du did not group into the 2 clades, but grouped sister to clade B. Isolate Du had the highest variability when compared to the available CP sequences on GenBank and to the other South African isolates. More research needs to be performed to determine if a third GFLV clade is present in South African vineyards. Although only the 1515 bp CP was used in phylogenetic analysis, sequence alignments of the ±1765 bp fragment showed a six nucleotide insertion in the 3'non-coding regions of all the isolates in clade B (appendix 3). No associations were found between the leaf symptoms displayed and the sequence variability, the geographic origin, or the cultivar. This led to the conclusion that other factors contribute to the severity and symptom expression. Multiple strain infections, synergistic infection with other grapevine viruses, viriods or phytoplasmas, could also play a roll in the severity of the symptom expression. The viral component responsible for symptom expression could also be linked to other parts of the genome. Pompe-Novak et al. (2007) speculated that the causative agent could be situated in the RNA2

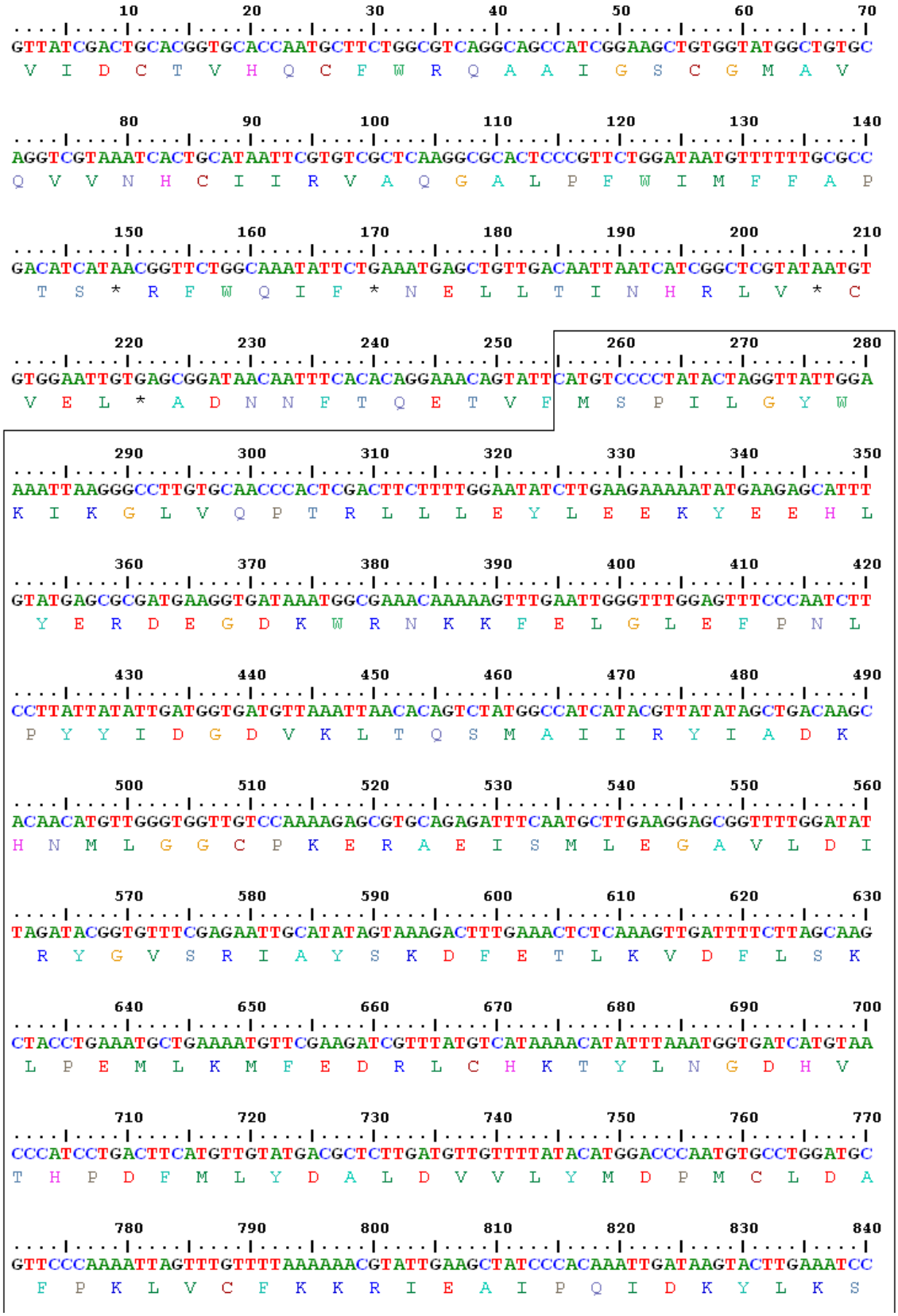
3’Non-coding region and/or the RNA1 genome of the GFLV. More research needs to confirm this hypothesis.

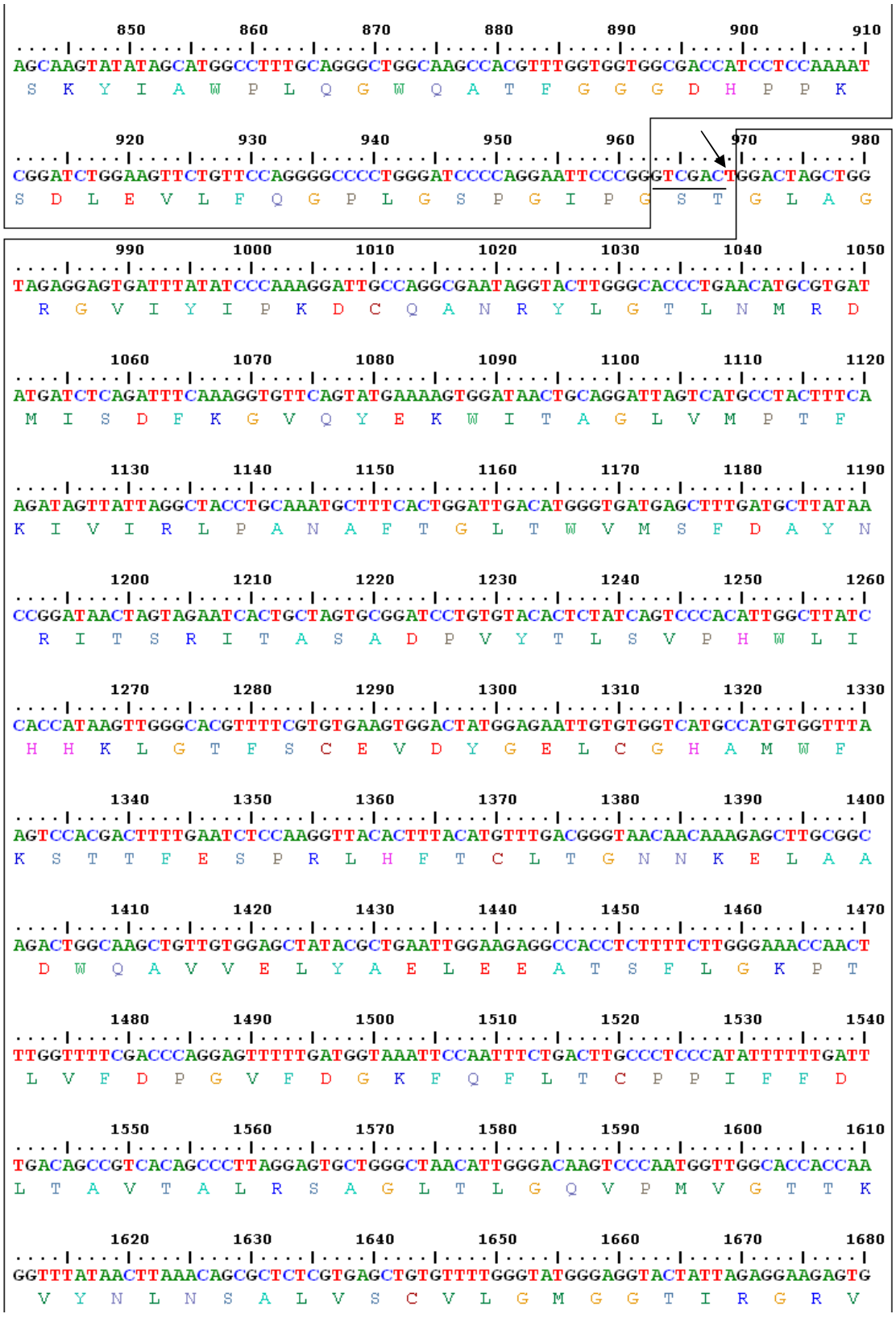
A rapid-direct-one-tube-RT-PCR that detected both the variant groups and the Du isolate was optimised. Primers were designed to detect the all known South African and International isolates by amplifying a region of low variability. The assay was compared to commercially available DAS-ELISA and ImmunoStrip tests (Agdia) and found to be the most sensitive and reliable diagnostic assay. Twenty one of the 23 samples tested, amplified the 348 bp coat protein fragment, 19 samples tested positive with the ImmunoStrips and 17 with the DAS-ELISA. Sample S1 and S3 that tested negative with all three assays were collected from symptomless vines. Thus the rapid-direct-one-tube-RT-PCR assay is approximately 20% more sensitive than the DAS-ELISA.

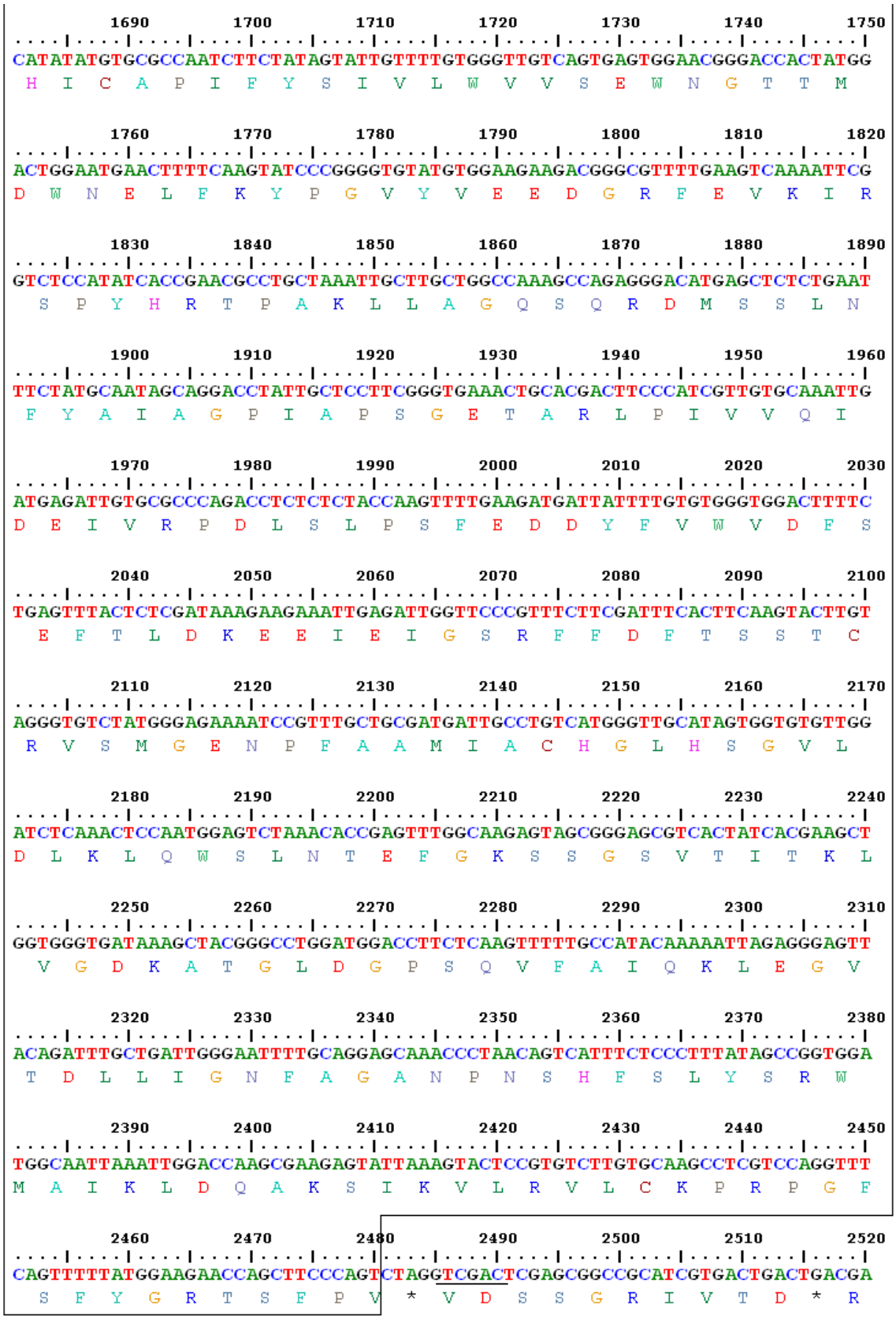
The rapid-direct-one-tube-RT-PCR assay uses crude plant sap eliminating the time consuming step of RNA extractions. The assay could be utilised for large scale testing and up to 96 samples can be tested per run, similar to the throughput of ELISA tests. Moreover, with the world wide expansion in the use of PCR, reaction components have become less expensive and comparable with that of DAS-ELISA diagnostic tests. With the rapid and accurate detection rate and elimination of false negatives, this assay is an asset to the industry, easily implementable and could contribute to the control of the virus.

Although we did not succeed in producing an ELISA diagnostic test, a sensitive and reliable rapid-direct-one-tube-RT-PCR diagnostic assay was developed and optimised using new primers designed to amplify a 348 bp region of low variability, detecting all known South African GFLV isolates.

APPENDIX 1: pGex-6P-2 cloning strategy




850 860 870 880 890 900 910
AGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAAT
S K Y I A W P L Q G W Q A T F G G G D H P P K
920 930 940 950 960 970 980
CGGATCTGGAAGTTCTGTTCCAGGGGCCCCTGGGATCCCCAGGAATTCCCGGGTCGACTGGACTAGCTGG
S D L E V L F Q G P L G S P G I P G S T G L A G
990 1000 1010 1020 1030 1040 1050
TAGAGGAGTGATTTATATCCCAAAGGATTGCCAGGCGAATAGGTACTTGGGCACCCTGAACATGCGTGAT
R G V I Y I P K D C Q A N R Y L G T L N M R D
1060 1070 1080 1090 1100 1110 1120
ATGATCTCAGATTTCAAAGGTGTTCAGTATGAAAAGTGGATAACTGCAGGATTAGTCATGCCTACTTTCA
M I S D F K G V Q Y E K W I T A G L V M P T F
1130 1140 1150 1160 1170 1180 1190
AGATAGTTATTAGGCTACCTGCAAATGCTTTCACTGGATTGACATGGGTGATGAGCTTTGATGCTTATAA
K I V I R L P A N A F T G L T W V M S F D A Y N
1200 1210 1220 1230 1240 1250 1260
CCGGATAACTAGTAGAATCACTGCTAGTGCGGATCCTGTGTACACTCTATCAGTCCCACATTGGCTTATC
R I T S R I T A S A D P V Y T L S V P H W L I
1270 1280 1290 1300 1310 1320 1330
CACCATAAGTTGGGCACGTTTTCGTGTGAAGTGGACTATGGAGAATTGTGTGGTCATGCCATGTGGTTTA
H H K L G T F S C E V D Y G E L C G H A M W F
1340 1350 1360 1370 1380 1390 1400
AGTCCACGACTTTTGAATCTCCAAGGTTACACTTTACATGTTTGACGGGTAACAACAAAGAGCTTGCGGC
K S T T F E S P R L H F T C L T G N N K E L A A
1410 1420 1430 1440 1450 1460 1470
AGACTGGCAAGCTGTTGTGGAGCTATACGCTGAATTGGAAGAGGCCACCTCTTTTCTTGGGAAACCAACT
D W Q A V V E L Y A E L E E A T S F L G K P T
1480 1490 1500 1510 1520 1530 1540
TTGGTTTTCGACCCAGGAGTTTTTGATGGTAAATTCCAATTTCTGACTTGCCCTCCCATATTTTTTGATT
L V F D P G V F D G K F Q F L T C P P I F F D
1550 1560 1570 1580 1590 1600 1610
TGACAGCCGTCACAGCCCTTAGGAGTGCTGGGCTAACATTGGGACAAGTCCCAATGGTTGGCACCACCAA
L T A V T A L R S A G L T L G Q V P M V G T T K
1620 1630 1640 1650 1660 1670 1680
GGTTTATAACTTAAACAGCGCTCTCGTGAGCTGTGTTTTGGGTATGGGAGGTACTATTAGAGGAAGAGTG
V Y N L N S A L V S C V L G M G G T I R G R V



Arrow indicates the inserted nucleotide. First block indicates the GST protein. Second block indicates the GFLV CP. Underlined nucleotides indicate the *Sal*I recognition sites.

APPENDIX 2: South African GFLV partial RNA2 sequences

EU702440, Isolate A1

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   1 ACCTTCTCTA TCAGGAGCCG CTCACGATCG GTGAGGATTG ACAGAAATGT TGATCTTCCC
  61 CAACTTGAGG CTGAACCCAG ATTGAGTTCA ACTGTGAGAG GACTAGCTGG TAGAGGAGTG
 121 ATTTATATCC CAAAGGATTG CCAGGCGAAT AGGTACTTGG GCACCCTGAA CATGCGTGAT
 181 ATGATCTCAG ATTTCAAAGG TGTTCAGTAT GAAAAGTGGA TAACTGCAGG ATTAGTCATG
 241 CCTACTTTCA AGATAGTTAT TAGGCTACCT GCAAATGCTT TCACTGGATT GACATGGGTG
 301 ATGAGCTTTG ATGCTTATAA CCGGATAACT AGTAGAATCA CTGCTAGTGC GGATCCTGTG
 361 TACACTCTAT CAGTCCCACA TTGGCTTATC CACCATAAGT TGGGCACGTT TTCGTGTGAA
 421 GTGGACTATG GAGAATTGTG TGGTCATGCC ATGTGGTTTA AGTCCACGAC TTTTGAATCT
 481 CCAAGGTTAC ACTTTACATG TTTGACGGGT AACAACAAAG AGCTTGCGGC AGACTGGCAA
 541 GCTGTTGTGG AGCTATACGC TGAATTGGAA GAGGCCACCT CTTTTCTTGG GAAACCAACT
 601 TTGGTTTTCG ACCCAGGAGT TTTTGATGGT AAATTCCAAT TTCTGACTTG CCCTCCCATA
 661 TTTTTTGATT TGACAGCCGT CACAGCCCTT AGGAGTGCTG GGCTAACATT GGGACAAGTC
 721 CCAATGGTTG GCACCACCAA GGTTTATAAC TTAAACAGCG CTCTCGTGAG CTGTGTTTTG
 781 GGTATGGGAG GTACTATTAG AGGAAGAGTG CATATATGTG CGCCAATCTT CTATAGTATT
 841 GTTTTGTGGG TTGTCAGTGA GTGGAACGGG ACCACTATGG ACTGGAATGA ACTTTTCAAG
 901 TATCCCGGGG TGTATGTGGA AGAAGACGGG CGTTTTGAAG TCAAAATTCG GTCTCCATAT
 961 CACCGAACGC CTGCTAAATT GCTTGCTGGC CAAAGCCAGA GGGACATGAG CTCTCTGAAT
1021 TTCTATGCAA TAGCAGGACC TATTGCTCCT TCGGGTGAAA CTGCACGACT TCCCATCGTT
1081 GTGCAAATTG ATGAGATTGT GCGCCCAGAC CTCTCTCTAC CAAGTTTTGA AGATGATTAT
1141 TTTGTGTGGG TGGACTTTTC TGAGTTTACT CTCGATAAAG AAGAAATTGA GATTGGTTCC
1201 CGTTTCTTCG ATTTCACTTC AAGTACTTGT AGGGTGTCTA TGGGAGAAAA TCCGTTTGCT
1261 GCGATGATTG CCTGTCATGG GTTGCATAGT GGTGTGTTGG ATCTCAAACT CCAATGGAGT
1321 CTAAACACCG AGTTTGGCAA GAGTAGCGGG AGCGTCACTA TCACGAAGCT GGTGGGTGAT
1381 AAAGCTACGG GCCTGGATGG ACCTTCTCAA GTTTTTGCCA TACAAAAATT AGAGGGAGTT
1441 ACAGATTTGC TGATTGGGAA TTTTGCAGGA GCAAACCCTA ACAGTCATTT CTCCCTTTAT
1501 AGCCGGTGGA TGGCAATTAA ATTGGACCAA GCGAAGAGTA TTAAAGTACT CCGTGTCTTG
1561 TGCAAGCCTC GTCCAGGTTT CAGTTTTTAT GGAAGAACCA GCTTCCCAGT CTAGAGTATC
1621 TGACTATAAA AGACTCAGGT GTACATATGT GCTTTAATAG TTGTGTGTAT TATTTTGTAT
1681 TGTAGTTTGC TTTAACTTGT TTACTGCTTT AGTGTGTTTA ATTTCATGCT TTTAGTGGCG
1741 ACAGTGTGTT GTTTGT
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EU702441, Isolate S1

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   1 ACCTTCTCTA TCAGGAGCCG CTCACGATCG GTGAGGATTG ACAGAAATGT TGATCTTCCC
  61 CAACTTGAGG CTGAACCCAG ATTGAGTTCA ACTGTGAGAG GACTAGCTGG TAGAGGAGTG
 121 ATTTATATCC CAAAGGATTG CCAGGCGAAT AGGTACTTGG GCACCCTGAA CATACGTGAT
 181 ATGATCTCAG ATTTCAAAGG TGTTCAGTAT GAAAAGTGGA TAACTGCAGG ATTAGTCATG
 241 CCTACTTTCA AGATAGTTAT TAGGCTACCT GCAAATGCTT TCACTGGATT GACATGGGTG
 301 ATGAGCTTTG ATGCTTATAA CCGGATAACT AGTAGAATCA CTGCTAGTGC GGATCCTGTG
 361 TACACTCTAT CAGTCCCACA TTGGCTTATC CACCATAAGT TGGGCACGTT TTCGTGTGAA
 421 GTGGACTATG GAGAATTGTG TGGTCATGCC ATGTGGTTTA AGTCCACGAC TTTTGAATCT
 481 CCAAGGTTAC ACTTTACATG TTTGACGGGT AACAACAAAG AGCTTGCGGC AGACTGGCAA
 541 GCTGTTGTGG AGCTATACGC TGAATTGGAA GAGGCCACCT CTTTTCTTGG GAAACCAACT
 601 TTGGTTCCCG ACCCAGGAGT TTTTGATGGT AAATTCCAAT TTCTGACTTG CCCTCCCATA
 661 TTTTTTGATT TGACAGCCGT CACAGCCCTT AGGAGTGCTG GGCTAACATT GGGACAAGTC
 721 CCAATGGTTG GCACCACCAA GGTTTATAAC TTAAACAGCG CTCTCGTGAG CTGTGTTTTG
 781 GGTATGGGAG GTACTATTAG AGGAAGAGTG CATATCTGTG CGCCAATCTT CTATAGTATT
 841 GTTTTGTGGG TTGTCAGTGA GTGGAACGGG ACCACTATGG ACTGGAATGA ACTTTTCAAG
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 961 CACCGAACGC CTGCTAAATT GCTTGCTGGC CAAAGCCAGA GGGACATGAG CTCTCTGAAT
1021 TTCTATGCAA TAGCAGGACC TATTGCTCCT TCGGGTGAAA CTGCACGACT TCCCATCGTT
1081 GTGCAAATTG ATGAGATTGT GCGCCCAGAC CTCTCTCTAC CAAGTTTTGA AGATGATTAT
1141 TTTGTGTGGG TGGACTTTTC TGAGTTTACT CTCGATAAAG AAGAAATTGA GATTGGTTCC
1201 CGTTTCTTCG ATTTCACTTC AAGTACTTGT AGGGTGTCTA TGGGAGAAAA TCCGTTTGCT
1261 GCGATGATTG CCTGTCATGG GTTGCATAGT GGTGTGTTGG ATCTCAAACT CCAATGGAGT
1321 CTAAACACCG AGTTTGGCAA GAGTAGCGGG AGCGTCACTA TCACGAAGCT GGTGGGTGAT
1381 AAAGCTACGG GCCTGGATGG ACCTTCTCAA GTTTTTGCCA TACAAAAATT AGAGGGAGTT
1441 ACAGATTTGC TGATTGGGAA TTTTGCAGGA GCAAACCCTA ACAGTCATTT CTCCCTTTAT
1501 AGCCGGTGGA TGGCAATTAT ATTGGACCAA GCGAAGAGTA TTAAAGTACT CCGTGTCTTG
1561 TGCAAGCCTC GTCCAGGTTT CAGTTTTTAT GGAAGAACCA GCTTCCCAGT CTAGAGTATC
1621 TGACTATAAA AGACTCAGGT GTACATATGT GCTTTAATAG TTGTGTGTAT TATTTTGTAT
1681 TGTAGTTTGC TTTAACTTGT TTACTGCTTT AGTGTGTTTA ATTTCATGCT TTTAGTGGCG
1741 ACAGTGTGTT GTTTGT
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EU702442, Isolate G2

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 121 ATCTATATCC CAAAGGATTG CCAGGCAAAT AGATATTTGG GCACCTTGAA CATACGTGAT
 181 ATGATCTCAG ATTTCAAAGG TGTTCAGTAT GAAAAGTGGA TAACTGCAGG ACTAGTCATG
 241 CCTACTTTTA AGATAGTTAT TAGGCTACCT GCAAATGCTT TCACTGGATT GACATGGGTG
 301 ATGAGCTTCG ATGCTTATAA CCGGATAACT AGTAGAATCA CTGCTAGTGC GGATCCTGTG
 361 TACACCCTAT CAGTCCCACA TTGGCTTATC CACCATAAGT TGGGCACGTT TTCGTGTGAA
 421 GTGGACTATG GGGAATTGTG TGGTCATGCC ATGTGGTTTA AGTCCACGAC TTTTGAATCT
 481 CCAAGGTTAC ACTTCACATG TTTAACGGGT AACAACAAAG AGCTGGCGGC AGACTGGCAA
 541 GCTGTTGTGG AGCTATATGC TGAATTGGAA GAGGCCACCT CTTTTCTTGG GAAACCAACT
 601 TTGGTTTTCG ACCCAGGAGT TTTTAATGGT AAATTCCAAT TTCTGACTTG CCCTCCCATA
 661 TTCTTTGACC TGACAGCCGT CACAGCCCTA AGGAGTGCTG GGCTGACATT GGGACAAGTC
 721 CCAATGGTCG GCACCACTAA GGTTTATAAC TTAAACAGCA CTCTCGTGAG CTGTGTTTTG
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 841 GTTTTGTGGG TTGTCAGTGA GTGGAACGGG ACCACTATGG ACTGGAATGA ACTTTTCAAG
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1081 GTGCAAATTG ATGAGATTGT GCGCCCAGAC CTCTCTCTAC CAAGTTTTGA AGATGATTAT
1121 TTTGTGTGGG TAGACTTTTC TGAGTTTACT CTCGATAAAG AAGAAATTGA GATTGGTTCC
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1721 ACAGTGTGTT GTTTGT
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EU702443 isolate W5

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 181 ATGATTTCAG ATTTTAAAGG TGTTCAGTAT GAAAAGTGGA TAACTGCAGG ACTAGTCATG
 241 CCTACTTTTA AGATAGTTAT TAGGCTACCT GCAAATGCTT TCACTGGATT GACATGGGTG
 301 ATGAGCTTTG ATGCTTATAA CCGGATAACT AGTAGAATCA CTGCTAGTGC GGATCCTGTG
 361 TACACCCTAT CAGTCCCACA TTGGCTTATC CACCATAAGT TGGGCACGTT TTCGTGTGAA
 421 GTGGACTATG GGGAATTGTG TGGTCATGCC ATGTGGTTTA AGTCCACGAC TTTTGAATCT
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 541 GCTGTTGTGG AGCTATATGC TGAATTGGAA GAGGCCACCT CTTTTCTTGG GAAACCAACT
 601 TTGGTTTTCG ACCCAGGAGT TTTTAGTGGT AAATTCCAAT TTCTGACTTG CCCTCCCATA
 661 TTCTTTGACC TGACAGCCGT CACAGCCCTA AGGAGTGCTG GGCTGACATT GGGACAAGTC
 721 CCAATGGTTG GCACCACTAA GGTTTATAAC TTAAACAGCG CTCTTGTGAG CTGTGTTTTG
 781 GGTATGGGAG GTACTATTAG AGGAAGAGTG CATATCTGTG CGCCAATCTT CTATAGTATT
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1381 AAAGCTATGG GCCTGGATGG ACCTTCTCAA GTTTTTGCCA TACAAAAACT AGAGGGAGTT
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1501 AGCCGATGGA TGGCAATTAA ATTGGACCAA GCAAAGAGTA TTAAAGTACT CCGTGTCTTG
1561 TGCAAGCCTC GTCCAGGTTT CAGTTTTTAT GGAAGAACCA GCTTCCCAGT CTAGAGTATC
1621 TGACTATAAA AGACTCAGGT GTACATATGT GCTTTAATAG TTGTATGTAT TATTTTGTAT
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1741 ACAGTGTGTT GTTTGT
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EU702444 Isolate W1

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 121 ATTTATATCC CAAAGGATCG CCAGGCAAAT AGATACTTGG GCACCCTGAA CATACGTGAT
 181 ATGATTTCAG ATTTTAAAGG TGTTCAGTAT GAAAAGTGGA TAACTGCAGG ACTAGTCATG
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1381 AAAGCTATGG GCCTGGATGG ACCTTCTCAA GTTTTTGCCA TACAAAAACT AGAGGGAGTT
1441 ACAGATTTGC TGATTGGGAA TTTTGCAGGA GCAAACCCTA ACAGTCATTT CTCCCTTTAT
1501 AGCCGATGGA TGGCAATTAA ATTGGACCAA GCAAAGAGTA TTAAAGTACT CCGTGTCTTG
1561 TGCAAGCCTC GTCCAGGTTT CAGTTTTTAT GGAAGAACCA GCTTCCCAGT CTAGAGTATC
1621 TGACTATAAA AGACTCAGGT GTACATATGT GCTTTAATAG TTGTATGTAT TATTTTGTAT
1681 TGTAGTTTGC TTTAACTTGT TTACTGCTTT AGTGTGTTTA ATTTCATGCT TTTAGTGGCG
1741 TCAGTGTGTT GTTTGT
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EU702445 Isolate D1

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   1 ACCTTCTCTA TCAGGAGTCG CTCACGATCG GTGAGGATTG ACAGAAATGT TGATCTTCCC
  61 CAACTTGAGG CTGAACCCAG ATTGAGTTCA ACTGTGAGAG GACTAGCTGG TAGAGGAGTG
 121 ATTTATATCC CAAAGGATTG CCAGGCAAAT AGGTACTTGG GCACCCTGAA CATACGTGAT
 181 ATGATCTCAG ATTTCAAAGG TGTCCAGTAT GAAAAGTGGA TAGCTGCAGG ATTAGTCATG
 241 CCTACTTTCA AGATAGTTAT TAGGCTACCT GCAAATGCTT TCACTGGATT GACATGGGTG
 301 ATGAGCTTTG ATGCTTATAA CCGGATAACT AGTAGAATCA CTGCTAGTGC GGATCCTGTG
 361 TACACTCTAT CAGTCCCACA TTGGCTTATC CACCATAAGT TGGGCACGTT TTCGTGTAAA
 421 GTGGACTATG GAGAATTGTG TGGTCATGCC ATGTGGTTTA AGTCCACGAC TTTTGAATCT
 481 CCAAGGTTAC ACTTTATATG TTTGACGGGT AACAACAAAG AGCTTGCGGC AGACTGGCAA
 541 GCTGTTGTGG AGCTATACGC TGAATTGGAA GAGGCCACCT CTTTTCTTGG GAAACCAACT
 601 TTGGTTTTCG ACCCAGGAGT TTTTGATGGT AAATTCCAAT TTCTGACTTG CCCTCCCATA
 661 TTTTTTGACT TGACAGCCGT CACAGCCCTT AGGAGTGCTG GGCTGACATT GGGACAAGTC
 721 CCAATGGTTG GCACCACCAA GGTTTATAAC TTAAACAGCG CTCTCGTGAG CTGTGTTTTG
 781 GGTATGGGAG GTACTATTAG AGGAAGAGTG CATATCTGTG CGCCAATCTT CTATAGTATT
 841 GTTTTGTGGG TTGTCAGTGA GTGGAACGGG ACCACTATGG ACTGGAATGA ACTTTTCAAG
 901 TATCCCGGGG TGTATGTGGA AGAGGATGGA AGCTTTGAAG TCAAAATTCG TTCTCCATAT
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1081 GTGCAAATTG ATGAAATCGT GCGCCCTGAT CTTTCTCTAC CAAGTTTTGA AGATGATTAT
1141 TTTGTATGGG TGGATTTTTC TGAGTTCACT CTTGATAAAG AAGAAATTGA GATTGGTTCT
1201 CGTTTCTTTG ATTTTACTTC AAACACTTGT AGAGTGTCTA TGGGAGAAAA CCCGTTTGCT
1261 GCAATGATTG CCTGTCATGG ATTGCATAGT GGTGTATTGG ATCTCAAACT GCAATGGAGT
1321 CTGAACACTG AATTTGGCAA GAGCAGCGGG AGCGTTACTA TCACGAAGCT GGTTGGCGAT
1381 AAAGCCATGG GTCTGGATGG GCCTTCACAA GTTTTTGCCA TACAAAAACT AGAGGGAGTC
1441 ACGGAACTGT TGATTGGGAA TTTTGCAGGA GCAAACCCAA ATACTCATTT CTCTCTCTAT
1501 AGTAGATGGA TGGCAATTAA ATTGGATCAA GCAAAGAGCA TTAAAGTGCT CCGTGTTTTG
1561 TGTAAACCCC GCCCAGGTTT CAGTTTTTAT GGAAGAACCA GCTTCCCAGT CTAGGGTATC
1621 TGACTTTAAA AGACCCAGGT GTACATGTGT ACTGTATTAG TAATGTACGT TATTTGGTGT
1681 TGTAATCTGC TTTAACTTGT TTACTGCTTT AGTGTGTTTA TTTTCATGCT TTTAGTGGCG
1741 ACAGTGTGTT GTTTGT
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EU702446 Isolate D12

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 241 CCTACTTTTA AGATAGTTGT TAGGCTACCT GCAAATGCTT TCACTGGATT GACGTGGGTG
 301 ATGAGCTTTG ATGCTTATAA TCGGATAACT AGTAGAATTA CTGCTAGTGC AGATCCTGTA
 361 TACACCCTGT CAGTCCCTCA TTGGCTTATC CACCATAAGT TGGGCACGTT TTCATGCGAG
 421 ATAGACTATG GAGAATTGTG TGGCCACGCC ATGTGGTTTA AGTCCACAAC ATTTGAATCT
 481 CCTAGGTTAC ATTTCACGTG TTTAACGGGT AACAACAAAG AACTAGCGGC AGACTGGCAA
 541 GCTGTCGTAG AGTTGTATGC CGAGTTGGAA GAGGCCACCT CTTTTCTTGG AAAACCAACT
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 661 TTTTTTGATT TGACAGCCGT TACGGCTCTC AAGAGTGCTG GGCTGACATT GGGTCAGGTC
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 781 GGTATGGGAG GTACTATTAA AGGGGAGGTT CACATTTGTG CGCCAATCTT TTATAGTATT
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 961 CACCGAACGC CTGCTAGATT GCTTGCTGGT CAAAGTCAAA GGGATATGAG CTCCCTAAAC
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1201 CGTTTCTTTG ACTTCACTTC AAGCACTTGT AAGGTTGCTA TGGGAGAGAA CCCATTTGCT
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1321 TTAAATACCG AGTTCGGCAA AAGTAGCGGG AGCATCACTA TTACGAAGCT GGTAGGTGAT
1381 AAGGCCATGG GCCTGGATGG ACCGTCTCAA GTTTTTGCGA TACAAAGACT GGAGGGAACC
1441 ACCGATTTGT TGATTGGGAA TTTTGCAGGA GCAAACCCAA ACACCCATTT CTCCCTCTAC
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1561 TGTAAGCCTC GTCCAGGTTT CAGTTTTTAT GGAAGAACCA GTTTCCCAGT CTAGGGTATC
1621 TGACTTTAAA AGACCCATAT GTACGTGGTT ATTTCTTTTG TTTTGATTGT GTACATTGCT
1681 TTGTGTTATA ATTTGTTTTA ACTTGTTTAC TGCTTTAGTG TGTTTAATTT CATGCTTTTA
1741 GTGGCGACAG TGTGTTGTTT GT
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EU702447 Isolate Du

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 241 CCTATTTTCA AAATTGTTGT TAGGCTACCC GCTAACGCGT TTACTGGGCT TACATGGGTT
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 961 CACCGAACGC CTGCTAGGCT GCTTGCTGGT CAAAGCCAGA GGGATATGAG CTCCCTAAAC
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1741 GTGGCGACAG TGTGTTGTTT GT
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EU702448 Isolate V2

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 421 ATAGACTATG GAGAATTGTG TGGCCACGCT ATGTGGTTTA AGTCCACAAC ATTTGAATCT
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1741 GTGGCGGCAG TGTGTTGTTT GT
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EU702449 Isolate V1

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EU702450 Isolate D7

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 421 ATAGACTATG GAGAATTGTG TGGCCACGCT ATGTGGTTTA AGTCCACAAC ATTTGAATCT
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 541 GCTGTCGTAG AGTTGTATGC CGAGTTGGAA GAGGCCACCT CTTTTCTTGG AAAACCAACT
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 721 CCAATGGTTG GCACTACCAA GGTTTATAAT CTGAACAGTG CTCTTGTAAG CTGTGTTCTG
 781 GGTATGGGAG GTACTATTAA AGGGAAGGTC CACATTTGTG CGCCAATCTT TTATAGTATT
 841 GTTTTATGGG TTGTCAGCGA GTGGAACGGG ACCACTATGG ATTGGAATGA ACTTTTCAAA
 901 TATCCCGGAG TGTATGTGGA AGAAGACGGA AGCTTTGAAG TTAAAATCCG CTCTCCATAT
 961 CACCGAACGC CTGCTAGATT GCTTGCTGGT CAAAGTCAAA GGGATATGAG CTCCCTAAAC
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1741 GTGGCGACAG TGTGTTGTTT GT
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EU702451 Isolate W8

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1741 GTGGCGACAG TGTGTTGTTT GT
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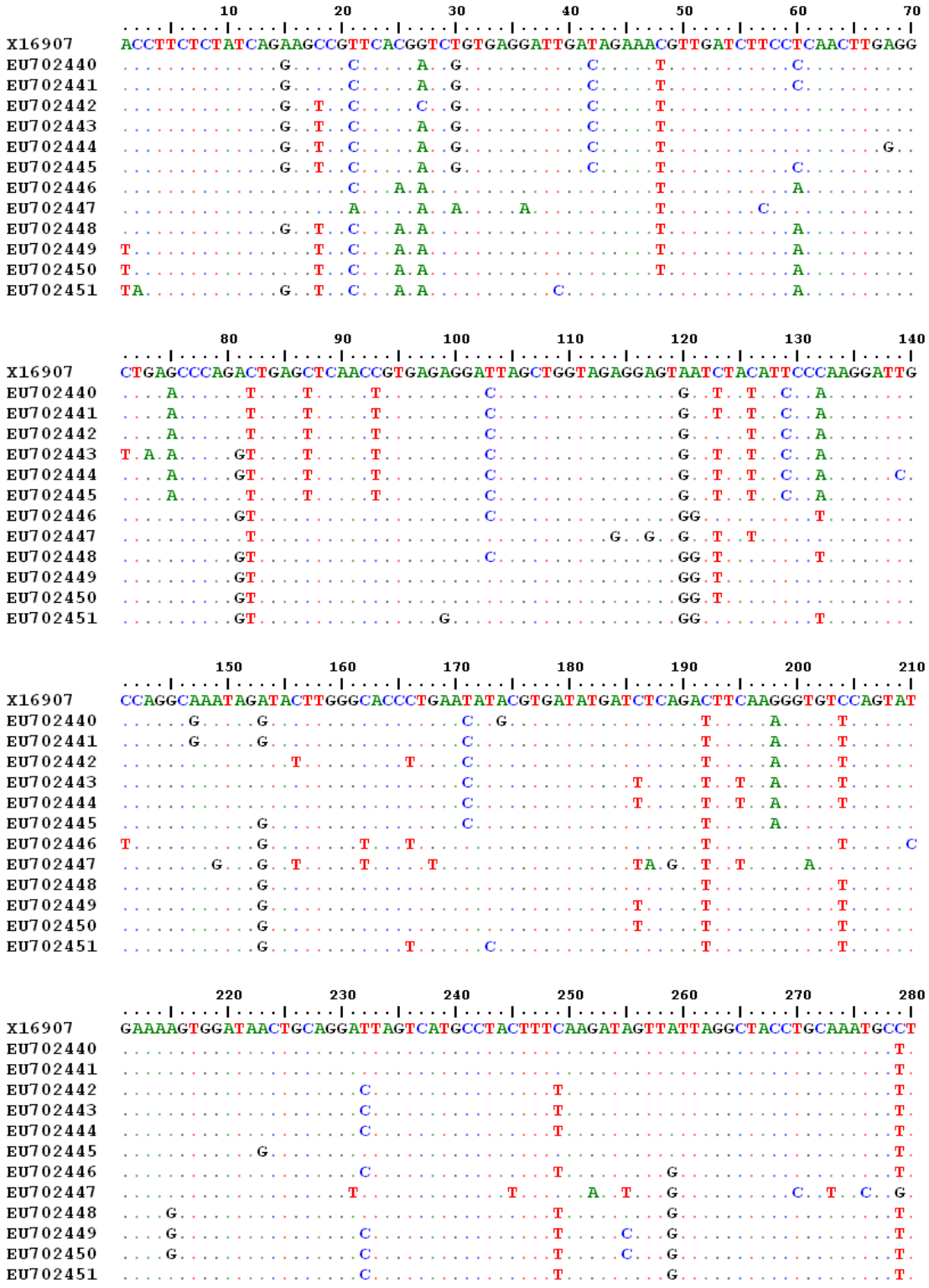
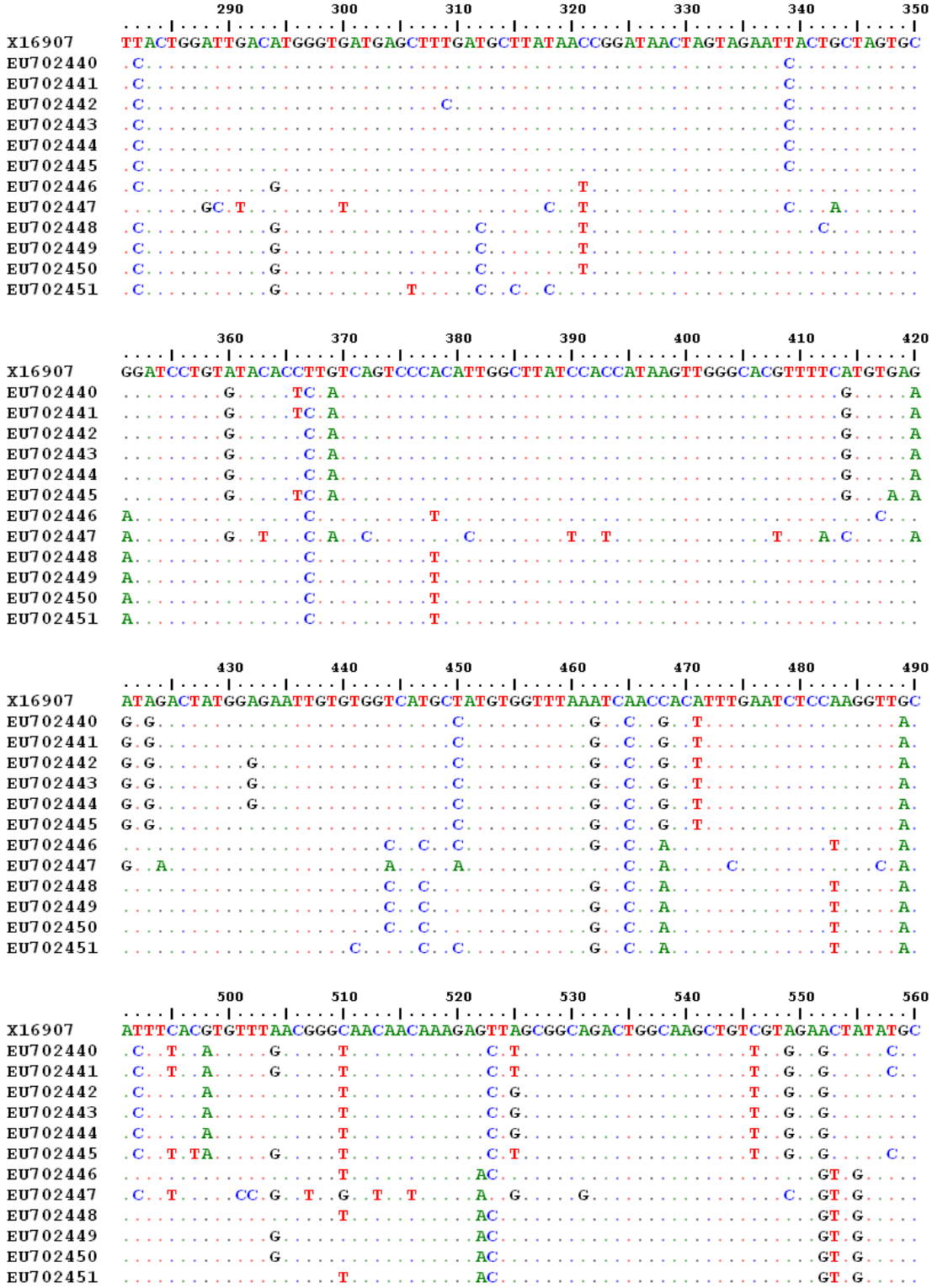
APPENDIX 3: Sequence alignment of the South African GFLV sequences with Isolate F13 (France)

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EU702449 .C.....G.....C.....T.....
EU702450 .C.....G.....C.....T.....
EU702451 .C.....G.....T....C..C..C.....
360 370 380 390 400 410 420
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EU702443 .....G....C.A.....G.....A
EU702444 .....G....C.A.....G.....A
EU702445 .....G....TC.A.....G...A.A
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EU702447 A.....G..T...C.A..C.....C.....T..T.....T...A.C.....A
EU702448 A.....C.....T.....
EU702449 A.....C.....T.....
EU702450 A.....C.....T.....
EU702451 A.....C.....T.....
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EU702441 G.G.....C.....G..C..G..T.....A.
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EU702443 G.G.....G.....C.....G..C..G..T.....A.
EU702444 G.G.....G.....C.....G..C..G..T.....A.
EU702445 G.G.....C.....G..C..G..T.....A.
EU702446 .....C..C..C.....G..C..A.....T.....A.
EU702447 G..A.....A.....A.....C..A.....C.....C.A.
EU702448 .....C..C.....G..C..A.....T.....A.
EU702449 .....C..C.....G..C..A.....T.....A.
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EU702451 .....C.....C..C.....G..C..A.....T.....A.
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EU702451 .....T.....AC.....GT.G.....


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EU702444
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EU702451
640 650 660 670 680 690 700
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EU702446
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EU702450
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EU702450
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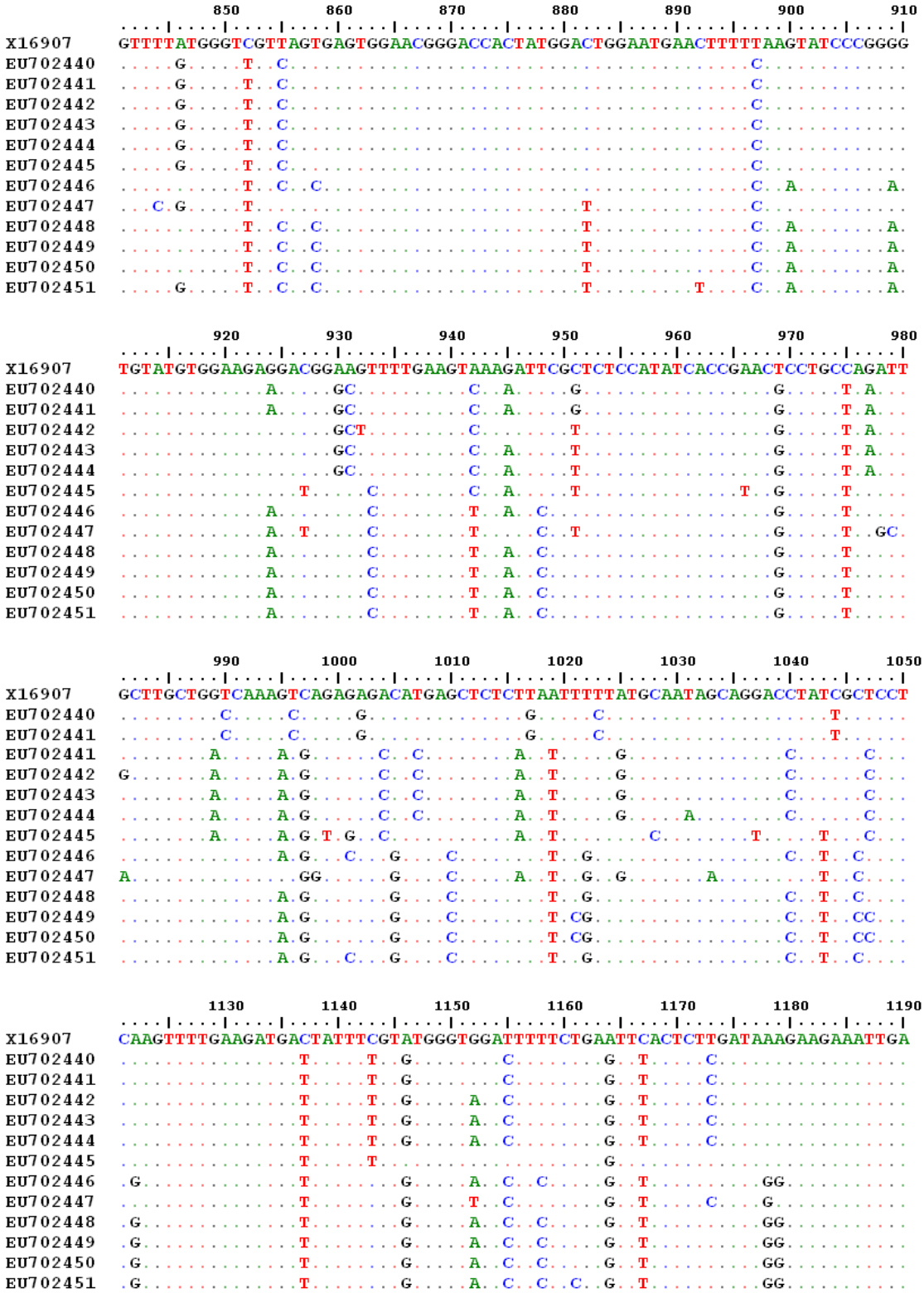
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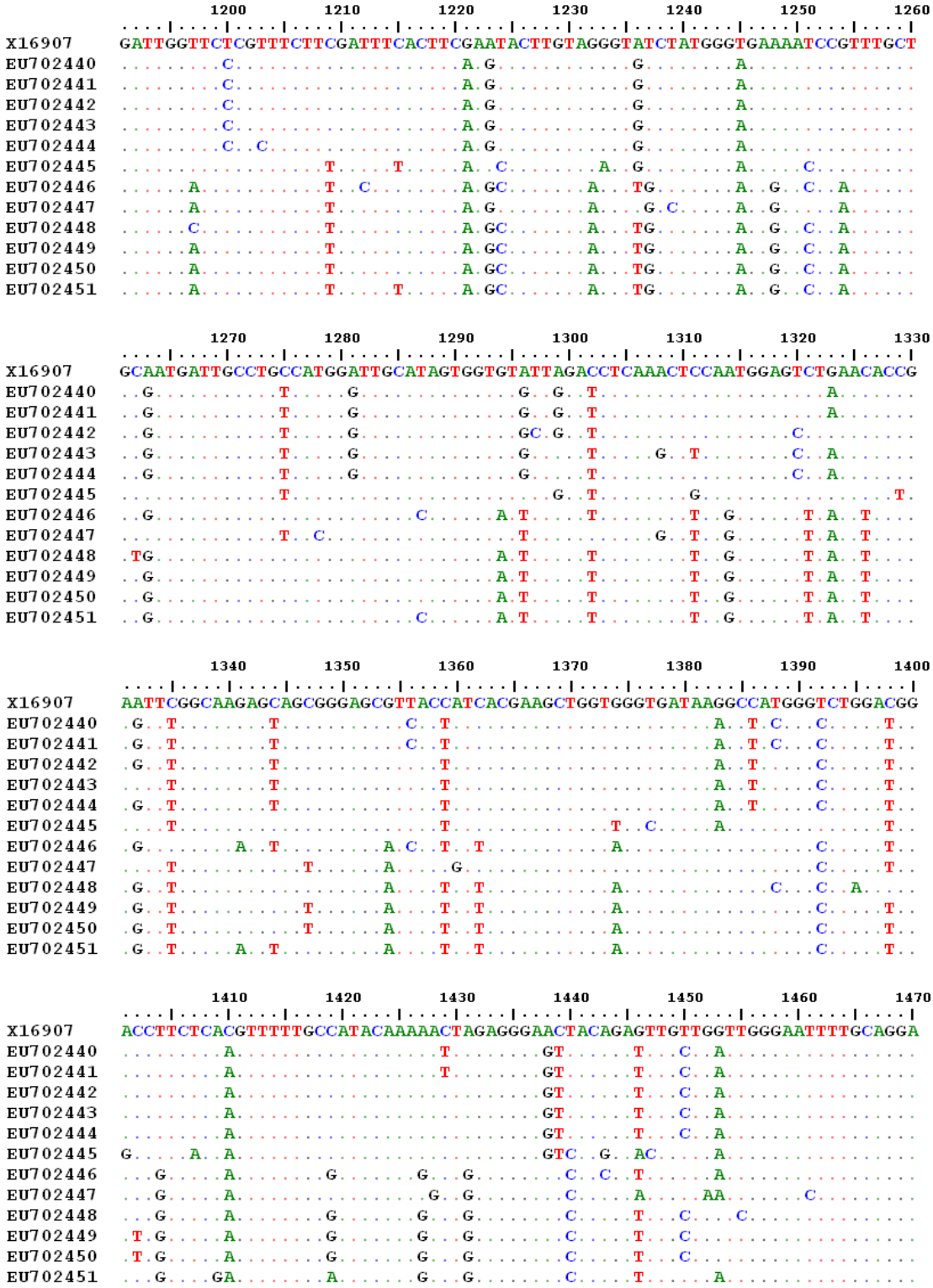
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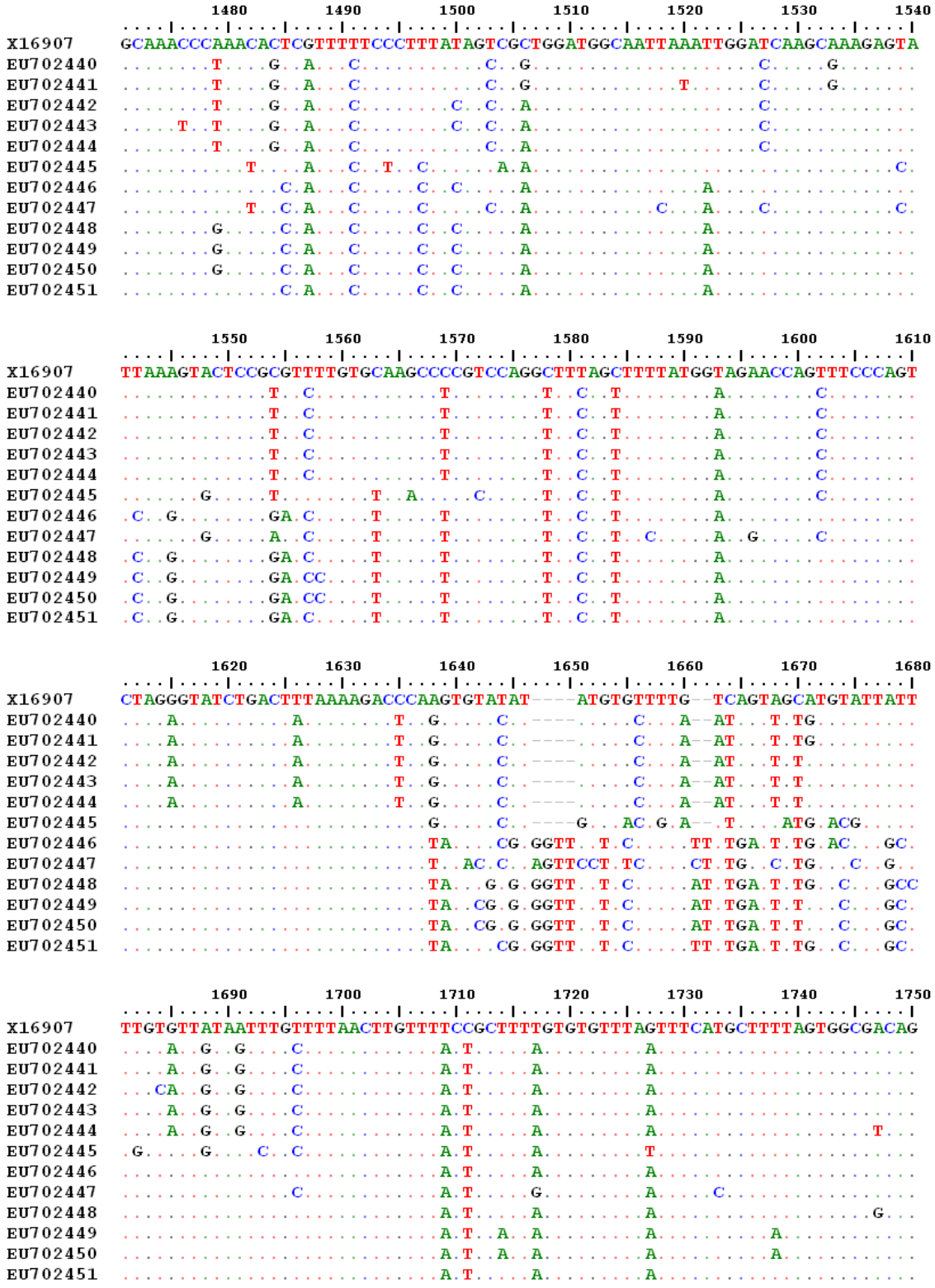
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EU702440 ....A..........A........T..G.....C..----.....C...A--AT...T.TG.........
EU702441 ....A..........A........T..G.....C..----.....C...A--AT...T.TG.........
EU702442 ....A..........A........T..G.....C..----.....C...A--AT...T.T..........
EU702443 ....A..........A........T..G.....C..----.....C...A--AT...T.T..........
EU702444 ....A..........A........T..G.....C..----.....C...A--AT...T.T..........
EU702445 ..............................G.....C..----G...AC.G.A---T.....ATG.ACG.....
EU702446 ..............................TA....CG.GGTT..T.C.....TT.TGA.T.TG.AC...GC.
EU702447 ..............................T..AC.C..AGTTCCT.TC....CT.TG..C.TG...C..G..
EU702448 ..............................TA...G.G.GGTT..T.C.....AT.TGA.T.TG..C...GCC
EU702449 ..............................TA..CG.G.GGTT..T.C.....AT.TGA.T.T...C...GC.
EU702450 ..............................TA..CG.G.GGTT..T.C.....AT.TGA.T.T...C...GC.
EU702451 ..............................TA....CG.GGTT..T.C.....TT.TGA.T.TG..C...GC.
1690 1700 1710 1720 1730 1740 1750
X16907 TTGTGTTATAATTTGTTTTAACTTGTTTTCCGCTTTTGTGTGTTTAGTTTCATGCTTTTAGTGGCGACAG
EU702440 ....A..G..G....C..........A.T.....A.........A.....................
EU702441 ....A..G..G....C..........A.T.....A.........A.....................
EU702442 ...CA..G..G....C..........A.T.....A.........A.....................
EU702443 ....A..G..G....C..........A.T.....A.........A.....................
EU702444 ....A..G..G....C..........A.T.....A.........A.....................T...
EU702445 .G.....G....C..C..........A.T.....A.........T.....................
EU702446 ..............................A.T.....A.........A.....................
EU702447 ................C...........A.T.....G.........A.....C...............
EU702448 ..............................A.T.....A.........A.....................G...
EU702449 ..............................A.T..A..A.........A..........A..........
EU702450 ..............................A.T..A..A.........A..........A..........
EU702451 ..............................A.T.....A.........A.....................

```
                1760
          ....|....|..
X16907    TGTGTTGTTTGT
EU702440  ............
EU702441  ............
EU702442  ............
EU702443  ............
EU702444  ............
EU702445  ............
EU702446  ............
EU702447  ............
EU702448  ............
EU702449  ............
EU702450  ............
EU702451  ............
```

APPENDIX 4: Buffer Composition

Buffer name	Buffer composition
Total RNA extraction buffer	3 M $NaClO_4$, 0.2 M Tris-HCl, pH 8.3, 5% (w/v) SDS, 8.5% (w/v) PVPP, 2% (w/v) PEG 6000, 1% (v/v) β-ME
TE buffer	10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.2% (v/v) β-ME
Grinding buffer	1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$ (pH 9.6), 2% (w/v) PVP 40, 0.2% (w/v) BSA, 0.05% (v/v) Tween 20, 1% (w/v) $Na_2S_2O_5$
TAE buffer	40 mM Tris, 0.114% (v\v) HOAc, 1 mM EDTA pH 8.0
GES buffer	100 mM glycine-NaOH pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X100
TEN50 Lysis buffer	20 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol, 0.2 mM DTT
TEN150 Lysis buffer	20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 5 mM DTT, 0.2% sarkosyl, 0.4% Triton X100
Buffer A	25 mM Tris, 5 mM $MgCl_2$, 5 mM β-ME
PBS-1	8.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 150 mM NaCl, pH 7.4
STE	10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 100 µg/ml PMSF
PBS-2	4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3
PBS-3	4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 300 mM NaCl, 2.7 mM KCl, pH 7.3
Elution buffer 2	75 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT, 0.1% (w/v) SDS
Glutathione reconstitution buffer	10, 25 or 50 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0
Resolving buffer	375 mM Tris-HCl, pH 8.8, 0.1% SDS
Stacking buffer	125 mM Tris-HCl, pH 6.8, 0.1% SDS
Protein loading buffer	100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT
Destaining buffer	(30% (v/v) methanol, 10% (v/v) HOAc
Electro-elution buffer	25 mM Tris-HCl, pH 8.5, 200 mM glycine
PreScission protease cleavage buffer	50 mM Tris-HCl, pH 7, 1 mM EDTA, 150 mM NaCl
Transfer Buffer	25 mM Tris, 192 mM glycine
Plasmid extraction solution 1	25 mM Tris-HCl pH 8, 500 mM Glucose, 100 mM EDTA
Plasmid extraction solution 2	200 mM NaOH, 1% (w/v) SDS
Plasmid extraction solution 3	3 M KOAc, 2 M HOAc, pH 4.8
TBS	20 mM Tris-HCl, 137 mM NaCl, pH 7.6
TBS-T	1 x TBS, 0.1% (v/v) Tween 20
SDS-PAGE running buffer	25 mM Tris-HCl, pH 8.3, 200 mM glycine, 0.1% SDS

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