

**THE DEVELOPMENT OF ENZYME-LINKED
IMMUNOSORBENT ASSAYS TO DETECT POTATO VIRUS Y
AND POTATO LEAF ROLL VIRUS USING RECOMBINANT
VIRAL COAT PROTEINS AS ANTIGENS**

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degree of Master of Science (Biochemistry)
at the University of Stellenbosch**



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Declaration

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

Signed:



Date:

Summary

Potato Virus Y (PVY) and Potato Leafroll Virus (PLRV) are two of the most destructive potato viruses capable of drastically diminishing crop yields by up to 80%. The presence of these viruses in planting material namely seed potato stocks are routinely diagnosed by enzyme-linked immunosorbent assay (ELISA) kits. The kits currently used by Potatoes South Africa are obtained from Europe. These kits have produced false positive and false negative results in the past. Potatoes South Africa required an ELISA that was reliable, cheap and specific for the detection of South African strains of the two respective viruses.

In this study the viral coat protein genes were amplified by RT-PCR from a South African source of infected plant material. The PVY and PLRV coat protein genes were subsequently cloned into pGEM-T Easy vector and sequenced. The sequences of the two viruses were aligned and compared to corresponding viral coat protein gene sequences obtained from Genbank. Subsequently the two amplified and cloned coat protein genes of PVY and PLRV were sub-cloned into an expression system (pET-14b) to induce and express the respective recombinant viral coat proteins. The induction of the cloned coat protein genes yielded successful production of the recombinant PVY coat protein but the induction and expression of the recombinant PLRV coat protein was unsuccessful.

The isolated recombinant PVY CP was then used to immunize a rabbit to produce highly specific anti-PVY CP immunoglobulins. The antiserum obtained from the rabbit was used to develop an ELISA to detect the presence of PVY in seed potato stocks in South Africa. The ELISA kit was subsequently used in preliminary trials to determine if the kit could detect PVY infected plant material. The initial results of the ELISA trials using PVY infected material obtained from Potatoes South Africa yielded positive results.

Opsomming

Aartappel Virus Y (PVY) en Aartappel Rolblad Virus (PLRV) is twee van die mees vernietigende aartappel virusse wat 'n oes tot 80% kan verlaag. Virus infeksie van plant materiaal teweete aartappelmoere word deur “enzyme-linked immunosorbent assay” (ELISA) toetsstelle bevestig. Die toetsstelle wat op die oomblik gebruik word deur Aartappels Suid-Afrika word in Europa vervaardig. Hierdie toetsstelle het vals positiewe en vals negatiewe resultate in die verlede gegee. Aartappels Suid-Afrika benodig toetsstelle wat betroubaar, goedkoop en spesifiek vir Suid-Afrikaanse virus stamme is.

In hierdie studie is besmette plantmateriaal vanuit Suid-Afrika gebruik vir die amplifisering van virale mantel proteïen gene met behulp van RT-PCR. Die PVY en PLRV mantel proteïen gene was daarna in die pGEM-T Easy vektor gekloneer en nukleotied volgordes is bepaal. Die nukleotied volgordes is met ander PVY en PLRV gene vanaf Genbank vergelyk. Die twee ge-amplifiseerde en gekloneerde mantel proteïen gene van PVY en PLRV is uitgesny en gekloneer in 'n ekspressie sisteem (pET-14b) om die mantel proteïen te produseer. Induksie van die gekloneerde mantel proteïen gene het gelei tot die suksesvolle produksie van 'n PVY mantel proteïen, maar produksie van die PLRV mantel proteïen was onsuksesvol.



Die geïsoleerde PVY mantel proteïen is vervolgens gebruik vir die immunisering van 'n konyn vir die produksie van konyn anti-PVY antiliggame. Die antiserum verkry vanaf die konyn is gebruik vir die ontwikkeling van 'n ELISA vir die identifisering van PVY infeksies in aartappelmoere. Voorlopige proewe is deurgevoer om te bepaal of hierdie ELISA PVY infeksies in plantmateriaal sou kon opspoor. Aanvanklike resultate toon dat die ELISA suksesvol PVY infeksies in plantmateriaal verkry vanaf Aartappels Suid-Afrika kan opspoor.

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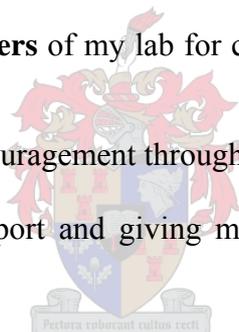
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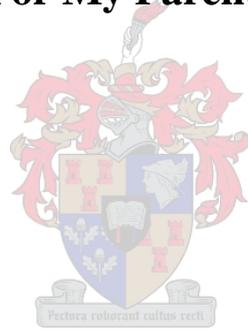
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For My Parents



ABBREVIATIONS

ABTS	2,2'-azino-bis-ethylbenzthiazoline-6-sulphonic acid
Anon	anonymous
AP	alkaline phosphatase
Abs	absorbance
bp	base pair
BSA	bovine serum albumin
Cbis	concentration N,N'-methylene-bis-acrylamide
cDNA	copied DNA
CP	Coat protein
CI	cylindrical inclusion
Da	Dalton
DAPSA	DNA and Protein Sequence Alignment
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
EDTA	ethylene diamine tetra-acetic acid di-sodium salt
EIA	enzyme immunoassays
ELISA	enzyme-linked immunosorbent assay
Fc	non-antigen binding fragment of immunoglobulin
h	hour
HC-Pro	helper component protease
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HC-Pro	helper component-protease
HRP	horseradish peroxidase
Ig	immunoglobulin
IPTG	isopropyl β -D-thiogalactopyranoside
kbp	kilo base pair
kDa	kilo Dalton
K_i	dissociation constant
K_m	Michaelis Menton
LB	Luria-Bertani
mA	milliampere
min	minutes
M_r	relative molecular mass
MCS	multiple cloning site
NB	naked bacteria
N1a	protein N1a

N1b	protein N1b
ORF	open reading frame
OD	optical density
OPD	O-phenylenediamine
ODia	O-dianisidine
PBS	phosphate buffered saline
PCR	polymerase Chain reaction
PLRV	Potato leafroll virus
PVP	polyvinylpyrrolidon
PVY	Potato Virus Y
P1	protein 1
P3	protein 3
RB	reaction Buffer
RdRp	ribosomal dependant RNA polymerase
R _f	relative mobility
RNA	ribonucleic acid
RT	room temperature
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	total concentration of acrylamide and N,N'-methylene-bis-acrylamide
TAE	Tris-base, glacial acetic acid, EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylendiamine
T _m	melting temperature
UV	ultra-violet
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-B-D-galactoside
5-AS	5-aminosalicylic acid
6K1	6 kilo Dalton protein 1
6K2	6 kilo Dalton protein 2

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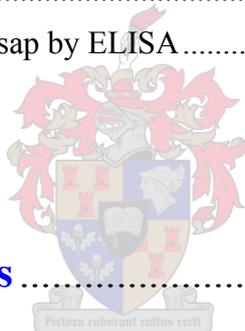
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Chapter 1

Introduction

1.1 Economic implications

Over the past ten years in South Africa there has been an average production of 1.578 million tons of potatoes, which is valued at R1.57 billion. The gross value of potato production is 43% of all major vegetables and accounts for 4% of the total agricultural production. It is therefore clear that potato production makes an important contribution to food production in South Africa. In order to maintain this production it is, however, of critical importance to control the diseases that can threaten the sustainability of potato production in South Africa.

Due to the fact that potato tubers are used as seed material in potato production, potato crops are vulnerable to a number of transmissible plant diseases. This can lead to a progressive build up of pathogens thereby making it almost impossible to reproduce from the same seed source for a long period of time. For this reason it is essential that disease free seed material is used, which can only be achieved by rigorous testing of seed material. In South Africa, potato farmers were originally represented by the government funded Potato Board prior to 1994. This organisation was subsequently privatised into the Potato Producers Organisation which since now been transformed into Potatoes SA. Potatoes SA plays a vital role in potato production in this country since one of the roles of Potatoes SA is to supply disease free seed material to seed potato growers (www.potatoes.co.za).

1.2 The certification of seed potato stocks

Although Potatoes SA as a body serving the South African potato producer, implements testing for diseases, the actual certification of seed potato tubers as disease free, is regulated by a statutory body, the Independent Certification Council for Seed Potatoes. Thus the South African potato certification scheme functions under the direct authority of the Independent Certification Council

for Seed Potatoes, which in turn has contracted the Potato Certification Service to manage the South African seed potato certification scheme. There are approximately 10000 hectares registered annually in South Africa alone for seed potato production and can be broken down to 400 separate registered seed growers which are continuously being supervised by the Potato Certification Service (www.potatoes.co.za).

These registered seed growers supply seed tuber planting material is subject to the requirements of and controlled by the Potato Certification Service to ensure that the farmer is provided with healthy planting material. The seed tubers are subjected to a number of tests to determine the presence of viruses and bacterial diseases before the crop is certified. The laboratories that perform the disease testing are registered with the Department of Agriculture and accredited by the Independent Certification Council for Seed Potatoes.

Disease free seed potatoes are initially produced *in vitro* and are labelled as generation 0 and each field reproduction will add a generation number to it. The number of field reproductions is always indicated on its certification labels. The certification of seed potatoes is allowed between generations 1-8 as consecutive generations are exposed to bacterial and viral infections, which increases the possibility of disease in the planting material. Within generations 1-8 the seed potatoes are sub-divided into three classes, Elite, Class 1 and Standard grade, which indicate the quality and infectious status of the tubers in its generation (www.potatoes.co.za).

When certifying seed potatoes the following points are taken into consideration: the *in vitro* multiplication of disease free seed potatoes and the production from it as generation 0, the identification of generations 1-8 and the dual phasing out of planting material. The first phase of the dual phasing out of planting material is the tracking of the potatoes generation, once past generation 8 the seed potatoes are phased out. The second phase of the scheme is to determine the quality of the potato and once its has been certified as Standard grade potato within generations 1-8 it may not be registered again for further production (www.potatoes.co.za).

All these procedures are implemented to ensure that the build up of disease in seed potatoes and the planting soils will be limited. The use of early generation planting material will also add to the minimizing of seed-born diseases that are present in seed potatoes. The practise of not allowing uncertified material to be planted in the same field as registered seed potatoes additionally ensures a decrease in the chance of infection for that crop.

The certification process involves a number of steps before the seed potatoes are certified. The crop must be registered 21 days after planting with two field inspections to determine disease infections and to sample the tubers for testing in a laboratory environment. Post-control samples are taken at the time of tuber inspection to confirm the quality of the seed potatoes (www.potatoes.co.za).

1.3 Potato disease testing in SA

There are at present five testing laboratories in South Africa that are situated in and around major seed growing areas. All the laboratories are registered with the South African Department of Agriculture and are run in accordance with the prescribed procedures and control actions. The role of the testing laboratories is to determine the presence and the level of infection of harmful diseases that are present in the seed potatoes. The laboratories test for a number of diseases namely for the viruses Potato virus Y (PVY), potato virus X (PVX), potato virus A (PVA), potato virus M (PVM), potato virus S (PVS), Potato leafroll virus (PLRV) and the tomato spotted wilt virus (TSWV). The tubers are also tested for *Ralstonia solanacearum* (bacterial wilt), and *Erwinia carotovora* (www.potatoes.co.za).

The viruses, PVY and PLRV are capable of drastically diminishing crop yields. These two viruses are routinely diagnosed by enzyme-linked immunoassay (ELISA) kits obtained from Europe. Due to the problems encountered with the use of these kits, Potatoes SA funded the development of local ELISA kits in this laboratory for the detection of these viruses, to overcome the problems that are encountered.

1.4 Objectives

The main objective of this study was to develop ELISAs to detect the presence of PVY and PLRV in seed potato stocks that do not give false positive or false negative results. False positives may be due to cross-reactivity of the antisera used in the assay. This may be caused by the co-isolation of plant proteins along with viral particles from infected plant material, which may have been used for immunisation, thereby producing antibodies against the virus as well as against plant proteins. The ELISAs should not give false negative results as well which may be caused by the lack of antibody specificity for the virus. This in turn, may be caused by mutations in the viral coat protein genes thereby avoiding detection by the antibodies.

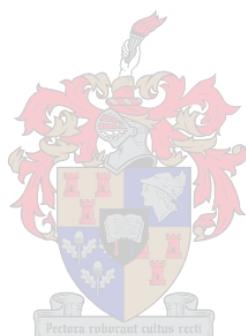
The objectives of the study were directed at circumventing these problems and thereby producing antibodies of the required specificity and the development of specific ELISAs to be used in the detection of SA strains of these viruses. The detailed objectives of this study are summarised below:

- a) To amplify, clone and sequence the viral coat protein (CP) genes of PVY and PLRV from South African infected material in order to avoid the possibility of false negatives.
- b) To induce and express the production of the recombinant viral CPs in *E. coli* preventing false positives by avoiding contamination by plant proteins.
- c) To isolate the viral CPs and subsequently to use them in the production of highly specific anti-viral CP antibodies.
- d) To use the antisera in the development of ELISA kits to be used to detect the presence of viruses in seed potato stocks.

In this thesis, an overview of the biology of PVY and PLRV is given in **Chapter 2**. The first objective to amplify, clone and sequence the viral CP genes of PVY and PLRV from South African infected potato leaves

(Sandveld) is addressed in **Chapter 3**. Virally contaminated South African potato material was used as a viral source to prevent the possibility of false negatives that ELISA kits obtained from suppliers outside South Africa might give due to mutations present in South African strains.

Attempts to express the recombinant PVY CP's cloned into *E. coli* are discussed in **Chapter 4**. In **Chapter 5** the production of the highly specific antibodies against the PVY CP is described. The final objective of developing an ELISA to detect PVY is presented in **Chapter 6**. The conclusions and results obtained of this study are discussed in **Chapter 7**.



Chapter 2

Review of Potato Virus Y (PVY) and Potato Leafroll Virus (PLRV)

2.1 Introduction

Potato virus Y (PVY) has been responsible for severe crop epidemics in the past as the virus spreads easily and can decrease a crop yield by up to 80% (Okamoto et al., 1996). The disease can therefore have a major economic impact on potato production.

Potato leaf roll virus (PLRV) along with PVY are considered to be the main cause of potato degeneration world wide and together are responsible for the drastic decreases in crop yield (de Souza-Dias et al., 1999). Plants that are infected with PLRV tend to have smaller tubers but a decrease in crop yield can depend on many different factors such as environmental conditions, variety and strain (de Bokx et al., 1972).

Both these plant viruses belong to a group of viruses, which have plus-sense RNA and among these various viruses four distinct super-groups are recognized. Until recently two super-groups namely the picorna-like and the alpha-like super-groups were recognized and now a further two, the sobemo-like and carmo-like super-groups have been added. PVY falls under the picorna-like super-group whereas PLRV is included under the sobemo-like super-group. The picorna viruses have a 5'-linked protein and encodes a single highly conserved polyprotein. The sobemo viruses also have a 5'-linked protein and have replicative domains. PVY belongs to the family *Potyviridae* and the genus *Potyvirus*. PLRV is included in the family *Luteoviridae* and the genus *Luteovirus*. The identification of a certain virus species is determined by a number of morphological properties, which include several characters and the extent of the relationships will decide the species demarcation. The characters that are used to distinguish between virus species

within the same genus are the genome sequence relatedness, natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, physico-chemical properties of virions and antigenic properties of viral proteins (Mandahar 1999).

2.2 Virology of PVY and PLRV

The PVY virus was first reported in *Solanum tuberosum* (potato) in 1931. Three main groups of PVY (PVY^O, PVY^N and PVY^C) have been characterised according to systemic symptoms and their severity. The great diversity in the strains of PVY is due to the lack of “proof reading” in the replication of RNA and the subsequent survival of the progeny (McDonald et al., 1996).

The PVY^O group (common strains), produce systemic symptoms of leaf crinkle, rugosity and leaf drop streak. The PVY^N group causes venal necrosis in tobacco plants and very mild mottling on potato cultivars. The PVY^C group causes stipple streak and can include mild mosaic on potato leaves (McDonald et al., 1996, de Bokx et al., 1972).

PLRV is a luteovirus and has a positive sense single stranded RNA, which is encapsulated by a small polyhedral viral coat protein about 23 nm in diameter. Symptoms can vary depending on strain, however, a general symptom affecting the leaves manifests in all strains. Plants that come from an infected tuber first show symptoms on the lower leaves, which gradually move upwards towards the top leaves. The infected leaves are rolled upwards and are brittle, the plants also become slightly yellow and stunted (Nolte et al., 2002).

When a healthy plant becomes infected with the viral pathogen the top leaves are the first to show symptoms of upward leaf rolling and take on a reddish appearance. The upward leaf rolling and reddish appearance are only visible much later in the season and the lower leaves of the plant usually take on a normal appearance but can vary from strain to strain (Johnson et al., 2002).

The tubers react to the virus by forming an internal necrosis known as net necrosis, which is visible once the tuber is cut open. The vascular tissue seems to be more susceptible to the virus than other tissues in the tuber. A PLRV infection also affects the stems and petioles where phloem necrosis is also a visible symptom (Johnson et al., 2002).

2.3 Hosts

PVY is a very common virus and is able to infect different crop species in the *Solanaceae* namely; potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*) and pepper (*Capsicum frutescens*).

2.4 Vectors

The most common manner in which PVY is spread in the field is by the peach aphid, *Myzus persicae*. Other aphids also responsible for the spread are: *Macrosiphum euphorbiae*, *Aphis fabae*, *A. frangulae*, *A. nasturtii*, *Cavariella pastinacae*, *Neomyzus circumflexus*, *Myzus cetus* and *Myzus ornatus* (de Bokx 1972).

The most efficient vector that transmits PLRV is the green peach aphid *Myzus persicae*. Other aphids that are able to transmit PLRV are: *M. ascalonicus*, *Neomyzus circumflexus*, *Aulacarthum solani*, *Macrosiphum euphorbiae* and *Aphis nasturtii* (de Bokx et al. 1972).

2.5 Transmission

PVY is a stylet borne virus and one of the proteins produced by PVY acts as a bridge connecting the virus to the stylet of the aphid whereby it is released into the next plant the aphid is feeding upon (Blanc et al. 1997). Once aphids have acquired the virus they are not carriers of the pathogen for life yet they can re-acquire the virus numerous times.

PLRV cannot be transmitted manually; the only mode of infection is by aphid transmission. The aphid first acquires the pathogen by feeding on an infected plant, after which the virus circulates from the gut into the circulatory system until it reaches the salivary glands from which it can be excreted into a healthy plant. Once an aphid acquires the virus it will remain infected for the rest of its life span. The process of aphid infection takes place within 24-28 hours and is known as a circulative virus (de Bokx et al. 1972).

2.6 The molecular biology of PVY and host interactions

The viral RNA of PVY is surrounded by a flexuous rod shaped coat protein which is 684 nm long and approximately 34 kDa. The virions contain one molecule of linear plus sense single stranded RNA. The 5'-end is covalently linked to a VPg protein and the 3'-end has a poly (A) tail (www.micro.msb.le.ac.uk). The flexuous rod shaped particles (Figure 2.2) consist of one protein which is the viral coat protein (CP) and a RNA molecule which has a VPg protein covalently bonded to the 5'-end and is polyadenylated at its 3'-end. PVY consists of a monopartite genome that is 10-11 kbp long depending upon the strain of the virus. The RNA molecule codes for a polyprotein between 3000-3400 amino acid residues in length, which is then cleaved by internal virus coded proteinases to release the individual viral products (Mandahar 1999).

The polyprotein produces 9 individual protein products after its cleaved by its internal proteinases. The products from the N-terminus to the C-terminus are: protein 1 (P1), helper component protease (HC-Pro), protein 3 (P3), 6 kilodalton protein 1 (6K1), cylindrical inclusion protein (CI), 6 kilodalton protein 2 (6K2), protein N1a (N1a), protein N1b (N1b) and the CP (Reichmann et al., 1992).

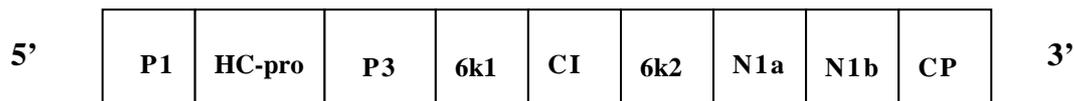


Figure 2.1 A representation of the PVY RNA molecule from its 5'-end to its 3'-end.

2.6.1 P1 Protein

P1 has two activities: the first being a protease activity and the second an activation activity. The *cis*-cleavage function cleaves the P1 and HC-Pro components from each other and is achieved by a chymotrypsin-like serine protease domain of P1 (Ryan et al., 1997, Verchot et al., 1995).

The P1 activation viral replication by binding to single stranded RNA suggests it might stimulate genome amplification in *trans* (Verchot et al., 1995).

2.6.2 HC-Pro Protein, P3 and 6K1

HC-Pro is a multifunctional protein. The C-terminus of HC-Pro has a papain-like cysteine protease function that cleaves the HC-Pro and P3 proteins (Ryan et al., 1997, Maia et al., 1996).

The second function of HC-Pro is its ability to aid aphid transmission of the virus. The helper component (HC) portion of the protein acts as a bridge between the virion and the stylet of the aphid and can be broken when the aphid feeds on another plant (Rojas et al., 1997, Blanc et al., 1997, Maia et al., 1996). The HC-Pro protein has a molecular mass of 58 kDa but its biological activity is correlated with a protein of about 100-150 kDa, which led to the assumption that the active form of HC is a homodimer (Rojas et al., 1997).

The central domain of the HC-Pro protein has two functions: the first being that it promotes genome amplification and the second that it assists in the

spread of the virus through the plant. The movement of PVY through the plant is the result of the ability of HC-Pro to modify the size exclusion limit of the plasmodesmata (Maia et al., 1996, Rojas et al., 1997, Kasschau et al., 1997, Carrington et al., 1996).

The functions of the third protein on the polyprotein P3 and the following protein 6K1 are still unclear but they do possess certain pathogenicity determinants of the potyvirus (Riechmann et al., 1992, Mandahar 1999).

2.6.3 CI and 6K2

The CI is needed for genome replication. It possesses a domain that functions as an ATPase-helicase and also exhibits an RNA helicase activity *in vitro* (Kadare et al., 1997, Riechmann et al., 1992). The CI is localized inside the plasmodesmata and is vital for cell to cell movement of the virus (Rodriguez-Cerezo et al., 1997).

The 6K2 protein has a central hydrophobic domain that associates with large vesicles derived from the endoplasmic reticulum. It has been postulated that the 6K2 targets replication complexes to the membranous sites of replication (Schaad et al. 1997).

2.6.4 N1a, N1b and Coat Protein

The last 3 proteins encoded by the single PVY polyprotein are the N1a, N1b and the CP. The N-terminal of the N1a protein codes for the VPg protein (24-27 kDa) that is covalently linked through a tyrosine residue to the 5'-end of the RNA molecule (Murphy et al. 1996). The VPg protein possibly interacts with the viral polymerase to initiate RNA synthesis. Molecule viral replication is abolished when there is a mutation in the tyrosine residue that binds VPg to the 5'-end of the RNA (Li et al. 1997, Murphy et al. 1996). The C-terminal region of N1a is a chymotrypsin-like cysteine protease that cleaves at several places on the polyprotein (Ryan et al., 1997).

The N1b protein possesses the vital function of the viral RNA-dependant RNA polymerase (RdRp). N1b is recruited by a specific interaction with N1a protease domain, coat protein and possibly VPg to act as the RNA polymerase for PVY (Li et al., 1997, Mandahar 1999).

Besides encapsulating the viral genomic RNA of the virus, the CP has two other functions. The N-terminus of the CP is a determinant of aphid transmission (Blanc et al. 1997) and mutations in the terminal regions showed a decrease in cell to cell movement (Carrington et al. 1996, Rojas et al. 1997), suggesting that the CP plays a vital role in virus transport. The viral RNA is embedded into the coat protein, which self assembles around the plus-sense RNA molecule and can be seen in Figure 2.2.

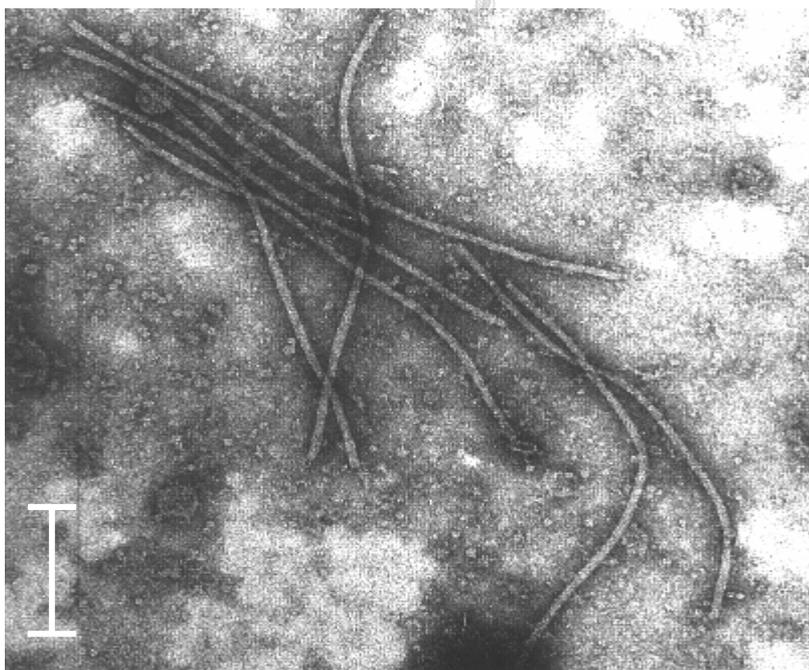


Figure 2.2 An electron micrograph of the PVY virus, each virus particle is approximately 684 nm in length (scale bar = 200 nm).

2.7 Molecular interactions of PLRV with its host

PLRV belongs to a super-group, which have a Vpg protein but do not have a polyadenylated tail at the 3'-end (Mandahar 1999).

The known luteoviruses evolved from a chance recombination of the luteovirus specific 3'-terminal with the sobemovirus-like 5' terminal. Luteoviruses of Subgroup 2 were included into the "Sobemo-like" supergroup as the genus *Polerovirus* (Mandahar 1999).

The genome of PLRV, which is covalently bonded to VPg at its 5' end, has seven open reading frames (ORF's). The genome is divided into two clusters, namely the 5' terminal replicative genes and the 3' terminal coat protein coding region (Rohde et al., 1994).

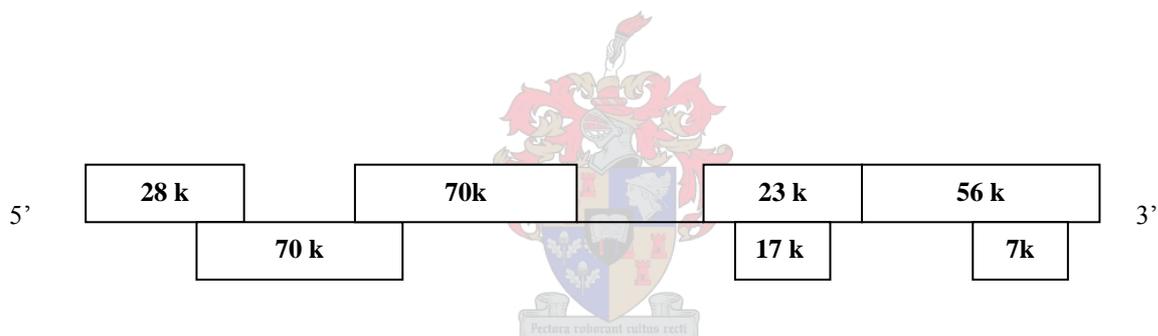


Figure 2.3 A graphic representation of the PLRV genome from its 5' end to its 3' end.

2.7.1 ORF's 0,1 and 2

ORF0 encodes a 28 kDa protein that is poorly conserved among all the poleroviruses and does not influence genome replication. The mechanism whereby the protein plays a role in determining the pathogenesis of the virus is not yet understood (van der Wilk et al., 1997).

ORF1 encodes a 70 kDa protein of which the N-terminus contains domains of highly hydrophobic amino acid residues homologous to those of the 6k2 protein of the PVY virus. Adjacent to the N-terminus is a chymotrypsin-like protease domain which catalyses the cleavage of the polyprotein. The C-

terminal of the 70 kDa protein encodes for the VPg protein (Ashoub et al., 1998, Mandahar 1999).

The ORF2 and ORF1 overlap and ORF2 are translated through a –1 frameshift as a polyprotein ORF1/ORF2. The expression of this fusion product produces the ribosomal dependant RNA polymerase (RdRp)(Ashoub et al., 1998).

2.7.2 ORF's 3 to 6

ORF3 encodes for the viral CP and suppression of its stop codon initiates a readthrough protein (ORF3/ORF5) that is a minor component of the viral capsid. Both these proteins do not assist in viral replication. The readthrough domain of the CP does, however play a role in aphid transmission by binding to an extracellular protein of the aphid (Rohde et al., 1994, Mandahar 1999). As with the PVY, the viral RNA of PLRV is embedded into the coat protein which self assembles to encapsulate the RNA (Figure 2.4).

ORF4 encodes for a movement protein (17 kDa) that functions in the cell to cell movement of the virus in its host. An additional ORF has been identified (ORF6) encoding for a protein of an unknown function (Ashoub et al., 1998).

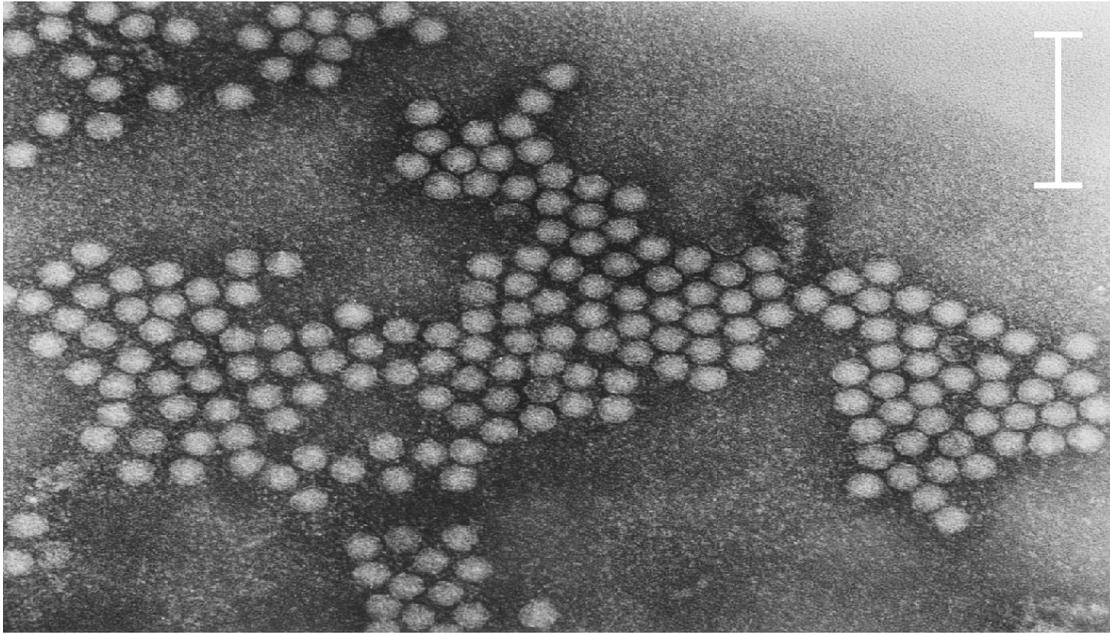
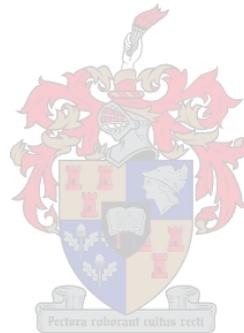


Figure 2.4 An electron micrograph of the PLRV virus. Each particle is approximately 23 nm in diameter (scale bar = 200 nm).



Chapter 3

The amplification and purification of the viral coat protein gene of PLRV and PVY

3.1 DNA amplification by polymerase chain reaction

DNA replication in a living cell is a highly complex procedure involving numerous proteins working together in completing the duplication of its genome. The polymerase chain reaction (PCR), that was first introduced by Saiki et al. (1985), uses the most basic of these enzymatic reactions to replicate this machinery *in vitro*. The PCR can replicate a small region of a specific target DNA template from a small amount to millions of copies within a matter of hours (McPherson and Moller, 2000).

The PCR uses a specific buffer system that copies the template DNA by using a DNA polymerase that incorporates deoxynucleotides into the new double stranded copied DNA (cDNA). The PCR buffer has a number of components such as deoxynucleotide triphosphates (dNTPs), oligonucleotide primers, a thermostable DNA polymerase, a buffer that contains $MgCl_2$ and the DNA template (Saiki, 1989). Vital to the buffer system is the inclusion of Mg^{2+} ions as they form a soluble complex with dNTPs, which is critical for their incorporation. The ions also increase the activity of DNA polymerase and raise the melting temperature (T_m) of the double stranded DNA and the primer template interaction (Newton and Graham 1997).

The original PCR amplifications used the Klenow fragment (DNA polymerase) which produced incomplete sequences as well as having a low optimum temperature of $37^\circ C$, which was easily inactivated thereby having to be replaced after every PCR cycle (Saiki et al. 1988). The discovery of a thermostable Taq DNA polymerase isolated from *Thermus aquaticus* allowed for the development of an automated PCR (Arnheim et al., 1992). Taq

polymerase has an optimum temperature of 72°C, thereby allowing for annealing and extension to take place at a higher temperature decreasing the chance of mismatch priming (Saiki, 1989).

The automation of the PCR relies on the use of heating and cooling to control the three different steps of the enzymatic reaction. The three steps of the PCR are denaturation, annealing and extension (McPherson and Moller, 2000).

Denaturation is usually achieved between 94-95°C. At this temperature the hydrogen bonds of the double stranded DNA start to break leaving single stranded DNA (McPherson and Moller, 2000).

In the following annealing step of the PCR cycle the temperature is lowered to the T_m of the specific oligonucleotide primers. The specific primers bind to the complementary sequence on the template DNA so that their 3'-hydroxyl ends face towards the target sequence to be amplified (McPherson and Moller, 2000).

In the final extension step of the cycle the temperature is increased once again to 72°C. At this temperature the DNA polymerase will bind to the primer-template complex and start incorporating free dNTPs from the buffer solution at the 3'-hydroxyl group of the specific primer. The extension along the template strand in the 3' direction will continue until the start of the next denaturing cycle (Arnheim et al., 1992).

The original DNA template along with new cDNA will now both be the template for the following PCR cycles thereby increasing the target DNA sequence exponentially (Arnheim et al., 1992). This process can be divided into three phases. The first phase is the screening phase where the primers search for complementary template DNA sequences in the early cycles of the PCR. Amplification is the second phase where the accumulation of target DNA increases exponentially as the previously synthesized products now become the preferred templates (Arnheim et al., 1992). The final plateau phase occurs in the late stages of the PCR when amplification becomes sub-

optimal. This occurs due to product accumulation and saturation of the enzyme active sites. The decrease in the primer-template ratio promotes self-annealing of the newly synthesized strands, which then blocks the primer from binding to the target sequence. This in turn starts the primers to bind to non-specific DNA, which is then amplified (McPherson and Moller, 2000).

3.2 Reverse Transcriptase PCR (RT-PCR)

Both PLRV and PVY are single stranded RNA viruses. The PCR cannot amplify RNA encoding the coat proteins of the respective viruses. The use of the single tube RT-PCR allows the use of viral RNA as the original template for amplification.

The RT-PCR is made possible by the introduction of reverse transcriptase, which is an RNA-dependant DNA polymerase. The reverse transcriptase, which is purified from *E. coli* containing the *pol* gene of Moloney Murine Leukemia virus (M-MuLV) synthesizes a complementary DNA strand using the viral RNA as the template. The RT-PCR and the following PCR can be carried out in the same tube to decrease contamination. In addition M-MuLV permits cDNA synthesis at higher temperatures preventing the formation of secondary structures in the RNA (McPherson and Moller, 2000).

3.3 Isolation of viral RNA from leaf samples

Leaf samples infected with PVY and PLRV used for the isolation of viral RNA were obtained from Potatoes SA. Leaf (0.1 g) samples were added to 2 ml grinding buffer, pH 9.6 (2% PVP 40, 0.2% BSA, 0.05% Tween 20) and shaken well.

The leaf extract, 4 μ l, was placed into a microcentrifuge tube containing 25 μ l of sterile 1 X GES (0.1 M Glycine-NaOH, pH 9, 50 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and incubated for 10 minutes at 95°C. The samples were subsequently placed on ice for 5 minutes. The samples were

stored at -80°C and 2 μl was used as template RNA in subsequent RT-PCR amplifications.

3.4 RT-PCR amplification of the viral coat protein gene

For the amplification of the viral coat protein gene of the PVY virus two oligonucleotide primers specific to the 5' and 3' ends of the gene encoding for the viral coat protein were designed. For future cloning purposes restriction enzyme sites included in the 5' and 3' ends of the primers to facilitate ligation into an expression vector. The forward primer (PVYf) contained a *Nco*I cleavage sites and the reverse primer (PVYr) contained a *Bam*H1 cleavage site. The PLRV forward primer (PLRVf) contained a *Nco*I cut site and the reverse primers (PLRVr) overhang contained a *Nde*I restriction enzyme site as shown in Table 3.1.

Table 3.1 The oligonucleotide primer sequences for PVY and PLRV. The red letters on the forward primer indicate the *Nco*I restriction enzyme site and on the reverse primer indicate the *Bam*H1 site

Primer and cut site	Sequence
PVYf/ <i>Nco</i> I	5'-GCACG CCATGG (CG) AAATGACACAAT(CT)GATGC-3'
PVYr/ <i>Bam</i> H1	5'-CTGG GGATCCT CACATGTT(CT)TTGACTC CAAG-3'
PLRVf/ <i>Nco</i> I	5'-GCACG CCATGG GTACGGTTCGTGGTTAAAGG-3'
PLRVr/ <i>Nde</i> I	5'-CTGCTG CCATATG CTATTTG GGGTTCTGCAAAGC-3'

The amplification reactions were carried out in a final volume of 25 μl (Table 3.2). All PCR amplifications were done in a Hybaid PCR Express Thermal Cycler. The RT-PCR conditions are shown in Table 3.2. The amplified DNA product was subsequently analysed by gel electrophoresis. The samples were added to a 0.1 volume of loading buffer (50% glycerol, 0.1% v/v bromophenol blue, 50 mM EDTA, 100 mM Tris-base, pH 8.0) and electrophoresed on a 1.4% agarose gel (Molecular grade agarose D1-LE, Whitehead Scientific) in 1 X TAE buffer (Tris-base, glacial acetic acid, 0.5 M EDTA, pH 8.0). For

ultraviolet detection of the DNA products 3 μl (0.175 $\mu\text{g}/\text{ml}$) of ethidium bromide was added to the gel (Figure 3.1, Figure 3.2).

Table 3.2 The RT-PCR mixture for the amplification of the PVY coat protein gene.

Volume (μl)	RT-PCR Components
12.5	Sterile Water
2.5	10x PCR Buffer
2.5	10x Sucrose/Cresol Red dye (20 % w/v sucrose, 1mM Cresol Red)
0.625	20 μM forward primer (PVYf)
0.625	20 μM reverse primer (PVYr)
1.25	0.1 M dithiothreitol (DTT)
0.75	50 mM MgCl_2
0.5	10 mM dNTPs
0.125	Superscript II (200 U/ μl)(Roche)
0.25	Taq DNA polymerase (5 U/ μl)(Bioline)
23 μl	Total Volume

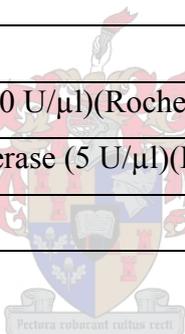


Table 3.3 The PCR conditions for the amplification of the PVY coat protein gene.

Temperature ($^{\circ}\text{C}$)	Time	Cycles
48 $^{\circ}\text{C}$	30 min	
94 $^{\circ}\text{C}$	30 sec	35 cycles
53 $^{\circ}\text{C}$	45 sec	
72 $^{\circ}\text{C}$	60 sec	
72 $^{\circ}\text{C}$ 4 $^{\circ}\text{C}$	7 min hold	

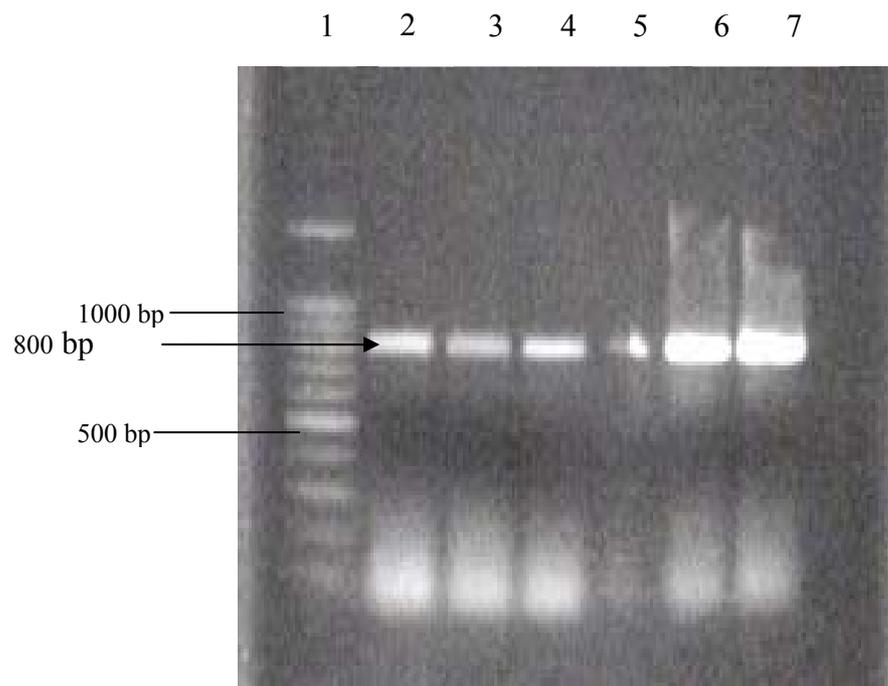


Figure 3.1 Analysis of the gel electrophoresis of PVY CP cDNA Lane 1 is DNA marker (Promega, 100 bp) and lanes 2-7 show amplification products of different plant sap samples infected with PVY.

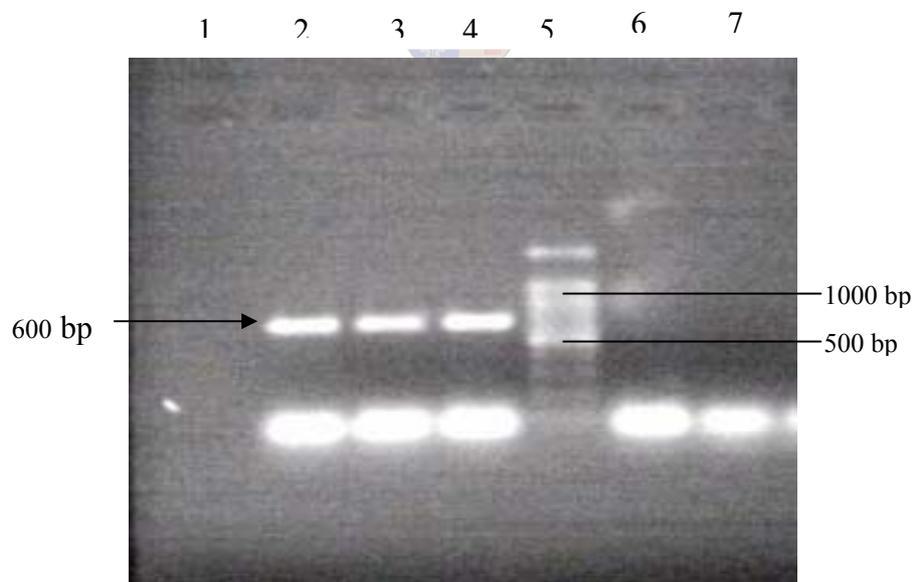


Figure 3.2 Analysis of the gel electrophoresis of the PLRV CP gene products from the RT-PCR, lanes 2-4: CP genes from PLRV containing samples; and lane 5: DNA marker (Promega 100 bp). Lane 6 and 7: unsuccessful RT-PCR reactions.

3.5 Cloning of the PCR products

Both cDNA CP PCR products of the PVY and PLRV viruses had to be confirmed by sequencing and compared to other strain sequences obtained from GenBank (www.ncbi.nlm.nih.gov/Genbank). A convenient vector for cloning and sequencing PCR products is pGEM-T Easy vector (Figure 4.3)(Promega). The plasmid vector has T7 and SP6 RNA polymerase promoter sequences on either side of a multiple cloning region within an α -peptide coding region coding for the enzyme β -galactosidase. The successful insertion of the PCR product inactivates the α -peptide coding sequence and colonies containing the insert are easily identified by colour screening on indicator plates. The pGem-T Easy vector is ligated most efficiently using a 1:1 ratio with a PCR product. The ligation was carried out according to the manufacturer's instructions as shown in Table 3.4.

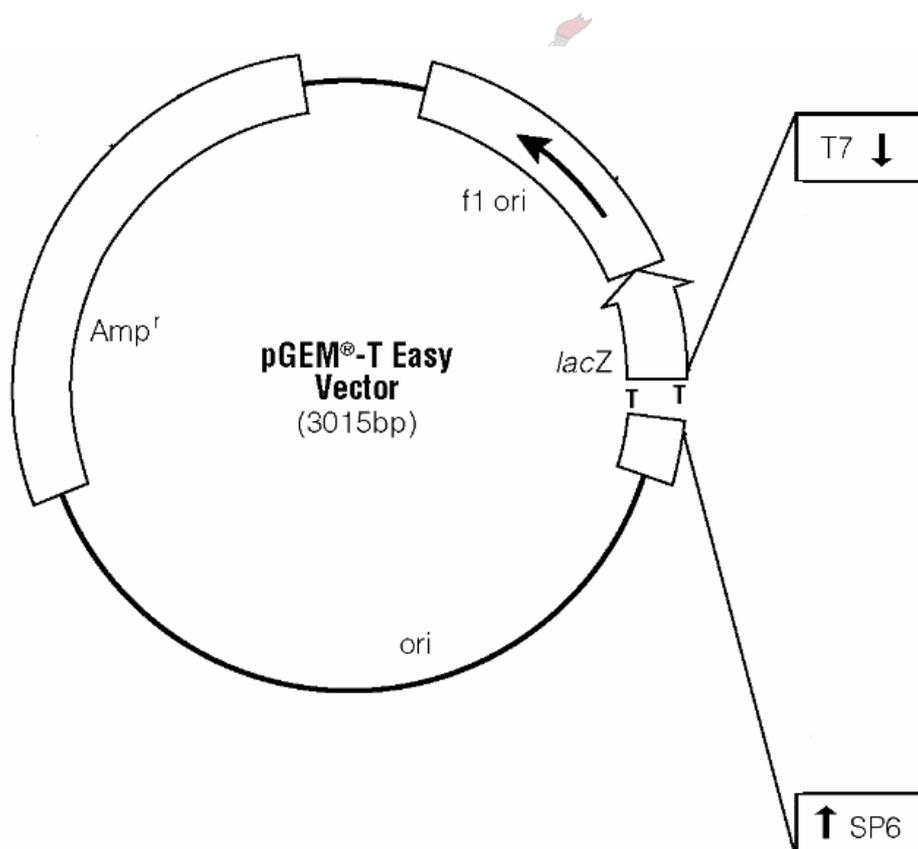


Figure 3.3 A diagram of the pGEM-T easy vector (Promega technical manual).

Table 3.4 The protocol for the standard ligation reaction of the PCR product into pGEM-T Easy vector.

Reaction components	Standard reaction	Positive control	Background control
2x rapid ligation buffer	5 μ l	5 μ l	5 μ l
PGEM-T Easy vector (50ng)	1 μ l	1 μ l	1 μ l
PCR product (1:1 molar ratio)	3 μ l	-	-
Control insert DNA	-	2 μ l	-
T4 DNA ligase (3 U/ μ l)	1 μ l	1 μ l	1 μ l
Deionized water to final Volume of	10 μ l	10 μ l	10 μ l

The reaction samples were incubated overnight at 4°C to obtain the maximum amount of ligated vector.

3.6 Transformation of *E. coli* with cloned PCR Products

For each ligation reaction two Luria-Bertani (LB) (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl, 11 deionized water, pH 7.0) plates (15 g agar/L of LB medium) with ampicillin (ampicillin 100 μ g/ml) were prepared. The plates were also coated with 20 μ l isopropyl β -D-thiogalactopyranoside (IPTG, 0.1 M) and 20 μ l of X-Gal (50 mg/ml, 100 mg 5-bromo-4-chloro-3-indolyl-B-D-galactoside, 2 ml N,N'-dimethyl-formamide, 2 ml dH₂O).

Two μ l of each ligation reaction was mixed with high efficiency competent *E. coli* cells (DH5 α) and placed in ice for 20 minutes, heat shocked for 50 seconds at 42°C and returned to ice for 2 minutes. LB medium (950 μ l) was added to each tube, which was incubated at 37°C for 1.5 hours with shaking at 150 rpm. A volume of 100 μ l of each transformation culture was plated out in duplicate onto the LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. A successful transformant will generally be a white colony due to the disruption in the coding sequence of the α peptide.

3.7 Diagnostic PCR

White colonies were subjected to direct PCR using T7 and SP6 promoter primers flanking the insert (Table 3.5), and a toothpick scrape of the colonies as template DNA (Table 3.6). The thermocycling conditions are given in Table 3.7. The products were electrophoresed on a 1% agarose gel to determine which colonies contained insert DNA (Figure 3.4).

Table 3.5 The primer sequences of the T7 and SP6 promoters.

Primer	Sequence
T7	5'-ATTATGCTGAGTGATATCCC -3'
SP6	5'-ATTTAGGTGACACTATAGAA -3'

Table 3.6 The PCR mix for colony diagnostic amplification.

Volume μ l	Components
6.9	Sterile Water
1	10x PCR Buffer
0.4	200 μ M dNTPs
0.5	20 μ M forward primer (PVYf)
0.5	20 μ M reverse primer (PVYr)
0.6	1.5 mM MgCl ₂
0.1	TAQ 0.05 U/ μ l (Bioline)
10 μ l	Final volume per tube

Table 3.7 The PCR cycle conditions for the colony diagnostic amplification.

Temperature (°C)	Time	Cycles
94°C	5 min	
94°C	30 sec	25 cycles
55°C	45 sec	
72°C	60 sec	
72°C 4°C	7 min hold	

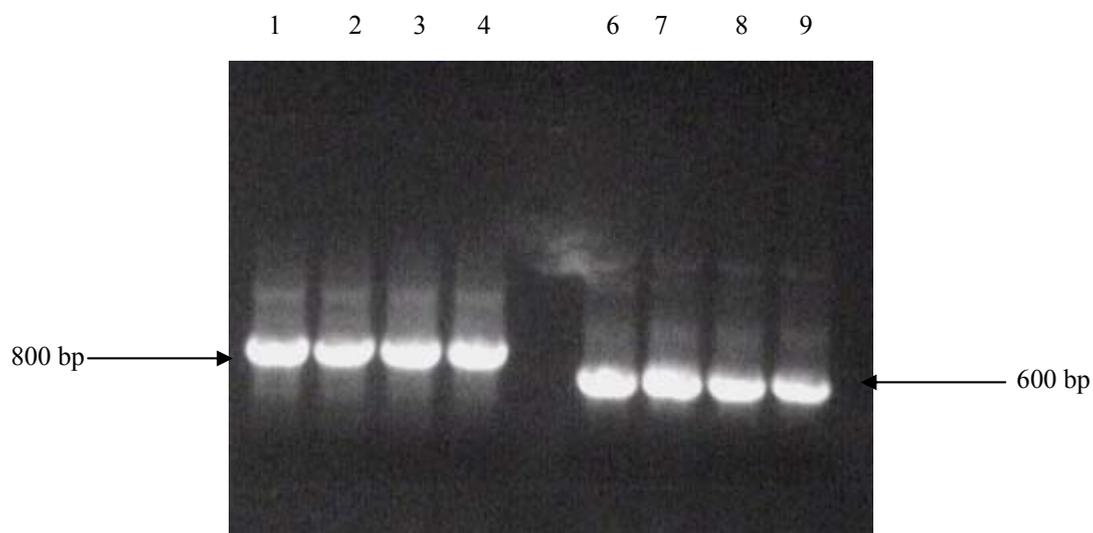


Figure 3.4 Analysis of the ligated inserts obtained by direct PCR. Lane 1: PVY CP RT-PCR product; lane 2-4: PVY DNA insert (pGEM/PVY); lane 6-8: PLRV DNA insert (pGEM/PLRV); lane 9: PLRV CP RT-PCR product. Band sizes were checked on a separate electropherogram.

3.8 Isolation of recombinant plasmid DNA

The recombinant colonies which contained insert DNA were inoculated in 5 ml of LB medium containing ampicillin (100 µg/ml) and incubated at 37°C in a shaking (160 rpm) incubator for 16 hours.

The plasmids were isolated using the Promega Wizard Plus SV Minipreps DNA purification system, according to the manufacturer's instructions. The

samples were electrophoresed against varying concentrations of DNA to determine the sample concentration (results not shown).

3.9 Sequencing of the transformed vector

The plasmid samples were concentrated on a Savant Speedvac to a concentration of 100 ng/ μ l. Plasmid DNA was sequenced using the ABI PRISM BigDyeTM Terminator v3.0 Sequencing Ready Reaction Kit (Table 3.8) with the T7 and SP6 primers. The subsequent PCR amplification was performed in a Hybaid PCR Express Thermal Cycler (Table 3.9).

Table 3.8 The sequencing reaction mixture of the plasmid preparations, pGEM-T Easy.

Volume (μ l)	Sequencing reaction components
2	Terminator Mix
1	Forward Primer T7 (0.8 μ mol/ μ l)
1	Reverse Primer SP6 (0.8 μ mol/ μ l)
5	5x Sequence dilution buffer
0.5	DNA sample (100 ng/ μ l)
1.5	Deionised water

The PCR products were analysed by an ABI PRISM 373 DNA Sequencer at the DNA sequencing facility of the University of Stellenbosch, Stellenbosch, South Africa.

Table 3.9 The PCR amplification conditions for the sequencing of the pGEM-T Easy vector.

Temperature	Time	Cycles
94°C	5 min	
94°C	30 sec	25 cycles
55°C	45 sec	
72°C	60 sec	
72°C 4°C	7 min hold	

3.9.1 Sequence analysis

The sequences obtained from the DNA sequencing facility were aligned and compared to other PVY and PLRV sequences obtained from GenBank using the DNA and Protein Sequence Alignment (DAPSA) program (Harley, 1998). The sequence alignment and comparison of the cloned PVY CP gene in pGEM-T Easy against other PVY strains is shown in Addendum 1. The cloned PLRV CP gene into pGEM-T Easy aligned with other PLRV strains is shown in Addendum 2.



The sequences that corresponded to the PVY and PLRV sequences from GenBank were subsequently used for the expression of the viral coat proteins as described in chapter 4. For simplicity, these will be referred to as PVY/pGEM and PLRV/pGEM in the rest of this thesis.

Chapter 4

Expression of the viral Coat Proteins for PVY and PLRV

4.1 Introduction

Once the cDNA was successfully cloned into the pGEM-T Easy vector the next step was to sub-clone it into an expression vector to obtain a recombinant viral coat protein. One of the most powerful expression vectors is the pET (Novagen) system, which utilises the T7 promoter to induce expression. Another advantage of the pET system is its wide range of vector types and host strains as no one strategy is infallible for the expression of recombinant proteins. The pET system also has a cloning site with a host of restriction enzyme cut sites, which allow for a wide range of ligation possibilities.

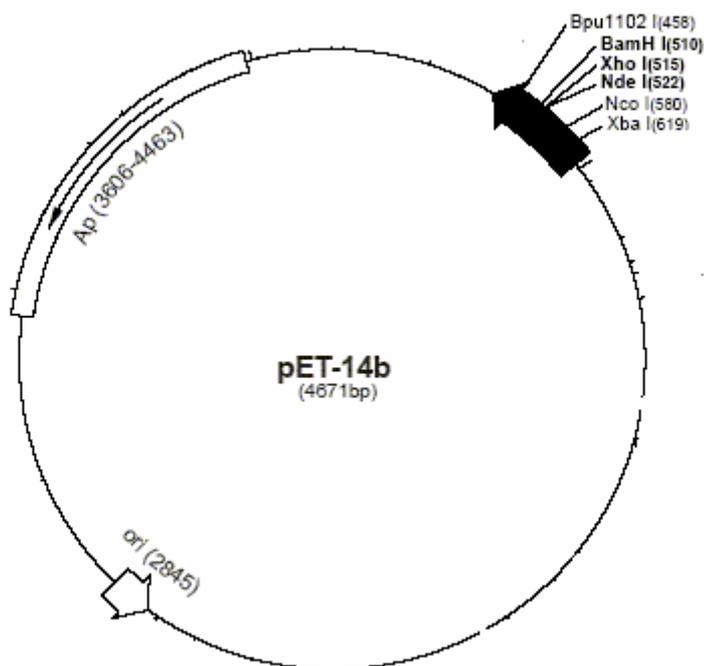


Figure 4.1 The Vector map of pET-14b (Novagen).

Basal transcription in pET is controlled by the T7 promoter which would in other uncontrolled instances slow the growth rate of the host *E. coli*. The *E. coli* RNA polymerase does not recognise the T7 promoter thereby crippling the expression step unless an alternative source of T7 RNA polymerase is produced. Once a successfully transformed plasmid is produced in a non-expression host it is then again transformed into a host strain that contains the T7 RNA polymerase gene (λ DE3 lysogen) for expression. The T7 RNA polymerase gene is under control of the lacUV5 promoter, which does express under the non-induced state but by using a host strain (pLysS) which contains plasmids that encode for T7 lysozyme that naturally inhibits the T7 RNA polymerase in non-induced cells, expression can be prevented. The final addition of IPTG induces the expression of T7 RNA polymerase thereby stimulating the subsequent expression of the target gene (Novagen).

4.2 Cloning viral CP in the pET expression plasmid

The target DNA cloned into the pGEM-T easy vector had to subsequently be cut out and ligated into pET-14b plasmid vector.

4.2.1 The digestion and purification of insert DNA

The vector and insert DNA was prepared by digesting the plasmid DNA with restriction enzymes. The pGEM/PVY and pET-14b vector was cut with *Nco*1 and *Bam*H1 restriction enzymes (Roche)(Table 4.1). The pGEM/PLRV and pET-14b vectors were both digested with *Nco*1 and *Nde*1 restriction enzymes (Roche) (Table 4.2).

The respective restriction enzyme mixtures were incubated for 1 hour at 37°C and then inactivated by incubation at 65°C for 15 min. The products electrophoresed on a 1% agarose (0.5 μ g/ml ethidium bromide) gel (Figure 4.2). The bands were visualised with a UV light source and the respective insert DNA and pET-14b vector were gel purified using the Wizard DNA isolation kit (Promega).

Table 4.1 Restriction digestion of pET-14b and pGEM/PVY with *Nco*I and *Bam*H1.

pET 14b Digest for PVY		pGEM/PVY	
15 μ l	pET-14b vector (3 μ g)	8 μ l	pGEM clone (100ng/ μ l)
3 μ l	10x buffer B	3 μ l	10x buffer B
1 μ l	<i>Bam</i> H1 (10 U)	1 μ l	<i>Bam</i> H1 (10 U)
1 μ l	<i>Nco</i> I (10 U)	1 μ l	<i>Nco</i> I (10 U)
1 μ l	Sterile Water	8 μ l	Sterile Water
20 μ l	Final Volume	20 μ l	Final Volume

Table 4.2 Restriction enzyme digestion of pET-14b and pGEM/PLRV clone with *Nco*I and *Nde*I.

pET-14b digest for PLRV		pGEM/PLRV	
15 μ l	pET-14b vector (3 μ g)	8 μ l	pGEM clone (100ng/ μ l)
3 μ l	10x buffer H	3 μ l	10x buffer H
1 μ l	<i>Nde</i> I (10 U)	1 μ l	<i>Nde</i> I (10 U)
1 μ l	<i>Nco</i> I (10 U)	1 μ l	<i>Nco</i> I (10 U)
1 μ l	Sterile Water	8 μ l	Sterile Water
20 μ l	Final Volume	20 μ l	Final Volume

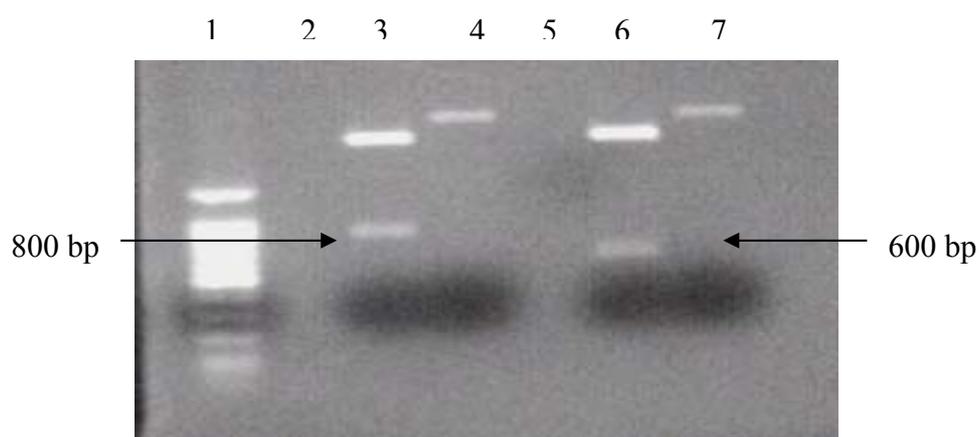


Figure 4.2 Analysis of the restriction enzyme digest products. Lane 1: DNA marker; Lane 3: pGEM/PVY; Lane 6: pGEM/PLRV, Lane 4: pET-14b digested vector for PVY; Lane 7: pET-14b digested vector for PLRV CP gene inserts.

4.2.2 The ligation into pET-14b vector

Ligation of the PVY (pEt/PVY) and PLRV (pET/PLRV) CP gene into pET-14b is a standard reaction that requires 50-100 ng of pET (determined by DNA concentration electrophoresis gel) vector mixed with 50 ng of insert. The ligation mixture (Table 4.3) was incubated overnight at 16°C.

Table 4.3 The standard protocol for the ligation of insert DNA into pET-14b.

Vol (µl)	Components
2 µl	10x Ligation buffer (200 mM Tris-HCl pH 7.6, 100 mM MgCl ₂ , 250 µg/ml acetylated BSA)
2 µl	DTT (100 mM)
1 µl	ATP (10 mM)
2 µl	pET-14b vector (50 ng/µl)
1 µl	T4 DNA ligase (0.4 U/µl)
6 µl	Target gene insert (0.2 pmol)
6 µl	Nuclease-free water

4.3 Transformation of pET/PVY and pET/PLRV into non-expression *E. coli* host

LB agar plates were prepared and coated with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). Competent cells (DH5α), 50 µl, were placed into 1.5 ml microcentrifuge tubes, 2 µl of each ligation reaction was added to the tubes and placed on ice for 20 min. The competent cells were heat shocked at 42°C for 50 sec and placed back on ice for 2 min. LB medium, 950 µl, containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml), was added to the cells and incubated for 1.5 hours at 37°C, 150 rpm. Each transformation, 100 µl, was spread onto the LB agar plates and incubated overnight at 37°C.

4.3.1 Screening of transformants

Single colonies were added to 5 ml LB medium containing the respective antibiotics and incubated overnight at 37°C in a shaking incubator, 150 rpm.

Plasmid DNA was isolated using the Promega Wizard Plus SV Minipreps DNA Purification System, according to the manufacturer's instructions.

The pET-14b cloning region is flanked by a T7 promoter and T7 terminator primer sequences (Table 4.4). Using these primers the isolated plasmid vectors were sequenced to identify positive clones and establish whether the CP sequence was in reading frame.

Table 4.4 The primer sequences for the T7 promoter and terminator regions.

Primer	Sequence
T7 promoter	5'-TAATACGACTCACTATAGGG -3'
T7 terminator	5'-GATCAATAACGAGTCGCCAC -3'

Sequencing reactions were prepared using as previously described. The sequences were aligned and compared to other PVY and PLRV sequences obtained on GenBank using the DNA and Protein Sequence Alignment (DAPSA) program (Harley, 1998). The sequence alignment and comparison of the cloned PVY CP gene in pET-14b against other PVY strains is shown in Addendum 3. The cloned PLRV CP gene into pET-14b aligned against other PLRV strains is shown in Addendum 4.

4.4 Transformation and expression of pET/PVY and pET/PLRV clones into the expression host, BL21 DE3 pLysS

For expression purposes positive clones were transformed into the BL21 DE3 pLysS host strain. Purified plasmid, 2 µl, was added to 50 µl competent cells and placed on ice for 20 min. The reaction mixture was heat shocked for 50 sec at 42°C and placed on ice for 2 min. LB medium, 950 µl, was added and incubated for 1.5 hours at 37°C, 150 rpm. The mixture, 100 µl, was plated out onto LB agar plates with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). The plates were then incubated overnight at 37°C. A single colony

was picked from the plate and added to 5 ml of LB medium with the respective antibiotics and incubated overnight at 37°C, 150 rpm.

The transformed cells, 100µl, were added to 42.5 ml induction mixture (TB, per litre: 12 g Bacto tryptone, 24 g yeast extract, 4 ml glycerol, 900 ml deionized water, autoclaved), 5 ml potassium phosphate buffer (per litre: 23.1 g KH_2PO_4 , 125.4 g K_2HPO_4 , autoclaved) and 2.5 ml of glucose (20% stock). The culture was incubated at 37°C, 150 rpm and grown to an optical density (OD) of $\text{OD}_{600} = 0.4-1$ and protein expression was induced with IPTG (final concentration of 0.4 mM).

Prior to induction, a 1 ml aliquot was taken and thereafter every hour for 4 hours. The samples were centrifuged in microcentrifuge tubes at 5000 x g for 5 min at 4°C, the supernatant discarded and the pellet resuspended in a 100 µl of sterile water. The same expression was done with a control sample of pET-14b vector in the expression host without insert DNA.

4.5 Analysis of expressed protein by SDS-PAGE

The cell samples were electrophoresed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine if the induction of the cells was successful. The samples electrophoresed with rainbow coloured protein molecular weight markers (Amersham, 14.3-220 kDa) to determine if the induced protein was of the correct size.

Cell samples, 20 µl, (1-4 hours and the control 2, 4 hours) were mixed with an equal volume of treatment buffer (0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% 2-mercapto-ethanol, pH 6.8) and 0.1% of the final volume of bromophenol blue (0.1% (w/v) in a 1.5 mM NaOH solution) and then boiled for 2 min in a water bath.

Electrophoresis was carried out on the cell samples in a 7 cm x 8 cm x 1.5 mm resolving gel (10% T, 2.7% Cbis, 0.1% SDS, 0.375 M Tris-HCl, pH 8.8) using a Hoefer SE 200 Mighty Small™ Vertical Gel Electrophoresis Unit.

The samples were added to a ten well stacking gel (4.5% T, 2.7% Cbis, 1% SDS, 0.125 M Tris-HCl, pH 8.3) that was placed in an electrode buffer (250 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3).

The rainbow coloured protein molecular weight markers (Amersham, 14.3-220 kDa) were used as the protein standards and were treated in the same way as the cell samples. The gel was stained overnight with Coomassie staining solution (0.025% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) methanol) and then placed in Destain 1 (50% (v/v) methanol, 10% (v/v) acetic acid) for 4 hours and then in Destain 2 (7% (v/v) acetic acid, 5% (v/v) methanol) for a further 4 hours (Figure 4.3). The molecular weight of the protein bands was calculated by comparing their relative mobilities (Rf-values) with those of the protein standards that were electrophoresed on the same gel.

The induction and expression of the PLRV CP was repeatedly performed but remained unsuccessful. The expression can be affected by many factors namely the type of expression host used and the insertion of the gene into the expression vector. Clearly the combination used here does not allow for the effective recombinant coat protein induction of PLRV. The use of different restriction enzyme cut sites and different expression hosts could lead to a successful expression of the PLRV CP.

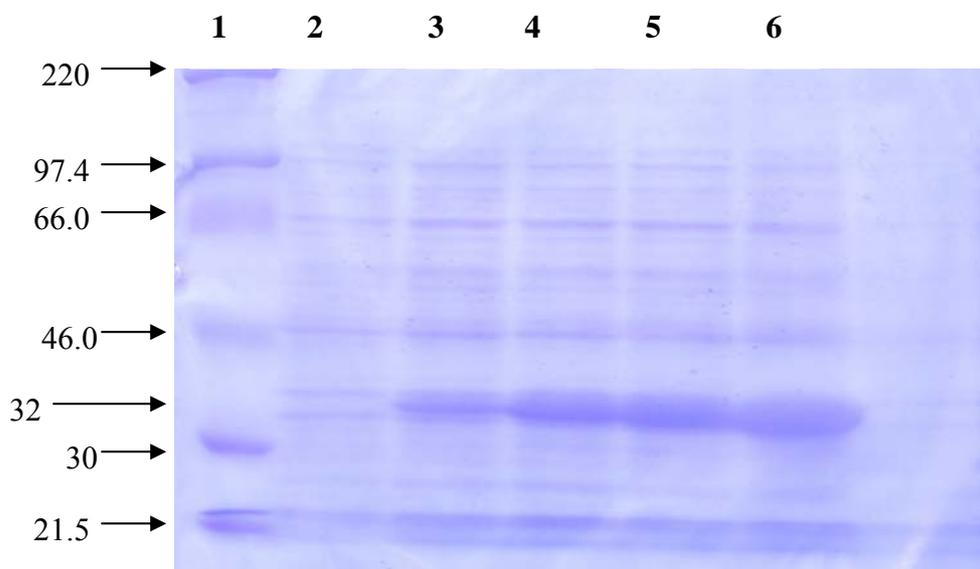


Figure 4.3 Analysis of the SDS-Page gel showing all the induced cell samples of pET/PVY. Lane 1: molecular protein markers; Lane 2: un-induced cell samples; lanes 3-6: 1-4 hour induced cell samples. The expressed recombinant protein which is possibly the PVY CP is present at 32 kDA.

The expression of the recombinant PVY CP was successful and the protein shown in Figure 4.3 was of the correct and expected molecular mass (32 kDA). Analysis shows an increase in CP expression over a 4 hour period. The viral coat protein of PVY present in Figure 4.3 was extracted from the SDS-PAGE gel and subsequently used for immunisation to produce specific antibodies against the PVY coat protein as described in chapter 5.

Chapter 5

The production of antibodies against heterologously expressed PVY CP

5.1 Isolation of recombinant PVY CP

The expressed protein was subsequently gel purified and isolated for immunisation purposes. To this end a preparative SDS-PAGE was performed. The first 2 wells were loaded with coloured rainbow (Amersham, 14.3-220 kDa) protein marker and a lysed cell sample (4th hour after induction), the third well was loaded with coloured protein marker and wells 4-10 contained induced cells (4th hour after induction). After electrophoresis, the first 2 lanes of the gel were removed, stained and aligned with the gel after destaining. Bands corresponding to the expressed protein were excised and placed in sterile water. The recombinant PVY CP was clearly visible on the stained portion of the gel as previously shown in figure 4.3 (Chapter 4).

The gel was cut up into fine slices and placed into a Biometra-Elucon (Omni-Science) apparatus containing running buffer (25 mM Tris, 192 mM glycerol, 0.1% SDS). The proteins were eluted at 200 V at 20 mA for 3 hours. Four, 100 µl, samples were removed and placed in microcentrifuge tubes at 4°C. The current was subsequently reversed and 100 µl samples were removed at two minute intervals for a total of 30 min.

5.2 Protein determination of Elucon fractions

The protein concentration was determined in each of the samples using the Bradford method (Bradford, 1976) that has been modified for use in microtitre plates. A standard solution of Bovine serum albumin (BSA, fraction V, 2 mg/ml) was used to prepare a standard dilution series (Table 5.1). Each standard and sample, 5 µl, was pipetted in duplicate into a microtitre plate and 250 µl of Bradford reagent (0.01% (w/v) Coomassie

Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid) was added and incubated for 2 min at room temperature.

The plate was placed into a Titertek Multiscan spectrophotometer and the absorbances were read at 620 nm. The BSA standards were used to determine a standard curve.

Table 5.1 The standard dilution series from a 2 mg/ml stock solution of BSA.

µg Protein	0	25	50	75	100	125	150
µl Buffer	100	87.5	75	62.5	50	37.5	25
µl BSA stock solution (2 mg/ml)	0	12.5	25	37.5	50	62.5	75

Table 5.2 The Absorbance results at 620 nm of the BSA dilution series.

Abs 620 nm	Concentration mg/ml
0	0
0.047	0.25
0.109	0.5
0.158	0.75
0.217	1
0.262	1.25
0.305	1.5

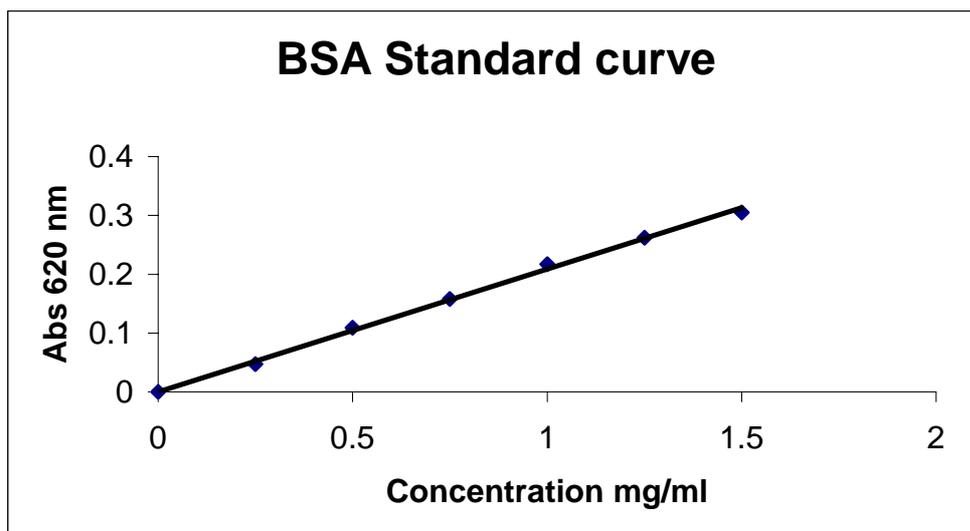


Figure 5.1 The Standard curve obtained from the dilution series with BSA.

Table 5.3 The concentration of the separate PVY coat protein isolation fractions.

Sample	Abs 620nm	Concentration mg/ml
1	0.016	0.0766224
2	0.113	0.5411457
3	0.214	1.0248246
4	0.026	0.1245114
5	0.021	0.1005669
6	0.27	1.293003
7	0.104	0.4980456
8	0.165	0.7901685
9	0.095	0.4549455
10	0.064	0.3064896
11	0.106	0.5076234
12	0.06	0.287334

The proteins were precipitated with saturated $(\text{NH}_4)_2\text{SO}_4$ (50% (v/v)) and incubated at 4°C overnight. The samples were centrifuged at 13 000 x g for 40 min, the supernatant discarded and the pellet resuspended in sterile water. A protein determination was performed on the samples to determine the final concentration of the protein and stored at 4°C for later use.

Table 5.4 Total amount of PVY coat protein in separate fractions.

Sample	Amount of protein mg	µg of protein (150 µl)
1	0.011493	11.49
2	0.081172	81.17
3	0.153724	153.72
4	0.018677	18.67
5	0.015085	15.08
6	0.019395	193.95
7	0.074707	74.7
8	0.118525	118.52
9	0.068242	68.24
10	0.045973	45.97
11	0.076144	76.14
12	0.0431	43.1
		Total Amount: 900.7921

5.3 Raising antibodies to the recombinant PVY CP in rabbits

For immunisation purposes the protein was firstly adsorbed to naked bacteria. *Salmonella minnesota* R595 bacteria cells were treated with mild acid hydrolysis, stripping the cells of their antigenic determinants (Bellstedt et al., 1986). The expressed protein can be adsorbed to the surface of the naked bacteria producing a complex to be used in the immunisation of rabbits. Small quantities of protein are required for this method of immunisation, which is advantageous when small amounts of protein are available (Bellstedt et al., 1986).

The optimal ratio by dry mass of protein to naked bacteria is 1 to 5. A standard 2 mg/ml solution of naked bacteria was prepared in sterile water for use in the adsorption to the antigenic PVY coat protein. Protein, 120 µg, was added to 250 µl of naked bacteria solution and homogenized with a loosely fitting Teflon plunger. The mixture was subsequently dried by rotary

evaporation on a Speedvac Savant and resuspended in PBS to the desired final concentration (40 μg of PVY coat protein adsorbed to 200 μg naked bacteria /0.5 ml PBS). Rabbits were immunised with the expressed protein as shown in Table 5.5.

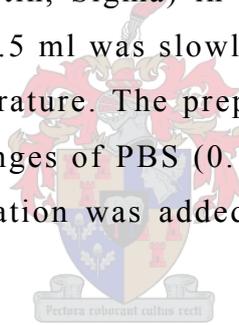
Table 5.5 The immunisation schedule followed to produce rabbit anti PVY coat protein antibodies.

Day	Dose of naked bacteria adsorbed to PVY coat protein	Volume of blood serum
1	240 μg	2 ml
4	240 μg	
7	240 μg	
12	240 μg	
15	240 μg	
18	240 μg	10 ml
28	240 μg	
42	240 μg	
46	240 μg	
49	240 μg	
56	240 μg	22 ml
84	240 μg	
91	240 μg	
98	240 μg	
105	240 μg	22 ml
127	240 μg	
130	240 μg	
134	240 μg	
141	240 μg	22 ml

5.4 Biotinylation of rabbit anti-PVY CP antibodies

A 1 ml sample of day 105 antiserum was added to 2 ml PBS and 3 ml saturated ammonium sulphate to precipitate the Ig fraction. The sample was incubated for 20 min at 4°C and centrifuged for 20 min at 15000 rpm. The pellet was redissolved in 2 ml PBS and a further 2 ml of saturated ammonium sulphate was added and incubated for 20 min at 4°C. The mixture was centrifuged for 20 min at 15000 rpm and the remaining pellet was redissolved to its original volume (1 ml) in PBS. The Ig fraction was dialyzed at 4°C overnight against two changes of carbonate buffer (0.1 M, pH 8.3).

The Ig concentration was determined by absorption at 280 nm. Carbonate buffer was added to the Ig fraction to obtain a 5 mg/ml concentration of rabbit anti-PVY CP antibodies. A standard solution of biotinamidocaproate N-hydroxysuccinimide ester (Biotin, Sigma) in N,N-dimethylformamide (DMF) (2 mg/ml) was prepared and 2.5 ml was slowly added to the Ig fraction while stirring for 2 h at room temperature. The prepared conjugate was dialyzed at 4°C overnight against two changes of PBS (0.15 M, pH 7.2). The biotinylated rabbit anti-PVY CP Ig preparation was added to glycerol in a 1:1 ratio and stored at -20°C.



5.5 Anti-PVY titre determination by ELISA

A 96 well microtitre plate (Nunc, Maxisorp with certificate) was coated with PVY coat protein (2 µg/ml, 100 µl/well) in carbonate buffer (50 mM, pH 9.6) overnight at 4°C. The PVY coat protein solution was decanted and the plate was blocked with Casein buffer (0.5% casein, 0.15 M NaCl, 0.01 M Tris, 0.02% thiomersal, pH 7.6, 200 µl/well) for 1 h at 37°C.

The rabbit sera from days 0, 18, 56 and 105 were prediluted 1:20 in casein-Tween (casein buffer mixed with 0.1% Tween 20) and a series dilution was made in casein-Tween (total, 100 µl/well) and incubated for 1 h at 37°C. The serum dilutions were then decanted and the wells were washed three times with PBS-Tween (PBS buffer with 0.1% Tween 20).

Goat anti-rabbit antibody was diluted (1:400) with casein-Tween and added to the wells (100 μ l/well). The plate was incubated for 1 h at 37°C after which the contents were decanted and the wells washed three times with PBS-Tween.

Rabbit peroxidase-anti-peroxidase (PAP, Sigma) was diluted (1:500) with casein-Tween, added to the plate (100 μ l/well) and incubated for 1 h at 37°C. After decanting the contents, the wells are washed three times with PBS-Tween and the substrate solution was added (0.05% 2,2'-Azino-bis[3-ethylbenzthiazoline-6-sulfonic acid](ABTS), 0.015% H₂O₂ in 0.1 M Citrate buffer, pH 5, 100 μ l/well) and incubated at room temperature for 10 min. The absorbance was measured at 405 nm on a Titertek Multiscan spectrophotometer.

The titre of the rabbit anti-PVY coat protein immunoglobulins was expressed as the dilution that has an absorbance value of 0.1 in the ELISA (Bellstedt et al., 1987). These titre values plotted against the days blood was collected, was used to assess the immune response over time (Figure 5.2). The rabbit anti-PVY coat protein immunoglobulins were used for the development of an ELISA to detect the presence of PVY in seed potato stocks as described in chapter 6.

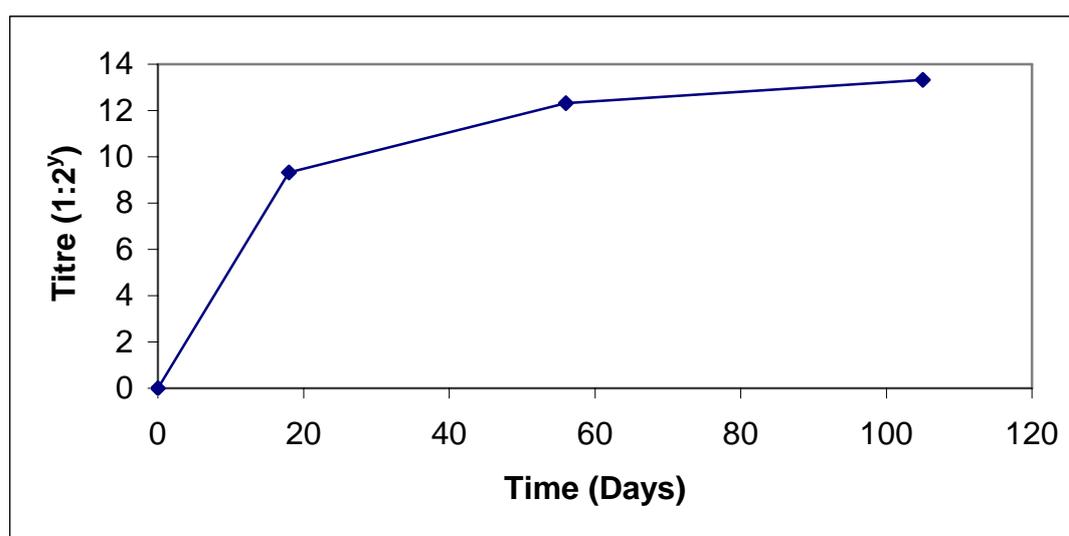


Figure 5.2 The immune response of the rabbit immunized with PVY CP adsorbed to naked bacteria.

5.6 Western blot analysis of rabbit anti-PVY CP antibodies

The induced pET/PVY cell samples (4th hour, for cell sample preparation see section 4.5) and rainbow coloured protein molecular weight markers were electrophoresed on a 10% SDS-PAGE gel as previously described. After electrophoresis the SDS-gel was placed onto blotting paper, which was subsequently covered with a nitrocellulose membrane (0.45 μm , Schleicher and Schuell) slightly larger than the gel, making sure of uniform contact. The gel and the membrane were sandwiched between blotting paper and scouring pads (Scotch Brite). Perspex plates were placed on either side of the sandwiched gel and membrane and inserted into a transfer chamber filled with transfer buffer (0.05 M Tris, 0.2 M glycine and 20% v/v methanol, pH 8.3). The SDS-gel was placed closer to the cathode and the membrane closer to the anode to electrophoretically transfer the proteins to the membrane (16 h, 120 mA at a constant current).

After 16 h the transfer was stopped and the membrane was removed and placed in casein buffer for 20 min (0.5% casein, 0.15 M NaCl, 0.01 M Tris, 0.02% thiomersal, pH 7.6) to prevent non-specific binding of proteins to the membrane. The membrane was subsequently incubated with rabbit anti-PVY CP antibodies, day 56, diluted 1:2000 with casein buffer and incubated for 1 h at 37°C. The membrane was washed three times (5 min each wash) in PBS-Tween (PBS and 0.1% Tween 20). The membrane was washed as previously described after each step. The membrane was placed into a solution of sheep anti-rabbit antibodies diluted 1:500 in casein buffer and incubated for 1 h at 37°C. Next the membrane was incubated (1 h, 37°C) in rabbit-PAP which was diluted 1:5000 in casein buffer. The final step of the western blot was the addition of substrate solution consisting of 4-chloro-1-naphtol (18 ng) dissolved in cold methanol (6 ml), which was added to 30 ml PBS (pH 7.2) containing 9 μl H₂O₂ (34%). The reaction was stopped after 30 min by washing the membrane with distilled water and drying it with tissue. The membrane was covered in foil and stored at 4°C. The band of expressed PVY CP (32 kDA) is clearly visible on the western blot nitrocellulose membrane (Figure 5.3), which indicates the recognition of the rabbit anti-PVY

antibodies for the viral CP. Bands of higher molecular weight proteins are also visible on the western blot membrane. These bands are most likely to be the result of antibodies produced against protein components of the NB, which were used as carriers for immunisation. As NB are prepared from Gram-negative *Salmonella minnesota*, which are closely related to *E. coli*, these antibodies in all likelihood also recognise *E. coli* proteins. These antibodies do not, however, pose a problem in the recognition of PVY in plant material as such bacteria do not occur in the plant material to be tested.

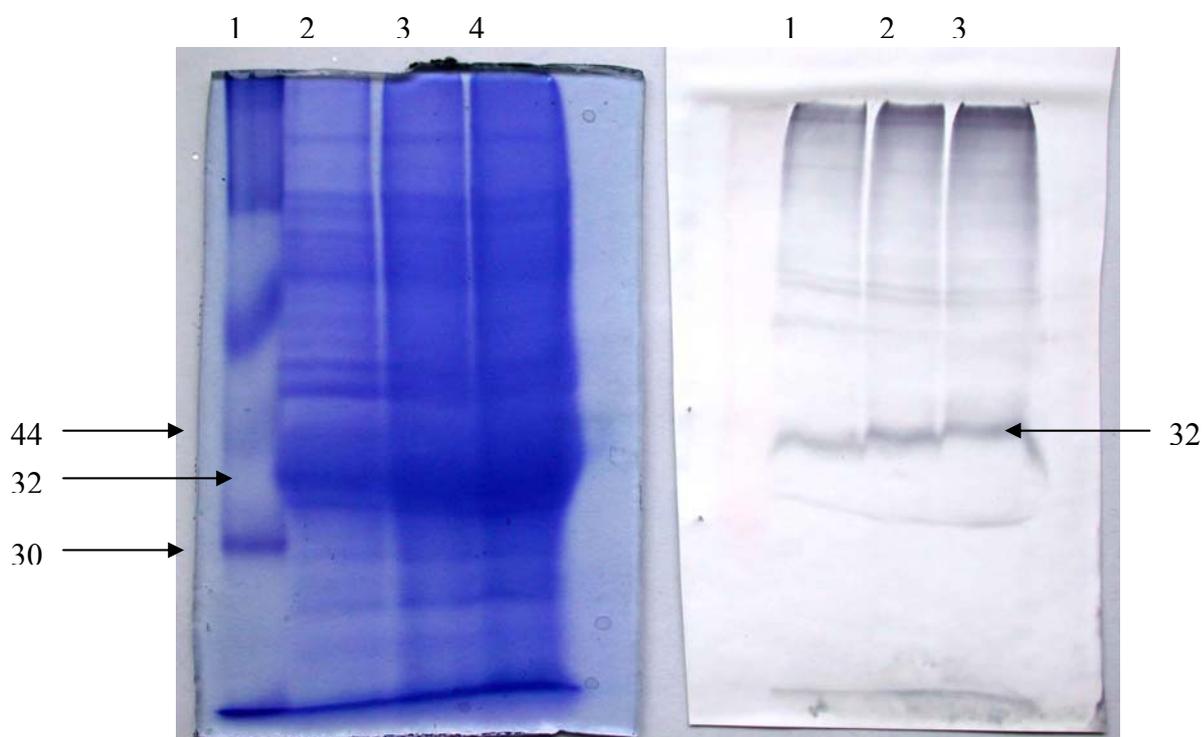


Figure 5.3 The SDS-PAGE (left) and Western blot (right) analysis of pET/PVY expressed PVY CP. SDS-gel lane 1: Rainbow molecular weight markers, size shown in kDA; lane 2: 10 μ l of pET/PVY cell sample; lane 3: 30 μ l of pET/PVY cell sample; lane 4: 50 μ l of pET/PVY cell sample. Western blot, lane 1: 10 μ l of pET/PVY cell sample protein transfer; lane 2: 30 μ l of pET/PVY cell sample protein transfer; lane 3: 50 μ l of pET/PVY cell sample protein transfer.

CHAPTER 6

The detection of PVY in seed potatoes by ELISA

6.1 Introduction

The main objective of this project was to produce a readily available and inexpensive test for testing seed potatoes for the presence of PVY and PLRV. The development of an ELISA to detect the virus in planting material was needed to determine the level of infection, critical for disease control in seed potatoes.

The advantages of using the ELISA as a testing method is: its specificity, sensitivity, rapidity and its low cost. Using the ELISA, low concentrations of antigens (0.1 ng/ml) can be detected. In addition to being a highly specific detection system, the ELISA can also provide quantitative results (Springer, 1999).

There are two types of enzymatic immunoassays (EIA) - the homogenous EIA where no separation of antibody or antigen from solution is required and the heterogeneous non-competitive indirect ELISA where separation is required (Springer, 1999). The heterogeneous ELISA can be further subdivided into three groups namely; the competitive binding test, immunoenzymometric test and the sandwich method (Springer, 1999). In these assays the reaction takes place in a liquid environment with one of the reagents adsorbed to a solid phase (Goers, 1993). The antigen or antibody is not revealed directly through the ELISA, but indirectly by an intermediate reagent. The sandwich method is the most commonly used ELISA as no purified antigen is required.

6.2 The sandwich ELISA

The sandwich ELISA (Figure 6.1) is the most versatile assay to determine proteins in a biological sample, as no purified antigen is required, only an antibody that is specific to the antigen (Goers, 1993). The first sandwich ELISA was introduced by Engvall and Perlman in 1971 and can be used to determine the levels of antibody or antigen in a given biological sample (Goers, 1993).

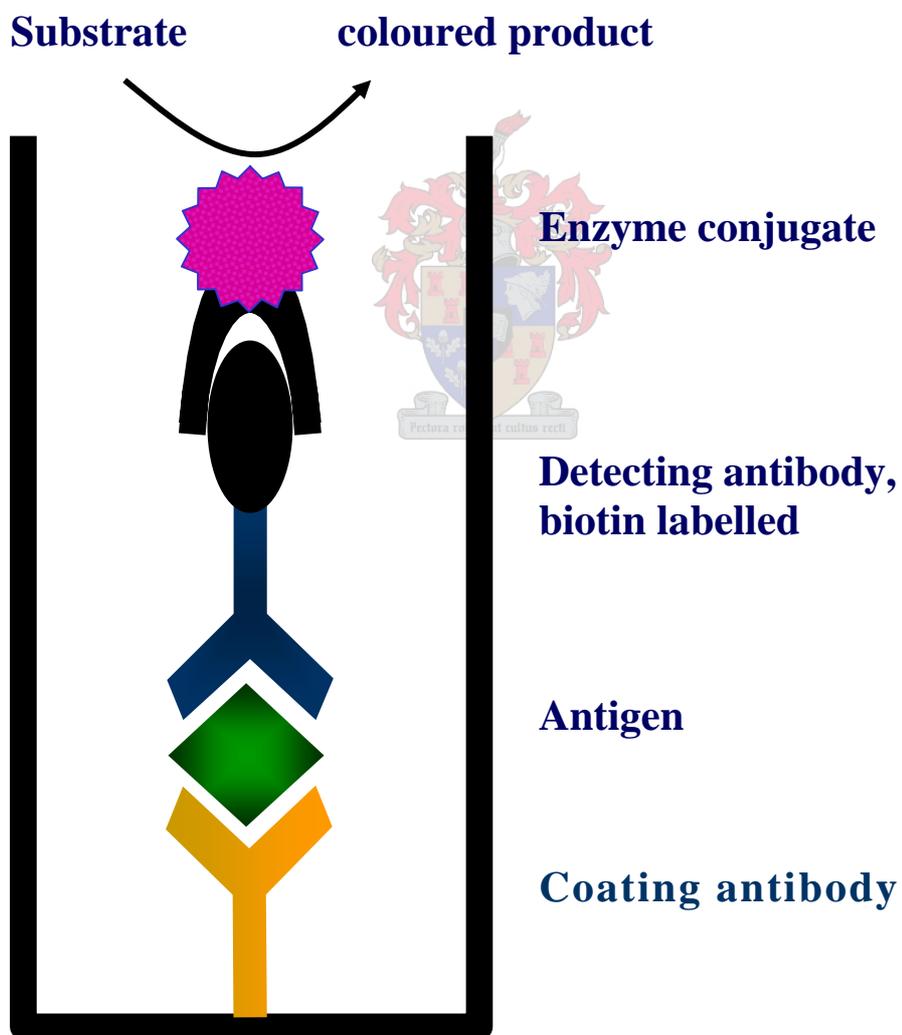


Figure 6.1 A typical sandwich ELISA

6.2.1 Binding of antigen or antibody to the solid phase

In this technique an antibody or antigen is firstly adsorbed to a solid phase by non-covalent bonding. This solid phase bonding allows for the washing and removal of any unbound agents in the solution.

Only multivalent antigens with repeating epitopes can be used, as the protein must be recognised by more than one antibody, which is essential in the detection of the antigen (Springer, 1999). Microtitre plates are used as the solid phase reactants in this assay as they can accommodate up to 96 samples (96 wells on a plate) and is standardised for most microtitre plate readers.

Direct coating or passive adsorption of the antigen or antibody to the plate is sufficient (Goers, 1993). The adsorption of the antigen or antibody to the polystyrene surface of the plate is due to the intermolecular attraction of van der Waal's forces. Covalent bonds are only formed between the reagents when chemically modified plates are used. The attraction forces of covalent and ionic bonds can be up to 100 times stronger than van der Waal's intermolecular bonds (Anon, 2004). Some microtitre plates can have chemically reactive groups added to the well surface. The addition of these reactive groups will change the binding properties of the well surface to increase the strength of binding of the antibody or antigen to the well surface (Larson et al., 1987). The antigen or antibody will remain bound during treatment with most detergents and moderate levels of pH and salt concentration (Goers, 1993).

After the adsorption process the wells of the microtitre plate are saturated with a blocking agent which binds to those areas that are not bound by antigen or antibody, preventing non-specific binding of serum proteins or secondary antibodies by the same forces involved with the coating (Springer, 1999).

6.2.2 Antigen detection

The following step in this ELISA process is the addition of the antigen or antibody which is incubated in the wells from as little as an hour to a day depending on the sensitivity of the test. The incubation buffer for this step contains a low concentration of non-ionic detergent such as Tween 20 which will aid to prevent non-specific binding (i.e hydrophobic interactions) (Springer, 1999). After incubation the buffer is discarded and a wash step is performed and any unbound antigen, antibody or serum proteins will be washed away. The affinity of the solid phase reagent for the antigen or antibody will prevent its removal. The washing process is essential in maintaining a low background as any cross-reacting antibodies or antigen with a low affinity will be removed more rapidly through the repeated wash steps (Springer, 1999).

6.2.3 Detecting antibody

The secondary antibody must bind the antigen at an epitope different to the one that is bound to the primary antibody, which is in turn adsorbed to the solid phase microtitre plate well. These secondary antibodies that are bound to the antigen in question are covalently bound to an enzyme that all ELISAs require to produce a final signal.

Direct coupling of the enzyme to the secondary antibody can cause steric hindrance, so as an alternative the secondary antibodies are covalently labelled with biotin, a water-soluble B-complex vitamin without changing any biological activity (Tijssen, 1985).

The biotin in turn links with avidin, which in turn is bound to the enzyme of choice. The biotin reagent is directly coupled to an aminocaproyl spacer which can reduce steric hindrance in binding avidin to the biotin reagent (<http://www.sigma-aldrich.com>). It is this indirect coupling that enables the amplification of the original antibody/antigen interaction. Avidin is a

glycoprotein obtained from egg whites and has a very high affinity for biotin (Springer, 1999). Streptavidin, produced by the bacterium *Streptomyces avidinii*, is preferred to avidin due to its favourable pI (5.5-6.5) and it is not glycosylated thereby avoiding less non-specific binding, resulting in less background (Tijssen, 1985). The biotin-antibody complex binds more than one enzyme-streptavidin conjugate molecule. The ratio of enzyme molecules to antigen-antibody complex is greater, which will greatly amplify the final signal increasing the immunoassay's sensitivity for the antigen (Springer, 1999).

6.2.4 Enzymes used in immunoassays for the detection of antibodies or antigens

In an ELISA the ideal enzyme of choice should have a high specific activity, a low K_m for the substrate but a high K_m for the product and a high K_i (Tijssen, 1985). The enzyme should have the following properties: be stable in its free or conjugated form during storage, easily prepared in pure form, easily detectable activity, absent from biological samples and active within the assay conditions. The most widely used enzymes are horseradish peroxidase (HRP), calf intestinal alkaline phosphatase (AP), B-galactosidase from *E. coli*, urease and glucose oxidase from fungal sources (Barrett, 1983).

6.2.5 Substrate

The substrates used in EIA are cleaved by an enzyme to produce a coloured product that can be measured spectrophotometrically. The product formed is directly proportional to the amount of antigen in the given sample (Springer, 1999).

As mentioned before, a single enzyme molecule can act on more than one substrate molecule, as it is not altered by the reaction enhancing the sensitivity of the assay. This reaction can, however, be influenced by temperature (Springer, 1999).

The use of a coloured product in the detection of the antigen is a cheap and simple detection system that is easy to handle with a great sensitivity. Since all enzymes and substrates give similar results EIA will vary as to which detection system is used.

A widely used enzyme, horseradish peroxidase, has several chromogenic substrates that it can act upon namely 2,2'-Azino-di(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) which forms a green product, O-phenylenediamine (OPD) and 5-Aminosalicylic acid (5AS) which both form yellow product and O-Dianisidine (ODia) which produces a red coloured product.

6.3 The detection of PVY in plant sap by ELISA

A 96 well microtitre plate (Nunc, Maxisorp with certificate) was coated with rabbit anti-PVY serum (1:800, 200 µl/well) in carbonate buffer (50 mM, pH 9.6) overnight at 4°C. The rabbit antiserum solution was decanted and the plate was incubated with casein buffer (0.5% casein, 0.15 M NaCl, 0.01 M Tris, 0.02% thiomersal, pH 7.6, 250 µl/well) for 1 h at 37°C.

The infected potato leaf sap samples were prediluted 1:2 in casein-Tween (casein buffer mixed with 0.1% Tween 20) and a series dilution was made in casein-Tween (total, 200 µl/well) and incubated for 2 h at 37°C. The potato leaf sap solutions were decanted and the wells were washed three times with PBS-Tween (PBS buffer with 0.1% Tween 20).

Biotinylated rabbit anti-PVY antibodies were diluted (1:100) with casein-Tween, added to the wells (200 µl/well) and incubated for 1 h at 37°C. The plates contents were decanted and washed three times with PBS-Tween.

Streptavidin peroxidase (AVPO) was diluted (1:100) with Casein-Tween, added to the plates (200 µl/well) and incubated for 1 h at 37°C. After decanting the contents, the plates were washed three times with PBS-Tween and the substrate solution, 200 µl/well, was added (0.05% 2,2'-Azino-bis[3-

ethylbenzthiazoline-6-sulfonic acid](ABTS), 0.015% H₂O₂ in 0.1 M Citrate buffer, pH 5). The plate was incubated for 30 min at 37°C and the absorbance measured at 405 nm on a Titertek Multiscan spectrophotometer.

Infected leaf samples obtained from the Sandveld and Pretoria were crushed in a plant press and the sap was gathered in vials and tested using the ELISA test described above. The results obtained from the preliminary testing of the ELISA can be seen in Table 6.1.

Table 6.1 The absorbance values obtained in the ELISA of the potato samples read after 30 min at 405 nm on a Titertek Multiscan spectrophotometer.

Dilution	1/2	1/4	1/8	1/16	1/32
Pretoria	0.984	0.473	0.164	0.141	0.06
Sandveld 1	0.789	0.641	0.46	0.104	0.05
Sandveld 2	0.996	1.045	0.408	0.115	0.016

Dilution	1/64	1/128	1/256	1/512	1/1024
Pretoria	0.036	0.012	0	0	0
Sandveld 1	0	0	0	0	0
Sandveld 2	0.028	0	0	0	0

The samples used in the ELISA had all been tested previously by the ELISA kits obtained from Europe in the laboratories of Potatoes SA in Piketberg (Sandveld 1 and 2 sample) or in Pretoria (Pretoria sample) and confirmed as PVY positive. An uninfected potato sample was also tested (results not shown) and absorbance values of below 0.05 were obtained at all dilutions. This therefore illustrates that the ELISA developed in this study specifically detects PVY. Further studies in which the ELISA developed in this study is compared on a large scale to the European kits that are currently

commercially used will now have to be performed before the ELISA developed here can be used routinely for PVY detection in South Africa.



CHAPTER 7

Discussion and conclusions

In this study the RNA encoding both PVY and PLRV CP was successfully amplified by RT-PCR and the resulting cDNA cloned into the pGEM-T easy plasmid. Subsequently the CP genes of both viruses were sequenced and compared with other PVY and PLRV CP gene sequences obtained from Genbank. The aligned CP gene sequences revealed that there were many differences (mutations) between the South African strains of PVY and PLRV as compared to the CP gene sequences of the overseas strains. The CP gene sequences were translated *in silico* into their respective amino acid sequences to determine if the genetic variation transferred itself into amino acid changes in the virus CP (Addendum 5 and 6). The sequence homology between the cloned PVY CP gene and other strains ranged between 89%-98,6%, the amino acid residue homology between the same sequences ranged from 91%-98%. The recombinant PLRV CP gene sequence homology ranged from 96%-97% compared to other PLRV CP sequences and the amino acid homology ranged between 96%-97%. However, the differences in amino acid changes between the South African strains of PVY (97% homology) and PLRV (98% homology) compared to those of overseas (88%-90% for PVY and 95%-96% for PLRV) strains further illustrated that the local strains differed from overseas strains. This sequence data indicates further studies on strain variation for viral mutation assessment are needed for a continual monitoring of viral drift in South Africa. The continual monitoring of the viral mutations and subsequent updating of the ELISA kits to effectively detect these respective viruses will help to eliminate false negative results caused by the mutations in the CP genes of PVY and PLRV. Further studies will also have to be done to determine if the ELISA developed in this study using a South African viral strain will detect PVY strains from overseas as well as all types of PVY strains in South Africa.

The two virus coat protein genes were sub-cloned into the protein expression system pET-14b and sequenced to determine the reading frame. The expression of the recombinant PVY CP was successful but the expression of the recombinant PLRV CP could not be achieved. Future research will have to be done to successfully induce and express the recombinant PLRV CP.

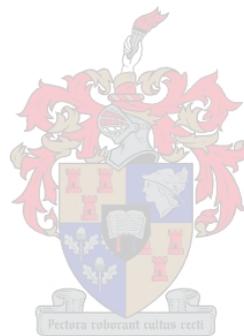
To obtain highly specific antibodies against PVY for the ELISA the expressed CP adsorbed to naked bacteria was used to raise rabbit antibodies by immunising PVY CP naked bacteria complexes. The immune response of the rabbit was determined against the isolated recombinant PVY CP to show that the antibody levels increased over time in response to the immunisation.

When producing antibodies using naked bacteria as an antigen carrier, antibodies are not only raised against the antigen i.e. PVY CP but also against the carrier bacteria. As these naked bacteria are prepared from Gram-negative *Salmonella minnesota* bacteria, antibodies raised against bacterial components have the potential of recognising Gram-negative bacteria on the potato plants to be tested. Antibodies against bacterial components were not viewed to be a problem because leaf material is tested primarily and bacterial cells are not present in high enough quantities to influence the ELISA. The presence of PVY in tubers is also detected by means of ELISA. Antibodies raised against NB which may potentially detect contaminating Gram-negative soil bacteria do not pose a problem as the skin of the tuber is removed before testing and only tuber material is placed in the microtitre plate during the ELISA.

The sequences of the PVY and PLRV CP genes are completely different and share no sequence homology as well as amino acid sequence homology as they belong to different viral families (see chapter 2). The antibodies raised against the recombinant PVY CP will not cross-react for this reason with the PLRV CP. Although specificity testing was not performed with other potato viruses that are more closely related to PVY than PLRV, cross-reactivity of the antibodies was not expected as other researchers have also found that no

cross-reactivity with these viruses occurs because they are still too distantly related to PVY to possess enough amino acid homology.

The rabbit antiserum obtained in this study was used to develop the ELISA to detect the presence of PVY in potato samples. Initial results showed the successful detection of PVY in infected plant material by means of the ELISA in which the antibodies raised against recombinant PVY CP were used. The ELISA kit developed in this study will now be used in parallel with the older kits obtained from Europe that Potatoes South Africa is currently using to determine if the new ELISA system can produce reliable results. Further comparisons of the European and South African ELISA kits must also be performed on a larger scale in order to verify that the kit developed in this study will be suitable for routine detection of PVY in South Africa.



References

- Anon (2004). www.nuncbrand.com, October 2004.
- Arnheim, N. and Erlich, H. (1992). Polymerase chain reaction strategy. *Annu. Rev. Biochem.*, 61: 131-156.
- Ashoub, A., Rohde, W. and Prufer, D. (1998). *In planta* transcription of a second subgenomic RNA increases the complexity of the subgroup 2 luteovirus genome. *Nucleic Acid Res.* Vol 26. No. 2: 420-426.
- Barker, H, Webster, K.D. and Reavy, B. (1993). Detection of PVY in potato tubers: a comparison of PCR and ELISA. *Potato Res.* 36:13-20.
- Barrett, J.T. (1983) Textbook of immunology, 4th edn., p.277-282, C.V. Mosby Company, London.
- Beczner, L., Horvath, J., Romhanyi, I. and Forster, H. (1984). Studies on the etiology of TNRD in potato. *Potato Res.* 27: 339-352.
- Blanc, S., Lopez-Moya, J., Wang, R., Garcia-Lampasona, S., Thornburg, D.W. and Pirone, T.P. (1997). A specific interaction between coat protein and HC correlates with aphid transmission of a potyvirus. *Virology.* 231: 141-147.
- Blanco-Urgoiti, B., Tribodet, M., Leelere, S., Ponz, F., Perez de San Roman, C., Legorburu, F.J. and Kerlan, C. (1998). Characterization of PVY isolates from seed potato batches. *Eur. J. Plant Path.* 104: 811-819.
- Botes, A. (1998). Preliminary investigations into the immunity of ostriches to Newcastle disease and *Clostridium perfringens* infections. M.Sc thesis, University of Stellenbosch.
- Botes, A. (2004) Immunological and epidemiological investigations in South African ostriches and penguins. Ph. D thesis, University of Stellenbosch.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

Cann, C.J. (2001). Principles in molecular virology, 3rd edn., pp. 1-70, Academic Press, London.

Carrington, J.C. and Freed, D.D. (1990). Cap independent enhancement of translation by a plant potyvirus 5' nontranslated region. *J. Virol.* Vol. 64. No.4: 1590-1597.

Carrington, J.C., Kasschau, K.D., Mahajan, S.K. and Schaad, M.C. (1996). Cell to cell and long distance transport of viruses in plants. *Plant Cell.* 8: 1669-1681.

Čeřovská, N. (1998). Production of monoclonal antibodies to PVY^{NTN} strain and their use for strain differentiation. *Plant Path.* 47: 505-509.

De Souza, J.A.C., Russo, P., Betti, J.A., Miller, L. and Slack, S.A. (1999). Simplified extraction method for ELISA and PCR Detection of Potato leafroll luteo virus primary infection in dormant potato tubers. *Amer. J. Potato Res.* 76: 209-213.

Ellis, P., Stace-Smith, R., Bowler, G. and Mackenzie, D.J. (1996). Production of monoclonal antibodies for detection and identification of strains of PVY. *Canadian J. Plant Path.* 18: 64-70.

Flis, B. (1995). Inheritance of extreme resistance to PVY in potato. *Potato Res.* 38:199-210.

Goers, J. (1993) Immunochemical techniques, p119-134, Academic Press, London.

Haldeman- Cahill, R., Daros, J. and Carrington, J.C. (1998). Secondary structures in the capsid protein coding sequence and 3' nontranslated region involved in amplification of the TEV genome. *J. Virol.* Vol. 72. No. 5: 4072-4079.

Herbert, E. and McDonald, J.G. (1973). Characterization of some proteins associated with viruses in the potato Y group. *Virology.* 56: 349-361.

Hoffman, K., Titus, G., Montibeller, J.A. and Finn, F.M. (1982). Avidin binding of carboxyl-substituted biotin and analogues. *Biochemistry.* 21: 978-984.

<http://www.life.anu.edu.au/viruses/ICTVdb/index.html>.

<http://www.micro.msb.le.ac.uk/335/plant.html>.

<http://www.ncbi.nih.gov/Genbank>

<http://www.potatoes.co.za>



<http://www.virustaxonomyonline.com>

Kadaré, G. and Haemmi, A. (1997). Virus encoded RNA helicases. *J. Virol.* Vol. 71, No. 4: 2583-2590.

Kasschau, K.D., Cronin, S. and Carrington, J.C. (1997). Genome amplification and long distance movement functions associated with the central domain of TEV helper component-proteinase. *Virology.* 228: 251-262.

Larson, P.H., Johansson, S.G.O., Anders, H. and Gothe, S. (1987). Covalent binding of proteins to grafted plastic surfaces suitable for immunoassays. *J. Immunol. Methods,* 98: 129-135.

Le Romancer, M., Kerlan, C. and Nedellec, M. (1994). Biological characterizations of various geographical isolates of PVY inducing superficial necrosis of potato tubers. *Plant Path.* 43: 138-144.

Li, X.H., Valdez, P., Olvera, R.E. and Carrington, J.C. (1997). Functions of the tobacco etch virus RNA polymerase (N1b). *J. Virol.* Vol.71, No. 2: 1598-1607.

Maia, I.G., Haemmi, A. and Bernadi, F. (1996). Potyviral HC-Pro a multifunctional protein. *J. Gen Virol.* 77: 1335-1341.

Mandahar, C.L. (ed)(1999). Molecular biology of plant viruses. Kluwer Academic, Boston.

McDonald, J.G. and Singh, R.P. (1996) Host range, symptomology and serology of isolates of PVY that share properties with both the PVY^N and PVY^O strain groups. *Amer. J. Potato Res.* 73: 309-313.

McPherson, M.J. and Moller, S.G. (eds.)(2000). PCR, 1-194, Bios Scientific Publishers Ltd, UK.

Meyer, B. (2003) Methods for serological and PCR detection of *Salmonella enteritidis* in chickens. M.Sc thesis, University of Natal.

Murphy, J.F., Klein, P.G., Hunt, A.G. and Shaw, J.G. (1996). Replacement of the tyrosine residue that links the a potyviral vpg to the viral RNA is lethal. *Virology.* 220: 535-538.

Newton, C.R and Graham, A. (eds)(1997). PCR, 2nd ed., pp. 1-28, BIOS Scientific Publishers Ltd., Oxford.

Okamoto, D., Nielsen, S., Albrechtsen, M. and Borkhardt, B. (1996). General resistance against PVY introduced into a commercial potato cultivar by genetic transformation with PVY^N coat protein gene. *Potato Res.* 39: 271-282.

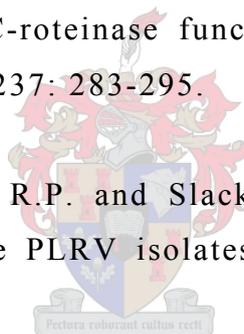
Reichman, J.L., Lain, S. and Garcia, J.A. (1992). Highlights and prospects of potyvirus molecular biology. *J. Gen Virol.* 73: 1-16.

Rodriguez-Cerezo, E., Findlay, K., Shaw, J.G., Lomonosoff, G.P., Qiu, S.G., Linstead, P., Shanks, M. and Risco. C. (1997). The coat and cylindrical inclusion proteins of a potyvirus are associated with connections between plant cells. *Virology.* 236: 296-306.

Rohde, W., Gramstat, A., Schmitz, J., Tacke, E. and Prufer, D. (1994). Plant viruses as model systems for the study of non-canonical translation mechanisms in higher plants. *J Gen Virol.* 75: 2141-2149.

Rojas, M.R., Zerbini, F.M., Allison, R.F., Gilbertson, R.L. and Lucas, W.L. (1997). Capsid protein an HC-roteinase functions as potyvirus cell to cell movement proteins. *Virology.* 237: 283-295.

Russo, P., Miller, L., Singh, R.P. and Slack, S.A. (1999). Comparison of nucleotide sequences from the PLRV isolates collected in Brazil. *Amer. J. Potato Res.* 76: 17-24.



Ryan, M.D. and Flint, M. (1997). Virus encoded proteinases of the picornavirus super-group. *J. Gen Virol.* 78: 699-723.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985). Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230: 1350-1354.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Schard, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988). Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239:487-491.

Saiki, R.K. (1989). The design and optimisation of the PCR. In: Erlich, H.A. (ed), *PCR Technology Principles and Applications for DNA Amplification*, pp7-16. Macmillan Publishers Ltd., England.

Schaad, M.C., Jensen, P.E. and Carrington, J.C. (1997). Formation of plant RNA virus replication complexes on membranes. *The EMBO J.* Vol. 16. No. 13: 4049-4059.

Springer. (1999) Antibody usage in the lab (Caponi, L & Migliorini, P. eds.), p1-32, Springer-Verlag, Germany.

Tijssen, P., (1985). Burdon, R.H., Knippenburg, P.H. (eds), Laboratory techniques in biochemistry and the molecular biology practice and theory of enzyme immunoassays, volume 15, Elsevier Science Publishers B.V., Amsterdam.

Verchot, J. and Carrington, J.C. (1995). Evidence that the potyvirus P1 proteinase functions in *trans* as an accessory factor for genome amplification. *J. Virol.* Vol. 69. No. 6: 3668-3674.



Van der Wilk, F., Houterman, P., Molthoff, J., Hans, F., Dekker, B., van den Heuvel, J., Huttinga, H. and Goldbach, R. (1997). Expression of the Potato Leafroll Virus ORF0 induces viral-disease-like symptoms in transgenic potato plants. *American Phytopath Soccity.* Vol 10. No. 2: 153-159.

Wu, X. and Shaw, J.G. (1998). Evidence that assembly of a potyvirus begins near the 5' terminus of the viral RNA. *J. Gen Virol.* 79: 1525-1529.

Addendum 1

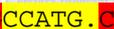
P4SP6san is the RT-PCR product cloned into pGEM-T easy and sequenced using the T7 and SP6 primers and aligned with other PVY CP DNA sequences

 The primer area designed for the 5' direction RT-PCR

 The restriction enzyme cut site for NcoI

 The primer area designed for the 3' direction RT-PCR

 The restriction enzyme cut site for BamHI

						60
PVY-N1	-----	-----	-----GGAA	ATGACACAAT	CGATGCAGGA	GGAAGCACTA
PVY-N2	-----	-----	-----	-----	T.....
PVY-N3	-----	-----	-----	-----	-----T.....
PVY-N5	-----	-----	-----C	-----	-----	.A.....GC.
PVY-O2	-----	-----	-----	-----	-----A..AC.
PVY-O3	-----	-----	-----C	-----	T.....	.A..A..G..
PVY-US1	-----	-----	-----C	-----	TA.....AC.
PVY-CH1	-----	-----	-----C	-----	-----	..G...GC.
PVY-H1	-----	-----	-----	-----	-----
PVY-O1	-----	-----	-----CT	T.....A..AC.
PVY-Lb	-----	-----	-----	-----	T.....
P4SP6san	CCGCGGGAAT	TCGATT	 GCAC	 GCCATG	 C.....

						120
PVY-N1	AGAAAGATGC	AAAACAAGAG	CAAGGTAGCA	TTCAACCAAA	TCTCAACAAG	GAAAAGGAAA
PVY-N2
PVY-N3G
PVY-N5CC	.C..GTT...	C.CG.....A	.G...A..T.
PVY-O2TCC	.C..GTT...	C.CG.....A	.G...A..T.
PVY-O3	G.....	G...CG..A	.G.....	.C..G.....	..CT.....	.G...T.
PVY-US1ACA.C	.C..GT...	C..G.T...A	.G...A..T.
PVY-CH1	...G..CA.	...G.C...AC..G.....	..CT.....	.G...C.
PVY-H1	...G.....T	.G.....
PVY-O1CA.C	.A..GT...	C..G.GT...	.G...A..T.
PVY-Lb	...G.....T
P4SP6san

180

PVY-N1	AGGACGTGAA	TGTTGGAACA	TCTGGAAGCTC	ACACTGTGAC	ACGAATTAAA	GCTATCACGT
PVY-N2C.
PVY-N3AC.
PVY-N5T.....	..C...T...G..A.	.T.....C.	GA....C..G
PVY-O2T.....	..C...T...G..A.	.T.....C.	GA....C..GT....
PVY-O3CC..T...A.	.T.....AC.	.A....A..GT....
PVY-US1	.A..T.....	..CC..C...G..A.C.	GA....C..G
PVY-CH1T...G.	.T..A...C.	GA....A...
PVY-H1T.....C.
PVY-O1	.A..T.....C...G..A.	.T.....C.	GA....C..G
PVY-LbT.....C.
P4SP6san	C.....C.

240

PVY-N1	CCAAAATGAG	AATGCCCAAG	AGTAAGGGTG	CAACTGTACT	AAATTTGGAA	CACTTACTCG
PVY-N2A
PVY-N3
PVY-N5A	..C....A.C..G..	...C..A...G..T.
PVY-O2A	..C....A.C..G..	...C..A...T.
PVY-O3T..A	..C..A..A.	.GG.C..G..	...CC.A...	..T..G..T.
PVY-US1A	..C....A.	..G.C..G..	G....A...G..T.
PVY-CH1	.A.....CA	..C..A..A.C.....	G..C..A...
PVY-H1AG.....A...G....
PVY-O1GA	..C....A.	T.G.C.CG..	C..C..A...G....
PVY-LbA
P4SP6san	G.....A..

300

PVY-N1	AGTATGCTCC	ACAGCAAATT	GACATCTCAA	ATACTCGAGC	AACTCAATCA	CAGTTTGATA
PVY-N2T...
PVY-N3
PVY-N5C.....A.....	..T..T...G..
PVY-O2A.....	..T..T...G..
PVY-O3	.A.....A..G..A	..T..T...G..
PVY-US1A.....	..T..T...G..C.
PVY-CH1C..G...	..T.....	..C.....
PVY-H1
PVY-O1A.....G..
PVY-LbC.....
P4SP6san

360

PVY-N1	CATGGTATGA	AGCAGTACAA	CTTGCATACG	ACATAGGAGA	AACTGAAATG	CCAAGTGTGA
PVY-N2
PVY-N3A.
PVY-N5	.G.....	G....G.GG	A.G.....G...
PVY-O2	.G.....	G....G.GG	A.G.....G...
PVY-O3	.G.....GG	..A.....G.....
PVY-US1	.G.....	G....G.GG	A.G.....	.T.....G...
PVY-CH1	.G.....G.G	G.....G..	.T.....
PVY-H1	.G.....	..G.....
PVY-O1	.G.....	G..G..G.GG	A.G.....C.G...
PVY-Lb	.G.....	..G.....	T.....G..C.....
P4SP6sanC.....

420

PVY-N1	TGAATGGGCT	TATGGTTTGG	TGCATTGAAA	ATGGAACCTC	GCCAAATATC	AATGGAGTTT
PVY-N2
PVY-N3
PVY-N5G..C.....
PVY-O2G..C.....
PVY-O3A..G..C.....
PVY-US1G..C.....
PVY-CH1C.....
PVY-H1C..C.....
PVY-O1C.....
PVY-LbC..C.....
P4SP6sanC

480

PVY-N1	GGGTTATGAT	GGATGGAGAT	GAACAAGTCG	AATACCCACT	GAAACCAATC	GTTGAGAATG
PVY-N2C..A..A....
PVY-N3
PVY-N5GA..T..	..G....GT.
PVY-O2GA..T..	..G....GT.
PVY-O3GA..T..	..T...GT.
PVY-US1GA..T..	..G....GT.
PVY-CH1GA..T..GT.A....
PVY-H1
PVY-O1GA..T..GT.T
PVY-Lb
P4SP6san

540

PVY-N1	CAAACCAAC	ACTTAGGCAA	ATCATGGCAC	ATTTCTCAGA	TGTTGCAGAA	GCGTATATAG
PVY-N2
PVY-N3
PVY-N5C..G..
PVY-O2C.....
PVY-O3G.G.C.
PVY-US1C.....
PVY-CH1G.....	..C.....G
PVY-H1
PVY-O1C.....C....
PVY-LbC.....
P4SP6san

600

PVY-N1	AAATGCGCAA	CAAGAAGGAA	CCATATATGC	CACGATATGG	TTTAGTTCGT	AATCTGCGCG
PVY-N2A.....
PVY-N3A.....
PVY-N5A.....A...AG..
PVY-O2A.....A...AG..
PVY-O3A.....A...G..
PVY-US1A.....A...AG..
PVY-CH1A.....A...AA..
PVY-H1A.....
PVY-O1A...AG..
PVY-LbA.....
P4SP6sanA.....A....

660

PVY-N1	ATGGAAGTTT	GGCTCGCTAT	GCTTTTGACT	TTTATGAAGT	TACATCACGG	ACACCAGTGA
PVY-N2C.....
PVY-N3
PVY-N5	...TGG...	A..G..T...	..C.....G..	C.....A
PVY-O2	...TGG...	A..G..T...	..C.....G..	C.....A
PVY-O3	...TGG..G.	A..G..T...	C.....A
PVY-US1	..ATGG...	A..G..T...G..	C.....A
PVY-CH1	..AT.....	A..G.....	..C..C..T.	.C.....G..C..A
PVY-H1G..	C.....A
PVY-O1	..AT....C.	A..G.....	..C.....	C.....AG....
PVY-LbG...G..	C.....A
P4SP6san

720

PVY-N1	GGGCTAGAGA	GGCACACATT	CAAATGAAGG	CCGCAGCTTT	AAAATCAGCT	CAATCTCGAC
PVY-N2T.....
PVY-N3
PVY-N5G..	A..G.....A..	G.....C	..C.....
PVY-O2G..	A..G.....A..	G.....C	..C.....
PVY-O3G..	A..G....AA..C.....
PVY-US1G..	A..G.....A..	G.....C	..C.....
PVY-CH1C..G..	A..G..T..CA.G..A..	G..G.....	..C.....
PVY-H1G..	A..G.....A..	G.....C
PVY-O1G..	A..G....CA..	G.....C	..C.....
PVY-LbG..	A..G....CA..	G.....C	..C.....
P4SP6san

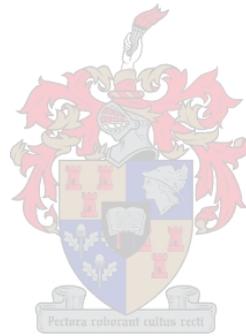
780

PVY-N1	TTTTCGGATT	GGATGGTGGC	ATTAGTACAC	AAGAGGAAAA	CACAGAGAGG	CACACCACCG
PVY-N2
PVY-N3C.....
PVY-N5G..	..C.....	..C.....
PVY-O2G..	..C.....G..
PVY-O3GC.	..C.....	..C.....G..
PVY-US1G..	..C.....	..C.....G..
PVY-CH1G..	..C.....	..C.....G..
PVY-H1G..	..C.....G..
PVY-O1G..	..C.....	..C.....G..
PVY-LbG..	..C.....	..C.....G..
P4SP6san

840

PVY-N1	AGGATGTTTC	TCCAAGTATG	CATACTCTAC	TTGGAGTGAA	GAACATGTGA	-----
PVY-N2T.....C..	-----
PVY-N3	-----
PVY-N5C..C..	-----
PVY-O2C..G.C..	A.....	-----
PVY-O3C..	..A....	-----
PVY-US1C..G.C..	-----
PVY-CH1C..C.C..	-----
PVY-H1C..C..	-----
PVY-O1G.GC.	A.....	-----
PVY-LbC..C..	-----
P4SP6sanC..	A.....	GGATCCGCAG

PVY-N1	-----	-----
PVY-N2	-----	-----
PVY-N3	-----	-----
PVY-N5	-----	-----
PVY-O2	-----	-----
PVY-O3	-----	-----
PVY-US1	-----	-----
PVY-CH1	-----	-----
PVY-H1	-----	-----
PVY-O1	-----	-----
PVY-Lb	-----	-----
P4SP6san	AATCACTAGT	GAATTC



Addendum 2

L1T7SAN is the PLRV CP (Sandveld) gene amplified by RT-PCR and cloned into pGEM-T easy and sequenced, aligned against other sequences of PLRV obtained from GenBank.

 The 5' primer used for the RT-PCR on the PLRV CP gene (Sandveld)

 The Restriction enzyme cut site for NcoI

 The 3' primer used for the RT-PCR on the PLRV CP gene (Sandveld)

 The restriction enzyme cut site for NdeI.

						60
PLRV-1	-----	-----	--ATGAGTAC	GGTCGTGGTT	AAAGGAAATG	TCAATGGTGG
PLRV-11	-----	-----
PLRV-CUB	-----	-----
PLRV-GER	-----	-----
PLRV-KOR	-----	-----
PLRV-ROD	-----	-----
PLRV-SCO	-----	-----
L1T7SAN	CGCGGGAATT	CGATT	 GCACG	 CC...G	

						120
PLRV-1	TGCACACCAA	CCAAGAAGGC	GAAGAAGGCA	ATCCCTTCGC	AGGCGCGCTA	ACAGAGTTCA
PLRV-11	..T...A...	..G.....
PLRV-CUB	..TG..A...
PLRV-GER	..T...A...G...
PLRV-KOR	..T...A...
PLRV-ROD	..AT...A...AG...AG..
PLRV-SCO	..T...A...T..
L1T7SAN	..T...A...A..

						180
PLRV-1	GCCAGTGGTT	ATGGTCACGG	CCCCTGGGCA	ACCCAGGCGC	CGAAGACGCA	GAAGAGGAGG
PLRV-11
PLRV-CUB
PLRV-GER
PLRV-KOR
PLRV-ROD
PLRV-SCO
L1T7SAN

						240
PLRV-1	CAATCGCCGC	TCAAGAAGAA	CTGGAGTTCC	CCGAGGACGA	GGCTCAAGCG	AGACATTTCGT
PLRV-11
PLRV-CUB
PLRV-GER
PLRV-KOR
PLRV-ROD
PLRV-SCO
L1T7SAN

						300
PLRV-1	GTTTACAAAG	GACAACCTCA	TGGGCAACTC	CCAAGGAAGT	TTCACCTTCG	GGCCGAGTCT
PLRV-11
PLRV-CUBG
PLRV-GER
PLRV-KORGA
PLRV-RODGT
PLRV-SCOGA
L1T7SANG

						360
PLRV-1	ATCAGACTGT	CCGGCATTCA	AGGATGGAAT	ACTCAAGGCC	TACCATGAGT	ATAAGATCAC
PLRV-11
PLRV-CUB
PLRV-GERT
PLRV-KORC
PLRV-ROD
PLRV-SCO
L1T7SAN

						420
PLRV-1	AAGCATCTTA	CTTCAGTTCG	TCAGCGAGGC	CTCTTCACC	TCCTCCGGTT	CCATCGCTTA
PLRV-11
PLRV-CUBA
PLRV-GERC
PLRV-KOR
PLRV-ROD
PLRV-SCO
L1T7SANTGC

						480
PLRV-1	TGAGTTGGAC	CCCCATTGCA	AAGTATCATC	CCTCCAGTCC	TACGTCAACA	AGTTCCAAAT
PLRV-11
PLRV-CUBA
PLRV-GER
PLRV-KOR
PLRV-RODA
PLRV-SCO
L1T7SANA

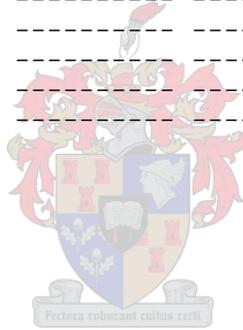
						540
PLRV-1	TACGAAGGGC	GGCGCCAAGA	TTTATCAAGC	GCGGATGATA	AACGGGGTAG	AATGGCACGA
PLRV-11AC
PLRV-CUBAC
PLRV-GERAC
PLRV-KORACAT
PLRV-RODTACC
PLRV-SCOAC
L1T7SANTACC

						600
PLRV-1	TTCTTCTGAG	GATCAGTGCC	GGATACTGTG	GAAGGGAAAT	GGAAAATCTT	CAGATACCGC
PLRV-11
PLRV-CUB
PLRV-GER
PLRV-KORT.
PLRV-ROD	...G.....T....	...A....C....
PLRV-SCOT....
L1T7SAN	...G.....A....CC....

						660
PLRV-1	AGGATCCTTC	AGAGTCACCA	TTAGGTTGGC	CTTGCAAAAC	CCCAAATAG-	-----
PLRV-11C...G....	T.....-	-----
PLRV-CUBC...G....	T.....-	-----
PLRV-GERC...G....	T.....-	-----
PLRV-KORC...G....	T.....-	-----
PLRV-RODC...G....	T...G...	...G....-	-----
PLRV-SCOC.A.G.A..	T.....-	-----
L1T7SANTC.AAG..	.T...G....	.C ATATGGCAGC	-----

						720
PLRV-1	-----	-----	-----	-----	-----	-----
PLRV-11	-----	-----	-----	-----	-----	-----
PLRV-CUB	-----	-----	-----	-----	-----	-----
PLRV-GER	-----	-----	-----	-----	-----	-----
PLRV-KOR	-----	-----	-----	-----	-----	-----
PLRV-ROD	-----	-----	-----	-----	-----	-----
PLRV-SCO	-----	-----	-----	-----	-----	-----
L1T7SAN	AGAATCACTA	GTGAAT	-----	-----	-----	-----

PLRV-1	-----	-----
PLRV-11	-----	-----
PLRV-CUB	-----	-----
PLRV-GER	-----	-----
PLRV-KOR	-----	-----
PLRV-ROD	-----	-----
PLRV-SCO	-----	-----
L1T7SAN	-----	-----



Addendum 3

PET7 is the aligned sequence of the PVY CP in pET14-b

 The 5' sequence of pET14b

 The restriction enzyme cut site for NcoI

						60
PET7	TTACTTTAAG	AANGAGATAT	ACCATGG CAA	ATGACACAAT	CGATGCAGGA	GGAAGCACTA
P4SP6 _{san}	CCG.GGG..T	TCGATTGC.C	G.....
PVY-N1	-----	-----	-----G..
PVY-N2	-----	-----	-----G..	T.....
PVY-N3	-----	-----	-----G..T....
PVY-N5	-----	-----	-----A...GC.
PVY-O2	-----	-----	-----G..A..AC.
PVY-O3	-----	-----	-----	T.....	..A..A..G..
PVY-US1	-----	-----	-----	TA.....AC.
PVY-CH1	-----	-----	-----G...GC.
PVY-H1	-----	-----	-----G..
PVY-O1	-----	-----	-----T..	T.....	..A..AC.
PVY-Lb	-----	-----	-----G..	T.....
						120
PET7	AGAAAGATGC	AAAACAAGAG	CAAGGTAGCA	TTCAACCAAA	TCTCAACAAG	GAAAAGGAAA
P4SP6 _{san}
PVY-N1
PVY-N2
PVY-N3G
PVY-N5C.....C.....	..C..GTT...	C.CG.....A	..G...A..T.
PVY-O2TC.....C.....	..C..GTT...	C.CG.....A	..G...A..T.
PVY-O3	G.....	G....CG..A	..G.....	..C..G.....	..CT.....	..G...T....
PVY-US1A.C.....	..A.C.....	..C..GT.....	C..G.T...A	..G...A..T.
PVY-CH1	...G..CA.	...G.C...AC..G.....	..CT.....	..G...C....
PVY-H1	...G.....T	..G...T....
PVY-O1C.....	..A.C.....	..A..GT...	C..G.GT...	..G...A..T.
PVY-Lb	...G.....T
						180
PET7	AGGACGTGAA	TGTTGGAACA	TCTGGAACTC	ACACTGTGCC	ACGAATTAAA	GCTATCACGT
P4SP6 _{san}	C.....
PVY-N1A.
PVY-N2
PVY-N3A.
PVY-N5	...T.....	..C...T...G..A.	..T.....	GA...C..G
PVY-O2	...T.....	..C...T...G..A.	..T.....	GA...C..GT....
PVY-O3CC..T...A.	..T.....A..	..A...A..GT....
PVY-US1	..A..T.....	..CC..C...G..A.	GA...C..G
PVY-CH1T...G.	..T..A.....	GA...A...
PVY-H1T.....
PVY-O1	..A..T.....C...G..A.	..T.....	GA...C..G
PVY-LbT.....

480

PET7	GGGTTATGAT	GGATGGAGAT	GAACAAGTCG	AATACCCACT	GAAACCAATC	GTTGAGAATG
P4SP6 _{san}
PVY-N1
PVY-N2C..A..A..
PVY-N3
PVY-N5GA..T..	.G.....GT.
PVY-O2GA..T..	.G.....GT.
PVY-O3GA..T..	...T..GT.
PVY-US1GA..T..	.G.....GT.
PVY-CH1GA..T..GT.A..
PVY-H1
PVY-O1GA..T..GT.T
PVY-Lb

540

PET7	CAAAACCAAC	ACTTAGGCAA	ATCATGGCAC	ATTTCTCAGA	TGTTGCAGAA	GCGTATATAG
P4SP6 _{san}
PVY-N1
PVY-N2
PVY-N3
PVY-N5C..G..
PVY-O2C.....
PVY-O3G.G	..C.....
PVY-US1C.....
PVY-CH1	...G.....	..C.....G
PVY-H1
PVY-O1C.....C..
PVY-LbC.....

600

PET7	AAATGCGCAA	CAAAAAGGAA	CCATATATGC	CACGATATGG	TTTAATTCGT	AATCTGCGCG
P4SP6 _{san}
PVY-N1G.....G.....
PVY-N2G.....
PVY-N3G.....
PVY-N5A	..G.
PVY-O2A	..G.
PVY-O3G..A	..G.
PVY-US1A	..G.
PVY-CH1A	..A.
PVY-H1G.....
PVY-O1G.....A	..G.
PVY-LbG.....

660

PET7	ATGGAAGTTT	GGCTCGCTAT	GCTTTTGACT	TTTATGAAGT	TACATCACGG	ACACCAGTGA
P4SP6 _{san}
PVY-N1
PVY-N2C.....
PVY-N3
PVY-N5	..TGG...A..G..T..	..C.....G..	C.....A
PVY-O2	..TGG...A..G..T..	..C.....G..	C.....A
PVY-O3	..TGG..G..A..G..T..G..	C.....A
PVY-US1	..ATGG...A..G..T..G..	C.....A
PVY-CH1	..AT.....A..G.....	..C..C..T..	..C.....G..C..A
PVY-H1G..	C.....A
PVY-O1	..AT...C..A..G.....	..C.....G..	C.....A	..G.....
PVY-LbG.....G..	C.....A

720

PET7	GGGCTAGAGA	GGCACACATT	CAAATGAAGG	CCGCAGCTTT	AAAATCAGCT	CAATCTCGAC
P4SP6san
PVY-N1
PVY-N2T.....
PVY-N3
PVY-N5G..	A..G.....A..	G.....C..	..C.....
PVY-O2G..	A..G.....A..	G.....C..	..C.....
PVY-O3G..	A..G....AA..C.....
PVY-US1G..	A..G.....A..	G.....C..	..C.....
PVY-CH1	...C.G..	A..G..T..CA..	...G.A..	G..G.....	..C.....
PVY-H1G..	A..G.....A..	G.....C..
PVY-O1G..	A..G....CA..	G.....	..C.....
PVY-LbG..	A..G....CA..	G.....C..	..C.....

780

PET7	TTTTTCGGATT	GGATGGTGGC	ATTAGTACAC	AAGAGGAAAA	CACAGAGAGG	CACACCACCG
P4SP6san
PVY-N1
PVY-N2
PVY-N3C.....
PVY-N5G..	..C.....	..C.....
PVY-O2G..	..C.....G..
PVY-O3GC.	..C.....	..C.....G..
PVY-US1G..	..C.....	..C.....G..
PVY-CH1G..	..C.....	..C.....G..
PVY-H1G..	..C.....G..
PVY-O1G..C.....G..
PVY-LbG..	..C.....	..C.....G..

840

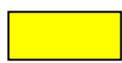
PET7	AGGATGTTTC	TCCAAGTATG	CATACTCTAC	TTGGAGTCAA	AAACATGTGA	GGATCCGN--
P4SP6sanCAG
PVY-N1G..	G.....	-----
PVY-N2T.....	G.....	-----
PVY-N3G..	G.....	-----
PVY-N5C..	G.....	-----
PVY-O2C..G..	-----
PVY-O3	G...A...	-----
PVY-US1C..G..	G.....	-----
PVY-CH1C..C..	G.....	-----
PVY-H1C..	G.....	-----
PVY-O1G..G..	-----
PVY-LbC..	G.....	-----

PET7	-----	-----
P4SP6san	AATCACTAGT	GAATTC
PVY-N1	-----	-----
PVY-N2	-----	-----
PVY-N3	-----	-----
PVY-N5	-----	-----
PVY-O2	-----	-----
PVY-O3	-----	-----
PVY-US1	-----	-----
PVY-CH1	-----	-----
PVY-H1	-----	-----
PVY-O1	-----	-----
PVY-Lb	-----	-----

Addendum 4

LENCO1T7 is the PLRV CP gene (Sandveld) sub-cloned into pET14b and sequenced.

 The 5' sequence of pET14b before the cloning site.

 The 3' sequence of pET14b after the cloning site.

						60
LENCO1T7	- ACTTTAAGA AGGAGATATA	CCATGGGTAC	GGTCGTGGTT	AAAGGAAATG	TCAATGGTGG	
L1SP6SAN	-G.GGG..TT C.ATTGC.CG	
PLRV-GER	-----	---.A....	
PLRV-CUB	-----	---.A....	
PLRV-KOR	-----	---.A....	.C.....	
PLRV-ROD	-----	---.A....G.....	
PLRV-SCO	-----	---.A....	
PLRV-1	-----	---.A....	
PLRV-11	-----	---.A....	

						120
LENCO1T7	TGTACAACAA CCAAGAAGGC	GAAGAAGGCA	ATCCCTTCGC	AGGCGCGCTA	ACAGAGTACA	
L1SP6SAN	
PLRV-GERG.....T..	
PLRV-CUB	..G.....T..	
PLRV-KORT..	
PLRV-ROD	.A.....AG.....A.....G..	
PLRV-SCOT.....T..	
PLRV-1	..C...C.....T..	
PLRV-11G.....T..	

						180
LENCO1T7	GCCAGTGGTT ATGGTCACGG	CCCCTGGGCA	ACCCAGGCGC	CGAAGACGCA	GAAGAGGAGG	
L1SP6SAN	
PLRV-GERT.....T.....	
PLRV-CUB	
PLRV-KORG.....	
PLRV-ROD	
PLRV-SCO	
PLRV-1	
PLRV-11	

						240
LENCO1T7	CAATCGCCGC TCAAGAAGAA	CTGGAGTTCC	CCGAGGACGA	GGCTCAAGCG	AGACATTTCGT	
L1SP6SAN	
PLRV-GER	
PLRV-CUB	
PLRV-KOR	
PLRV-ROD	
PLRV-SCO	
PLRV-1	
PLRV-11	

300

LENCO1T7	GTTTACAAAG	GACAACCTCG	TGGGCAACTC	CCAAGGAAGT	TTCACCTTCG	GGCCGAGTCT
L1SP6SAN
PLRV-GERA
PLRV-CUB
PLRV-KORA
PLRV-RODT
PLRV-SCOA
PLRV-1A
PLRV-11A

360

LENCO1T7	ATCAGACTGT	CCGGCATTCA	AGGATGGAAT	ACTCAAGGCC	TACCATGAGT	ATAAGATCAC
L1SP6SAN
PLRV-GERT
PLRV-CUB
PLRV-KORC
PLRV-ROD
PLRV-SCO
PLRV-1
PLRV-11

420

LENCO1T7	AAGTATCTTA	CTTCAGTTCG	TCAGCGAGGC	CTCTTCCACC	TCCGCCGGCT	CCATCGCTTA
L1SP6SAN
PLRV-GER	..C.....T.....
PLRV-CUB	..C.....T...AT.
PLRV-KOR	..C.....T...T.
PLRV-ROD	..C.....T...T.
PLRV-SCO	..C.....T...T.
PLRV-1	..C.....T...T.
PLRV-11	..C.....T...T.

480

LENCO1T7	TGAGTTGGAC	CCCCATTGCA	AAATATCATC	CCTCCAGTCC	TACGTCAACA	AGTTCCAAAT
L1SP6SAN
PLRV-GERG.....
PLRV-CUBG.....A.....
PLRV-KORG.....
PLRV-ROD
PLRV-SCOG.....
PLRV-1G.....
PLRV-11G.....

540

LENCO1T7	TACGAAGGGC	GGCGCTAAAA	CCTATCAAGC	GCGGATGATA	AACGGGGTAG	AATGGCACGA
L1SP6SAN
PLRV-GERC....	..T....
PLRV-CUBC....	..T....
PLRV-KORC....	..T....	..A....	..T....
PLRV-ROD
PLRV-SCOC....	..T....
PLRV-1C..G.	TT.....
PLRV-11C....	..T....

600

LENCO1T7	TTCGTCTGAG	GATCAGTGCC	GGATACTGTG	GAAAGGAAAT	GGAAAATCTT	CAGACCCCGC
L1SP6SAN
PLRV-GER	...T.....G.....TA....
PLRV-CUB	...T.....G.....TA....
PLRV-KOR	...T.....T.G.....TA....
PLRV-RODT.....T....
PLRV-SCO	...T.....G.....TT....
PLRV-1	...T.....G.....TA....
PLRV-11	...T.....G.....TA....

660

LENCO1T7	AGGATCCTTT	AGAGTCACCA	TCAAAGTGGC	TTTGCAGAAC	CCCAAATAGC	ATATGCTCGA
L1SP6SANGCA.C
PLRV-GERCGG.....	...A...-	-----
PLRV-CUBCGG.....	...A...-	-----
PLRV-KORCGG.....	...A...-	-----
PLRV-RODCGG.....G....-	-----
PLRV-SCOCG.A...	...A...-	-----
PLRV-1CT.GGT...	C...A...-	-----
PLRV-11CGG.....	...A...-	-----

720

LENCO1T7	GGATCCGGCT	GCTAACAAAG	CCG-----	-----	-----	-----
L1SP6SAN	A..AT.ACTA	.TG..TTCGC	GGCCGCCTGC	AGGTCGACCA	TATGGGAGAG	CTCCCAACGC
PLRV-GER	-----	-----	-----	-----	-----	-----
PLRV-CUB	-----	-----	-----	-----	-----	-----
PLRV-KOR	-----	-----	-----	-----	-----	-----
PLRV-ROD	-----	-----	-----	-----	-----	-----
PLRV-SCO	-----	-----	-----	-----	-----	-----
PLRV-1	-----	-----	-----	-----	-----	-----
PLRV-11	-----	-----	-----	-----	-----	-----

LENCO1T7	-----	-----
L1SP6SAN	GTTGGATGCA	TAGCTG
PLRV-GER	-----	-----
PLRV-CUB	-----	-----
PLRV-KOR	-----	-----
PLRV-ROD	-----	-----
PLRV-SCO	-----	-----
PLRV-1	-----	-----
PLRV-11	-----	-----



Addendum 5

The aligned protein sequence of the PVY CP (PET7) in pET-14b that was expressed and used to immunize a rabbit with. PET7 is aligned against other PVY strains obtained off Genbank.

						60
PET7	MANDTIDAGG	STKKDAKQEQ	GSIQPNLNKE	KEKDVNVGTS	GTHTVPRIKA	ITSKMRMPKS
PVY-Lb	-G.....I.....
PVY-N1	-G.....T.....
PVY-N2	-G.....K.....
PVY-N3	-G.....S.....
PVY-N5E	.S.....P..L.P..G	.D....A...
PVY-O2	-G.....	NN...V.P..L.P..G	.D....A...
PVY-O3E	NSR....P..P..G	.D....A...
PVY-US1N...	.N...T.P..	S...S..I.G	.D....A...
PVY-CH1S...T.P..P..G	.D.....T.
PVY-H1	-G.....NG
PVY-O1	NN.....P..	S...S..S.G	.D.....R.

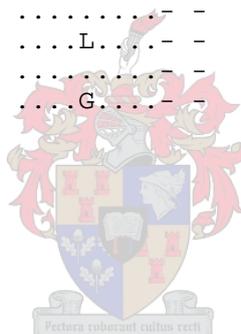
						120
PET7	KGATVLNLKH	LLEYAPQQID	ISNTRATQSQ	FDTWYEAVQP	AYDIGETEMP	TVMNGLMVWC
PVY-LbE.L	..V.....
PVY-N1E.L
PVY-N2E.LL
PVY-N3E.K..L
PVY-N5E.RM
PVY-O2E.RM
PVY-O3	..A...E.RLA...
PVY-US1	..A...E.RM
PVY-CH1E.RVS...
PVY-H1	..A.....L
PVY-O1	..VAA...E.RMQ...

						180
PET7	IENGTSPNIN	GVRVMMDGDE	QVEYPLKPIV	ENAKPTLRQI	MAHFSDVAEA	YIEMRNKKEP
PVY-LbW.....P...
PVY-N1W.....
PVY-N2W....N.
PVY-N3W.....
PVY-N5V.	..W....N.
PVY-O2V.	..W....N.
PVY-O3	.K.....V.	..W....N.S...
PVY-US1V.	..W....N.
PVY-CH1W....N.
PVY-H1W.....
PVY-O1W....N.

240

PET7	YMPRYGLIRN	LRDGLSLARYA	FDIFYEVTSTRT	PVRAREAHIQ	MKAAALKSAQ	SRLFGLDGGI
PVY-LbV..	P.....
PVY-N1V..
PVY-N2V..V..
PVY-N3V..
PVY-N5VG..	P.....
PVY-O2VG..	P.....
PVY-O3V..VGV..	P.....
PVY-US1MG..	P.....
PVY-CH1I..	P.....
PVY-H1V..
PVY-O1I..	P.....

PET7	STQEENTERH	TTEDVSPSMH	TLLGVKNM*G	S
PVY-Lb-	-
PVY-N1-	-
PVY-N2S..-	-
PVY-N3-	-
PVY-N5-	-
PVY-O2-	-
PVY-O3K.-	-
PVY-US1-	-
PVY-CH1L.-	-
PVY-H1-	-
PVY-O1G.-	-



Addendum 6

The protein sequence of the PLRV CP (LENCO1T7) cloned into pET-14b aligned against other PLRV CP sequences obtained from Genbank.

						60
LENCO1T7	MGTVVVKGNV	NGGVQQPRRR	RRQSLRRRAN	RVQPVVMVTA	PGQPRRRRRR	RGGNRRSRRT
PLRV-ROD	.S....R...	...I...K..
PLRV-1	.S.....	...AH.....
PLRV-11	.S.....
PLRV-CUB	.S.....
PLRV-GER	.S.....	S.....
PLRV-SCO	.S.....M.
PLRV-KOR	.S.L.....E.....
						120
LENCO1T7	GVPRGRGSSE	TFVFTKDNLV	GNSQGSFTFG	PSLSDCPAFK	DGILKAYHEY	KITSILLQFV
PLRV-ROD
PLRV-1M
PLRV-11M
PLRV-CUB
PLRV-GERM
PLRV-SCOT
PLRV-KORT
						180
LENCO1T7	SEASSTSAGS	IAYELDPHCK	ISSLQSYVNK	FQITKGGAKT	YQARMINGVE	WHDSSDQCR
PLRV-RODS..
PLRV-1S..	V.....I
PLRV-11S..	V.....
PLRV-CUBSD.	V.....
PLRV-GERS..	V.....
PLRV-SCOS..	V.....
PLRV-KORS..	V.....Q.....
LENCO1T7	ILWKGNGKSS	DPAGSFRVTI	KVALQNP*KH	MLEDPAANKA		
PLRV-ROD	R.....R.-	-----		
PLRV-1T.....	RL.....-	-----		
PLRV-11T.....	R.....-	-----		
PLRV-CUBT.....	R.....-	-----		
PLRV-GERT.....	R.....-	-----		
PLRV-SCOS.....-	-----		
PLRV-KORT.....	R.....-	-----		

