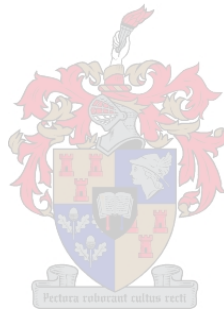# A study of genomic variation in and the development of detection techniques for Potato Virus Y in South Africa

Johan Christiaan Visser

Thesis presented in fulfillment of the requirements for the
degree of Master of Science (Biochemistry)
at the University of Stellenbosch

Supervisor: Prof. D. U. Bellstedt

Department of Biochemistry

University of Stellenbosch

March 2008

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) is responsible for considerable yield losses in the South African potato industry. The incidence of this virus has greatly increased over the past few years. Even more worrying is the variation of symptoms observed during PVY infection and the recent appearance of the more virulent PVY^{NTN} strain in local fields. This project aimed to investigate the possible genetic variation within the viral genome and to establish the origin of strains. The project also aimed to establish a dependable, area specific enzyme-linked immunosorbent assay (ELISA) to replace the currently used ELISAs. Currently seed potato certification is done using ELISA kits imported from Europe. These kits were developed for the detection of overseas variants of PVY and the use thereof in South Africa has in the past lead to false negatives. Finally, this project set out to develop, optimize and establish a sensitive and reliable real-time reverse transcriptase polymerase chain reaction (qRT-PCR) detection protocol for PVY.

In the first part of the study the coat protein (CP) gene of PVY isolates from plant material obtained from various parts of South Africa was amplified using RT-PCR. The resulting cDNA was then sequenced directly or cloned into a vector and then sequenced. The resulting sequences were aligned in a data matrix with international reference sequences, analyzed and grouped according to strain. Examination of the CP gene within this matrix as well as phylogenetic analysis revealed six main groups of PVY. These six groups included the traditional PVY^{N} and PVY^{O} groups and a recombinant group. Furthermore it also revealed variants of PVY^{N} and PVY^{O}. These mutants and recombinants pose a threat as they may lead to South African strains of PVY expressing coat proteins which vary from those found overseas. This may render the currently used European ELISA method of detection less effective and subsequently result in an increase in viral prevalence. This reinforced the need for a detection method based on local viral strains. Phylogenetic and Simplot analysis also confirmed that a recombinant strain between PVY^{N} and PVY^{O} had evolved and that PVY^{NTN} was such a recombinant.

The second part of the study aimed to develop and establish detection methods based on local variants of PVY. This included the development of ELISA and qRT-PCR detection methods of PVY. Previously amplified cDNA of the PVY CP gene was cloned into an expression vector and successfully expressed. Antibodies produced against the recombinant protein, when used in ELISA, however, failed to achieve the required levels of sensitivity. This prompted the development of qRT-PCR detection methods for PVY. Primer combinations for PVY were designed using the previously established CP gene data matrix. A reliable and sensitive SYBR® Green I based qRT-PCR assay was developed for the detection of PVY. The assay effectively detected all known South African variants of PVY. Furthermore, a Taqman® assay was developed for the detection of all variants of PVY. The Taqman® assay was 10 fold less sensitive and does not allow for amplicon verification through melting curve analysis, but it does add more specificity due to the addition of the probe. Although these qRT-PCR detection methods are still too expensive to replace the routine diagnostics done with ELISA, they do offer the opportunity to screen valuable mother material and confirm borderline cases in seed certification.

Opsomming

Aartappel virus Y (PVY) is verantwoordelik vir aansienlike opbrengsverliese in die Suid-Afrikaanse aartappelindustrie. Die insidensie van infeksie deur die virus het drasties toegeneem oor die afgelope jare. Wat egter meer kommerwekkend is, is die groter variasie in simptome van PVY infeksie en die onlangse voorkoms 'n meer virulente ras, PVY^{NTN}. Hierdie projek poog om moontlike genetiese variasie van PVY te ondersoek en om die oorsprong van rasse op te spoor. Die projek het ook gepoog ook om 'n bruikbare, betroubare en area spesifieke "enzyme-linked immunosorbent assay" (ELISA) toets te ontwikkel om die huidige ingevoerde ELISA te vervang. Hierdie toetse is ontwikkel om oorsese variante van PVY op te spoor en die gebruik daarvan het in die verlede gelei tot vals negatiewes. Verder is daar ook ondersoek ingestel na die ontwikkeling van 'n sensitiewe en betroubare "real-time reverse transcriptase polymerase chain reaction" (qRT-PCR) protokol vir die opsporing van PVY.

In die eerste deel van die studie is die mantelproteïen geen van PVY isolate vanuit plant materiaal geamplifiseer deur die gebruik van RT-PCR. Hierdie materiaal is vanaf verskeie streke in Suid-Afrika ontvang. 'n Volgordebepalingsreaksie is uitgevoer op gekloneerde of ongekloneerde cDNA verkry uit die RT-PCR. DNA volgordes is in 'n data matriks geplaas en vergelyk met internationale volgordes om die plaaslike isolate te analiseer en te groepeer. Deur vergelyking en filogenetiese ontleding kon ses hoofgroepe van PVY geïdentifiseer word, wat tradisionele PVY^N en PVY^O, sowel as 'n rekombinante ras en variante binne die tradisionele PVY^N en PVY^O groepe ingesluit het. Rekombinante en mutante kan veroorsaak dat Suid-Afrikanse rasse van PVY mantelproteïene uitdruk wat afwyk van die oorsese rasse wat tot gevolg mag hê dat die ELISAs van oorsee minder effektief kan wees en kan lei tot verhoogde virus voorkoms. Die realiteit en gevaar versterk die gedagte dat 'n deteksie metode gebaseer op plaaslike virusse absoluut krities is. Filogenetiese sowel as Simplot analise het bevestig dat 'n mutante ras tussen PVY^N en PVY^O ontstaan het en dat PVY^{NTN} 'n rekombinante ras is.

Die tweede deel van die studie was daarop gemik om deteksie metodes te ontwikkel wat gebaseer was op plaaslike variante van PVY. Dit sluit die ontwikkeling van ELISA sowel as qRT-PCR deteksie van PVY in. Voorheen geamplifiseerde cDNA is in 'n ekspressievektor gekloneer en suksesvol uitgedruk. Teenliggaampies teen die rekombinante proteïen, indien in ELISA aangewend, kon egter nie die nodige sensitiwiteit oplewer nie. Dit het aanleiding gegee tot ontwikkeling van qRT-PCR deteksie metodes. Inleier kombinasies vir PVY was ontwikkel deur die gebruik van die bestaande mantelproteïen geen data matrikse. 'n Betroubare en sensiewe SYBR$^®$ Green I qRT-PCR deteksie protokol was ontwikkel vir die effektiewe deteksie van alle bekende Suid-Afrikanse rasse van PVY. Verder is 'n sogenaamde "Taqman$^®$" protokol ook ontwikkel vir deteksie van alle rasse. Die "Taqman$^®$" protokol was 10 voudiglik minder gevoelig and laat nie bevestiging deur smeltkurwe analise toe nie, maar verleen meer spesifisiteit deur die toevoeging van die "Taqman$^®$ probe". Hierdie qRT-PCR deteksie metodes is tans te duur om as roetine diagnostiese toetse te gebruik en kan dus nie ELISA vervang nie, maar hulle bied wel die geleentheid om waardevolle moeder materiaal te toets en grensgevalle in aartappelsaad sertifisering te bevestig.

Acknowledgements

Prof. D.U. Bellstedt for affording me the opportunity to work under your guidance.

Coral de Villiers for making sure the laboratory runs smoothly and always having a caring word.

Dr. Annelise Botes for technical assistance.

Dr. Benny Bytebier and Margaret de Villiers for your help with the phylogenetic analysis.

Staff members and fellow students for assistance and invaluable input.

Potatoes South Africa for funding.

My parents, Johnny and Elna Visser, as well as family and friends for supporting me throughout my studies.

Torrin Theron for your love and support.

Abreviations

ARC-VOPI	Agricultural Research Council-Vegetable and Ornamental Plant Institute
CBS	Coen Bezuidenhout Seed Testing Center
CI	consistency index
CIb	cylindrical inclusion body
CIREs	cap-independent translation regulatory elements
CP	coat protein
DAS-ELISA	double antibody sandwich enzyme-linked immunosorbent assay
ELISA	enzyme-linked immunosorbent assay
G0	generation 0
HC-Pro	helper component proteinase
HSP70	heat shock protein 70
IRES	internal ribosome entry site
kDa	kiloDalton
NIa-Pro	nuclear inclusion protein a proteinase
NIb	nuclear inclusion protein b
NTR	non-translated region
P1	P1 protein
P3	P3 protein
PAUP	phylogenetic analysis using parsimony
PLRV	potato leaf roll virus
PTNRD	potato tuber necrotic ringspot disease
PVA	potato virus A
PVIP	viral genome-linked protein interacting protein
PVM	potato virus M
PVS	potato virus S
PVY	potato virus Y
PVX	potato virus X
qRT-PCR	real time reverse transcriptase polymerase chain reaction
RdRp	RNA dependant RNA polymerase
RI	retention index
RT-PCR	reverse transcriptase polymerase chain reaction
TSWV	tomato spotted wilt virus
VPg	viral genome-linked protein
5'-NTR	5'-non-translated region
6K1	6-kDa protein 1
6K2	6-kDa protein 2

TABLE OF CONTENTS

Chapter 1

Introduction

South Africa may only be responsible for the production of a mere 0.5% of the world's potatoes but the industry grosses in the order of R1.6 billion per year (Anonymous, 2007). This forms 43% of the total income generated by major vegetables in South Africa. There are approximately 1 700 potato farmers (of which 400 are seed growers) active in South Africa and these employ a total of about 66 600 farm workers (Anonymous, 2007). These farmers utilize approximately 55 600 hectares of land for potato cultivation. On average 157.8 million 10 kg pockets are produced per year and in the region of 20.5 million of these pockets are earmarked for use as seed potatoes (Anonymous, 2007). The majority of this is produced in the Sandveld region located in the Western Cape Province. The Sandveld is responsible for production of seed potatoes as well as a large quantity of potatoes for human consumption. Currently a substantial percentage of potatoes produced by the South African potato producer is infected by various pathogens. This has a negative impact on the quality and quantity of potatoes and thus results in a substantial loss of income.

Two of the most important plant viruses affecting potato production in South Africa are *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV). PVY infection of potato plants results in a variety of symptoms depending on the strain present in the plant tissue. The mildest of these symptoms is production loss but the most detrimental of these symptoms is what is referred to as potato tuber necrotic ringspot disease (PTNRD). Necrotic ringspots render potatoes unmarketable and can therefore result in a significant loss of income. PLRV infection of potato plants causes starch retention in leaves of the plant which in turn results in smaller tuber formation (Rich, 1983). Both these viruses are transmissible by aphid vectors but may also remain dormant in seed potatoes. This means that using the same line of potato for production of seed potatoes for several consecutive generations will lead to a progressive increase in viral load and subsequent loss of crop. An increase in potato plant infection with viruses over the past few years has lead to considerable losses to the South African potato industry. The increased rate of infection may be attributed to several factors. These include a marked decrease in the effectiveness and administration of chemicals used in vector control, the use of infected seed potatoes in cultivation, incorrect irrigation and farming methods as well as a lack of a sensitive, rapid and reliable method of detection (Coetsee, 2005). An increase in the average temperature of winters as a consequence of global warming has also lead to an increase in aphid numbers, which in turn has lead to an increase in viral distribution (Coetsee, 2005).

The increase of infection has prompted an intensified search for more reliable and sensitive methods of viral detection as these are crucial in the process of certifying potatoes as disease free and thus the successful management of the prevalence of potato plant pathogens. There are five laboratories for seed certification in South Africa: the Coen Bezuidenhout Seed Testing Center (Pretoria), the Piketberg Seed Testing Center (Piketberg), the Western Free State Seed Testing Center (Christiana), the Kwazulu Natal Seed Testing Center (Pietermaritzburg) and Northern Cape Laboratory services (Douglas). The Coen Bezuidenhout Seed Testing Centre is central to these test centers and acts as a controlling laboratory for

seed testing and pathogen screening (Anonymous, 2007). These laboratories test seed potatoes for *Potato virus Y* (PVY), *Potato leaf roll virus* (PLRV), *Potato virus X* (PVX), *Potato virus M* (PVM), *Potato virus A* (PVA), *Potato virus S* (PVS), *Tomato spotted wilt virus* (TSWV) as well as bacterial wilt (*Ralstonia solanacearum*) and *Pectobacterium* (*Pectobacterium carotovora* subsp. *carotovora*, *Pectobacterium carotovora* subsp. *atroseptica* and *Pectobacterium chrysanthemi*) infection. Newly certified potatoes, in most instances originating from tissue culture material, are categorized as Generation 0 (G0). For each field reproduction a generation is added and the resulting potatoes are tested for the above mentioned pathogens. Potatoes up to G8 may be used as seed potatoes, after which the line is terminated. Subsequent to certification, each batch of seed potatoes are graded based on the results of a variety of screenings. These include the degree of infection with the above mentioned pathogens but also black dot (*Colletotrichum coccodes*), silver scurf (*Helminthosporium solani*) and a host of other minor potato pathogens. There are three different grades in which seed potatoes may fall. These are: Elite, Grade 1 and Standard grade. Both Elite and Grade 1 potatoes are acceptable for use as seed potatoes while the Standard grade potatoes cannot be utilized as seed potatoes.

Currently imported enzyme-linked immunosorbent assay (ELISA) kits are used for routine viral detection in seed potato screening and certification. These kits are based on the detection of viral CP in potato plant material. A preliminary study showed that there was genetic variation in SA strains of PVY (Visser *et al.,* unpublished). The current study had two objectives: the first was to determine the extent to which genetic diversity occurs within South African strains of PVY. The second was to develop detection protocols for PVY. To this end recombinant PVY CP was expressed in *Escherichia coli* for production of anti-PVY antibodies which would subsequently be used in ELISA detection. Additionally, rapid and sensitive real-time reverse transcriptase polymerase chain reaction (qRT-PCR) detection protocols were also developed. The sequence data matrices generated during the first stage of the project were used for primer development for qRT-PCR for PVY detection.

A brief description of the molecular composition of PVY, transmission thereof, the consequences of infection as well as recombination within the PVY CP gene is given in Chapter 2 of this thesis. In this chapter background information on the molecular methods for the detection PVY, which were utilized in this project, are outlined. These include the tried and tested workhorse, the ELISA, as well as more recent techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) and the qRT-PCR. In Chapter 3 the methodology used in the generation of viral CP gene sequences as well as the phylogenetic analysis thereof is described. The molecular variability and genetic recombination patterns within PVY is also discussed. Immunological and PCR-based techniques developed and used for the detection of South African strains of PVY are described in Chapter 4. These techniques include detection of PVY using ELISA and qRT-PCR. This is followed by a conclusion and future intentions chapter and an addendum containing the nucleotide sequences of all of the PVY CP genes sequenced in this project and used for primer development.

References

Anonymous (2007). Available: http://www.potatoes.co.za (Access: Continuous).

Coetsee, J. (2005). Virusse bedreig hele aartappelbedryf, Landbouweekblad, **61637**: 44-45.

Visser, J.C., Rothmann, A.H. and Bellstedt, D.U. (Unpublished). An assessment of recombination patterns in South African strains of potato virus Y (PVY). Honours thesis.

Chapter 2

Molecular nature and detection of Potato Virus Y

2.1   Pathology, transmission and molecular biology of Potato Virus Y

2.1.1   PVY hosts, strains and symptoms

Potato virus Y (PVY) belongs to the potyvirus genus. The potyvirus genus is currently the largest of the plant virus groups and is thought to be the one of the most destructive families of plant viruses affecting potato crops (Ward and Shukla, 1991). The genus includes more than 200 members that bring about significant losses in the agricultural arena (Jawaid *et al.*, 2002). PVY infects many economically important plant species. These include potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill) and pepper (*Capsicum frutescens* L.) (McDonald and Singh, 1996). The level of damage to crop is determined by the strain of PVY infecting the plants, the viral load, the time at which infection occurs as well as the tolerance the host possesses toward the virus (Warren *et al.*, 2005). Resistance to PVY infection by hosts is low in many cases. Infection of a potato field with PVY may ultimately result in 10-100% loss in yield (Warren *et al.*, 2005).

It has been shown that the PVY has different isolates according to the symptoms they induce in various potato plant species (Delgado-Sanchez and Grogan, 1970). Extensive biological, serological and molecular variability of PVY isolates makes the classification of isolates as particular strains particularly difficult. Occurrence of a variety of symptoms and the emergence of the necrotic PVY[NTN] has lead to a search for more reliable classification tools than simple serological identification.

Traditionally three chief strains of PVY are recognized: PVY[C], PVY[N] and PVY[O]. PVY[C], originally known as Potato Virus C, was the first to be recognized and was identified in the 1930s (Salaman, 1930 as referred to in Jawaid *et al.*, 2002). PVY[C] induces hypersensitive reactions in a wide range of potato cultivars. These reactions include the formation of mild mosaic patterns or stipple streak. Unlike the other strains of PVY, some PVY[C] strains are non-aphid transmissible (Blanco-Urgoiti *et al.*, 1998). Previous studies by Visser *et al.* (Unpublished) did not identify any of the local isolates as being PVY[C] but it has been reported to occur to in South Africa (Brunt, 2001; De Bokx, 1981). A second strain of PVY is PVY[N] (Smith and Dennis, 1940, as referred to in Jawaid *et al.*, 2002). This strain was described in tobacco plants growing close to potato plants (Crosslin *et al.*, 2005). PVY[N] results in leaf necrosis and mild or even no damage to the tubers (figure 1a). The ordinary strain of PVY is denoted as PVY[O]. Infection of a potato plant with the PVY[O] strain results in mild tuber damage and does not cause leaf necrosis (Boonham *et al.*, 2002). Both PVY[N] and PVY[O] are aphid transmissible and occur in South Africa. In Europe these two strains have been shown to have recombined to form PVY[NTN] (Boonham *et al.*, 2002; Lorenzen *et al.*, 2006). The PVY[NTN] has been accredited with the ability to induce potato tuber necrotic ringspot disease (PTNRD) (figure 1b) (Boonham *et al.*, 2002). Tubers damaged by PTNRD become unmarketable and infection by PVY[NTN] thus results in a larger economic impact than infection by the other strains.
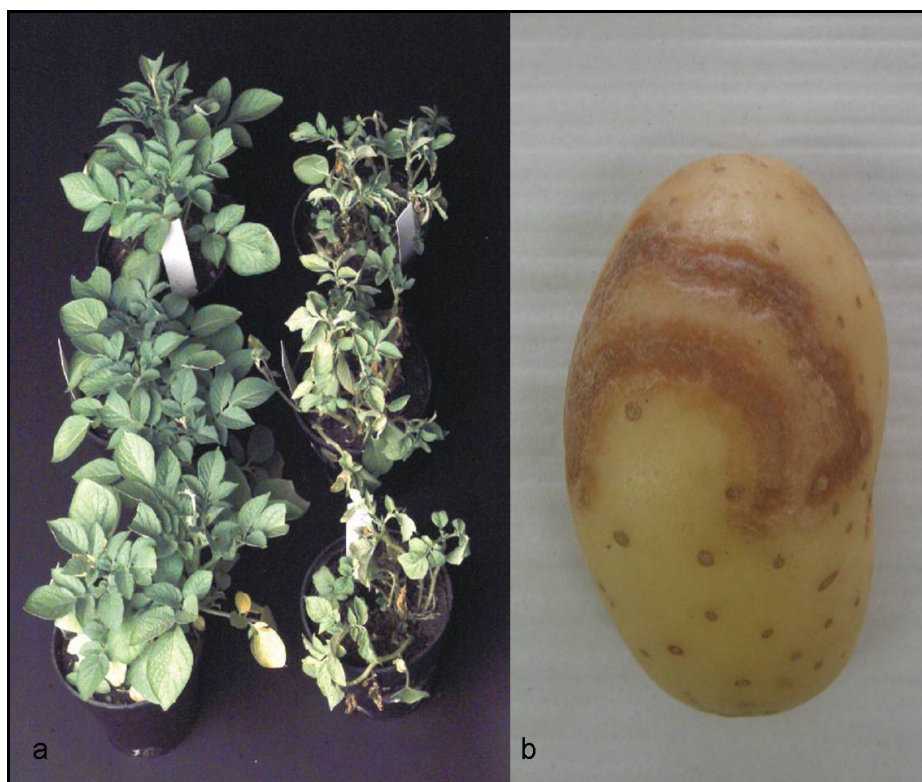
Figure 1. (a) Effect of PVYN and PVYO infected potato plants (right) in comparison to healthy plants (left). (b) PVY^{NTN} infection resulting in the formation of necrotic ringspots on tubers.

2.1.2   PVY transmission

PVY may be transmitted to potato plants through grafting, plant sap inoculation and through aphid transmission. The most common manner of PVY infection of plant material in the field is through the aphid and although aphids on their own can directly damage potato plants it is their role as viral vectors which has the greatest economic impact (Halbert *et al.*, 2003; Radcliffe and Ragsdale, 2002; Radcliffe, 1982). In cold climates aphids spend the winter either as wingless aphids giving birth to live young (viviparae) or as eggs. Hosts such as weeds and other crops serve as breeding grounds for these aphids and form a temporary area of colonization before the aphids migrate to the potato fields (Radcliffe and Ragsdale, 2002). In moderate climates, such as in South Africa, aphids are thought to reproduce asexually on weeds, other crops, indigenous plants and garden plants. This means that there are a number of aphids present year-round. The importance in effective and stringent monitoring of aphid populations is stressed in a review by Radcliffe and Ragsdale (2002) as PVY virions are introduced to potato fields almost solely by winged aphids from a virus source outside these fields. Wingless aphids have not yet been linked to the spread of PVY in potato fields (Ragsdale *et al.*, 1994).

The green peach aphid, *Myzus persicae*, has been found to be most effective in its role as viral vector (Halbert *et al.*, 2003; Van Hoof, 1980; Warren, 2005), but others such as *Aphis fabae, Aphis gossypii, Aphis nasturtii, Macrosipihum euphorbiae, Myzus (Nectarosiphon) certus, Myzus (Phorodon) humuli* and *Rhopalosiphum insertum* are also strongly associated with viral transmission (Halbert *et al.*, 2003; Van Hoof, 1980). The Agricultural Research Council-Vegetable and Ornamental Plant Institute (ARC-VOPI)

of South Africa identified twenty five species of aphid able to function as PVY vectors (Thompson, 1997). The efficiencies of some of these aphids to function as PVY vectors were also established (Ragsdale *et al.*, 2001) and were found to vary between the different species. In South Africa *Aphis fabae, Aphis gossypii* and *Aphis nasturtii* are the most common and efficient PVY vectors found in the field (Warren, 2005). Apart from being classed according to efficiency as vectors, aphids can also be divided into two subgroups, namely colonizing and non-colonizing species. Colonizing aphids are aphids which reproduce and establish themselves on potato plants, specifically, while non-colonizing aphids do not reproduce nor establish colonies on potato plants. Colonizing aphids are better adapted to life on potato plants and are thus generally considered as better PVY vectors than non-colonizing aphids. Non-colonizing aphids do not primarily feed on potato plants but do occasionally feed on them while searching for a more suitable host. Their lower efficiency as PVY vector is cancelled out by the sheer numbers in which they occur (Radcliffe, 1982; Robert *et al.,* 2000). Because of this, all aphids present in and around potato fields must be considered as possible vectors and their numbers carefully monitored.

Transmission of PVY by aphids occurs in a non-persistent, non-circulative manner which suggests a less intimate interaction between virion and vector than is the case of circulative virions (Gray, 1996). The fact that the virions are transmitted in a non-persistent fashion means that viral replication does not occur within the aphid vector and that, unless the aphid feeds on infected plants, it loses its ability to infect plants after two to three feedings (Bradley, 1953; Warren, 2005). The virions attach to the aphid stylet in a matter of seconds and may remain infectious for four to seventeen hours (Harrison, 1984; Kostiw, 1975). The distance over which the virions can be transmitted is limited due to the short period for which they remain infectious (Robert *et al.*, 2000). Although the short life span outside plants inhibits long distance viral transmission it does not reduce the transmission efficiency bestowed by the quick rate of viral acquisition and inoculation within a field.

2.1.3   Molecular description of PVY

Potyvirus virions consist of non-enveloped filamentous structures that are 680 – 900 nm in length and 11 to 15 nm in width (figure 2) (Edwardson, 1947, as referred to in Jawaid *et al.*, 2002). Morphologically the potyvirus consists of approximately 2 000 copies of coat protein (CP) which forms a cylindrical inclusion body (CIb) (Talbot, 2004). The CIb is considered to be the single most important phenotypic criterion for distinguishing a potyvirus from other virus groups.

Figure 2. An electron micrograph of PVY virions (Brunt *et al.*, 1996).

The CIb encapsulates a single strand of positive sense RNA which is in the order of 10 kb in length and has a nontranslated 5'-terminal region (5'-NTR) as well as a 3'-poly-A tail (Dougherty and Carrington, 1988; Van der Vlugt *et al.*, 1989). The positive sense genome contains a single extended open reading frame and acts directly as mRNA (figure 3). The 144 nucleotide 5'-NTR is particularly rich in adenine residues and has very few guanine residues. Rather than a conventional cap structure the 5'NTR is associated with a Viral genome linked protein (VPg) which is said to act as an enhancer of transcription (Carrington and Freed, 1990).
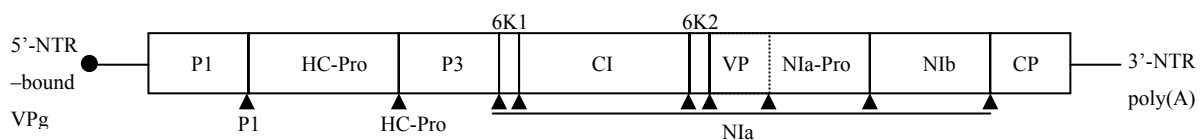
Figure 3. A schematic representation of the PVY genome illustrating the areas of enzymatic cleavage of the various arts of the polyprotein (Kennedy *et al.*, 1962).

The 5'-leader sequence has an internal ribosome entry site (IRES) and cap-independent translation regulatory elements (CIREs) (Dallaire *et al.*, 1994; Niepel and Gallie, 1999). The IRES directs cap-independent translation through a mechanism similar to that used by eukaryotes (Niepel *et al.*, 1999). The extended open reading frame encodes for a 350 kDa polyprotein (Dougherty and Carrington, 1988). This polyprotein is proteolytically processed by viral proteases (NIa, HC-Pro and P1) and undergoes co- and posttranslational cleavage to yield several multi-functional proteins. These include the following: P1 (P1 Protein), HC-Pro (Helper Component Proteinase), P3 (P3 Protein), 6K1 (6-kDa Protein 1), CIb (Cylindrical Inclusion body), 6K2 (6-kDa Protein 2), VPg (Viral Genome-linked Protein), NIa-Pro (Nuclear Inclusion Protein a, Proteinase domain), NIb (Nuclear Inclusion Protein b) and the CP (Coat Protein) (Talbot, 2004; Dougherty and Carrington, 1988).

The functions of these proteins have been studied in *in vitro* systems as well as in *Escherichia coli*. P1 is a proteinase which is derived from the N-terminal region of the potyvirus polyprotein and is the least conserved of all the potyvirus proteins. The P1 proteinase is similar to chymotrypsin-like serine proteinases and has an enzymatic triad consisting of Histidine-214, Asparagine-223 and Serine-256. This enzymatic site cleaves the polyprotein at the P1 C-termini (Jawaid *et al.*, 2002). It has been shown that proteolytic separation of P1 from HC-Pro is essential for viral infectivity of plants. This may be attributed to the RNA silencing suppressive properties of HC-Pro rather than the proteolytic activity of P1 in isolation. The P1 protein may thus also be described as an accessory factor for the suppression of RNA silencing (Pruss *et al.*, 1997). It may also be involved in virion replication and cell-to-cell transport of virions, however, extensive research has failed to prove either one of these proposed functions. The HC-Pro protein is the second of three proteinases and cleaves the polyprotein in an autocatalytic manner at a Glycine-Glycine bond at its own C-termini. It has also been shown that the HC-Pro protein promotes cell-to-cell as well as vascular movement (Cronin *et al.*, 1995; Rojas *et al.*, 1997). The protein also suppresses RNA silencers (Anandalakshmi *et al.*, 1998), partakes in aphid transmission (Maia *et al.*, 1996) and interacts with a calmodulin-like protein which in turn assists in the suppression of posttranscriptional gene silencing (Maia *et al.*, 1996). The exact function of the P3 protein remains unknown although some studies suggest a possible role in regulating the proteolytic processing of the viral polyprotein as well as viral RNA replication (Jawaid *et al.*, 2002). As in the case of the P3 protein, the exact role of 6K1 protein remains unclear but it is thought to play a role in virion RNA replication and serve as molecular anchor for virion amplification machinery. In a recent study it was proposed that 6K1 may be involved in cell-to-cell movement of the virions. The CI protein has a helicase function (Laín *et al.*, 1990) and also assists in cell-to-cell movement (Carrington *et al.*, 1998). The 6K2 protein is associated with vascular movement (Rajamaki and Valkonen, 1999). VPg partakes in vascular as well as cell-to-cell movement (Rajamaki and Valkonen, 1999; Schaad *et al.*, 1997) but also interacts with the translation initiation factors eIF4E and eIF(iso)4E (Wittman *et al.*, 1997). It also interacts with the plant protein PVIP (VPg-interacting protein) which in turn enhances viral movement through the plant (Dunoyer *et al.*, 2004). NIa-Pro acts as a proteinase which is responsible for proteolytic cleavage of a large portion of the polyprotein. It cleaves the potyviral polyprotein at seven different sites (P3/6K1, 6K1/CI, CI/6K2, 6K2/VPg, VPg/NIa-Pro, NIa-Pro/NIb and Nib/CP). NIa-Pro cleaves the polyprotein in a *cis-* and *trans*-manner, but *cis*-cleavages are more prevalent. The rate at which the polyprotein is cleaved can be regulated, thus providing an indirect control over potyviral protein activity. NIb is a RNA-dependant RNA polymerase and interacts with the poly-A binding protein (Wang *et al.*, 2000). CP encloses the viral RNA, assists in vascular as well as cell-to-cell movement (Dolja *et al.*, 1994, 1995) and is involved in aphid transmission (Atreya *et al.*, 1990). The 3'NTR plays a role in symptom induction.

The HC-Pro and CP components of PVY are thought to play a crucial role in aphid assisted viral transmission (Kassanis and Govier, 1971). HC-Pro, in its active form, is a soluble homodimer which is approximately 100 kDa in size (Blanc, 1997). According to the hypothesized bridge theory the HC-Pro protein binds both to the aphid stylet as well as the viral CP thereby temporarily binding the viral particle

to the stylet (Kassanis and Govier, 1971). Electron microscope images have shown HC-Pro associated with both virions as well as the stylet of the aphid vector to support the theory. Site directed mutagenesis studies have revealed two highly conserved motifs within the central and N-terminal portion of the viral HC-Pro protein which affect the transmissibility of PVY. These motifs are the Proline-Threonine-Cysteine (PTK) motif (amino acid numbers 655-657, GenBank reference M95491) as well as the Lysine-Isoleucine-Threonine-Cysteine (KITC) motif (amino acid numbers 397-400, GenBank reference M95491) (Atreya *et al.*, 1990, 1991; Granier *et al.*, 1993). HC-Pro is theorized to interact with the Aspartic acid-Alanine-Glycine (DAG) (amino acid numbers 2863 - 2865, GenBank reference M95491) motif of the CP (Atreya *et al.*, 1990, 1991; Granier *et al.*, 1993). The DAG motif is situated in a conserved region near the end of the CP N-terminal and is located on the surface of an assembled virion (Shukla and Ward, 1989). Pure extracts of PVY particles were found to be unable to infect plants while extracts containing both the HC-Pro component as well as the viral particles caused infection (Govier and Kassanis, 1974a, 1974b; Pirone and Blanc, 1996). Past studies revealed that mutations within the HC-Pro PTK and/or KITC motifs or in and around the CP DAG motif result in a decrease in transmissibility as well as the overall virulence (Atreya *et al.,* 1991, 1995).

To investigate the influence of a mutation within the KITC motif the HC-Pro of a natural occurring variant of PVY, PVY^C, was compared to the wild type. The KITC domain of the HC-Pro gene of PVY^C carries a mutation which causes a lysine to be substituted with a glutamic acid residue. Varying concentrations of the mutant HC-Pro were dot-blotted onto nitrocellulose membranes. Nonspecific binding to these membranes was blocked and subsequently incubated in a solution containing purified PVY virions. Probing the membrane with anti-PVY antibodies confirmed an interaction between the HC-Pro and the virion particle. This provided evidence that a mutation within the KITC motif does not influence interaction between the HC-Pro and the virion. To investigate the influence of this mutation on interaction between the aphid stylet and the HC-Pro, aphids were allowed to ingest a mixture containing virions as well as either PVY^O HC-Pro or PVY^C HC-Pro. The stylets were dissected and probed using anti-HC-Pro antibodies. Sixty percent of the aphids exposed to the wildtype HC-Pro were infected while none of the aphids exposed to the mutant HC-Pro demonstrated any interaction. These results indicate that the KITC motif within the HC-Pro plays a crucial role in attachment to the aphid stylet rather than to the virion. It also demonstrates that a highly basic residue such as lysine or arginine is required at amino acid position 307 in the HC-Pro gene for virus transmission to take place successfully.

To study the effect of mutations within the PTK motif several HC-Pro mutants were created and their ability to assist in PVY transmissibility tested. Substituting the lysine with a glutamic acid in the PTK motif lead to a 40% reduction in transmissibility whilst substituting the proline with an alanine lead to a complete loss of transmissibility (Peng, 1998). It is thought that this motif interacts with the DAG motif located in the CP. Mutations within the DAG motif lead to a decrease in transmissibility. These mutational studies proved that the interactions between the HC-Pro and the CP on the one hand and the HC-Pro and the aphid stylet on the other hand are crucial for virus transmission.

Upon entrance into the plant cell, the virus coat protein disassembles and releases its RNA genome. The viral RNA serves as mRNA and although little is known about the translation thereof, it is believed that the 5' non-coding region functions as an enhancer of translation (Carrington and Freed, 1980). The translated mRNA results in a polyprotein which is processed into mature proteins. Each polyprotein is then cleaved into ten different proteins which are believed to be multifunctional. These proteins, along with host proteins, assemble to form a replication complex. This complex performs negative-strand RNA synthesis, using the positive strand of viral RNA as a template. Once the additional RNA copies have been produced, they code for the synthesis of various proteins, as mentioned before, as well as coat proteins. These coat proteins will now enclose the newly formed genomes to give rise to new virions. It has been suggested that enclosure of the newly formed virions is initiated by the interaction of the coat proteins with the 5'terminus and that the coat protein is built up towards the 3'terminus (Wu and Shaw, 1998). The entire process of viral replication occurs within the endoplasmic reticulum. These newly synthesized viral particles are subsequently transported through the plasmodesmata to adjacent plant cells via several assisting potyvirus proteins. Distribution of viruses within the plant occurs according to the source-sink relationship between maturing- and growing tissues (Talbot, 2004). Virus concentration throughout the plant is high and this greatly increases the chance of uptake by aphids. Infection of plants by potyviruses can be varied in the symptoms shown. Infection can include veinal necrosis, mosaic symptoms as well as leaf malformation (Boonham *et al.*, 2002). Infected plants that do not show symptoms may have infected canopies and will yield lower quality products than their healthy counterparts. The viral defense mechanisms of plants will primarily try to restrict the movement of the virus. In failing this, it may attempt to induce cell death in infected tissue, thereby preventing the spread of virions (Bagnall and Bradley, 1958). Although the precise mechanism of disease induction by potyviruses in plants is unknown, it is known that these viruses cause a significant shutdown of host gene expression during viral replication (Bushell and Sarnow, 2002; Pompe-Novak *et al*., 2006). A disruption in gene expression disrupts the normal cellular function of cells. This could very well be the cause of the physical symptoms that the plant demonstrates.

2.1.4   Genetic variation and recombination within PVY

In the past most emphasis has been placed on studying and understanding evolution of RNA viruses within animal cells. These studies focused chiefly on studying human pathogenic viruses. In recent times research into plant virus evolution has intensified. Research on plant cell viruses has the advantage that an unlimited number of genetically identical hosts, or clones, can be produced without the difficulties brought about in animal cell tissue cultures (Roossinck, 2003). Using plant cells also helps avoid the ethical issues and concerns accompanying animal cell studies. The major driving force behind plant cell based viral studies may, however, rather be because of concerns brought about by the appearance of new viral strains as well as plant viruses overcoming previously established viral resistance in plants. New strains and newly acquired resistance may be caused by mutations, inversions, deletions, insertions or recombination in the genetic material of traditional viral strains.

A large number of factors may contribute to the emergence of new viral strains. These include an expanding range of hosts and vectors, climate changes, high throughput farming methods as well as a general increase in global human population and an increase in the mobility of these populations. Recombination and mutation are the evolutionary reasons for strain development and adaptation. Since the RNA dependent RNA polymerase (RdRp) of RNA virions lack proof-reading capability the rate of mutation within these types of virions is high (Domingo and Holland, 1997; Roossinck, 1997). Mutations such as inversions, deletions and insertions are common and may ultimately generate novel virus strains. Genetic evolution could lead to a change in the physical properties of virions. An example of a physical change would be a recombination or series of mutations within the CP gene. This would consequently cause an alteration of the CP. This may have major implications for immuno-based detection of virions. Genetic recombination and mutations may also trigger a change in the physical response of a plant to the infection. The PTNRD strain of PVY, PVY^{NTN}, is thought to have originated from recombination between the PVY^N and PVY^O strains. Cluster analysis show that PVY^{NTN} is categorized in the same cluster as PVY^N when the 5'-region of the CP gene and 5'-NTR are considered while the recombinant is clustered with PVY^O when the 3'-region of the CP gene and 3'-NTR are considered (Boonham *et al.*, 2002; Jawaid *et al.*, 2002). By comparing the sequences, it was discovered that the CP N-terminal and core regions of PVY^{NTN} concurred with those found in PVY^N (Revers *et al.*, 1996). However, it was found that PVY^{NTN} isolates cluster with PVY^O upon comparison of the CP gene and the 3'-NTR region (Boonham *et al.*, 2002). Restriction fragment length polymorphism (RFLP) analyses showed that there were other recombination events within PVY^{NTN} as well. When considering the 5'-NTR region up to the HC-Pro gene as well as the sequence from the NIa gene up to nearly the first 50% of the CP gene, the PVY^{NTN} sequence closely resembles that of PVY^N. Conversely, the PVY^{NTN} sequence correlates to that of the PVY^O from P3 up to the 6K2 gene and from the furthest part of the middle of the CP gene up to the 3'-extremity (Boonham *et al.*, 2002, Unpublished data as referred to in Jawaid *et al.*, 2002). The recombination event is also graphically illustrated when the two parent strains, PVY^N and PVY^O, are compared to the suspected recombinant using similarity plot analysis (Lorenzen *et al.*, 2006). Data acquired by Lorenzen *et al.* (2006) shows that there is a clear recombination event within the PVY CP gene at base pair 608 of the CP gene and base pair 9170 when the entire genome is considered (GenBank reference AY884982). At this point in the gene the nucleotide sequence changes from being similar to PVY^N to being similar to PVY^O.

Studies also illustrate that a further two recombination events can occur within the PVY genome (Lorenzen *et al.*, 2006). The first occurs between the HC-Pro and P3 gene at 2412 bp (GenBank reference AY884982) and the second between 6K2 and NIa genes at 5897 bp (GenBank reference AY884982) (Glais *et al.*, 2002). PVY^{NTN} resembles PVY^N in the gene region 5'-NTR to HC-Pro and from the NIa to the middle of the CP gene. The gene regions from P3 up to 6K2 and the 3'-region of the CP gene up to the end of the genome is PVY^O related.

2.2 Diagnostic techniques for detection of Potato Virus Y

2.2.1 ELISA

In the past, crops were inspected visually to determine whether or not they were disease free. Visual inspection was also used as a basis for seed certification. Determination of viral status through visual inspection is incredibly difficult as the symptoms may be masked or the infection latent (Robert *et al.*, 2000). As a result, post season tests and inspections were introduced. These tests involved the cultivation of previously harvested material in greenhouses. The resulting plants were inspected for a more accurate estimate of viral status. Although this method of screening did offer some degree of monitoring of viral presence it was subjective and highly ineffective. Enzyme-linked immunosorbent assay (ELISA) screening of crops and seed potatoes replaced visual inspection in the early 1970s. The use of ELISA offered routine diagnostic laboratories a quick, effective and sensitive method of screening for a wide range of potato plant viruses.

Detection of pathogens using ELISA relies on the interaction between the antigen and specific antibodies and has become a popular and cost-effective means of routine detection. In an ELISA the solid phase can be coated with the sample of interest containing the antigen (Tijssen, 1985). The efficiency to which the antigen binds to the solid phase is dependant on temperature, length of exposure as well as concentration (Tijssen, 1985). Solid phases used include nitrocellulose membranes, paper, glass, agarose and polystyrene or polyvinylchloride microtiter plates. Microtiter plates are the most widely used solid phase due to the fact that they are easy to handle, allow for automation and for analysis using microtiter plate readers. A drawback of these plates is that they are highly absorptive and this increases the incidence of non-specific binding of components used in the ELISA. Non-specific binding to the plates is reduced through the use of buffers containing proteins such as casein and non-ionic detergents such as Tween 20. After coating, excess sample is removed and the plate typically treated with a 1% casein containing solution. Subsequent to this the solid phase is treated with antibodies raised against the antigen of interest. After each incubation step the plate is washed with Tween 20 containing PBS. These washing steps are aimed to wash away any non-specifically bound components (Wilson and Walker, 2000). Non-specifically bound components are less strongly bound than the specific bound ones. Detection is achieved either through the addition of an enzyme-coupled antibody or the addition and detection of a biotinylated antibody. In a system using an enzyme-coupled antibody the subsequent addition of an appropriate substrate results in the formation of a colour proportional to the amount of antigen (Wilson and Walker, 2000). Alternatively the plate can be coated with antibody followed by incubation with the sample that is to be detected. This, in turn, can be detected as described above and is then referred to as the double antibody sandwich (DAS) ELISA. Both of these systems, however, have a disadvantage in that coupling of the enzyme to the antibody may result in stearic hindrance which in turn may result in a loss in function of the antibody and/or the enzyme (Blake and Gould, 1984). This may be overcome through the use of a biotin-avidin or biotin-streptavidin bridge. In this type of system biotin is coupled to the antibody. The biotin molecule has no influence on the working of the antibodies and is easily detected

using avidin or streptavidin conjugated to a suitable enzyme. Streptavidin has an extremely high affinity for biotin which results in even a higher degree of specificity than a system in which the enzyme is coupled directly the antigen. To establish whether or not the antigen is present, a substrate specific for the enzyme used is added. The enzyme then converts the substrate to a coloured product and the colour intensity can be correlated to the amount of antibodies bound and thus the amount of antigen present. A DAS-ELISA has the advantage that it can increase the specificity of the ELISA and reduce the occurrence of non-specific binding. As a result the DAS-ELISA principle is commonly employed in ELISA's for the detection of plant pathogens in plant sap without prior purification of the pathogen.

The ELISA is considered to be a safe, inexpensive and rapid method for detection of plant viruses. The inexpensive nature and relative simplicity thereof allows for it to be used as a workhorse within the agricultural sector and is used to screen thousands of samples per year. Unfortunately ELISAs are not completely failsafe. Virus levels within potato tubers, which are screened by ELISA for use as seed potatoes, are normally low while the tubers are dormant. ELISA detection of viruses in these potatoes is difficult and absorbance values may fall below the set cut-off value. For this reason, seed tuber screening is performed on sprouting rather than dormant tubers. Although this results in more reliable readings than direct tuber testing, it does delay the certification of seed potatoes (Gugerli and Gehringer, 1980). Another disadvantage of an immuno-based detection method is that changes at the gene level may have an influence on the immunogenicity of the antigen to be detected. In terms of potato plant viruses, mutations within the CP gene may cause the CP to undergo conformational changes rendering antibodies produced against the previously present virus less effective.

2.2.2   Reverse transcriptase PCR

RT-PCR has become a powerful and effective method for detection of potato plant viruses within potato plant material and even dormant potatoes. Only a minute piece of plant material is required for analysis using RT-PCR. Considering the protocol described within this thesis, 0.1 g of plant material is enough for 14 500 separate reactions. During a RT-PCR specific target RNA sequences are amplified exponentially into DNA copies. For this to occur, however, the RNA of the virus must first be transcribed to DNA by means of a reverse transcriptase polymerase. This polymerase synthesizes a DNA strand using the RNA as template. This results in a DNA/RNA complex. For synthesis of a DNA strand from the RNA template only the reverse primer is required since the RNA is a single strand arranged from 5' to 3'. Subsequently the newly synthesized DNA strand is used as a template for traditional PCR.

Different types of reverse transcriptase polymerases are available to suite different needs and reaction conditions. Reverse transcriptase enzymes commonly used include AMV RT, SuperScript™ III, ImProm-II™, Omniscript, Sensiscript and *Tth* RT. At the end of the RT step the polymerase enzyme is heat-activated. It could also be that the reverse transcriptase polymerase and DNA polymerase is one and the same enzyme and that the enzyme only requires a DNA polymerase activation step after the RT step. An example of such an enzyme is *Tth* polymerase. This enzyme has both RNA-dependent reverse transcriptase and DNA-dependent polymerase activity. However, the active center of the DNA

polymerase is covered by dedicated oligonucleotides, called Aptamers. At temperatures below the optimal reaction temperature of the DNA-dependent polymerase component of *Tth* remains covered by the Aptamers. At these temperatures the *Tth* enzyme only synthesizes a DNA copy of the RNA template. Once the reaction temperature is raised to 95°C, the Aptamers are removed and the DNA-dependent polymerase component will start to amplify the target sequence.

PCR amplification of the DNA target occurs in three steps: denaturation, annealing and extension (Wilson and Walker, 2000). Each of theses steps occur at a specific temperature for a fixed period of time. Denaturation is normally allowed to occur between 90 and 95°C and leads to the dissociation of DNA strands. After this the reaction is cooled to between 40 and 70°C to allow the primers to associate with their respective target sequences. This step is known as the annealing step and is primer specific. The temperature at which the primers anneal is critical. Too high temperatures would not allow the primers to associate with the DNA, resulting in no or poor amplification. Too low annealing temperature would ultimately lead to non-specific binding of the primers and non-specific amplification (Wilson and Walker, 2000). Primers bound to the regions flanking the target DNA provide 3'-hydroxyl groups for DNA polymerase catalyzed extension. The most commonly used DNA polymerase is *Taq*, a thermo-stable enzyme isolated from the thermophilic bacterium, *Thermus aquaticus* (Chein *et al.*, 1976). The DNA polymerase synthesizes new DNA strands along the template strands, using the primers as starting points. During the extension step the strands are amplified beyond the target DNA. This means that each newly synthesized strand of DNA will have a region complimentary to a primer. There is an exponential increase in the amount of DNA produced as the three above mentioned steps are repeated in a cyclic fashion. In a traditional PCR these steps might be repeated 20 to 55 times. A problem, however, with PCR amplification is that the temperature required for DNA strand dissociation also results in DNA polymerase denaturation. This is partially overcome by the bioengineering of polymerases which are more thermal stable and have longer half-lives.

Even though RT-PCR is technically more difficult to perform and more expensive than ELISA, it has the ability to allow for the detection of low viral loads. RT-PCR is considered to be 10^2 to 10^5 fold more sensitive than traditional ELISA (Spiegel and Martin, 1993; Dietzgen, 2002; Mumford *et al.*, 2004). RT-PCR also allows for the detection of several viral targets in the same reaction through the use of several primer combinations. This is called multiplexing. Although multiplexing is technically more demanding than a traditional simplex reaction it allows for a higher throughput in that a single sample can be tested for several viral strains in a single reaction. Primers used for multiplexing are chosen in such a manner that they result in amplicons of various sizes. This allows for post RT-PCR analysis using gel electrophoresis. Although RT-PCR saves time, allows for multiplexing and is more sensitive than ELISA, the reagents and instrumentation needed are expensive and require a higher level of technical expertise. Also, end product analysis using gel electrophoresis is laborious, relatively more expensive, time consuming and does not lend itself to automation. For these reasons the use of RT-PCR for routine screening is not feasible and has not replaced ELISA. It does, however, provide the industry with the opportunity to screen borderline cases, especially in the case of seed potato certification.

2.2.3   Real-time PCR

In most traditional PCRs the resulting products are analyzed after the PCR has been completed. This is called end-point analysis and is normally qualitative of nature rather than being quantitative. For this sort of analysis, products are mostly analyzed on an agarose gel and visualized using ethidium bromide as a fluorescent dye. Direct correlation between signal strength and initial sample concentration is not possible using end-point analysis since PCR efficiency decreases as the reaction nears the plateau phase. Real-time PCR, however, offers an accurate and rapid alternative to traditional PCR. Real-time PCR offers the researcher the opportunity to amplify and analyze the product in a single tube using fluorescent dyes. This is known as homogenous PCR. During a real-time PCR the increase in fluorescence is correlated with the increase in product. Through the use of different specific, dyes real-time PCR can be used to distinguish between different strains of a virus and even to detect point mutations. The major advantage of real-time PCR is that analysis of resulting products using gel electrophoresis is not required. This means that real-time PCR can be implemented as a high-throughput technique for sample screening.

2.2.3.1 DNA-binding fluorescent dyes

The use of an intercalating dye for real-time detection of a target sequence is the simplest and most inexpensive of techniques for this purpose. This method of detection is also known as sequence-independent detection which implies that the dye binds to dsDNA regardless of the sequence. The most widely used intercalating dyes used today are ethidium bromide, YO-PRO-1 and SYBR® Green I (Higuchi, 1993; Ishiguro, 1995; Tseng, 1997; Morrison, 1998). These dyes fluoresce at a specific wavelength when bound to dsDNA and are excited by an appropriate wavelength. Of the previously mentioned chemistries, SYBR® Green I is the most widely used dye for general use. SYBR® Green I emits little fluorescence when unbound and in solution. Its fluorescence, measured at 530 nm, increases significantly once bound to dsDNA. Although SYBR® Green protocols are generally easy to design and optimize, correct primer design is absolutely essential. Poor primer design may lead to non-specific amplification as well as primer dimerization and will ultimately result in the generation of a false positive signal. Primer dimerization, along with specific amplification, is associated with the reaction going into its plateau phase. Primer dimers can be distinguished from the full-length amplicon through the use of a melting curve analysis. Melting curve analysis relies on the variation in T_M between specific amplicons and non-specific amplifications such as primer dimers. Sensitivity of DNA-binding fluorescent dyes is limited at low template concentrations where primer dimerization overshadows template amplification. Currently SYBR® Green I cannot be used to detect single nucleotide polymorphisms (SNPs) since the intercalation of SYBR® Green I is not uniform throughout dsDNA, but with the introduction of uniformly distributed dyes, would in the near future allow for SNP detection.

2.2.3.2 Taqman®

An alternative the use of intercalating dyes for the detection of target DNA is by means of so-called Taqman® probes. Also known as a hydrolysis probe, Taqman® probes rely on the 5' exonuclease activity

of *Taq* DNA polymerase. The use of such a probe was first described in 1991 and formed the basis for fluorogenic probes in homogenous PCR reactions (Holland *et al.*, 1991). When intact, the probe consists of an oligonucleotide, normally in the order of 20 to 40 bp in length, linking a reporter and quencher dye. Other requirements for a well designed hydrolysis probe are: a GC content of 40-60%, no runs of a single nucleotide, no repeats of motifs and no overlapping of primer binding sites or hybridization with primers (Landt, 2001). Also of critical importance is that the probe has a T_M 5°C higher than that of the primers. This would ultimately cause the probe to associate with the template before primer extension occurs. While in close proximity the quencher dye suppresses the fluorescent signal emitted by the reported dye (Mackay *et al.*, 2002). The probe is designed to bind specifically within the amplicon of interest. Suppression of the reporter is ended as soon as the probe is digested by *Taq* during the extension phase of the reaction. Once the probe is digested and the reporter and quencher are separated, fluorescence at a specified wavelength can be detected. The number of probes hydrolyzed is directly proportional to the number of amplifications. This makes hydrolysis probes an excellent choice for quantification studies. A drawback of hydrolysis probes, however, is that melting curve analysis cannot be performed since the probe is hydrolyzed. This is, however, compensated for by the increased specificity provided by the fact that both primers and probe must be bound for fluorescence to occur. This makes Taqman[®] probe based real-time PCR (qPCR) more specific than fluorescent dye based qPCR but the sensitivities of the two types of detection systems differ depending on the amplicon length.

2.3   References

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H. and Vance V.B. (1998). A viral suppressor of gene silencing in plants. *Proc. Natl. Acad. Sci. USA*, **95**: 13079-13084.

Atreya, C.D., Raccah, B. and Pirone, T.P. (1990). A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. *Virol.,* **178**: 161-165.

Atreya, P.L., Atreya, C.D. and Pirone, T.P. (1991). Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. *Proc. Natl. Acad. Sci. USA*, **88**: 7887-7891.

Atreya, P.L., López-Moya, J.J., Chu, M., Atreya, C.D. and Pirone, T.P. (1995). Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids. *J. Gen. Virol.*, **76**: 265-270.

Bagnall, R.H. and Bradley R.H.E. (1958). Resistance to virus Y in the potato. *Phytopathology,* **48**: 61-120.

Blake, C. and Gould, B.J. (1984). Use of enzymes in immunoassay techniques. *Analyst*, **109**: 533-547.

Blanc, S., Lopez-Moya, J.J., Wang, R., Garcia-Lampasona, S., Thornsbury, D.W. and Pirone, T.P. (1997). A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus. *Virol.,* **231**: 141-147.

Blanco-Urgoiti, B., Tribodet, M., Leclere, S., Ponz, F., Perez dé San Roman, C., Legorburu, F.J. and Kerlan, C. (1998). Characterization of potato potyvirus y isolates from seed potato batches. Situation of the NTN, Wilga and Z isolates. *Eur. J. Pl. Path.,* **104**: 811-819.

Boonham, N., Walsh, K., Hims, M., Preston, S., North, J. and Barker, I. (2002). Biological and sequence comparisons of Potato virus Y isolates associated with potato tuber necrotic ringspot disease. *Pl. Path.,* **51**: 117-126.

Boonham, N., Walsh, K., Preston, S., North, J., Smith, P. and Barker, I. (2002). The detection of tuber necrotic isolates of Potato Virus Y, and the accurate discrimination of PVY^O, PVY^N and PVY^C strains using RT-PCR. *J. Virol. Meth.,* **102**: 103–112.

Bradley, R.H.E. and Rideout, D.W. (1953). Comparative transmission of potato virus Y by four aphid species that infect potatoes. *Can. J. Zool.,* **31**: 333-341.

Brunt, A.A. (2001). Potyviruses. In: Loebenstein G., Berger, P.H., Brunt, A.A. and Lawson, R.H. (eds), Virus and virus-like diseases of potatoes and production of seed-potatoes. Kluwer Academic Publishers, Dordrecht, pp 77-86.

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L. and Zurcher, E.J. (eds.) (1996 onwards). `Plant Viruses Online: Descriptions and Lists from the VIDE Database. Version: 20th August 1996. Downloaded from the worldwide web: http://biology.anu.edu.au/Groups/MES/vide/

Bushell, M. and Sarnow, P. (2002). Hijacking the translation apparatus by RNA viruses. *J. Cell Biol*., **158**: 395-399.

Carrington, J.C. and Freed, D.D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. *J. Virol.*, **64**: 1590-1597.

Carrington, J.C., Jensen, P.E. and Schaad, M.C. (1998). Genetic evidence for an essential role of potyvirus CI protein in cell-to-cell movement. *Plant J.,* **14**: 393-400.

Chien, A., Edgar, D.B. and Trela, J.M. (1976). Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J. Bacteriol.,* **174**: 1550-1557.

Cronin, S., Verchot, J., Haldenman-Chalin, R., Schaad, M.C. and Carrington, J.C. (1995). long distance movement factor: a transport function of the potyvirus helper component proteinase. *Plant Cell*, **7**: 549-559.

Crosslin, J., Hamm, P., Shiel, P., Hane, D., Brown, C. and Berger, P. (2005). Serological and Molecular Detection of Tobacco Veinal Necrosis Isolates of Potato Virus Y (PVY^N) from Potatoes Grown in the Western United States. *Amer. J. Pot. Res.,* **82**: 263-269.

Dallaire, B.J., Charest, P.J., Devantier., Y. and Laliberté, J.-F. (1994). Evidence for an internal ribosome entry site within the 5' non- translated region of turnip mosaic potyvirus RNA. *J. Gen. Virol.,* **75**: 3157-3165.

De Bokx, J.A. (1981). CMI/AAB Descriptions of plant viruses. Potato virus Y. **37:** 242. Downloaded from the worldwide web: www.dpvweb.net/dprv/showdpv.php?dpvno=242

Delgado-Sanchez, S. and Grogan, R.G. (1970). Potato virus Y. *CMI/AAB Descriptions of plant viruses.* **37**: CMI/AAB, Kew, Surrey, England, 4 pp.

Dietzgen, R.G. (2002). Application of PCR in plant virology. In: *Plant viruses as molecular pathogens*. Khan, J.A. and Dijkstra, J. (eds.), The Haworth Press, United States of America, pp. 471-500.

Dolja, V.V., Haldeman, R., Montgomery, A.E., Vandenbosch, K.A. and Carrington, J.C. (1995). Capsid protein determinants involved in cell-to-cell and long distance movement of tobacco etch potyvirus. *Virol.,* **206**: 1007-1016.

Dolja, V.V., Haldeman, R., Robertson, N.L., Dougherty, W.G. and Carrington, J.C. (1994). Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. *EMBO J.,* **13**: 1482- 1491.

Domingo, E. and Holland, J.J. (1997). RNA virus mutations and fitness for survival. *Annu. Rev. Microbiol.*, **51**: 151-178.

Dougherty, W.G. and Carrington, J.C. (1988). Expression and function of potyviral gene products. *Annu. Rev. Phytopathol.,* **26**: 123-143.

Dunoyer, P., Thomas, C., Harrison, S., Revers, F. and Maule, A. (2004). A cysteine-rich plant protein potentiates Potyvirus movement through an interaction with the virus genome-linked protein VPg. *J. Virol.*, **78**: 2301-2309.

Edwardson, J.R (1947). Some Properties of the Potato Virus Y Group. Florida Agricultural Experiment Stations Monograph Series, **4**: 398.

Glais, C. Kerlan, M. Tribodet, S. Astier-Manifacier and C. Robaglia (1996). Molecular characterization of potato virus YN isolates by PCR-RFLP. *Eur. J. Plant Pathol.*. **102**: 655–662.

Glais, L., Tribodet, M. and Kerlan, C. (2002). Genomic variability in Potato potyvirus Y (PVY): evidence that PVY^{NW} and PVY^{NTN} variants are single to multiple recombinants between PVY^O and PVY^N isolates. *Arch. Virol.,* **147** (2002), 363–378.

Govier, D.A. and Kassanis, B. (1974a). Evidence of that a component other than the virus particle is needed for aphid transmission of potato virus Y. *Virol.*, **57**: 285-286.

Govier, D.A. and Kassanis, B. (1974b). A virus-induced component of plant sap needed when aphids acquire potato virus Y from purified preparations. *Virol.*, **61**: 420-426.

Granier, F., Durand-Tardif, M., Casse-Delbart, F., Lecoq, H. and Robaglia, C. (1993). Mutations in zucchini yellow mosaic virus helper component protein associated with loss of aphid transmissibility. *J. Gen. Virol.*, **74**: 2737-2742.

Gray, S.M. (1996). Plant virus proteins involved in natural vector transmission. *Trends Microbiol*. **4**: 259-264.

Gugerli, P. and Gehriger, W. (1980). Enzyme-linked immunosorbent assay (ELISA) for the detection of potato leafroll virus and potato virus Y in potato tubers after artificial break of dormancy. *Pot. Res.*, **23**: 353–359.

Harrison, B.D. (1984). CMI/AAB Descriptions of plant viruses. Potato leafroll virus 291 (no. 36 revised). www.dpvweb.net/dpv/showdpv.php?dvpno=291.

Halbert, S.E., Corsini, D.L. and Wiebe, M.A. (2003). Potato virus Y transmission efficiency for some common aphids in Idaho. *Amer. J. Pot. Res.,* **80**: 87-91.

Higuchi R., Dollinger, G., Walsh, P.S. and Griffith, R. (1992) Simulutaneous amplification and detection of specific DNA sequences. *Biotech.* (NY), **10**: 413–417.

Hong, Y. and Hunt, A.G. (1996). RNA Polymerase Activity Catalyzed by a Potyvirus-Encoded RNA-Dependent RNA Polymerase. *Virol.*, **226**: 146–151.

Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'-3'exonocleuase activity of Thermus aquaticus. *Proc. Natl. Acad. Sci. USA*, **88**: 7276-7280.

Ishiguro T., Saitoh, J., Yawata, H., Yamagishi, H., Iwasaki, S. and Mitoma, Y. (1995) Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. *Anal. Biochem.*, **229**: 207–213.

Jawaid, A. Khan A.J and Dijkstra J. (2002). Plant Viruses as Molecular Pathogens. Food Products Press, The Haworth Press Inc., N.Y.

Kassanis, B. and Govier, D.A. (1971). The role of the helper virus in aphid transmission of potato aucuba mosaic virus and potato virus C. *J. Gen. Virol.*, **13**: 221-228.

Kennedy, J.S., Day, M.F. and Eastop, V.F. (1962). A conspectus of aphids as vectors of plant viruses, *Viruses. Commonwealth Institute of Entomology*. 114.

Kostiw, M. (1975). Investigation on the retention of potato viruses M and Y in two species of aphids (*Myzus persicae* Sulz. and *Aphis nasturtii* Kalt.). *Pot. Res.*, **18**: 637–640.

Laín, S., Riechmann, J.L. and García, J.A. (1990) RNA helicase: a novel activity associated with a protein encoded by a positive strand NA virus. *Nucl. Acid Res.,* **18**: 7003-7006.

Landt, O. (2001). In: Meuer, S., Wittwer, C. and Nakagawara, K. (eds), Rapid Cycle Real-time PCR: Methods and Applications. Springer Verlag, Germany, pp. 35–41.

Lorenzen, J.H., Meacham, T., Berger, P.H., Shiel, P.J., Crosslin, J.M., Hamm, P.B. and Kopp, H. (2006). Whole genome characterization of Potato virus Y isolates collected in the western USA and their comparision to isolates from Europe and Canada. *Arch. Virol.*, **151**: 1055-1074.

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

Mackay, I.M., Arden, K.E. and Nitsche, A. (2002). Survey and Summary: Real-time PCR in virology. *Nucl. Acid. Res.*, **30**: 1292-1305.

McDonald, J.G. and Singh, R.P. (1996). Host range, symptomology and serology of isolates of Potato virus Y (PVY) that share properties with both the PVY[N] and PVY[O] strain groups. *Amer. Pot. J.*, **73**: 309-314.

Morrison T.M., Weis, J.J. and Wittwer, C.T. (1998) Quantification of low-copy transcripts by continuous SYBR® Green I monitoring during amplification. *Biotech.* (NY), **24**:, 954–962.

Mumford, R.A., Fisher, T., Elmore, J., Vickers, D., Swan, H., Walsh, K., Barker, I. and Boonham, N. (2004). The development of a routine direct tuber testing method as a rapid and reliable alternative to the traditional growing-on test. 12th EARP Virology Section Meeting Rennes, France, 2004: abstracts of oral presentations and poster presentation. Available: http://www.rennes.inra.fr/eapr2004/abstracts.htm

Niepel, M. and Gallie, D.R. (1999). Identification and characterization of the functional elements within the tobacco etch virus 5' leader required for cap-independent translation. *J. Gen. Virol.,* **79**: 897-904.

Peng, Y., Kadoury, D., Gal-On, A., Huet, H., Wang, Y. and Raccah, B. (1998). Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions. *J. Gen. Virol.*, **79**: 897-904.

Pirone, T.P. and Blanc, S. (1996). Helper-dependent vector transmission of plant viruses. *Ann. Rev. Phytopathol.*, **34**: 227-247.

Pompe-Novak, M., Gruden, K., Baebler, S., Krečič-Stres, H., Kovač, M., Jongsma, M. and Ravnikar, M. (2006). Potatao virus Y induced changes in the gene expression of potato (Solanum tuberosum L.). *Physio. and Mol. Pl Path.*, **67**: 237-247.

Pruss, G., Ge, X., Shi, X.M., Carrington, J.C. and Vance, V.B. (1997). Plant synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell*, **9**: 859-868.

Radcliffe, E.B. (1982). Insect pests of potato. *Ann. R. Ento.,* **27**: 173-204.

Radcliffe, E.B. and Ragsdale, D.W. (2002). Aphid-transmitted potato viruses: The importance of understanding vector biology. *Amer. J. Pot. Res.* **79**: 353-386.

Ragsdale, D.W., Radcliffe, E.B., DiFonzo, C.D. (1994). Action thresholds for an aphid vector of potato leaf roll virus, pp. 99-110. In: Zehnder, G.W., Powelson, M.L., Jansson, R.K. and Raman, K.V. [ed.], Advances in potato pest biology and management. American Phytopathological Society, Minnesota, USA.

Ragsdale, D.W., Radcliffe, E.B. and DiFonzo, C.D. (2001). Epidemiology and field control of PVY and PLRV, pp. 237-270. In: Loebenstein, G., Berger, P.H., Brunt, A.A. and Lawson, R.H. [ed.]. Virus and virus-like diseases of potatoes and production of seed-potatoes. Kluwer Academic publishers, Dordrecht.

Rajamaki, M.L. and Valkonen J.P. (1999). The 6K2 protein and the VPg of potato virus A are determinants of systemic infection in *Nicandra physaloides*. *Mol. Plant Microbe Interact.,* **12**:1074-81.

Revers, F., Le Gall, O., Candresse, T., Le Romancer, M., and Dunez, J. (1996). Frequent occurrence of recombinant potyvirus isolates. *J. Gen. Virol.,* **77**: 1953-1965.

Rich, A.E. (1983). *Potato diseases*. Academic press, New York, **95**: 113-118.

Robert, Y., Woodford, J.A.T. and Ducray-Bourdin, D.G. (2000). Some epidemiological approaches to the control of aphid-borne virus diseases in seed potato crops in northern Europe. *Vir. Res.* **71**: 33-47.

Rojas, M.R., Zerbini, F.M., Allison, R.F., Gilbertson, R.L. and Lucas, W.J. (1997). Caspid protein and helper component-proteinase function as potyvirus cell-to-cell movement proteins. *Virol.*, **237**: 283-295.

Roossinck, M.J. (1997). Mechanisms of plant virus evolution. *Annu. Rev. Phytopathol.,* **35**: 1953-1965.

Salaman, R.N. (1930). Virus diseases of potato: Streak. *Nature*, **126**: 241.

Schaad, M.C., Lellis, A.D. and Carrington, J.C. (1997). VPg of tobacco etch potyvirus is a host genotype-specific determinant for long-distance movement. *J. Virol.,* **71**: 8624-8631.

Shukla, D.D. and Ward, C.W. (1989). Structure of potyvirus coat proteins and its application in taxonomy of the potyvirus group. *Adv. Virus Res.*, **36**: 273-314.

Smith, K.M. and Dennis, R.W.G. (1940). Some notes on suspected variant of S*olanum* virus 2 (potato virus Y). *Ann. Appl. Biol.*, **27**: 65-70.

Spiegel, S. and Martin, R.R. (1993). Improved detection of potato leaf roll virus in dormant potato tubers and microtubers by the polymerase chain reaction and ELISA. *Ann. Appl. Biol.*, **122**: 493-500.

Talbot, N.J. (2004). Plant-Pathogen Interaction. Blackwell Publishing. CRC Press.

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

Tseng S.Y., Macool, D., Elliott, V., Tice, G., Jackson, R., Barbour, M. and Amorese, D. (1997) An homogeneous fluorescence polymerase chain reaction assay to identify *Salmonella*. *Anal. Biochem.*, **245**: 207–212.

Thompson, G.J. (1997). Study and control of virus disease of potatoes. In: Landbounavorsingsraad Roodeplaat: Aartappelnavorsing 1996/1997. Agricultural Research Council, Pretoria.

Vance, V.B, Moore, D., Turpen, T.H., Bracker, A. and Hollowell, V.C. (1992). The complete nucleotide sequence of Pepper mottle virus genomic RNA: comparison of the encoded polyprotein with those of other sequenced potyviruses. *Virol.*, **191**: 19-30.

Van der Vlugt, R., Allefs, S., De Haan, P. and Goldbach, R. (1989). Nucleotide sequence of the 3'-terminal region of potato virus Y^N RNA. *J. Gen. Virol.,* **70**: 229-233.

Van Hoof, H.A. (1980). Aphid vectors of potato virus YN. *Neth. J. Pl. Path.,* **86**: 159.

Visser, J.C., Rothmann, A.H. and Bellstedt, D.U. (Unpublished). An assessment of recombination patterns in South African strains of potato virus Y (PVY). Honours thesis.

Wang, X. Ullah, Z. and Grumet, R. (2000). Interaction between Zucchini Yellow Mosaic Potyvirus RNA-Dependent RNA Polymerase and Host Poly-(A) Binding Protein. *Virol.,* **275:** 433-443.

Ward, C.W. and Shukla, D.D. (1991). Taxonomy of potyviruses: current problems and possible solutions. *Intervirology,* **32:** 269-296.

Warren, M., Krüger, K. and Schoeman, A.S. (2005). Potato virus Y (PVY) and potato leaf roll virus (PLRV): Literature review for potatoes South Africa. Department of Zoology and Entomology, Faculty of Natural and Agricultural Sciences, University of Pretoria.

Wilson, K. and Walker, J. (2000). Practical biochemistry: Principles and techniques. (5th ed). The Press Syndicate, University of Cambridge, Cambridge, U.K.

Wittmann, S., Chatel, H., Fortin, M.G. and Laliberte, J.F. (1997). Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. *Virol.,* **234**: 84-92.

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.

Chapter 3

An assessment of molecular variability and recombination patterns in the coat protein gene of South African strains of Potato Virus Y (PVY)

Visser, J.C. & Bellstedt, D.U.

Department of Biochemistry, The University of Stellenbosch, Private Bag X1, Stellenbosch, 7602,

South Africa

Summary

The coat protein (CP) gene of 95 South African potato virus Y (PVY) isolates was amplified using the reverse-transcriptase polymerase chain reaction (RT-PCR). The resulting cDNA products were cloned and sequenced or directly sequenced. These sequences were aligned and used to divide the isolates into specific groups which, when compared to reference sequences, belonged to the PVY^N, PVY^O, PVY^R and PVY^{NTN} strains. The CP gene of a number of isolates exhibited variations unique to South African strains of PVY. The arbitrarily named $PVY^{Super\ N}$ isolate was found to demonstrate a significant homology to Chinese variants of PVY and may be an indication of the import of infected material. In Europe, PVY^{NTN} developed as a result of recombination between PVY^N and PVY^O. Phylogenetic and Simplot analysis showed that some South African isolates developed as a result of recombination which occurs between PVY^N and PVY^O and that some of these exhibit the PVY^{NTN} phenotype. These variations in the PVY CP gene may have future implications for the detection of PVY in South Africa using antibody-based techniques.

Introduction

Currently a substantial percentage of potatoes produced by the South African potato producer is infected and thus affected by viral organisms, primarily potato virus Y (PVY) and potato leaf roll virus (PLRV) (Coetsee, 2005). The increased rate of infection can be attributed to several factors. These include a marked decrease in the effectiveness and administration of chemicals used in host control, the use of infected propagation material in cultivation, as well as incorrect irrigation and farming methods (Coetsee, 2005). An increase in the average temperature of winters has also lead to an increase in host number, which in turn has lead to an increase in viral distribution (Coetsee, 2005). In addition, the increased viral infection maybe the result of viral mutation and the evolution of highly virulent new viral strains.

PVY has been classified into different strains according to the symptoms they induce in potatoes and tobacco (Delgado-Sanchez and Grogan, 1970). Potato Virus C, now known as PVY^C, was the first to be recognized and was identified in the 1930s (Salaman, 1930 as referred to in Jawaid *et al.*, 2002). PVY^C induces hypersensitive reactions in a wide range of potato cultivars including the formation of mild mosaic patterns or stipple streak. Unlike the other strains of PVY, some PVY^C strains are non-aphid transmissible (Blanco-Urgoiti *et al.*, 1998). The second strain of PVY is denoted as PVY^O. Infection of a potato plant with the PVY^O strain results in mild tuber damage and does not cause leaf necrosis

(Boonham *et al.*, 2002). A third strain of PVY is PVY[N] (Smith and Dennis, 1940 as referred to in Jawaid *et al.*, 2002). This strain was described in tobacco plants growing close to potato plants (Crosslin *et al.*, 2005). PVY[N] results in leaf necrosis and mild or even no damage to the tubers. Both PVY[O] and PVY[N] are aphid transmissible. In Europe these two strains have been shown to have recombined to form PVY[NTN] (Boonham *et al.*, 2002). The PVY[NTN] has been accredited with the ability to induce potato tuber necrotic ringspot disease (PTNRD) (Boonham *et al.*, 2002a). As tubers damaged by PTNRD become unmarketable, infection by PVY[NTN] has a larger economic impact than infection by the other strains of PVY. Multiple alignments of the CP and the 5'-NTR sequences indicates recombination between the two main strains, PVY[N] and PVY[O], to form this substrain. By comparing the sequences, it was discovered that the 5'core regions and CP N-terminal of PVY[NTN] concurred with those found in PVY[N] (Revers *et al.*, 1996). However, it was found that PVY[NTN] isolates cluster with PVY[O] upon comparison of the CP gene and the 3'-NTR region (Boonham *et al.*, 2002a). Restriction fragment length polymorphism (RFLP) and SimPlot analyses showed that there were other recombination events within PVY[NTN] as well (Glais *et al.,* 1996; Lorenzen *et al.*, 2006). The 5'-NTR region up to the HC-Pro gene of PVY[NTN] as well as the NIa gene up to nearly the first 50% of the CP gene closely resembles that of PVY[N]. Conversely, the PVY[NTN] sequence correlates to that of the PVY[O] from P3 up to the 6K2 gene and from the furthest part of the middle of the CP gene up to the 3'-extremity (Unpublished data as referred to in Jawaid *et al.*, 2002).

Past studies have described that the three strains of PVY: PVY[C], PVY[N] and PVY[O] occur in South Africa (Brunt, 2001; De Bokx, 1981). Although all three strains have been reported, PVY[N] and PVY[O] seem to be the dominant strains in South Africa. Recently the so-called PVY[NTN] strain has been detected in South Africa (Pieterse, 2005).

However, no detailed study of variation of South African strains of PVY has been undertaken in recent years. The objective of this research project was to establish which strains of PVY are prevalent within South Africa and whether or not there is genetic variation within these strains. Strain variation in PVY in South Africa was assessed by determination of the CP sequences of a large number of South African isolates followed by phylogenetic analysis of the complete CP gene sequences of the different strains. Furthermore an attempt was made to establish whether or not South African PVY[NTN] strains have emerged due to recombination of PVY[N] and PVY[O]. Cluster analysis of the 5' 606 bp and the 3' 201 bp of the PVY CP gene was performed using phylogenetic analysis to assess recombination patterns. A SimPlot analysis was performed to scan the PVY CP gene for recombination events.

Materials and methods

Sample collection – PVY infected potato plant leaves were obtained from various regions in South Africa by CBS (Coen Bezuidenhout Seed Testing Center, Pretoria). Tubers of potato plants which were confirmed to be infected with PVY by ELISA, were planted at CBS. When they had grown to a height of approximately 20 cm, the terminal growth (10 cm) was shipped to Stellenbosch for further analysis. The samples used in this study are listed in table 1.

Table 1. Alphabetical list of samples (95) used in this study. In the majority of cases at least three sequences were obtained from each sample. Amplification of the CP gene of certain isolates resulted in truncated sequences. These twenty three partially truncated isolates (*) were not included in the final phylogenetic analysis. Generated sequences have not yet been submitted to GenBank.

Sample Code	Area	Classification
CC24(5)	Koue Bokkeveld, Western Cape, SA	N
CC24(7)*	Koue Bokkeveld, Western Cape, SA	O
CC55(8)	Koue Bokkeveld, Western Cape, SA	O
CC62(20)	Koue Bokkeveld, Western Cape, SA	NTN
CC66(91)	Koue Bokkeveld, Western Cape, SA	O
CC73(12)*	Koue Bokkeveld, Western Cape, SA	R
CC73(18)*	Koue Bokkeveld, Western Cape, SA	R
CC9(12)	Koue Bokkeveld, Western Cape, SA	NTN
CC9(30)	Koue Bokkeveld, Western Cape, SA	O415G
CC9(47)	Koue Bokkeveld, Western Cape, SA	R
CC9(48)	Koue Bokkeveld, Western Cape, SA	O415G
DD019(141)	Modderrivier, Douglas, SA	O
DD020(92)	Modderrivier, Douglas, SA	R
DD037F(31)	Swarthaak, Douglas, Northern Cape, SA	R
DD037F(35)	Swarthaak, Douglas, Northern Cape, SA	R
DD037F(9)	Swarthaak, Douglas, Northern Cape, SA	O
DD037F(96)	Swarthaak, Douglas, Northern Cape, SA	R
DD103A(101)	Droogevallei, Northern Cape, SA	O
DD103A(184)	Droogevallei, Northern Cape, SA	N
DD103A(80)	Droogevallei, Northern Cape, SA	O
DD122A(25)	Northern Cape, SA	O415G
DD122A(34)	Northern Cape, SA	O415G
DD122A(36)	Northern Cape, SA	R
GG517(128)	Dwarsweg, George, Western Cape, SA	O415G
GG517(170)	Dwarsweg, George, Western Cape, SA	O415G
GG517(93)	Dwarsweg, George, Western Cape, SA	N
NN297A(20)*	Underberg, Kwazulu Natal, SA	N
NN300(155) 19	Boston, Kwazulu Natal, SA	N
NN300(155) 22	Boston, Kwazulu Natal, SA	N
NN300(156)*	Boston, Kwazulu Natal, SA	N
NN300(41)	Boston, Kwazulu Natal, SA	N
NN300(60)	Boston, Kwazulu Natal, SA	Super N
NN300(76)	Boston, Kwazulu Natal, SA	NTN
NN300(98)	Boston, Kwazulu Natal, SA	N
NN300(99)	Boston, Kwazulu Natal, SA	Super N
NN305F(9)*	Underberg, Kwazulu Natal, SA	N
NN333B(28)	Mooi Rivier, Kwazulu Natal, SA	NTN
NN333B(87)	Mooi Rivier, Kwazulu Natal, SA	R
NN445(22)*	Underberg, Kwazulu Natal, SA	N
NN459A(14)	Nottingham, Kwazulu Natal, SA	O415G
NN459A(25)	Nottingham, Kwazulu Natal, SA	N
NN470A(20)*	Mooi Rivier, Kwazulu Natal, SA	R
NN470A(39)*	Mooi Rivier, Kwazulu Natal, SA	R
NN473A(6)*	Boston, Kwazulu Natal, SA	R
NN71(111)	Underberg, Kwazulu Natal, SA	N
NN98A(22)*	Underberg, Kwazulu Natal, SA	R
NN98A(35)*	Underberg, Kwazulu Natal, SA	R
PP003(119) 85	Reitz, East Free State, SA	R
PP003(119) 133	Reitz, East Free State, SA	R
PP014(18)*	Fouriesburg, Free State, SA	O
PP014(21)*	Fouriesburg, Free State, SA	O

PP026B(184)	Bethlehem, East Free State, SA	R
PV026(3)*	Reitz, Free State, SA	Super N
PVY[NTN] Sample 1	Marble Hall, Mpumalanga, SA	O
PVY[NTN] Sample 3.3	Petrusburg, South West Free State, SA	R
PVYNTN(4.1)*	Petrusburg, South West Free State, SA	R
PVYNTN(4.2)*	Petrusburg, South West Free State, SA	R
SS082A(171)	Sandveld, Western Cape, SA	N
SS082A(194)	Sandveld, Western Cape, SA	R
SS082A(88)	Sandveld, Western Cape, SA	N
SS121(166)	Roodepoort, Eastern Cape, SA	O
SS121(53)	Roodepoort, Eastern Cape, SA	R
SS121(154)	Sandveld, Western Cape, SA	O
SS121(197)	Sandveld, Western Cape, SA	R
SS121(82)	Sandveld, Western Cape, SA	R
SS147(144)	Roodepoort, Eastern Cape, SA	R
TT014B(184)	Middelburg, Mpumalanga, SA	R
TT019A(107)	Morgenzon, Mpumalanga, SA	N
TT026B(195)	Middelburg, Mpumalanga, SA	N
TT026B(86)	Middelburg, Mpumalanga, SA	O415G
TT026B(88)	Middelburg, Mpumalanga, SA	O415G
TT042A(3)*	Middelburg, Mpumalanga, SA	R
TT138D(111)	Morgenzon, Mpumalanga, SA	Super N
TT138D(13)	Morgenzon, Mpumalanga, SA	R
TT138E(102)	Morgenzon, Mpumalanga, SA	O415G
TT138E(111)	Morgenzon, Mpumalanga, SA	O415G
TT138E(113)	Morgenzon, Mpumalanga, SA	O415G
TT141A(76)	Morgenzon, Mpumalanga, SA	O415G
TV115B(3)*	Morgenzon, Mpumalanga, SA	O415G
TV120(6)*	Voorsorg, Hendriena	R
WW002(22)	Warrenton, Free State, SA	R
WW002(74)	Warrenton, Free State, SA	R
WW002(82)	Warrenton, Free State, SA	R
WW010(146)	Vaallaagte, Christiana, Western Cape, SA	O
WW010(147)	Vaallaagte, Christiana, Western Cape, SA	O415G
WW010(70)	Vaallaagte, Christiana, Western Cape, SA	O415G
WW073B(18)*	Christiana, Western Free State, SA	NTN
WW154A(175)	Christiana, Western Free State, SA	O415G
WW154A(62)	Christiana, Western Free State, SA	O
WW202B(21)	Pontplaas , Christiana, Western Cape, SA	R
WW202B(24)	Pontplaas, Christiana, Western Cape, SA	R
WW282E(3)	Bloemhoff, Western Free State, SA	O
WW655(11)*	Middelburg, Western Free State, SA	R
WW672(35)*	Christiana, Free State, SA	R
Z26	Cradock, Eastern Cape, SA	NTN

Plant material homogenization – Upon receival, leaf samples were stored in 1.5 ml Eppendorf tubes at -80°C in a Cascade Cooling unit (Snijders Scientific, Tilburg, The Netherlands). An autoclaved mortar and pestle was used to grind 0.1 g of a specific sample to a pulp. Thereafter 2 ml of grinding buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 2% (w/v) PVP40 (Sigma), 0.2% (w/v) BSA (Fluka), 0.05% (v/v) Tween 20, 1% (w/v) sodium meta bisulphide, pH 9.6) was added and the grinding process resumed until no sizable particles were visible. One milliliter of the ground leaf sample was transferred to a 1.5 ml Eppendorf tube. The homogenized plant extract was centrifuged in a Picofuge (Stratagene) for 15 seconds

causing remaining plant debris to pellet. Four microliters of the clear supernatant was added to 25 µl GES buffer (0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% (v/v) Triton X-100) and the entire volume incubated at 95°C for 10 minutes in a digital dry bath (Labnet International, Inc.). The samples were placed on ice immediately after this incubation period and kept on ice for no less than 5 minutes. Remaining extracts were stored at -80°C for future reference.

Amplification of the PVY CP gene using Reverse Transcriptase-PCR - Viral RNA within the plant material was amplified by means of RT-PCR. To this end 2 µl of homogenized leaf sample was added to 23 µl of a standard RT-PCR mixture. This mixture consisted of 2.5 µl 10 × PCR-buffer, 1.25 µl 0.1 M DTT, 1.5 µl 25 mM $MgCl_2$, 0.625 µl 20 µM forward primer, 0.625 µl 20 µM reverse primer, 1 µl 5 mM dNTPs (Bioline), 0.25 µl 5 U/µl Taq™ DNA polymerase (Bioline), 0.125 µl 200 U/µl SuperScript™ III (Invitrogen) and 15.125 µl Milli-Q® water. The primers used had the following sequences: forward primer (PVY(f), 03-1882), 5'-GCACGCCATGG(CG)AAATGACACAAT(CT)GATGC-3' and reverse primer (PVY(r), 02-1079), 5'-CTGCGGATCCTCACATGTT(CT)TTGACTCCAAG-3'. Both these primers were synthesized by the DNA Synthesis Laboratory, Department of Molecular and Cell Biology, University of Cape Town, South Africa. All RT-PCR amplifications were done in a Labnet Multi Gene II Thermal Cycler using the following program: a single reverse transcription step of 48°C for 30 minutes, followed by 35 cycles of DNA amplification at 94°C for 30 seconds, 64°C for 45 seconds and 72°C 60 seconds as well as an extension completion period at 72°C for 7 minutes. A holding temperature of 15°C was used for short periods of storage while samples were kept at 4°C for overnight storage. The amplicon of the above specified primer set is 807 bp in length, corresponding to the complete length of the PVY CP.

RT-PCR Product Purification - Although samples could be stored at either 15°C or 4°C for extended periods, they were generally used upon completion of the RT-PCR to reduce the likelihood of cDNA degradation. The total volume of the RT-PCR was mixed with a loading buffer (0.25% (w/v) bromophenol blue, 57.5% (v/v) glycerol, 0.5 M EDTA, 1 M Tris, pH 8.0, Milli-Q® water) and loaded on an 1% agarose gel. The gel was immersed in a 1 × TAE electrophoresis buffer (0.48% (w/v) Tris-base, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0) and allowed to equilibrate. Electrophoresis was performed at 100-110 V for 70 to 80 minutes using an EasyVolt, Stratagene (Whitehead Scientific) or an EPS 601 (Amersham Biosciences) electrophoresis setup. The agarose gels were stained in a 1 µg/ml ethidium bromide solution made up in Milli-Q® water and the RT-PCR products were visualized through the use of an E-Box CN-1000 UV transilluminator and portable darkroom (Vilber Lourmat). RT-PCR products were excised from the agarose gel through the use of a sterile scalpel and purified using the Wizard® SV and PCR Clean-up System (Promega) or the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to the protocols supplied by the respective manufacturers. Cleaned cDNA samples were concentrated to approximately two fifths of the original volume (from 50 µl to 20 µl) using a SpeedVac Concentrator (Savant Instruments, Farmingdale, N.Y.) and stored at -20°C in nuclease free Eppendorf tubes. Two microliters of the concentrated samples was analyzed on a 2% agarose gel.

Preparing chemically competent JM-109 cells for cloning – A 500 µl aliquot of JM-109 *Escherichia coli* cells was added to 4.5 ml of sterile LB growth medium (1% (w/v) Bacto® tryptone (Difco laboratories), 0.5% (w/v) Yeast extract (Merck), 1% (w/v) NaCl, pH 7.0) containing no antibiotics. The bacterial culture was grown at 37°C for 16 hours on a shaker set at 200 rmp. Subsequent to this 16 hour incubation step the cell culture was subcultured into Erlenmeyer flasks. Two and a half milliliters of overnight culture was added to 250 ml of sterile LB medium. Subcultures were allowed to grow at 37°C until an OD_{600} of between 0.500 and 0.600 was reached. Optical density was measured using a DU 650 spectrophotometer (Beckman). Cell cultures were placed on ice to halt growth once the desired optical density was achieved. The cultures were centrifuged in sterile pre-cooled JA20 centrifuge tubes at 3 024 × *g* for 10 minutes at 4°C using a J2-21 Beckman preparative centrifuge. The supernatant was discarded and the remaining pellets were carefully resuspended in a final volume of 50 ml of ice cold 100 mM $MgCl_2$. The suspension was incubated on ice for 30 minutes after which it was centrifuged at 1 935 × *g* at 4°C for 10 minutes. The supernatant was discarded and the resulting pellet gently resuspended in 10 ml of ice cold 100 mM $CaCl_2$ containing 15% (v/v) glycerol. The suspension was aliquoted into pre-cooled 1.5 ml Eppendorf tubes and flash frozen using ethanol cooled to -80°C.

Transformation of JM-109 cells with recombinant pGEM®-T Easy plasmids - Purified cDNA samples were cloned into pGEM®-T Easy vectors using the pGEM®-T and pGEM®-T Easy Vector Systems kit (Promega) following the manufacturer's protocol. The ligation reaction was kept at 4°C for 16 hours before being used in a transformation reaction. These plasmids were then transformed into chemically competent *Escherichia coli* (JM-109, 1×10^7 cfu/µg DNA) according to the pGEM®-T Easy protocol. Transformed cell cultures were plated out in duplicate (50 µl and 150 µl) on LB/agar plates supplemented with ampicillin (100 µg/ml) (Sigma), IPTG (160 mM) and X-Gal (48 µg/ml) and the plates incubated at 37°C for 19 hours. This was found to be sufficient for blue colour development but an additional 30 to 60 minutes at 4°C may be added to assist in the blue/white screening process.

Diagnostic PCR with SP6 and T7 primers - White colonies were subjected to a diagnostic PCR to establish whether or not the colony of interest contains a vector with an insert. The diagnostic PCR is a very crude but effective method of insert verification. The PCR mixture consisted of 1 µl 10 × PCR-buffer, 0.6 µl 25 mM $MgCl_2$, 0.5 µl 20 µM SP6, 0.5 µl 20 µM T7, 0.4 µl 5 mM dNTPs (Bioline), 0.1 µl 5 U/µl Taq™ DNA polymerase (Bioline) and 6.9 µl Milli-Q® water. All diagnostic PCR amplifications were done in a Labnet Multi Gene II Thermal Cycler with the following program: 94°C for 5 minutes, followed by 25 cycles of the amplification at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds and a single cycle at 72°C for 7 minutes. A holding temperature of 15°C was used. Products were loaded on a 1% agarose gel and electrophoresis and visualization was done as described with RT-PCR products.

Cultivation of selected clones and plasmid isolation - The remaining parts of the selected colonies were inoculated in 14 ml Falcon tubes in 5 ml of LB containing ampicillin (100 µg/ml) on a G24 Environmental Incubator/Shaker (New Brunswick Scientific Co., Inc., Edison, N.J., U.S.A.) set at 200

rpm at 37°C for 16 hours. For each isolate a total of three clones were selected for inoculation. Cloned plasmids were purified from the resulting cell cultures using a Plasmix Miniprep kit (Talent). A volume of 3.6 ml of the inoculant was used for plasmid isolation using the Plasmix Miniprep kit (Talent) (results not shown) while 500 µl of the inoculant was stored in 40% (v/v) glycerol at -80ºC for future use.

Sequencing of PCR inserts - The inserts in the purified plasmid DNA were amplified during sequencing PCR. For each insert containing plasmid DNA sample two sequencing PCRs were performed. The SP6 (5'-ATTTAGGTGACACTATAGAA-3') and T7 primers (5'-TAATACGACTCACTATAGGG-3') were used. These primers were supplied by the DNA Synthesis Laboratory, Department of Molecular and Cell Biology, University of Cape Town, South Africa. Each sequencing reaction consisted of 4 µl Terminator mix, 3 µl of either SP6 or T7 primer (3.3 µM) and 3 µl plasmid DNA. A Terminator Mix was utilized to save on Terminator Dye and consists of one part Terminator Dye (BigDye® Terminator v3 Cycle Sequencing kit, Applied Biosystems) and four parts Half Dye Mix (Bioline). A Labnet Multi Gene II Thermal Cycler was used and programmed as follows: 35 cycles of 96°C for 10 seconds, 52°C for 30 seconds and 60°C for 4 minutes followed by a final extension completion cycle of 60°C for 10 minutes. A holding temperature of 15°C was used. The products of cycle sequencing were analyzed using an ABI® 3100 Genetic Analyser (Applied Biosystems, Foster City, USA) in the Central Analytical Facility, University of Stellenbosch.

Direct sequencing of CP RT-PCR amplification products – Instead of cloning RT-PCR products first, direct sequencing of RT-PCR was also attempted. The PCR program and thermal cycler remained identical to those described for a standard sequencing PCR. Although the program was left unaltered, a different sequencing mix was used for direct sequencing. The sequencing mix for a single reaction consisted of 5 µl 5 × sequencing dilution buffer (Applied Biosystems), 2 µl Terminator Dye (BigDye® Terminator v3 Cycle Sequencing kit, Applied Biosystems), 1 µl Milli-Q® water, 1 µl primer (0.8 µM) and 1 µl of the cDNA sample. The same set of PVY specific primers used in the RT-PCR was used in this sequencing PCR. The products of cycle sequencing were analyzed using an ABI® 3100 Genetic Analyser in the Central Analytical Facility, University of Stellenbosch.

Nucleotide sequence analysis and alignment - Resulting electropherograms were edited using Chromas (v 2.23, Technelysium, Pty., Ltd.). The generated PVY CP gene nucleotide sequences were compared to sequences downloaded from GenBank (table 2) utilizing the software package BioEdit (v 7.0.5.2, Tom Hall). These PVY CP gene sequences and the sequences from pepper mottle virus (PMV) (M96425) were aligned using the Clustal W (v 1.4) alignment function embedded within the BioEdit package and finer alignment was performed by eye.

Phylogenetic analysis – Phylogenetic analysis of the aligned sequences was done using PAUP (4.0b10) (Swofford, 2002). A total of 72 complete sequences (Table 1) and 15 GenBank sequences (Table 2) were used in the final phylogenetic analysis. PMV was used as the outgroup in this analysis. The aligned matrix was trimmed at the 5' and 3' ends so as to exclude missing characters. A heuristic search (1 000 replicates) using TBR branch swapping was used with all 816 characters weighted equally to establish the

shortest possible trees from the data matrix. A bootstrap analysis (1 000 replicates) using TBR branch swapping was performed to establish clade support. Branches with bootstrap values $\geq$ 75% were considered as well supported whilst values between 75% and 50% were considered as moderately supported. Values below 50% were considered weakly supported and in line with traditional phylogenetic analysis are not indicated on phylograms. Additionally, a reduced but representative matrix was generated to investigate the suspected recombination event. This matrix included two sequences of each of the main strains, i.e. PVY^O, PVY^N, PVY^{NTN} and PVY^R. Phylogenetic analysis of this matrix was performed in two parts. Bases 1 to 615 (606 bp and three gaps of 2, 4, and 3 bp each) were included in the first analysis while bases 616 to 817 were used for the second analysis.

Table 2. An alphabetical summary of PVY CP gene sequences used in the phylogenetic analysis.

Isolate	GenBank accession number	Published country of origin	Published classification	Host
AY742729	AY742729	China	PVY^N	Tobacco
AY742731	AY742731	China	PVY^N	Tobacco
AY841258	AY841258	China	PVY^N	Tobacco
AY841267	AY841267	China	PVY^N	Tobacco
PVY-O2	X68226	USA	PVY^O	Potato
PVY-CH1	X68221	Chile	PVY^O	NA
PVY-O1	X14136	Argentina	PVY^O	Potato
PVY-US1	X68222	USA	PVY^O	Potato
PVY-JC1	U25672	China	PVY^O	Potato
PVY-N2	AF126258	Canada	PVY^N	Potato
PVY-N	AB025415	Japan	PVY^N	Potato
PVY-H1	X54611	Hungary	PVY^{NTN}	Potato
PVY-NTN3	AB042812	Japan	PVY^N	Potato
PVY-NTN2	X79305	Austria	PVY^{NTN}	Potato
Pep-Mot	M96425	California	NA	Pepper

Determination and analysis of possible recombination events – The CP gene sequence was scanned for possible recombination events using SimPlot (v 3.5.1). A window of 200 bp and a step size of 20 bp was used in the analysis. Parent strains, the so-called traditional PVY^O and PVY^N strains, were compared to suspected recombinant strains. The SimPlot package queries sequence data against a reference sequences and subsequently gives a similarity score which is then plotted on a graph to result in a graphic display of similarity.

Results

Amplification of the PVY CP gene using Reverse Transcriptase-PCR – The 807 bp CP gene of 95 isolates was successfully amplified using the RT-PCR protocol without prior RNA extraction. Resulting products were purified using either the Wizard® SV and PCR Clean-up System (Promega) or the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Samples were electrophoresed on a 2% agarose gel and visualized under UV-light after being stained with ethidium bromide. The resulting gel showed that there was a single RT-PCR product at 807 bp (figure 1). The 2% gel also allowed for an

approximation of cDNA concentration which was critical for downstream applications such as cloning and sequencing.

Transformation of JM-109 cells with recombinant pGEM®-T Easy plasmids and isolation thereof – PVY CP genes were successfully cloned into pGEM®-T Easy plasmids using the pGEM®-T Easy cloning kit (Promega). The presence of the insert was confirmed by use of a T7/SP6 diagnostic PCR (Results not shown). Plasmids containing the CP gene were successfully isolated from the bacterial cultures. Plasmid concentration was determined using a agarose gel electrophoresis and a molecular standard, 1 kb DNA ladder (BioLabs).
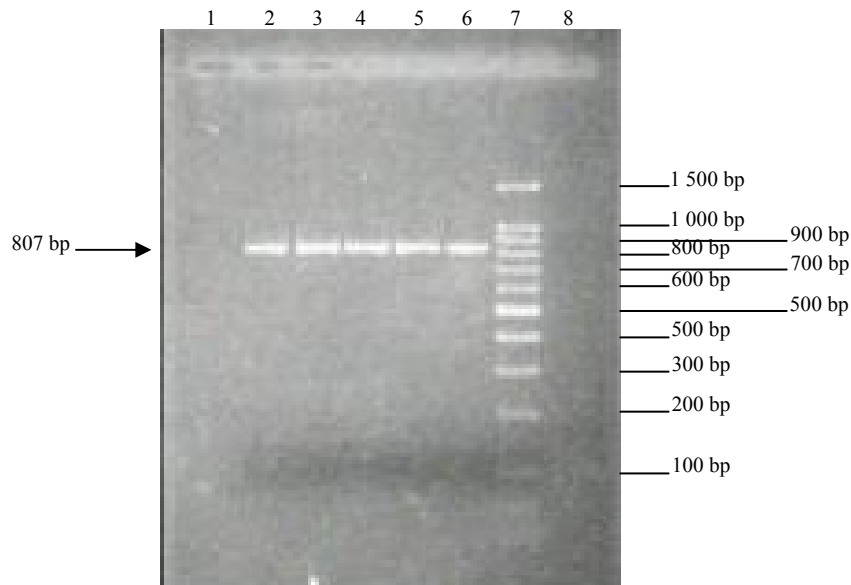
Figure 1. A digital image of RT-PCR products on a 2% agarose gel stained in ethidium bromide viewed under UV-light. Lanes 2-6: RT-PCR amplicons (807 bp) of various isolates of PVY, lane 7: 100 bp DNA ladder (Promega). Lanes 1 and 8 are empty.

Nucleotide sequence analysis of generated PVY CP gene sequences – Cloned PVY CP genes were successfully sequenced using the SP6 and T7 primer combination. The use of both forward and reverse sequences resulted in a sequence overlap which allowed for sequence verification and complete sequences were generated. Direct sequencing in some instances resulted in loss of 20 to 30 base pairs of nucleotide information at the 5' and 3' sides of the CP gene, but had no effect on the quality of rest of the electropherograms. Twenty three of the final 95 sequences, were truncated in this way.

All sequences could be aligned without gaps to generate a matrix from which the isolates could be identified by sequence comparison. Isolates could be identified as either PVY^N, PVY^O or PVY^R through the use of the sequence matrix and reference sequence data. A total of 21 isolates were identified as PVY^N while 32 isolates were identified as PVY^O. Four of the PVY^N isolates diverged significantly from the rest. These four isolates (NN300(60), NN300(99), PV026(3) and TT138E(111)) display only 95% sequence homology to the traditional PVY^N isolates. For this reason, these isolates were arbitrarily called PVY$^{Super N}$. The four PVY$^{Super N}$ isolates are nearly identical to one another which nullifies the possibility

that the observed sequence variation is due to reverse transcriptase or sequencing errors. However, the PVY$^{Super N}$ isolates identified from South African fields demonstrated a 98.8% sequence similarity to Chinese isolates sequenced by Na (Unpublished, Direct submission to GeneBank, 2004). At amino acid level the PVY$^{Super N}$ isolate differs by four amino acids from traditional PVY^N isolates such as NN71(111).

The PVY^O group consisted of traditional PVY^O strains as well as a novel isolate arbitrarily called PVY^{O415G} variant. PVY^{O415G} is similar to the parent strain except for a point mutation at base pair 415. Seventeen out of 32 PVY^O isolates were found to contain this point mutation. At this position an adenine is replaced by a guanine residue. This is a point mutation which results in an amino acid change in which asparagine is replaced by aspartate. Both these amino acids are polar and hydrophilic but unlike asparagine which is neutral, aspartate is negatively charged.

In addition to these strains, 43 recombinants were also identified. In the CP gene sequence of isolates belonging to this group the 5' 606 bp are characteristic of PVY^N and the 3' 201 bp are characteristic of PVY^O. A careful visual inspection of the PVY^R types showed that 6 isolates were true recombinants whilst 37 isolates showed a back mutation from PVY^O type to the PVY^N type in position 691. At amino acid level this makes the back-mutated recombinant identical to the parent PVY^N rather than a true PVY^N/PVY^O recombinant. Only those isolates identified as true recombinants were found to exhibit the PVY^{NTN} phenotype.

Phylogenetic analysis of generated PVY CP gene sequences – For the phylogenetic analysis, alignment of the PVY CP gene matrix with the PMV outgroup required the introduction of three gaps of 4, 2 and 3 base pairs respectively. The 23 truncated sequences of isolates were excluded from the final phylogenetic analysis. The heuristic search retrieved 820 trees with a tree length of 654. The analysis revealed that 450 of the 816 included characters were constant. Two hundred and eleven (26%) characters were found to be parsimony uninformative while 155 (19%) characters were parsimony informative. Tree statistics revealed a consistency index (CI) of 0.679 and a retention index (RI) of 0.928. One of these trees is represented in figure 2.

Phylogenetic analysis of the first 615 base pairs (including 9 gaps) of the matrix resulted in 9 trees of which the shortest was 261 in length (figure 3). Of the 615 characters, 387 were constant, 157 were parsimony uninformative and 71 were parsimony informative. Tree statistics revealed a CI of 0.954 and a RI of 0.888. In this analysis PVY^R and PVY^{NTN} isolates grouped with the PVY^N isolates. The PVY^O isolates formed a separate clade which did not collapse in the strict consensus analysis and was fully supported with a bootstrap value of 100.

Phylogenetic analysis of base pairs 616 to 817 of the matrix resulted in a single tree with a length of 67 (figure 4). Of the 201 characters 142 were constant, 43 were parsimony uninformative and 16 were parsimony informative. The tree had a CI of 0.985 and a RI of 0.957. In this tree, however, PVY^R and PVY^{NTN} grouped with PVY^O to form a clade. PVY^N forms a separate clade and is supported by a bootstrap value of 87.
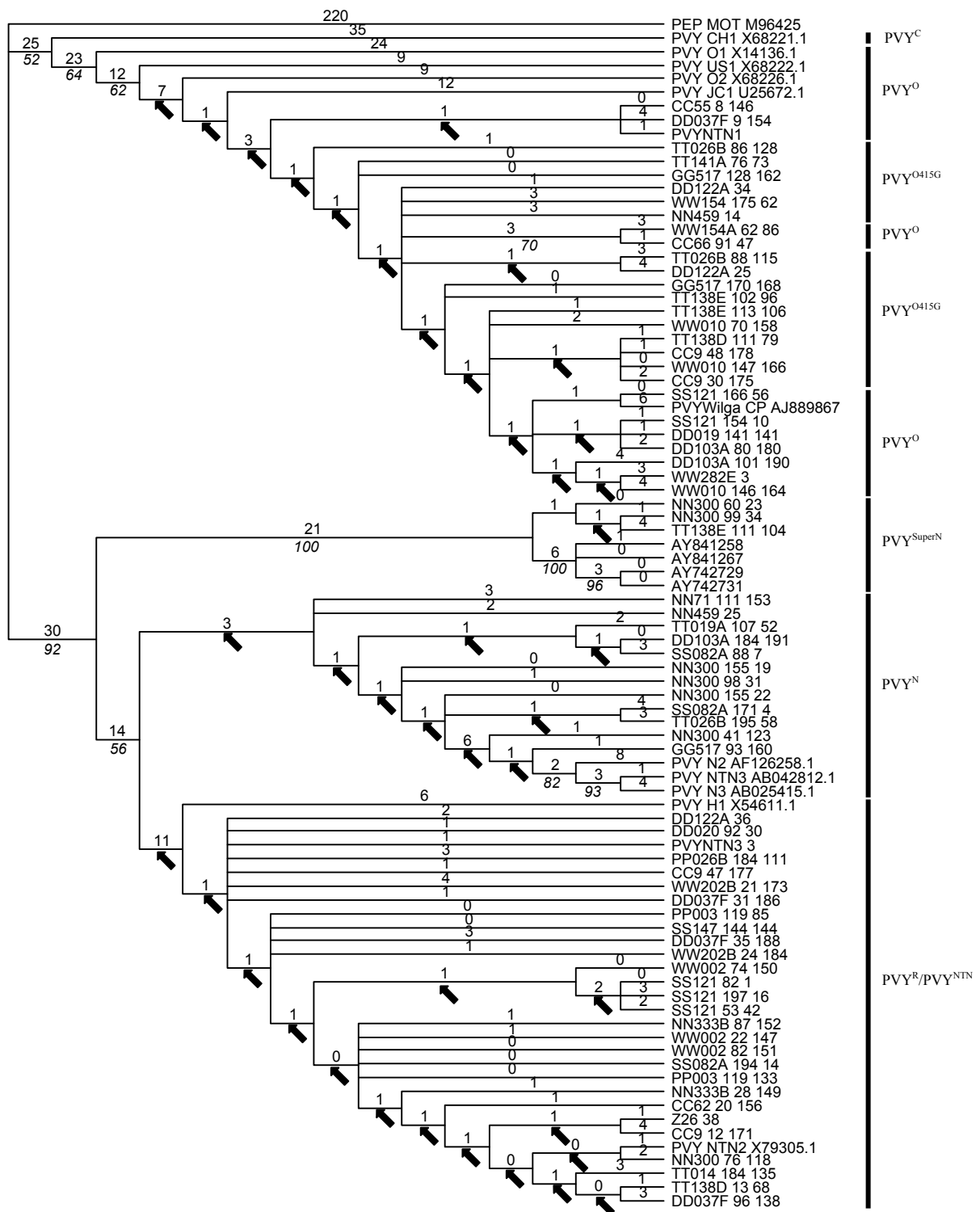
Figure 2. The shortest tree of a heuristic search performed on the PVY CP data matrix. Branch lengths are shown above branches and bootstrap values are indicated beneath in italics. Arrows indicate nodes which collapse in the strict consensus tree.
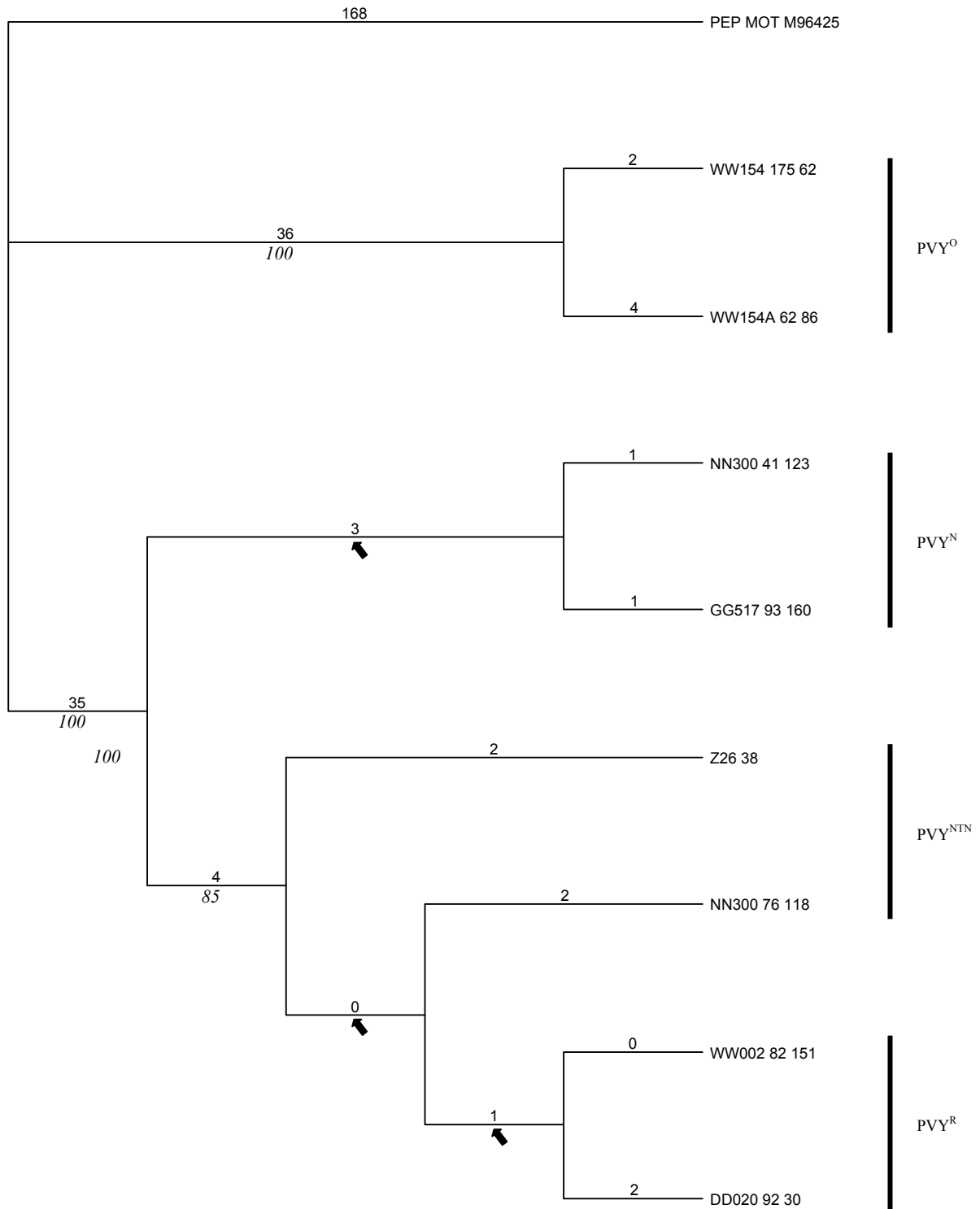
Figure 3. The shortest tree of a heuristic search performed on the bases 1 to 615 of the PVY CP data matrix. Branch lengths are shown above branches and bootstrap values are indicated beneath in italics. Arrows indicate nodes which collapse in the strict consensus tree.
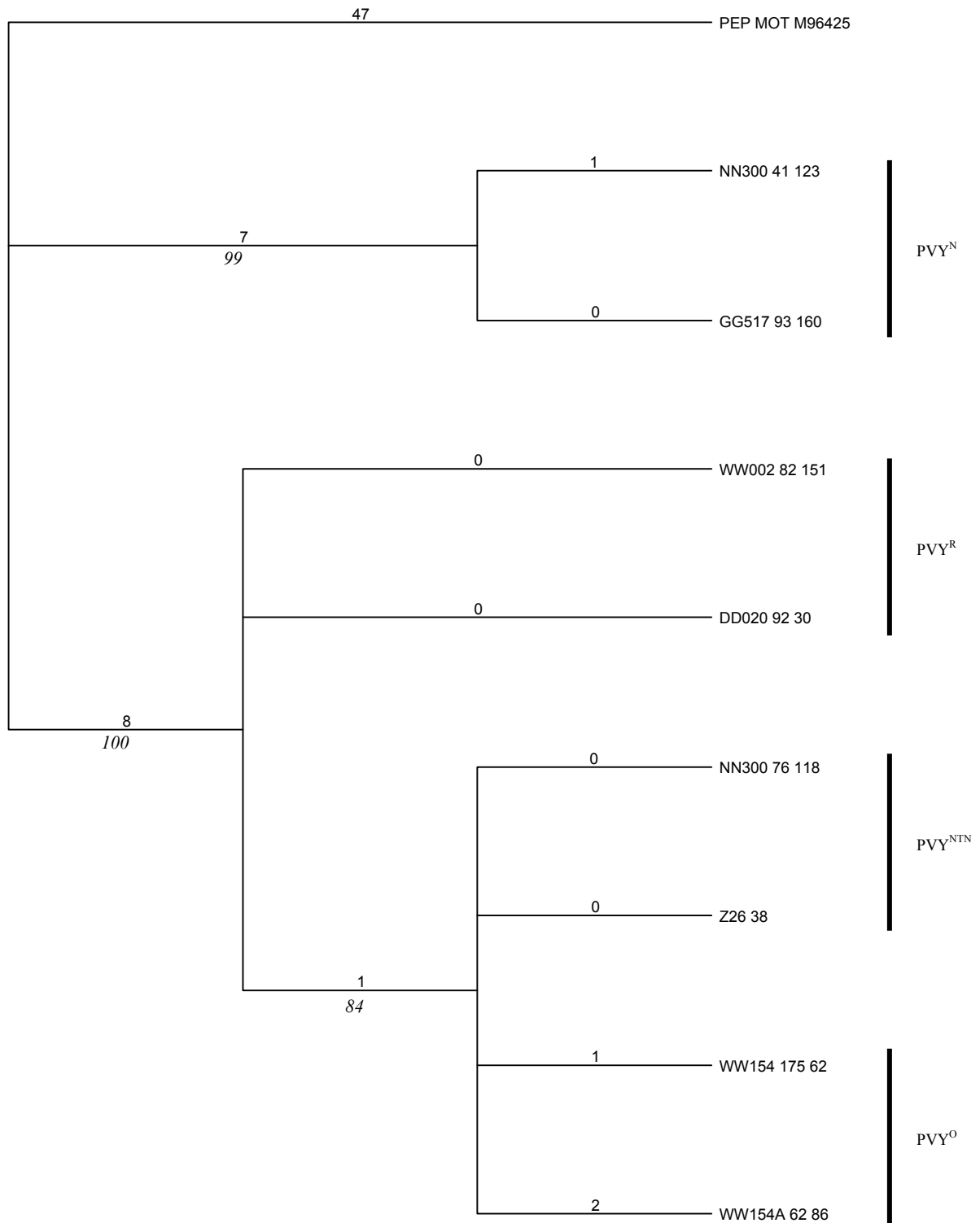
Figure 4. The shortest tree of a heuristics performed on bases 616 to 807 of the PVY CP data matrix. Branch lengths are shown above branches and bootstrap values are indicated beneath in italics.

Determination and analysis of possible recombination events – Both BioEdit and SimPlot analysis of the sequence data revealed a single point of recombination within the PVY CP gene. Comparison of a suspected recombinant isolate, Z26 (PVY[NTN]), with two of the traditional parent strains, GG517:93 (PVY[N]) and SS121:166 (PVY[O]), revealed a recombination event at 608 bp of the aligned matrix. SimPlot and BioEdit analyses of the gene clearly illustrates the 5' similarity of the recombinant with PVY[N] and the 3' similarity of the recombinant with PVY[O]. The precise sire of recombination is, however, difficult to pinpoint as no sequence variation is found in the aligned matrix from base pair 601 to 608.
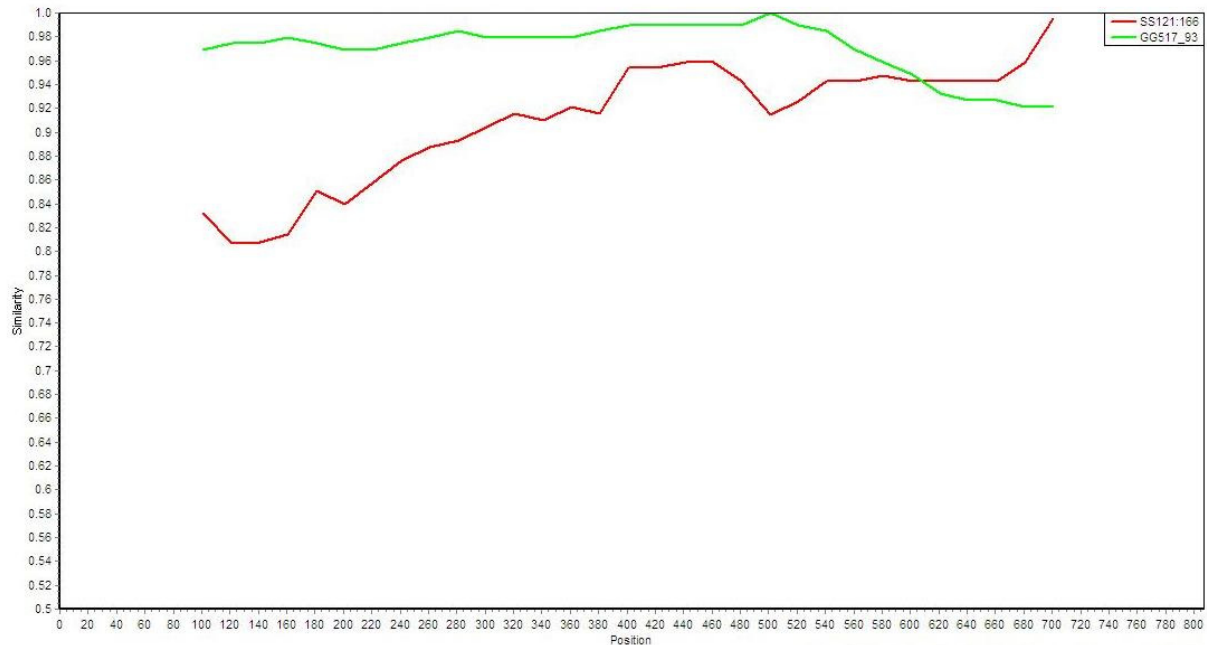
Figure 5. Analysis of the suspected recombination event within the CP gene of PVY using SimPlot (v 3.5.1). The suspected recombinant Z26 serves as the reference to which the parent strains SS121_166 (PVY[O]) and GG517_93 (PVY[N]) are compared. A window of 200 bp and step size of 20 bp was used.

Discussion

This study established that South African potatoes are infected with both PVY[N] and PVY[O] strains. The PVY[C] strain could not be identified in this study although it has been isolated before in South Africa (Brunt, 2001; De Bokx, 1981). A lack of aphid transmissibility is in all likelihood the limiting factor to the distribution of PVY[C] and the most likely cause for its absence. During the course of this study a total of 21 PVY[N] isolates and 32 PVY[O] isolates were identified confirming that both traditional strains of PVY[N] and PVY[O] as well as variants of these parent strains occur within South Africa. Four so-called PVY[Super N] isolates were identified which showed significant divergence from the other PVY[N] strains. The four isolates all originate from a central part of eastern South Africa. Two of the four, NN300(60) and NN300(99), originate from Kwazulu Natal, the third, PV026(3), is from the Free State while the fourth, TT138E(111), was obtained from Mpumalanga. It may be that all four isolates were obtained from the same seed producer since the regions of origin of the isolates are in close proximity to one another.

However, the PVY[Super N] isolates demonstrated significant sequence similarity to Chinese isolates (Na, Unpublished, Direct submission to GeneBank, 2004). According to the Department of Agriculture, South Africa does not import seed-potatoes and it can therefore be seen as an island in terms of potato import (Coetsee, 2005). There is therefore little chance of potato viruses entering the South African crop environment and contamination of local potato varieties. This, however, is contradicted by the occurrence of the PVY[Super N] isolates rather indicating that this variant of PVY[N] has been imported recently from China. Amongst the PVY[O] strains identified in South Africa traditional PVY[O] strains as well as a novel PVY[O415G] variant were identified.

CP gene sequence comparison and phylogenetic analysis revealed significant genetic variation within the various PVY strains in South Africa. Almost half the isolates analyzed in this study were recombinant strains of PVY[N] and PVY[O], indicating that recombination is occurring commonly in South Africa. Phylogenetic and SimPlot analysis further confirmed that recombination had occurred in these isolates. This is in agreement with the world-wide tendency towards recombination as exhibited in Europe, the United States of America and Asia (Li *et al*., 2006; Lorenzen *et al*., 2006). An interesting finding was that true recombinants and back-mutated recombinants occur in South Africa but that only the true recombinants exhibit the PVY[NTN] phenotype. However, other PVY isolates identified in this study, also exhibited the PVY[NTN] phenotype yet they did not show this recombination pattern. These PVY[NTN] strains may possess recombination events elsewhere in the genome. These would not be detected since only the CP gene was analyzed in this study. Future studies to determine the nature of PVY[NTN] types occurring in South Africa should therefore include the sequencing of greater regions of the PVY genome.

The most worrying outcomes of this study are that considerable variation occurs in PVY and the common occurrence of recombination in PVY. The considerable genetic variation within these PVY isolates may ultimately cause the virus to become more virulent and may also lead to a virus which is more easily aphid transmissible. Because of this possibility urgent attention must be given to current farming practices and seed potato certification procedures. Increased control of aphid populations and regulation of year-round farming is necessary to minimize viral load and exchange of viruses between plants. The associated amino-acid changes in CP composition may ultimately also have severe implications for immuno-based detection of mutated PVY isolates, in particular the PVY[NTN] and recombinant isolates. More sensitive non-immuno-based seed potato screening methods should therefore be developed to lessen the chances of infected tubers being used as seed potatoes, to lessen the chances of viral recombination and thus to reduce the viral presence in the field.

References

Blanco-Urgoiti, B., Tribodet, M., Leclere, S., Ponz, F., Perez dé San Roman, C., Legorburu, F.J. and Kerlan, C. (1998) Characterization of potato potyvirus Y isolates from seed potato batches. Situation of the NTN, Wilga and Z isolates, *Euro. J. Pl. Path.,* **104**: 811-819.

Boonham, N., Walsh, K., Hims, M., Preston, S., North, P. and Barker, I. (2002a). Biological and sequence comparisons of potato virus Y isolates associated with potato tuber necrotic ringspot disease. *Pl. Path.,* **51**: 177-126.

Boonham, N., Walsh, K., Preston, S., North, J., Smith, P. and Barker, I. (2002b). The detection of tuber necrotic isolates of potato virus Y and the accurate discrimination of PVY[O], PVY[N] and PVY[C] strains using RT-PCR. *J. Virol. Meth.,* **102**: 103-112.

Brunt, A.A. (2001). Potyviruses. In: Loebenstein G., Berger, P.H., Brunt, A.A. and Lawson, R.H. (eds), Virus and virus-like diseases of potatoes and production of seed-potatoes. Kluwer Academic Publishers, Dordrecht, pp 77-86.

Coetsee, J. (2005). Virusse bedreig hele aartappelbedryf, *Landbouweekblad*, **61637**, 44-45

Crosslin, J., Hamm, P., Shiel, P., Hane, D., Brown, C. and Berger, P. (2005). Serological and molecular detection of tobacco venial necrosis isolates of potato virus Y (PVY N) from potatoes grown in the western United States. *Amer. J. Pot. Res.* **82**: 263-269.

De Bokx, J.A. (1981). CMI/AAB Descriptions of plant viruses. Potato virus Y. **37:** 242. Downloaded from the worldwide web: www.dpvweb.net/dprv/showdpv.php?dpvno=242

Delgado-Sanchez, S. and Grogan, R.G. (1970). Potato virus Y, *CMI/AAB Description of plant viruses.* **37**, CMI/AAB, Kew, Surrey, England, pp 4.

Glais, C. Kerlan, M. Tribodet, S. Astier-Manifacier and C. Robaglia (1996). Molecular characterization of potato virus Y[N] isolates by PCR-RFLP. *Euro. J. Pl. Path.,* **102**: 655–662.

Jawaid, A., Khan, A.J. and Dijkstra, J. (2002). Plant viruses as molecular pathogens. Food products press, The Haworth Press Inc., N.Y.

Li, N., Wang, X., Zhou, G. and Dong, J. (2006). Molecular variability of the coat protein gene of potato virus Y from tobacco in China. *Acta Virol.*, **50**: 107-113.

Lorenzen, J.H., Meacham, T., Berger, P.H., Shiel, P.J., Crosslin, J.M., Hamm, P.B. and Kopp, H. (2006). Whole genome characterization of Potato virus Y isolates collected in the western USA and their comparision to isolates from Europe and Canada. *Arch. Virol.*, **151**: 1055-1074.

Na, L. (Unpublished, Direct submission to GeneBank, 2004). Molecular variability of the coat protein gene of potato virus Y. Plant Virus Department, Plant Protection Institute, CAAS, Yuan, Ming West Road, Beijing 100094, P.R. China.

Pieterse, B. (2005). A new variant of PVY in South Africa. Available: http://www.potatoes.co.za/ home.asp?pid=219 (Access: Continuous).

Revers, F., Le Gall, O., Candresse, T., Le Romancer, M. and Dunez, J. (1996). Frequent occurrence of recombinant potyvirus isolates. *J. Gen. Virol.,* **77**: 1953-1965.

Salaman, R.N. (1930). Virus diseases of potato: Streak. *Nature*, **126**: 241.

Swofford, D.L. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Smith, K.M. and Dennis, R.W.G. (1940). Some notes on suspected variant of Solanum virus 2 (potato virus Y). *Annals Appl. Biol.,* **27**: 65-70.

Chapter 4

Development of molecular detection methods for PVY

Visser, J.C. & Bellstedt, D.U.

Department of Biochemistry, The University of Stellenbosch, Private Bag X1, Stellenbosch, 7602,

South Africa

Summary

Sensitive and accurate methods of detection are of critical importance in the management of potato virus Y (PVY). In South Africa, in commercial potato production, ELISA kits from Europe are currently utilized for the routine screening of potato plant material and potato seed certification. However, due to considerable divergence in South African isolates of this virus in comparison to European isolates, there is a definite need for the development and implementation of an ELISA kit and/or another high throughput detection assay based specifically on South African isolates of PVY. For this purpose the coat protein (CP) gene of a South African isolate of PVY was cloned and expressed in BL21(DE3)pLysS using the pET14 system. The expressed recombinant CP was isolated using SDS-PAGE, purified through electrophoretic elution and $(NH_4)_2SO_4$ precipitation and used for antibody production. Antibodies were raised against the recombinant CP and when employed in an ELISA for PVY detection were less sensitive than the European kits. As an alternative, CP gene sequences of PVY isolates were used to design real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) assays. This led to the design of a sensitive SYBR[®] Green and Taqman[®] assay for the detection of PVY. The qRT-PCR assays proved to be 10^3 fold more sensitive than commercially available ELISA kits.

Introduction

Potato virus Y (PVY) is recognized as having a considerable economic impact within agricultural industries worldwide. PVY infection of potato plants may result in a loss in yield of between 10 and 100% (Warren *et al.*, 2005). Even low levels of infection in seed potatoes can have a detrimental effect on following generations. It has been shown that viral loads of 0.4% within a seed potato may increase to as high as 97% after four generations (Ragsdale *et al.*, 2001). Although potatoes with a viral load of between 1 and 4% may still be planted for commercial use they can no longer be used to produce seed potatoes (Warren *et al.*, 2005).

Currently routine screening of seed potatoes for the presence of viruses is performed using ELISA kits. In South Africa, these kits are imported from Europe where they were developed using European virus strains. Given the fact that considerable genetic variation in PVY in South Africa has been detected (Visser and Bellstedt, unpublished) this raises the question of whether or not these kits are able to detect South African isolates of PVY as effectively as the overseas strains.

Traditionally ELISA kits for viral detection are produced using viral particles purified from plant material. This method, however, is technically taxing, time consuming and results in low yields of viral

particles. An alternative method is the use of recombinant gene technology whereby the gene is cloned into a suitable vector and expressed in a host organism. Following reverse transcriptase polymerase chain reaction (RT-PCR) amplification of the viral CP gene it may be expressed using an expression host such as the *Escherichia coli* strain Bl21(DE3)pLysS. The recombinant protein may then be purified and used for immunization. Resulting antibodies should then theoretically be able to allow detection of the virus of interest. This approach has been used successfully in the development of ELISA kits for the detection of several potato plant viruses including potato virus A (PVA) (Čeřovská *et al.*, 2002) and potato mop-top virus (PMTV) (Hélias *et al.*, 2003). Previous attempts to produce ELISA kits for the detection of South African isolates of PVY and PLRV using recombinant protein methodology were relatively unsuccessful since these kits had limited sensitivity (Matzopoulos, 2005; Rothmann., 2007). Failure of locally produced ELISA kits and the possibility of misdetection by imported kits prompted a search for an alternative method which would allow for sensitive and reliable detection of the above mentioned viruses.

Detection of PVY through the use of RT-PCR is relatively well established but post-run analysis using agarose gel electrophoresis is expensive, laborious and time consuming. An alternative to traditional RT-PCR is real-time reverse-transcription polymerase chain reaction (qRT-PCR). Although this method of detection is more expensive than traditional RT-PCR it does offer a higher level of sensitivity, is quicker, allows for strain differentiation and eliminates the need for post-run sample analysis.

This study aimed to produce functional detection systems for South African of PVY isolates. In the first part of the study the coat protein gene of a South African isolate of PVY was amplified using RT-PCR. The resulting cDNA was then cloned into the expression host BL21(DE3)pLysS using the pET14 cloning system. The expressed protein was used for immunization and the resulting antibodies used to construct an ELISA. In the second part of this study, an investigation into qRT-PCR detection of PVY was launched. Previously established CP gene data matrices were used to design primer pairs for SYBR[®] Green based qRT-PCR detection of PVY. Primers and a fluorescent hydrolysis probe were designed for the so-called Taqman[®] detection of PVY. The designed qRT-PCR assays were used to screen samples from various regions of South Africa as well as tissue culture samples obtained from the South African National Potato Cultivar Collection.

Materials and methods

Sample collection – Potato plant leaves shown to be infected using the BIOREBA ELISA were obtained from various regions in South Africa by CBS (Coen Bezuidenhout Seed Testing Center, Pretoria). The following samples were used: CC9(12), CC34A(16), CC55(198), CC62(156), DD019(141), DD020A(22), DD020(92), DD103A(101), DD072C(98), DD122A(15), NN300(99), NN300(155), NN333B(87), PP003(137), PP002(126), SS082A(88), SS121(151), SS277(125), TT019A(98), TT026B(88), TT138E(102), TT138E(111), WW016(10), WW026B(24), WW282E(19), Z25 and Z26.

Amplification of the PVY CP gene through Reverse Transcriptase-PCR and cloning into pGEM®-T Easy – The PVY CP gene within the infected samples was amplified by means of RT-PCR (Visser and

Bellstedt, unpublished, see Chapter 3 of this thesis). Following RT-PCR product purification the PVY CP gene was cloned into pGEM®-T Easy (Promega) as described (Visser and Bellstedt, unpublished, see Chapter 3 of this thesis).

All plant samples were also subjected to this RT-PCR, followed by agarose gel electrophoresis of amplicons, to confirm their infection status.

Cloning of the CP gene of SS082A(88) into the pET14b expression vector – The PVY CP gene of the isolate SS082A(88) was removed from the cloning vector, pGEM®-T Easy, using the restriction enzymes Nco I and BamH I (New England Biolabs) in a double restriction digest. A single 20 µl digest reaction consisted of 3.5 µl Milli-Q® water, 2 µl 10 × NE Buffer for BamH I, 14 µl pGEM-T Easy plasmid with PVY CP insert (~34 ng/µl), 0.2 µl NcoI (10 U/µl) and 0.1 µl BamH I (20 U/µl). Simultaneously an expression vector, pET14b, was subjected to a similar double digest to result in an open vector. A single double digest reaction for the pET14b vector consisted of 7.5 µl Milli-Q® water, 2 µl 10 × NE Buffer for BamH I, 10 µl pGEM-T Easy plasmid with PVY CP insert (~50 ng/µl), 0.2 µl Nco I (10 U/µl) and 0.1 µl BamH I (20 U/µl). Reactions were allowed to proceed for three hours at 37°C and the enzymes inactivated by incubating the reaction mixtures at 65°C for 15 minutes. The restriction digest mixtures were separated on a 1% agarose gel. Bands of interest were excised and purified using the Wizard® SV and PCR Clean-up System (Promega) according to the manufacturer's instructions. The resulting DNA solutions were concentrated to a final volume of 15 µl using a SpeedVac Concentrator (Savant Instruments, Farmingdale, N.Y.). The concentrations were estimated through the use of 2% agarose gel by comparing gel band intensities to those of the 100 bp DNA ladder (Promega).

The purified PVY CP gene fragment was cloned into the pET14b vector using the reagents supplied with the pGEM®-T Easy cloning system (Promega) according to the instructions supplied by the manufacturer to produce the recombinant plasmid SS082A(88)-pET14b. Ten microliters of the recombinant plasmids were transformed into chemically competent *Escherichia coli* (JM-109, 1×10^7 cfu/µg) according to the pGEM®-T Easy protocol. Transformed cell cultures were plated out in duplicate (50 µl and 150 µl) on a LB/agar plate supplemented with ampicillin (50 µg/ml) (Sigma), IPTG (160 mM) and X-Gal (48 µg/ml) and incubated at 37°C for 19 hours. This incubation period was found to be sufficient for blue colour development but an additional 30 to 60 minutes at 4°C was sometimes added to enhance in the blue/white screening process.

Diagnostic PCR with SP6 and T7-terminator primers – White colonies were subjected to diagnostic PCR to establish whether or not the colony of interest contained a vector with an insert. The PCR mixture consisted of 1 µl 10 × PCR-buffer, 0.6 µl 25 mM MgCl$_2$, 0.5 µl 20 µM SP6 primer, 0.5 µl 20 µM T7 primer, 0.4 µl 5 mM dNTPs (Bioline), 0.1 µl 5 U/µl Taq™ DNA polymerase (Bioline) and 6.9 µl Milli-Q® water. These primers were supplied by the DNA Synthesis Laboratory, Department of Molecular and Cell Biology, University of Cape Town, South Africa. All diagnostic PRC amplifications were performed in a Labnet Multi Gene II Thermal Cycler with the following program: 94°C for 5 minutes, followed by 25 cycles of the amplification at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds and a

single cycle at 72°C for 7 minutes. A holding temperature of 15°C was used. Products were loaded on a 1% agarose gel and electrophoresed at 100 V for 60 minutes. Visualization of the resulting bands was done as described above. Insert orientation was confirmed through the use of cycle sequencing.

Sequencing plasmid inserts using SP6 and T7-terminator primers – The plasmids isolated from colonies confirmed to contain inserts were sequenced by cycle sequencing to confirm that the insert was ligated in the correct orientation. For each insert containing plasmid DNA sample two sequencing PCRs were performed. The SP6 and T7-terminator primers were used. Each sequencing reaction consisted of 4 μl Terminator mix, 3 μl of either SP6 or T7 primer (3.3 μM) and 3 μl plasmid DNA. A Terminator Mix was utilized to save on Terminator Dye and consists of one part Terminator Dye (BigDye® Terminator v3 Cycle Sequencing kit, Applied Biosystems) and four parts Half Dye Mix (Bioline). A Labnet Multi Gene II Thermal Cycler was used and programmed as follows: 35 cycles of 96°C for 10 seconds, 52°C for 30 seconds and 60°C for 4 minutes followed by a final extension completion cycle of 60°C for 10 minutes. A holding temperature of 15°C was used. The products of cycle sequencing were analyzed using an ABI® 3100 Genetic Analyser (Applied Biosystems, Foster City, USA) in the Central Analytical Facility, University of Stellenbosch.

Nucleotide sequence analysis and alignment - Resulting electropherograms were edited using Chromas (v 2.23, Technelysium, Pty., Ltd.). The generated PVY CP gene nucleotide data was compared to previously sequenced gene sequences utilizing the software package BioEdit (v 7.0.5.2, Tom Hall) to confirm orientation.

Cultivation of selected recombinant clones and subsequent plasmid isolation – The remaining parts of the selected colonies were inoculated in 14 ml Falcon tubes in 5 ml of LB containing ampicillin (50 μg/ml) on a G24 Environmental Incubator/Shaker (New Brunswick Scientific Co., Inc., Edison, N.J., U.S.A.) set at 200 rpm at 37°C for 16 hours. Cloned plasmids were purified from the resulting cell cultures using a Plasmix Miniprep kit (Talent).

Transformation of BL21(DE3)pLysS cells with recombinant SS082A(88)-pET14b plasmids – Ten microliters of purified recombinant SS082A(88)-pET14b plasmids were transformed into chemically competent BL21(DE3)pLysS cells in a similar fashion to the transformation of pGEM®-T Easy into JM-109 cells. Transformed cell cultures were plated out in duplicate (50 μl and 150 μl) on LB/agar plates supplemented with ampicillin (50 μg/ml), chloramphenicol (34 μg/ml) as well as 1% (v/v) glucose and incubated at 37°C for 19 hours.

Expression of the PVY CP – Following transformation, as many colonies as possible were swabbed from a single bacterial plate using a toothpick and deposited into a 14 ml Falcon tube containing 4.75 sterile LB medium supplemented with 1% (v/v) glucose, ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). This culture was grown for 16 hours at 37°C on a shaker set to 200 rpm. The resulting cultures were diluted fifty times in a TB induction medium (1.2% (w/v) Bacto® tryptone (Difco laboratories), 2.4% (w/v) yeast extract (Merck), 0.4% (v/v) glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) containing 1% (v/v) glucose as well as ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). These subcultures were grown

at 37°C on a shaker set at 200 rpm. Samples were taken from each of the subcultures to measure the OD_{600} and expression was induced by the addition of 40 mM IPTG at an OD_{600} of 0.6. Two negative controls, one which contained a plasmid with no insert but was induced and the other which contained a recombinant plasmid but was not induced, were also included to serve as controls for protein expression in later analyses. Subsequent to induction, the cultures were grown for four hours at 37°C on a shaker set at 200 rpm. Samples were taken from the induced subcultures as well as the negative controls on an hourly basis. After four hours the cultures were placed on ice to halt expression and growth. The cultures were transferred to sterile JA20 tubes and centrifuged for 5 minutes at 5 000 × g at 4°C. The supernatant was discarded and the pellet resuspended in 1 × TEN 50 buffer (20 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 0.2 mM DTT, 0.1% (v/v) Triton X 100, 50 mM NaCl, 10% (v/v) glycerol) using a tenth of the original cell culture volume. Samples taken at different time points before, during and after the induction were treated in a similar fashion. All resuspended cell cultures were stored at -80°C analysis or purification purposes.

SDS-PAGE analysis of protein expression – Samples were prepared for SDS-PAGE analysis by means of a freeze/thaw method. The samples were placed in a -80°C Cascade Cooling unit (Snijders Scientific, Tilburg, The Netherlands) for 10 minutes and then heated to 37°C for 5 minutes in a hybridization oven/shaker (Amersham Biosciences). This process was repeated three times. Subsequent to the freeze/thaw cycles, the samples were forced through a series of decreasing syringe needles (Nipro Medical Corporation) using a 10 ml syringe. To establish whether the PVY CP gene was in the soluble or insoluble fraction of the lysed cell suspension, a fraction was centrifuged at 13 000 × g for 5 minutes. The supernatant was separated from the pellet and the pellet resuspended in a tenth of the original volume of 1% (w/v) SDS. Twenty microliters of the supernatant and resuspended pellet were treated with an equal volume of sample treatment buffer (0.125 M Tris-HCl, 4% SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercapto-ethanol, pH 6.8) as well as one fifth of the total volume bromophenol blue (0.1% (w/v) in 1.5 mM NaOH). The buffered samples were incubated on a digital dry bath (Labnet International, Inc.) at 90°C for 10 minutes. After the incubation period the samples were cooled on ice. Cooled samples were loaded on a discontinuous SDS-PAGE gel. The gel consisted of a ten well 4.5% stacking gel portion (4.5% (w/v) acrylamide (BDH), 2.7% (w/v) N, N'-methylenebisacrylamide (Merck), 0.1% (w/v) SDS (Sigma), 0.125 M Tris-HCl, pH 6.8) and a separating gel segment (12% (w/v) acrylamide (BDH), 2.7% (w/v) N, N'-methylenebisacrylamide (Merck), 0.1% (w/v) SDS (Sigma), 0.375 M Tris-HCl, pH 8.8). For protein band size estimation, five microliters of Rainbow[TM] molecular weight marker (Amersham Biosciences) was treated in a similar fashion as the samples and also electrophoresed. The acrylamide gels were submerged vertically in an electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS (Sigma), pH 8.3). The run was performed at a constant 20 mA for approximately two hours after which the stacking gel portion was removed and the separating gel segment stained. The separating gel segment was stained in Coomassie staining solution (0.125% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol, 10% (v/v) acetic acid) at 37°C in a hybridization oven/shaker (Amersham Biosciences) for an hour. It was subsequently destained in Destain I (50% (v/v) methanol, 10% (v/v) acetic acid) at

37°C for an hour and overnight in Destain II (5% (v/v) methanol, 7% (v/v) acetic acid). After the overnight destain step the gel was rinsed using Milli-Q® water, sealed in a plastic bag to prevent it from drying and kept as a reference.

Elucon purification of the PVY CP – The lysed bacterial extract was run on several acrylamide gels, as previously described, along with the Rainbow™ marker. Each gel was loaded with Rainbow™ marker in lanes 1 and 3. Lanes 2 and 4 through to 10 contained 25 µl of the insoluble fraction of the lysate dissolved in 1% (w/v) SDS. After electrophoresis, lanes 1 and 2 were separated from the rest of the gel and stained as previously described and then used as a reference to find gel bands of interest on the unstained gel fragments. Bands of interest were excised using a sterile scalpel blade and sliced into fine pieces. The Elucon (Biometra) electroelution apparatus was filled with electrode buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS (Sigma), pH 8.6) and the acrylamide pieces carefully loaded in the elution channels. The Elucon was run at 20 mA for two hours after which the polarity was reversed. One hundred microliter fractions were collected from the wells every 10 seconds for the first minute and every 30 seconds for the following 8 minutes. Elucon samples were pooled, precipitated with 50% (v/v) $(NH_4)_2SO_4$ for 2 – 16 hours at 2°C and then centrifuged at 16 000 × g for 40 minutes in a refrigerated Heraeus Biofuge Fresco set at 4°C. The resulting supernatant was discarded and the pellet allowed to resuspend in 1/10 × PBS, pH 7.2, overnight at 4°C. The resuspended protein solution was transferred to a cellulose dialysis tube (Sigma Aldrich) and dialyzed overnight against two changes of 1/10 × PBS, pH 7.2, at 4°C to remove salts and SDS. The dialyzed samples were removed from the cellulose tubing, concentrated by means of a SpeedVac Concentrator (Savant Instruments, Farmingdale, N.Y.) and stored at -20°C.

Protein concentration determination – A modified Bradford protein determination protocol and a standard bicinchoninic acid (BCA) (Pierce) assay were used to estimate the protein concentration of the above mentioned samples. Protein concentrations were only determined after dialysis since high concentrations of SDS and salts within samples may result in false positives or false negatives. The micro Bradford assay is based on the spectrophotometric shift which occurs due to the electrostatic interaction between the Bradford dye and certain amino acids. Interaction is primarily with arginine but the dye also shows some interaction with the positively charged side chains of histidine, lysine, tyrosine, tryptophan as well as phenylalanine. Amino acid composition directly derived from DNA sequence data revealed that the PVY CP had none of these amino acids present. For the micro Bradford assay, a 2 mg/ml solution of BSA fraction V (Roche) was made in 1/10 × PBS, pH 7.2, of which a dilution series was prepared. Five microliters of each standard dilution and sample was pipetted in duplicate into separate wells of a Greiner microtiter plate and 250 µl micro Bradford solution (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid) was added to each. The contents of the wells were mixed by gently tapping the plate. The absorbance readings were taken at 620 nm using a Titertek Multiscan microtiter plate spectrophotometer subsequent to a 2 minute incubation period at room temperature.

Due to lack of amino acids reactive to Bradford solution used in the initial estimation of PVY CP concentration, determination by means of the BCA method was also employed. The BCA assay is based

on the reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline environment (the reaction volume) and the colourimetric detection of the cuprous ion at 562 nm. Chelation of two BCA molecules with one cuprous ion results in a purple-coloured reaction. The structure of the protein, the number of peptide bonds as well as the presence of cysteine, cystine, tryptophan and tyrosine have been described as the responsible components for colour development. A standard 2 mg/ml BSA fraction V solution was made in $1/10 \times$ PBS, pH 7.2. From this standard, a dilution series was set up. Ten microliters of each standard or sample was pipetted in duplicate into a Greiner microtiter plate. Two hundred microliters of the working reagent (50 parts BCA Reagent A : 1 part BCA Reagent B) was added to each well and mixed well for 30 seconds by gentle shaking. The plate was covered and incubated at 37°C for 60 minutes. Colour development is continuous and no true end point is reached but the 37°C incubation period slows the rate of change sufficiently to allow for accurate comparison between samples. The microtiter plate was cooled to room temperature and the absorbance measured at 562 nm using a Titertek Multiscan spectrophotometer.

Antibody production against recombinant PVY CP – Purified PVY CP proteins were adsorbed to naked bacteria to evoke an elevated immune response (Bellstedt *et al*., 1987). A 2 mg/ml solution of naked bacteria, prepared from *Salmonella minnesota* R595, was prepared in sterilized Milli-Q® water. One hundred and twenty micrograms of the PVY CP in 100 µl of $1/10 \times$ PBS, pH 7.2, were added to 250 µl (500 µg) of the naked bacteria solution. The mixture was dried using a SpeedVac Concentrator (Savant Instruments, Farmingdale, N.Y.) and the resulting powder resuspended in 1.25 ml $1 \times$ PBS, pH 7.2. To aid in the resuspension process and to break up large particles, the solution was subjected to a 10 second burst in a sonicator. Five hundred microliters of the suspension (40 µg of the recombinant PVY CP adsorbed to 200 µg naked bacteria in 500 µl $1 \times$ PBS, pH 7.2) was used per immunization. The rabbit was immunized on days 0, 3, 13, 16, 21, 28, 31, 35, 42, 49, 53 as well as 63 and 2 ml of blood was drawn on days 0, 28 and 63. A larger bleed (20 ml) was collected on day 42. The blood was incubated at 37°C for 30 minutes and stored overnight at 4°C. The following day, the blood samples were centrifuged at low speed to separate the serum from the blood clot. Serum was then carefully transferred to sterile Eppendorfs and stored at -20°C.

Detection of PVY CP by means of a Western blot – A 12% SDS-PAGE gel was prepared as previously described and the two halves thereof loaded with samples in such a way that they form mirror images of each other. Electrophoresis was performed as previously described. Afterwards the two halves were separated using a sterile scalpel and the one half stained as previously described. The remaining half was transferred to a 0.45 µm Protan nitrocellulose transfer membrane (Schleicher and Schuel) using wet transfer setup filled with transfer buffer (0.05 M Tris, 0.2 M glycine, 20% (v/v) methanol, pH 8.3). Protein transfer was carried out at a constant 120 mA for 18 hours. The nitrocellulose membrane was stained using Ponceau S (Sigma-Aldrich) to verify whether protein transfer did indeed occur. The membrane was stained for five minutes at room temperature while being gently agitated. Excess Ponceau S was washed off using Milli-Q® water and the membrane partially destained in Milli-Q® water for 2 minutes. The membrane was fully destained after three washes of five minutes each in an excess of Milli-Q® water. To reduce non-specific binding, the membrane was submerged in 50 ml casein buffer (10 mM

Tris-HCl, 0.15 mM NaCl, 0.5% (w/v) casein (BDH), 0.02% (w/v) thiomersal, pH 7.6) for 20 minutes at room temperature. The casein buffer was decanted and 20 ml of a 1/1000 dilution of day 63 antiserum containing rabbit anti-PVY antibodies added. The membrane was incubated at 37°C for one hour while subjected to gentle agitation. This incubation step was followed by a wash step of five minutes in 20 ml PBS-Tween (0.15 M PBS, pH 7.2, 0.1% (v/v) Tween-20) which was repeated three times. Ten milliliters of a 1/500 dilution of goat anti-rabbit serum in casein buffer was added after decanting the wash buffer. The membrane was once more incubated at 37°C for one hour while subjected to gentle agitation. After the incubation, the membrane was washed as previously described and incubated for one hour at 37°C in 10 ml of a 1/5 000 dilution of rabbit peroxidase-anti-peroxidase complex in casein buffer. The solution was decanted and the membrane washed with 30 ml of $1 \times$ PBS buffer, pH 7.2. The substrate was prepared in two parts. The first part contained 18 mg 4-chloro-1-naphtol dissolved in ice cold methanol and the second 9 μl H_2O_2 in 30 ml PBS, pH 7.2. These two volumes were mixed immediately before being added to the membrane. Colour development was allowed to proceed at room temperature. The colour development reaction was terminated by means of three two minute washes in an excess of Milli-Q® water. To preserve the colour, the membrane was stored wrapped in aluminum foil and kept at 4°C.

Isolation and biotinylation of anti-PVY antibodies for DAS-ELISA – One milliliter of day 63 serum was added to 2 ml $1 \times$ PBS, pH 7.2, and mixed well. Three milliliters of saturated $(NH_4)_2SO_4$ was added to the solution and the volumes mixed by gentle inversion. The mixture was incubated at 4°C for 20 minutes and centrifuged at 27 000 $\times$ g for 20 minutes at 4°C using a J2-21 Beckman preparative centrifuge fitted with JA20 rotor. The supernatant was decanted and the pellet resuspended in 2 ml $1 \times$ PBS, pH 7.2, using a glass rod and gentle swirling. Two milliliters of saturated $(NH_4)_2SO_4$ was added to the suspension and the solution mixed gently but thoroughly. Following a 20 minute incubation at 4°C the solution was centrifuged at 27 000 $\times$ g for 20 minutes at 4°C. The supernatant was decanted and the resulting pellet resuspended in 1 ml $1 \times$ PBS, pH 7.2, using a glass rod and gentle swirling. The resulting solution was dialyzed overnight at 4°C against two changes of 0.1 M carbonate buffer, pH 8.3. The optical density of the solution was measured at 280 nm using a DU 650 spectrophotometer (Beckman). Absorbance was used to calculate the protein concentration within the solution and the solution diluted to 5 mg/ml using 0.1 M carbonate buffer, pH 8.3. Biotinamidocaproate N- hydroxysuccinimide ester (Sigma) was dissolved in N,N-dimethylformamide (Merck) (2 mg/ml). Two hundred and fifty microliters of the biotinylation solution was added to every 1 ml of isolated Ig and the mixture stirred gently for two hours at room temperature. The biotinylation mixture was dialyzed overnight against two changes of $1 \times$ PBS, pH 7.2, and the dialyzed solution mixed well with sterilized glycerol (50% (v/v)). The resulting solution was stored at -20°C.

Sample preparation for DAS-ELISA detection of PVY – For the effective detection of PVY in plant material it is essential that fresh material is used. Storage of plant material leads to degradation of viral particles and the reduction of available antigen. Two hundred milligrams of fresh potato leaf material confirmed to be infected with PVY and uninfected control material was covered in liquid nitrogen and ground to a fine powder using a sterile mortar and pestle. The powder was allowed to thaw and mixed

well in 1 ml grinding buffer (0.1 M Tris, 0.01 M $MgSO_4 \cdot 7H_2O$, 2% (v/v) Triton X-100, 4% (w/v) PVPP, 0.2% (v/v) mercaptoethanol). The leaf pulp was transferred to an Eppendorf tube and centrifuged in a Picofuge (Stratagene) for 15 seconds to remove large pieces of plant material. The clear supernatant was transferred to a new tube and diluted five fold in casein-Tween buffer (10 mM Tris-HCl, 0.15 mM NaCl, 0.5% (w/v) casein (DMV International), 0.02% (w/v) thiomersal, 0.1% (v/v) Tween, pH 7.6).

DAS-ELISA detection of PVY in plants – All ELISA tests were done in Nunc Maxisorb 96 well microtiter plates (Nunc, Denmark). Columns 1 to 11 were coated with day 63 serum diluted 1/400 in 50 mM carbonate buffer, pH 9.6, and incubated overnight at 4°C. Column 12 was left uncoated. From this point on forward all buffers were preheated for 30 minutes at 37°C. The coating solution was decanted and the plate blocked with casein buffer (10 mM Tris-HCl, 0.15 mM NaCl, 0.5% (w/v) casein (DMV International), 0.02% (w/v) thiomersal, pH 7.6). The plate was incubated at 37°C for one hour, the contents decanted and the plate washed four times with PBS-Tween (0.15 M PBS, pH 7.2, 0.1% (v/v) Tween-20). One hundred microliters of plant sap was added to columns 2, 3 and 12 as previously described. A dilution series was made from column 3 to 11 and the plate incubated at 37°C for one hour. The plant sap solution was decanted and the plate washed as previously described. A 1/100 dilution of biotinylated anti-PVY CP antibodies in casein-Tween was added to all the wells and the plate incubated at 37°C. The contents of the wells were once again decanted and the plate washed as described before. One hundred microliters of streptavidin peroxidase (AVPO, Zymed, U.S.A), diluted 1/100 in casein-Tween, was added to each well and the plate incubated at 37°C for one hour. Subsequent to this incubation, the contents of the wells were decanted, the plate washed as previously described and 100 μl of the substrate (0.05% (w/v) 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid] (Roche), 0.05% (v/v) H_2O_2 diluted in 0.1 M citrate buffer, pH 5) added to each well. The plate was incubated at 37°C and absorbance readings taken at 405 nm at fifteen minute intervals for one hour.

Development of primers for SYBR® Green detection of PVY using the Roche LightCycler® – Initial development of primers for SYBR® Green detection of PVY was done on the previously generated CP gene sequence matrix (Visser and Bellstedt, unpublished) using AlelleID (v 4.01) (Premier Biosoft). Subsequent evaluation and modification of primer design was done manually. Two primer pairs for SYBR® Green based detection of PVY were synthesized by the DNA Synthesis Laboratory, Department of Molecular and Cell Biology, University of Cape Town, South Africa. The initial pair was synthesized in duplicate and the one set HPLC purified. This primer pair, PVY SG I(f) and PVY SG I(r), was used to establish the sensitivity of SYBR® Green in viral detection, the influence of HPLC purification of primers on SYBR® Green detection as well as to determine the degree of background fluorescence in non-template containing samples. The second set of primers, PVY SG II(f) and PVY SG II(r), was also synthesized at the University of Cape Town but was not HPLC purified.

SYBR® Green detection of PVY in plant material – Samples were prepared as described for traditional RT-PCR. All reactions were done in a LightCycler 1.5 (Roche Molecular Diagnostics) with 20 μl capillaries using the LightCycler® RNA Master SYBR® Green I kit (Roche Molecular Diagnostics). A

single 20 µl reaction consisted of 8.2 µl PCR grade water, 1.3 µl 50 mM Mn(OAc)$_2$, 2 µl 3 µM primer mix as well as 7.5 µl 2.7 × LightCycler[®] RNA Master SYBR[®] Green I and 1 µl crude sample. The cycling conditions for amplification were as follows: reverse transcription at 61ºC for 20 minutes, RT inactivation and PCR activation at 95ºC for 30 seconds, 55 cycles of denaturation at 95ºC for 5 seconds, annealing at 55ºC for 10 seconds and extension at 72ºC for 10 seconds. Amplification was followed by a melting curve analysis consisting of denaturation at 95ºC for 0 seconds, annealing at 65ºC for 15 seconds and a melting step of 95ºC (0.1ºC/seconds slope) for 0 seconds. The rotor was cooled to 40ºC upon completion of the run. All reactions were additionally analyzed on a 2% agarose gel. The amplicon was sequenced and aligned with the existing CP gene database.

Development of a primer pair and Taqman[®] probe detection of PVY – The two primer pairs (Pot Y F, Pot Y S, Pot Y A, Pot Y R) and Taqman[®] probe (Pot Y TM) were designed *in silico* using BioEdit (v 7.0.5.2, Tom Hall). Evaluation of the designs as well as production of the oligos was done by TIB MolBiol Syntheselabor GmbH, Berlin, Germany.

Taqman[®] detection of PVY in plant material – Samples were prepared as described for traditional RT-PCR. All reactions were done in a LightCycler 1.5 (Roche Molecular Diagnostics) with 20 µl capillaries using the QuantiTect Probe RT-PCR kit (Qiagen). The primers and probe designed and produced by TIB MolBiol were used in various combinations to establish which combination works most efficiently. A single reaction consisted of 10 µl 2 × QuantiTect Probe RT-PCR Master Mix, 1 µl of each primer at 20 µM, 0.4 µl 10 µM probe, 0.2 µl QuantiTect RT Mix, 6.4 µl PCR grade water and 1 µl crude sample. Cycling conditions were as follows: reverse transcription at 50ºC for 20 minutes, RT-PCR inactivation and PCR activation 95ºC for 15 minutes as well as a two step amplification step of 55 cycles with denaturation at 95ºC for 0 seconds and annealing at 60ºC for 60 seconds. Reactions were analyzed on a 2% agarose gel and the amplicon sequenced as described before.

BIOREBA DAS-ELISA versus SYBR[®] Green detection of PVY – This ELISA was performed according to the protocol as described by BIOREBA (Switzerland) and 200 µl of reagent volumes were added to each well in each of the following steps. PVY specific IgG was diluted 1 000 × in BIOREBA coating buffer (15 mM Na$_2$CO$_3$, 34.8 mM NaHCO$_3$, 3.08 mM NaN$_3$, pH 9.6) and used to coat a MaxiSorp F96 Immuno plate (Nunc). The plate was covered and incubated overnight at 4ºC. Subsequently the wells were washed four times using PBS-Tween (0.15 M PBS, pH 7.4, 0.05% (w/v) Tween-20). Plant samples were ground in BIOREBA extraction buffer (19.8 mM Tris, 136 mM NaCl, 2% (w/v) PVP K25, 0.05% (w/v) Tween 20, 2.68 mM KCl, 3.08 mM NaN$_3$, pH 7.4). The sample was diluted 1:1 in the extraction buffer and added to the plate. The plate was incubated overnight at 4ºC and washed as previously described. An alkaline phosphotase-labeled antibody was diluted 1 000 × in conjugate buffer (19.8 mM Tris, 136 mM NaCl, 2% (w/v) PVP K25, 0.05% (w/v) Tween 20, 2.68 mM KCl, 0.2% (w/v) BSA, 1 mM MgCl$_2$·6H$_2$O, 3.08 mM NaN$_3$, pH 7.4) and added to the plate. The plate was incubated at 4ºC for five hours and washed as previously described. Substrate (p-nitrophenyl phosphate (1 mg/ml) in 9.7% (v/v) diethanolamine, 3.08 mM NaN$_3$, pH 9.8) was added and incubated at room temperature for 30 minutes. Spectrophotometric

readings were taken at 405 nm. SYBR® Green detection was performed as previously described using the same plant material.

Table 1. Primers used for the detection of PVY using the LightCycler 1.5 (Roche). The primers and Taqman® probe targeted the CP gene region.

Primer name	Primer sequence (5' → 3')	Nucleotide position
PVY SG I (f)	ATGCCAACTGTGATGAATG	8893-8911
PVY SG I (r)	AATGTGCCATGATTTGCC	9041-9058
PVY SG II (f)	GCTTATGGTTTGGTGCATTG	8913-8932
PVY SG II (r)	AATGTGCCATGATTTGCCTA	9039-9058
Pot Y F (f)	GACRTAGGAGAAACTGARATGCCAAC	8875-8900
Pot Y S (f)	GARATGCCAACTGTGATGAATGGG	8890-8913
Pot Y A (r)	AGAAATGTGCCATGATTTGCCTAA	9038-9061
Pot Y R (r)	CGCATTTCWATATACGCTTCTGCAA	9068-9092
Pot Y TM	FAM-TATGGTTTGGTGCATTGARAATGGAACCTC-BBQ	8916-8945

§ Nucleotide positions are based on Genbank sequence X97895

Degenerate base pairs are indicated by R (A, G) and W (A, T)
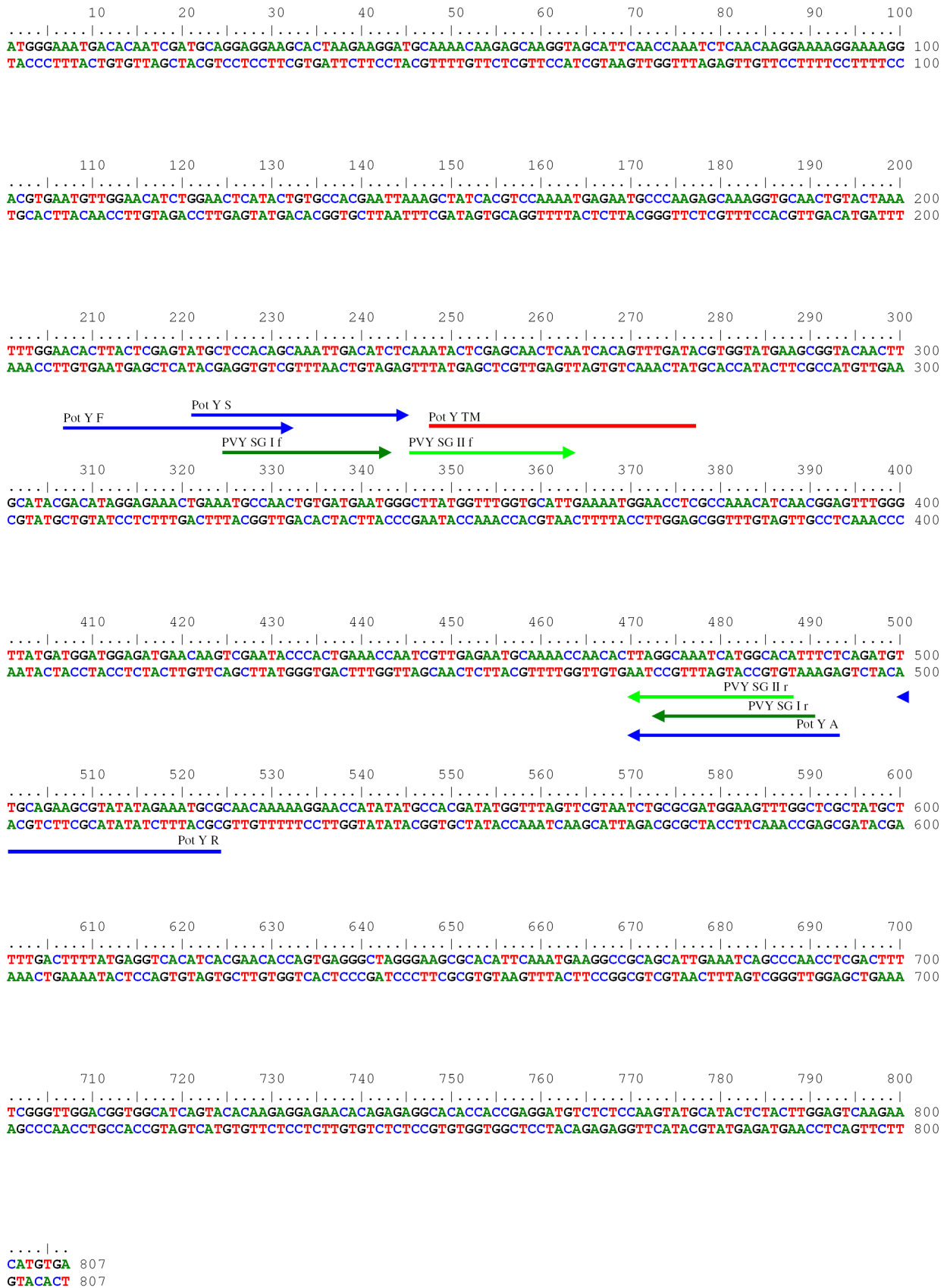
Figure 1. A graphic illustration displaying target regions the SYBR® Green and Taqman® primers and probe were based on. The primers were designed to bind to areas in PVY CP gene which are relatively conserved.

Results

Amplification of the PVY CP through Reverse Transcriptase-PCR and cloning into pGEM®-T Easy – This procedure was successfully completed as previously described (Visser and Bellstedt, unpublished, see Chapter 3 of this thesis). RT-PCR detection of viral infection revealed that all plant samples gave RT-PCR products, i.e. could be confirmed as being infected with the exception of CC34A(16), CC55(198), CC62(156), DD020A(22), DD072C(98), DD122A(15), PP003(137), PP002(126), SS121(155), SS277(125), TT019A(98), WW016(10), WW026B(24), WW282E(19) and Z25.

Restriction enzyme digestion using BamHI and NcoI for subcloning into pET14b – The CP gene of SS082A(88) was enzymatically cleaved from the TA-cloning vector pGEM®-T Easy using a combination of NcoI and BamHI. The expression vector pET14b was cleaved with the same restriction enzyme pair. All enzyme digestions were verified on a 2% agarose gel and found to be complete (figure 2). The PVY CP gene was subsequently cloned into the expression vector pET14b to result in the recombinant construct SS082A(88)-pET14b.
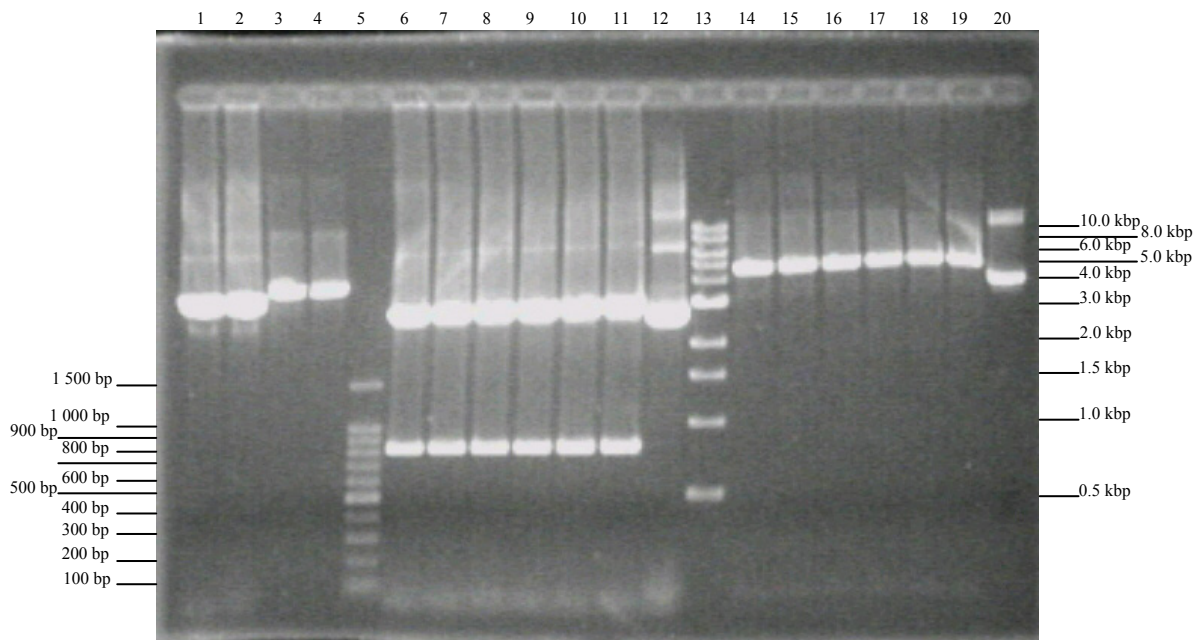
Figure 2. An image of a 2% agarose gel stained in ethidium bromide viewed under UV-light. Lane 1: NcoI digest of PVYCP/pGEM-T, Lane 2: BamHI digest of PVYCP/pGEM-T, Lane 3: NcoI digest of pET14b, Lane 4: BamHI digest of pET14b, Lane 5: 100 bp DNA ladder (Promega), Lane 6: NcoI/BamHI digest of SS082A(88)-pGEM-T, Lane 7: NcoI/BamHI digest of SS082A(88)-pGEM-T, Lane 8: NcoI/BamHI digest of SS082A(88)-pGEM-T, Lane 9: NcoI/BamHI digest of SS082A(88)-pGEM-T, Lane 10: NcoI/BamHI digest of SS082A(88)-pGEM-T, Lane 11: NcoI/BamHI digest of SS082A(88)-pGEM-T, Lane 12: SS082A(88)-pGEM-T undigested, Lane 13: 1 kb DNA ladder (BioLabs), Lane 14: NcoI/BamHI digest of pET14b, Lane 15: NcoI/BamHI digest of pET14b, Lane 16: NcoI/BamHI digest of pET14b, Lane 17: NcoI/BamHI digest of pET14b, Lane 18: NcoI/BamHI digest of pET14b, Lane 19: NcoI/BamHI digest of pET14b, Lane 20: PVYCP/pET14b undigested.

The resulting recombinant construct was subjected to PCR using the SP6 and T7-terminator primer pair. Sequence analysis revealed that the insert was complete and had the correct orientation (results not shown).

*Expression of PVY CP in BL21(DE3)pLysS using the pET14b cloning system –*Analysis of bacterial extracts of the recombinant construct SS082A(88)-pET14b expressed in the BL21(DE3)pLysS strain of *Escherichia coli* using the pET14b cloning system with SDS-PAGE revealed expression of a protein at 32 kDa, the expected size of the PVY CP (figure 3). Various time points taken after induction revealed that the concentration of expressed protein was at its highest four hours after induction. The cellular suspension was separated in a soluble and insoluble phase as described earlier and the product analyzed with SDS-PAGE (figure 4). The expressed PVY CP was found to be contained in the insoluble phase of the cellular extract. The PVY CP was isolated from the insoluble phase of the cellular extract by electro-elution which was shown using SDS-PAGE and was found to be pure but present in low concentrations (results not shown).
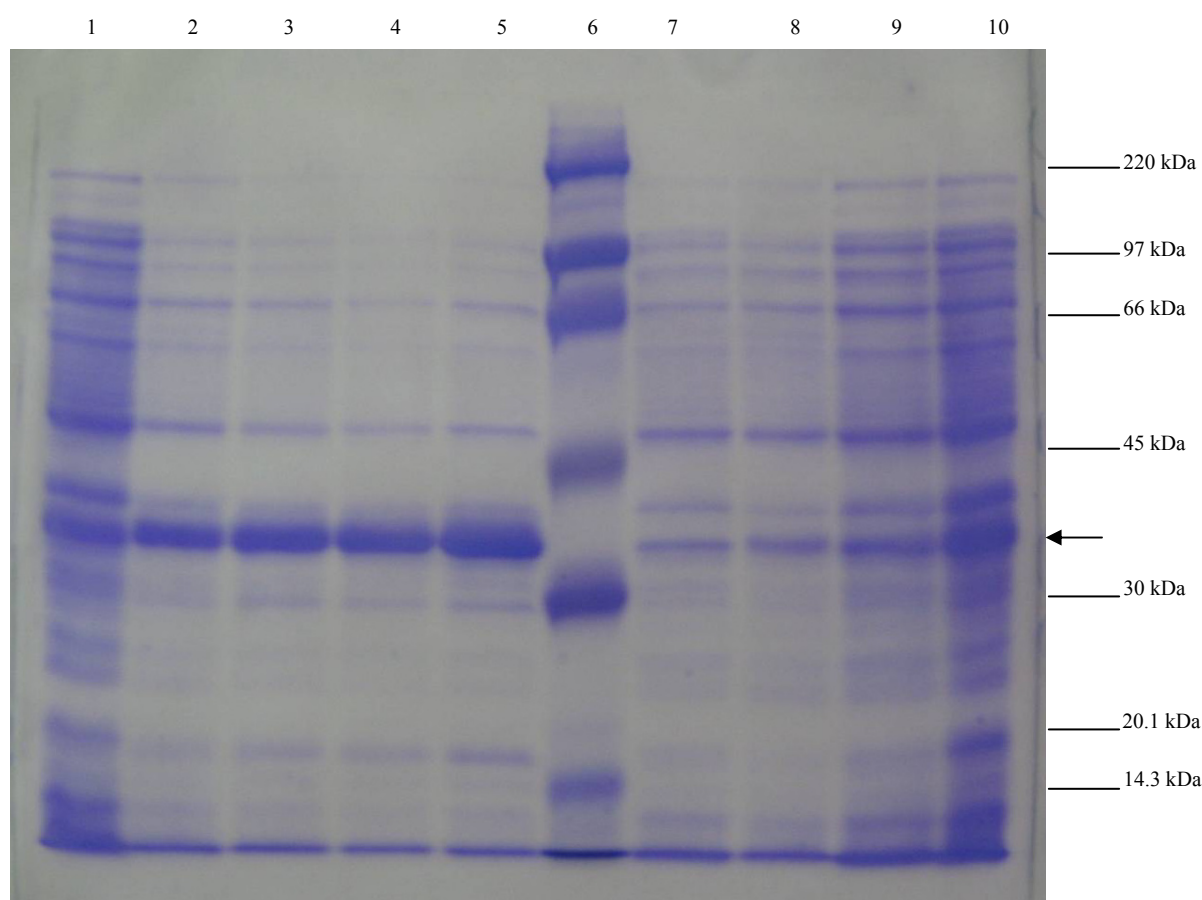
Figure 3. SDS-PAGE analysis (stacking gel 4.5% acrylamide, resolving gel 12% acrylamide) of protein expression within BL21(DE3)pLysS using the pET protein expression system. Lane 1: SS082A(88)-pET14b 1 before induction, Lane 2: SS082A(88)-pET14b 1 hour after induction, Lane 3: SS082A(88)-pET14b 2 hours after induction, Lane 4: SS082A(88)-pET14b 3 hours after induction, Lane 5: SS082A(88)-pET14b 4 hours after induction, Lane 6: Rainbow molecular protein marker (Amersham), Lane 7: pET14b before induction, Lane 8: pET14b 4 hours after induction, Lane 9: SS082A(88)-pET14b before induction, Lane 10: SS082A(88)-pET14b 4 hours no induction. The arrow indicates the position of the recombinant PVY CP at 32 kDa.
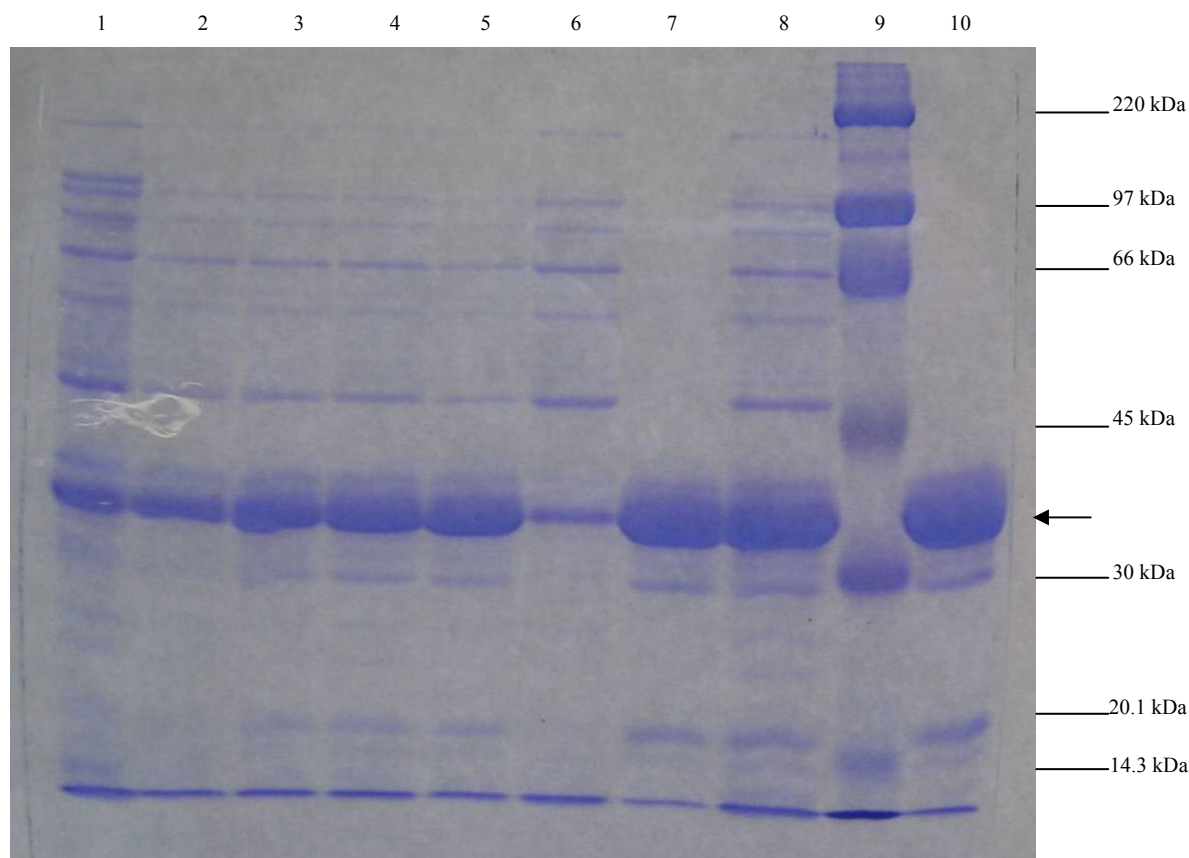
Figure 4. SDS-PAGE analysis (stacking gel 4.5% acrylamide, resolving gel 12% acrylamide) of the soluble and insoluble phases of the BL21(DE3)pLysS with SS082A(88)-pET14b cell extract. Lane 1: SS082A(88)-pET14b before induction, Lane 2: SS082A(88)-pET14b 1 hour after induction, Lane 3: SS082A(88)-pET14b 2 hours after induction, Lane 4: SS082A(88)-pET14b 3 hours after induction, Lane 5: SS082A(88)-pET14b 4 hours after induction, Lane 6: Soluble phase of SS082A(88)-pET14b 4 hours after induction, Lane 7: Insoluble phase of SS082A(88)-pET14b 4 hours after induction, Lane 8: Soluble and insoluble phase of SS082A(88)-pET14b 4 hours after induction, Lane 9: Rainbow molecular protein marker (Amersham), Lane 10: Insoluble phase of SS082A(88)-pET14b 4 hours after induction. The arrow indicates the position of the recombinant PVY CP at 32 kDa**.**

Antibody production against recombinant PVY CP – Antibody production against PVY CP was not specifically confirmed by ELISA or a similar technique. Instead the antiserum was used directly for Western blot analysis or for ELISA development for PVY detection.

Detection of PVY CP expression by means of Western blot – Cellular extracts and purified PVY CP were subjected to SDS-PAGE separation and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau S and confirmed successful protein transfer (figure 5a). The Ponceau S stain revealed an extremely faint band in the lane containing the purified protein at approximately 32 kDa. Once the nitrocellulose membrane had been destained it was subjected to Western blot analysis using serum obtained form the rabbit immunized with the recombinant PVY CP (figure 5b). The Western blot revealed several bands in different positions on the membrane. In lane 2, containing the purified PVY CP, a very faint band was present in the region of 32 kDa. In lane 3, containing the cellular extract of the induced cell line, the area expected to contain the PVY CP is only weakly stained in spite of the fact that Ponceau S staining confirmed that a large amount of recombinant protein was present.
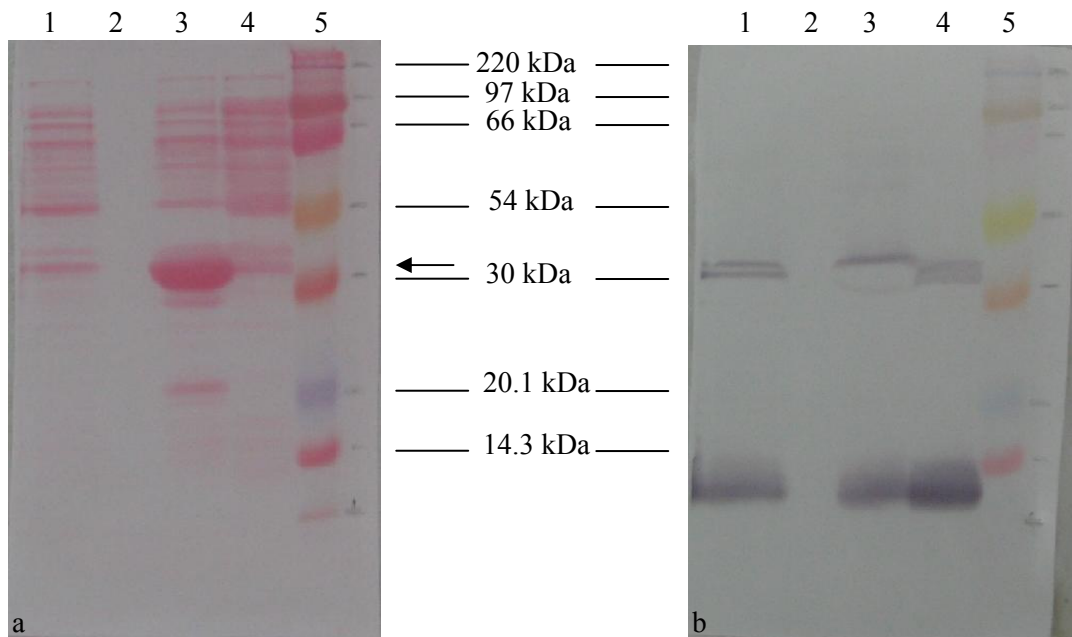
Figure 5. a: An image of a Ponceau S stain of proteins transferred to nitrocellulose. b: An image of a Western blot done with anti-PVY CP antibodies. For both a and b: Lane 1: pET14b 4 hours after induction, Lane 2: Purified recombinant PVY CP, Lane 3: SS082A(88)/pET14b 4 hours after induction, Lane 4: SS082A(88)/pET14b 4 hours with no induction, Lane 5: Rainbow molecular protein marker (Amersham).

DAS-ELISA detection of PVY – The isolated anti-PVY IgG used for biotinylation had an OD_{280} of 0.619 indicating a protein concentration of 11.34 mg/ml. The ELISA was able to discriminate between infected and uninfected material but the resulting absorbance values were relatively low (maximum absorbance: 0.25, figure 6). Samples could also not be diluted very much before the absorbance value in the ELISA dropped below the cutoff value of 0.16 as shown in figure 6.
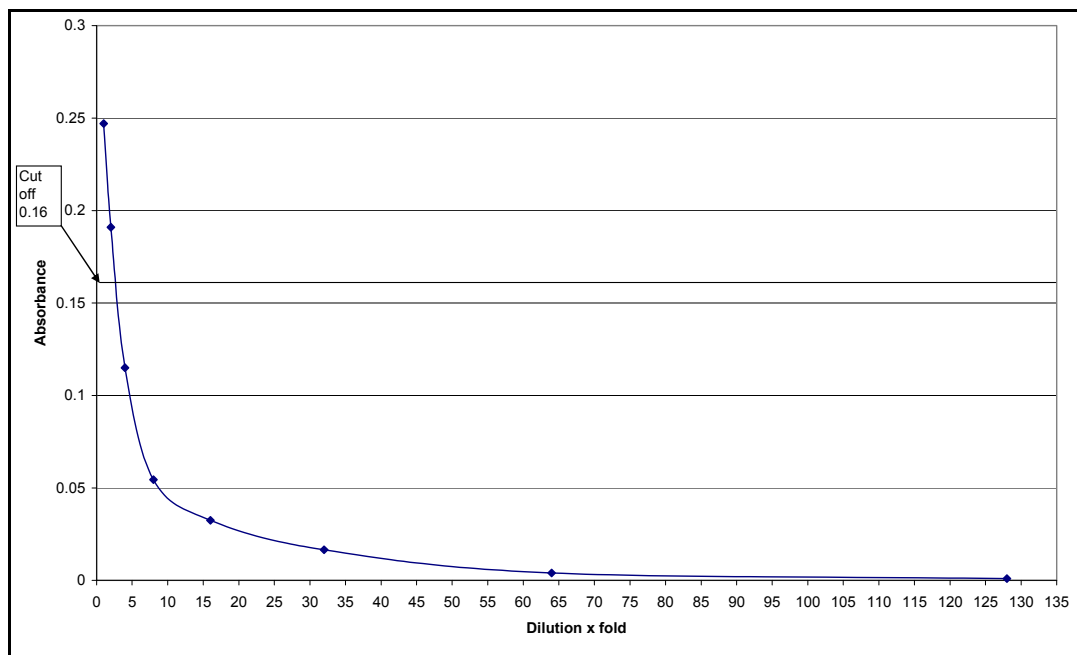
Figure 6. Absorbance values versus dilution illustrating the sensitivity of the ELISA produced based on recombinant protein technology. A cutoff value of 0.16, identical to that implemented in the industry, was used

Detection of PVY in potato plant material using SYBR® Green - The PVY SG I primer combination was used in preliminary protocol development but resulted in a high level of background and non-specific amplification (results not shown). The primers were redesigned and the resulting primers, PVY SG II, used in following investigations. Non-specific amplification was nullified by using the newly designed primer pair. A sample dilution of 10^6 was successfully detected using the PVY SG II primer combination with the LightCycler® RNA Master SYBR® Green I kit (Roche Molecular Diagnostics) (Figure 7 a and b). Resulting products were analyzed on a 2% agarose gel to confirm product size and also sequenced to confirm amplification of the correct target (results not shown). Agarose gel and sequence analyses verified an amplicon of 146 bp in length and generated sequences were identical to existing PVY CP gene sequences (results not shown). There was no noticeable difference in the results obtained when using purified versus the use of unpurified primers (results not shown) and hence the higher cost of HPLC purification of primers made HPLC purification unjustifiable.
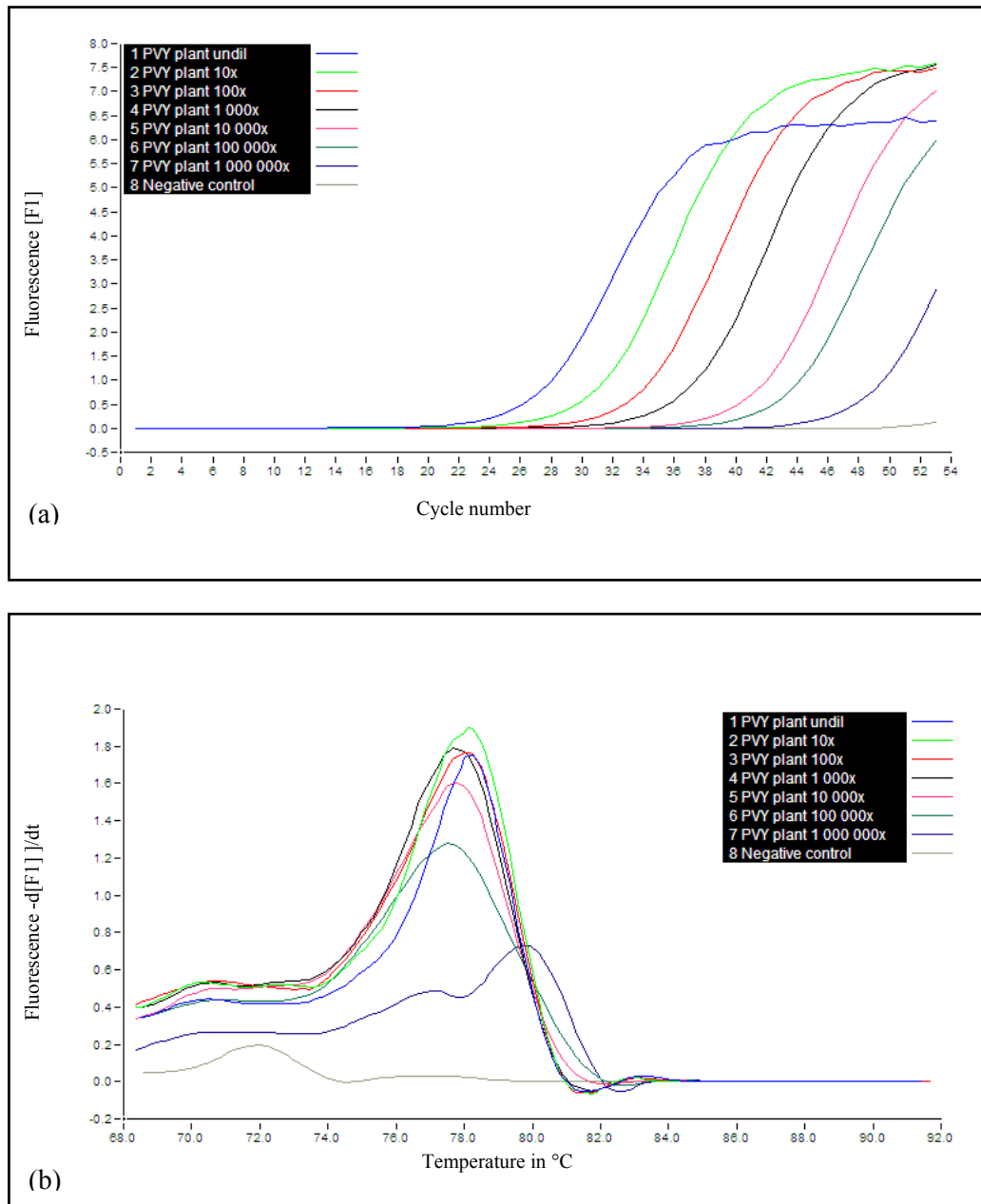
Figure 7. Sensitivity determination of the SYBR Green® qRT-PCR for PVY. Sensitivity of the assay was tested using a dilution series. (a) Increase in fluorescence with increasing cycle number. (b) Melting curve analysis of the qRT-PCR products shown in (a).

Two isolates representing each of the six major variants of PVY found in South Africa (PVY^N, PVY$^{Super\ N}$, PVY^O, PVY^{O415G}, PVY^R and PVY^{NTN}) were included in a qRT-PCR run to determine whether strain variation would negatively impact on detection or sensitivity. All variants were successfully detected and no major differences in melting curve analysis of the different variants was detected (figure 8a and b).

(a)

(b)
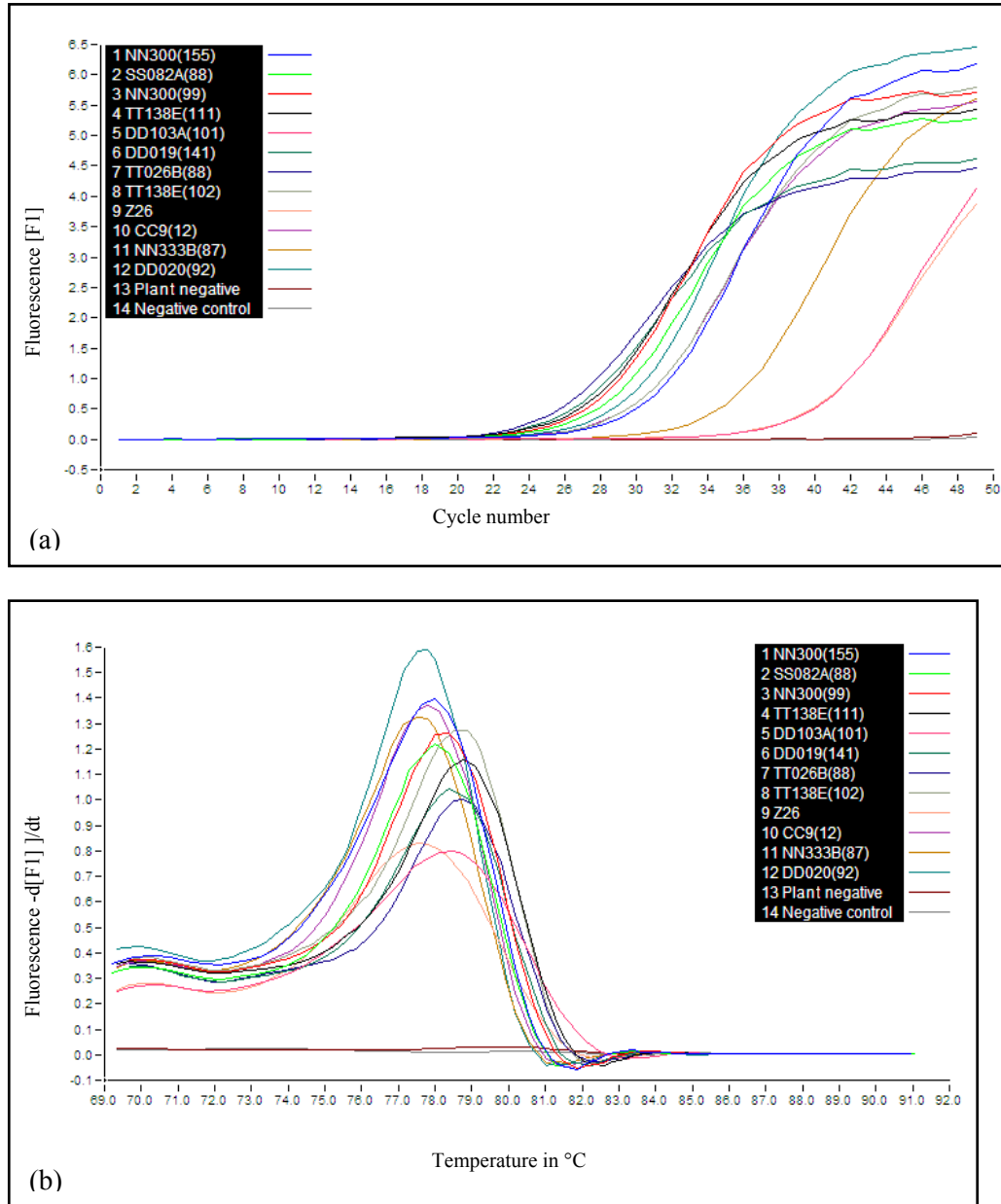
Figure 8. Effect of strains variation on SYBR$^®$ Green qRT-PCR. (a) Increase in fluorescence with increasing cycle number. (b) Melting curve analysis of the qRT-PCR products shown in (a).

During RT-PCR analyses of the samples some isolates did not result in a product, i.e. tested negative for infection. These samples were retested using the developed SYBR® Green II qRT-PCR protocol (figure 9a and b). Ten of the fifteen samples previously classified as negative, tested positive when subjected to qRT-PCR analysis. The remaining five proved to be negative and were also proven so using melting curve analysis and gel electrophoresis. These samples were PP003(137), PP002(126), CC62(156), DD122A(15) and DD020A(22).
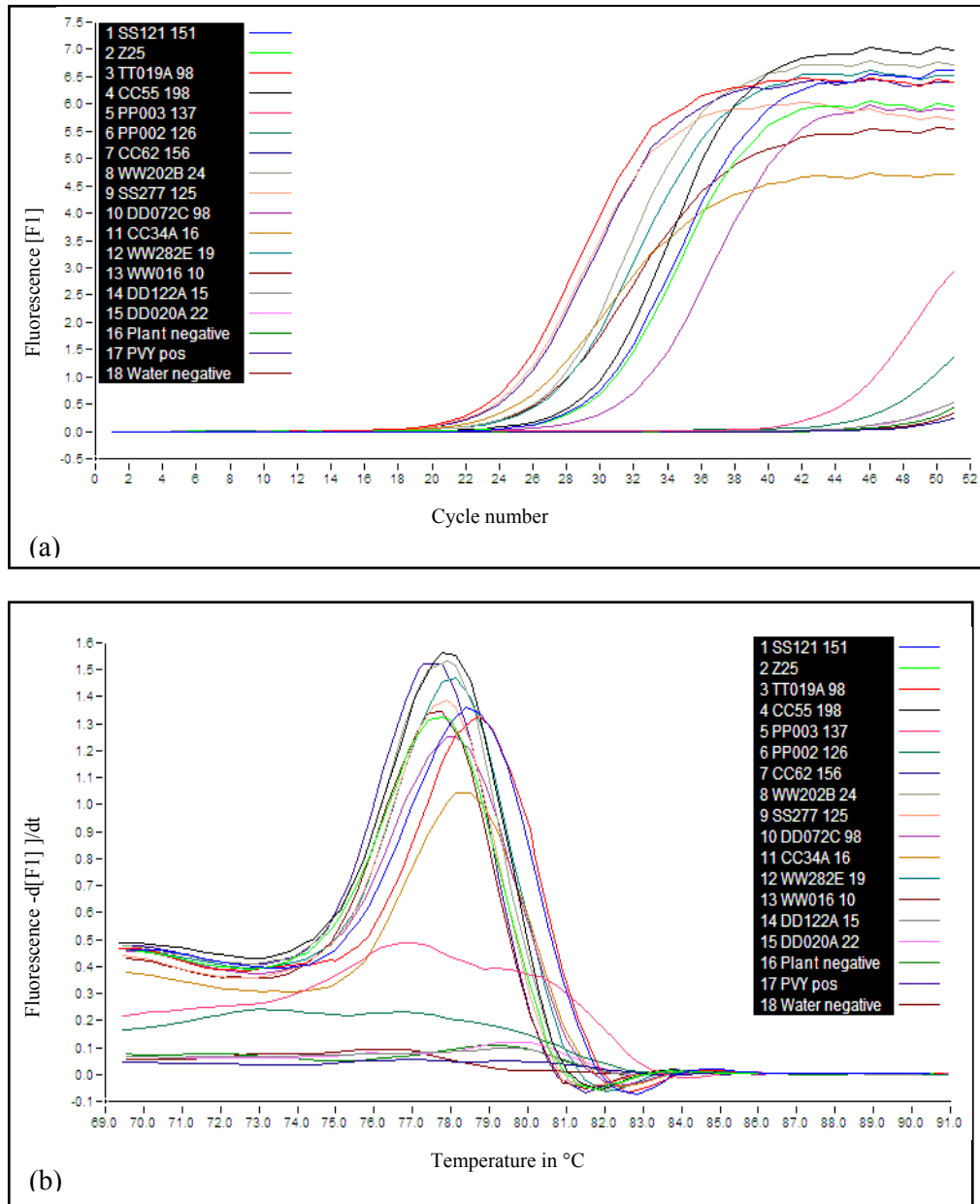
Figure 9. Sensitivity determination of the SYBR® Green qRT-PCR compared to RT-PCR. (a) Increase in fluorescence with increasing cycle number. (b) Melting curve analysis of the qRT-PCR products shown in (a).

Detection of PVY using Taqman® probe technology – Primers Pot Y S and Pot Y A were found to work well in combination with the Taqman® probe (results not shown). The Taqman® probe was tested in a dilution series similar to the one setup for the SYBR® Green protocol described earlier. Unlike the SYBR® Green protocol, which resulted in detection of the target amplicon in each dilution, the Taqman® probe only gave a signal up to 10^5 fold dilution. The negative controls, the water and uninfected plant samples, did not result in any fluorescence whatsoever (figure 10).
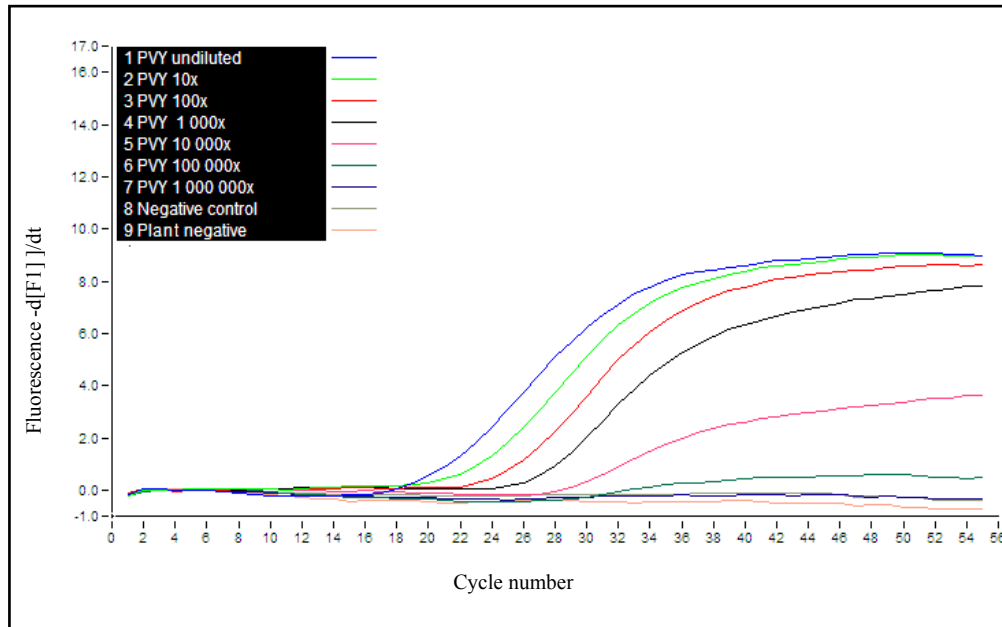
Figure 10. Sensitivity determination of the Taqman® probe. Sensitivity of the assay was tested using a dilution series.

The Taqman® probe was also tested on the various strains of PVY as well as the variants found within those strains (figure 11). The same samples used for the evaluation of SYBR® Green for strain detection were used in this analysis. The Taqman® probe successfully detected all the described strains and again did not result in a fluorescent signal in either the water or negative plant controls.
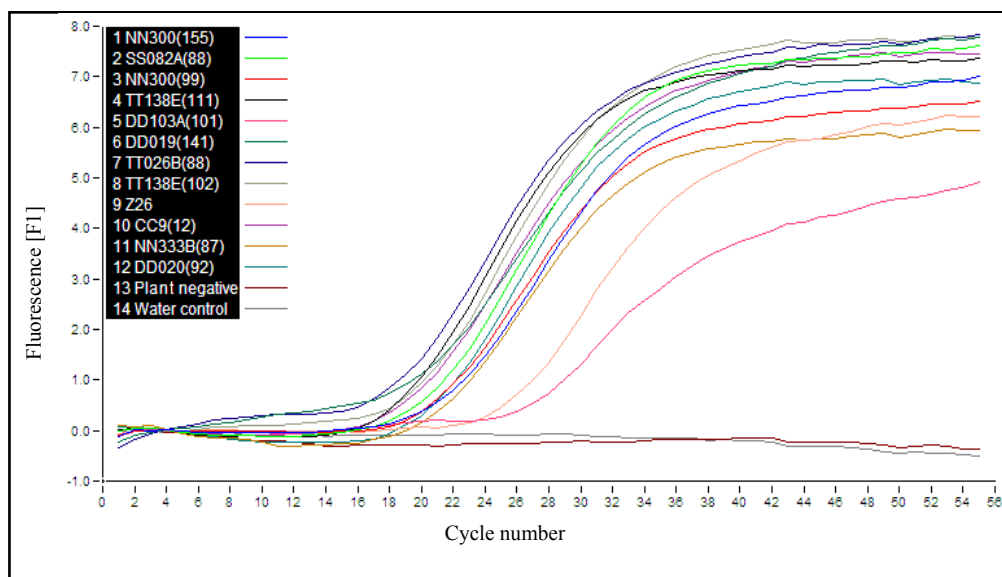
Figure 11. Effect of strains variation on the Taqman® qRT-PCR assay.

Comparison of BIOREBA DAS-ELISA with SYBR® Green detection of PVY – Using the standardized cutoff absorbance value of 0.16 it was found that the BIOREBA ELISA kit was able to detect all three samples from different regions of South Africa up to a 2^{10} dilution ($\sim 10^{3.01}$) (figure 12). Dilution series of the samples used for ELISA screening were made and used for qRT-PCR analysis.
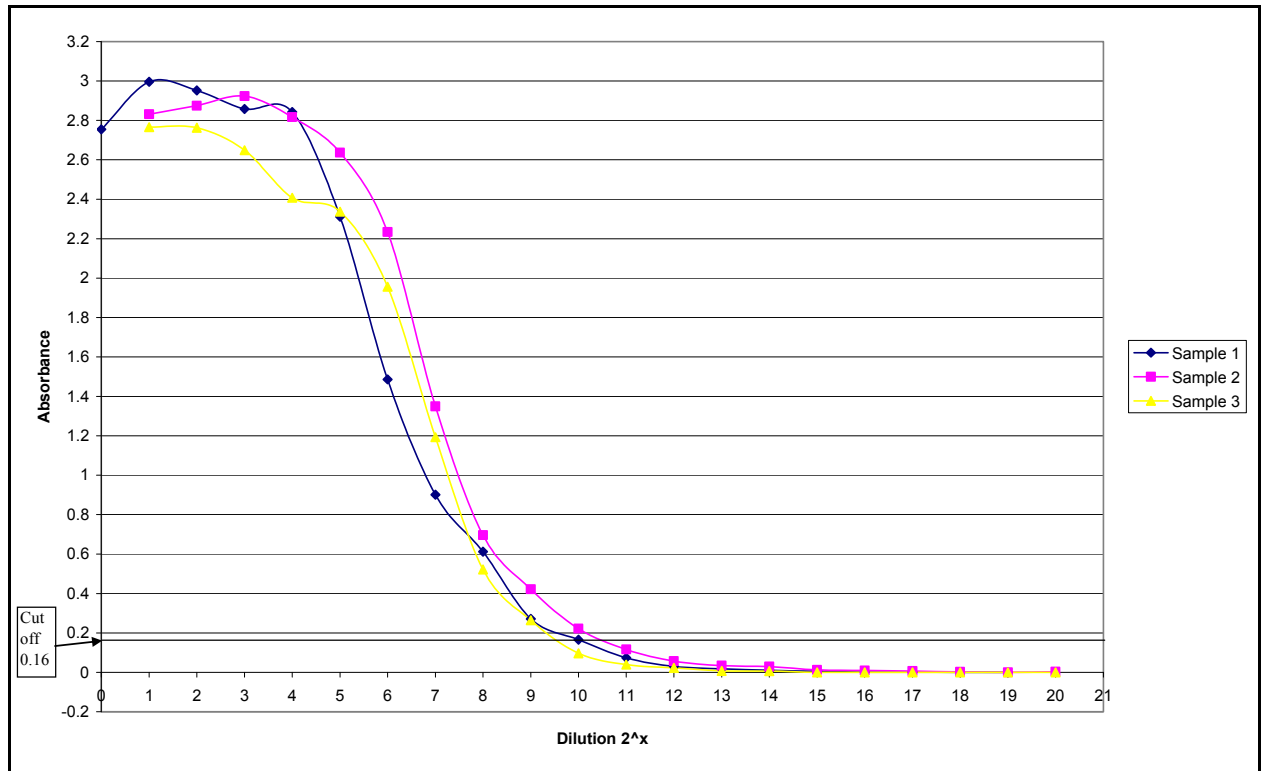
Figure 12. Absorbance values versus dilution illustrating the sensitivity of the commercially available BIOREBA ELISA kit for the detection of PVY in potato plant material. Three different samples were obtained from different regions within South Africa and tested on the same plate using the same reagents. A cutoff value of 0.16, identical to that implemented in the industry, was used.

qRT-PCR analysis of sample 1 and 2 resulted in effective detection of PVY up to a 10^6 dilution (figures 13a and b). Analysis of sample 3 only resulted in effective detection of a 10^4 fold dilution in qRT-PCR (figure 13c). Melting curve analysis of these samples was not possible since the computer running the LightCycler 1.5 suffered a hardware failure at the beginning of the melt. Samples were, however, analyzed using agarose gel electrophoresis. The gel revealed successful amplification of the target amplicon in all of the dilutions and no amplification within the negative controls (figure 14). Agarose gel electrophoresis of these samples reiterated the importance of melting curve analysis of SYBR® Green products and also revealed amplicons at 10^5 and 10^6 fold dilutions of sample 3.
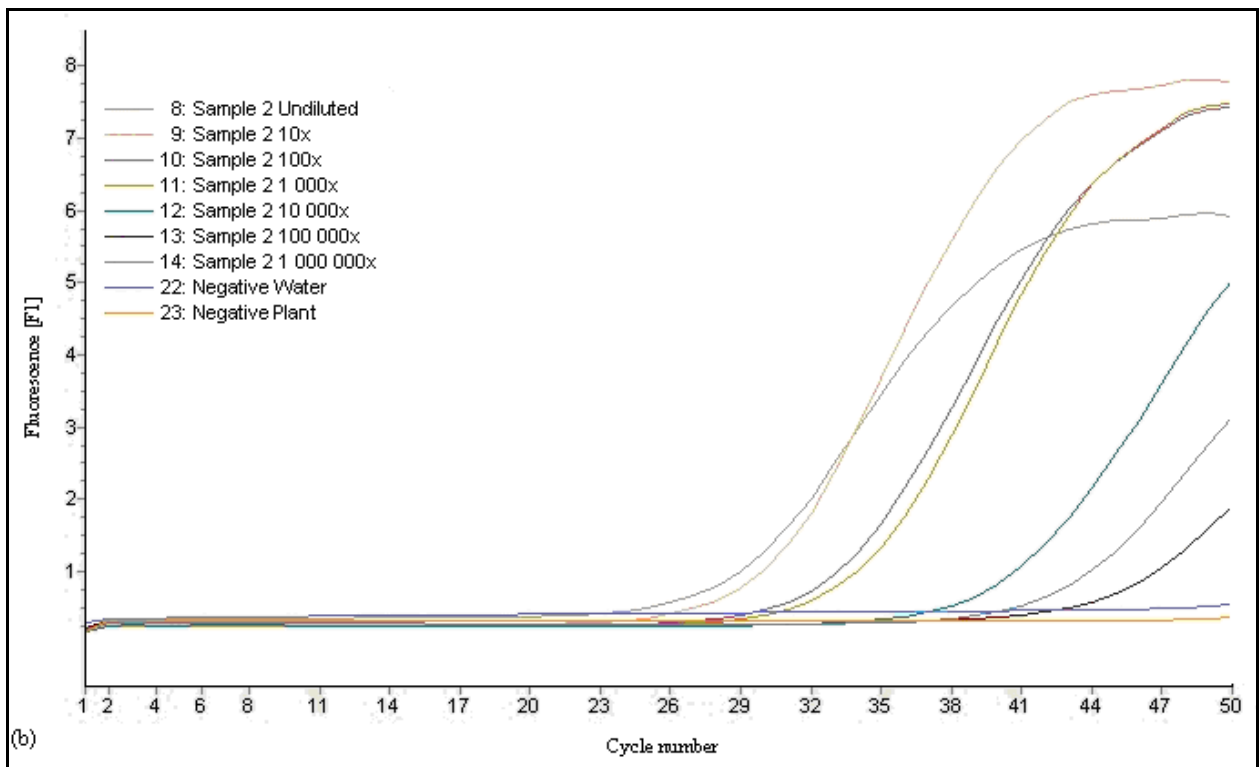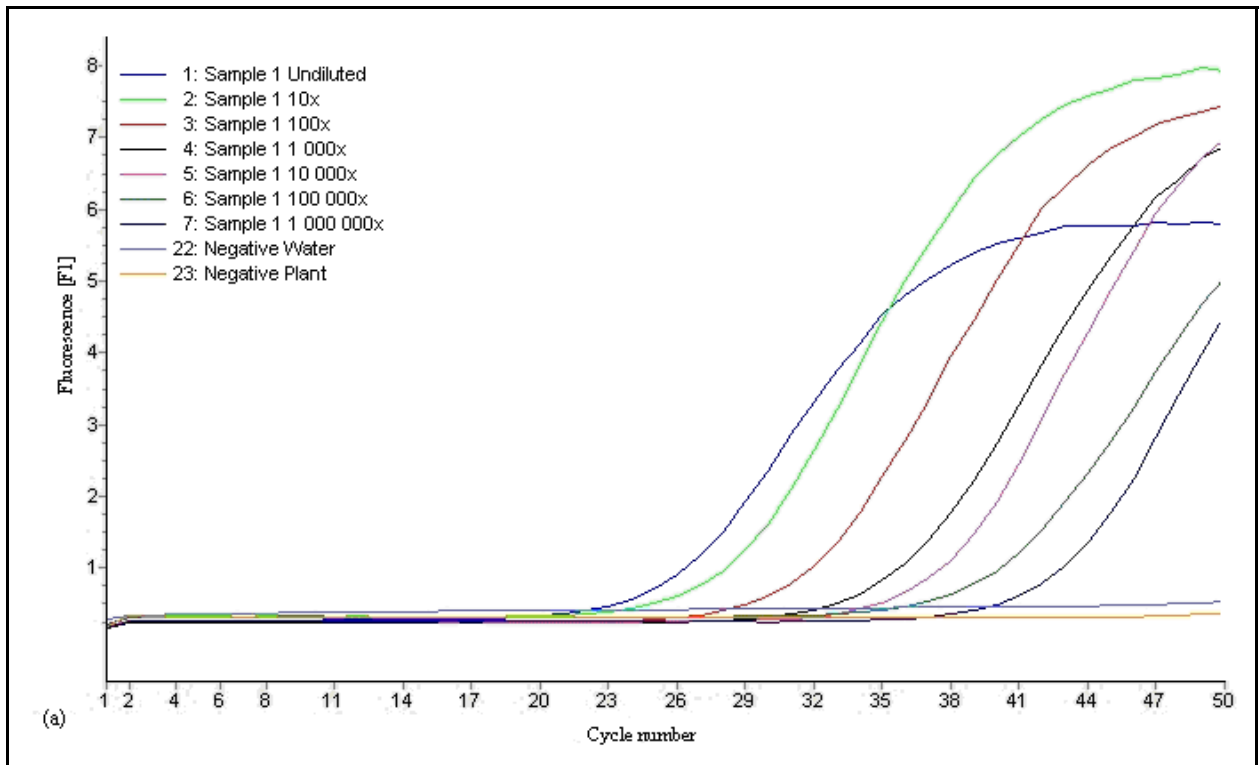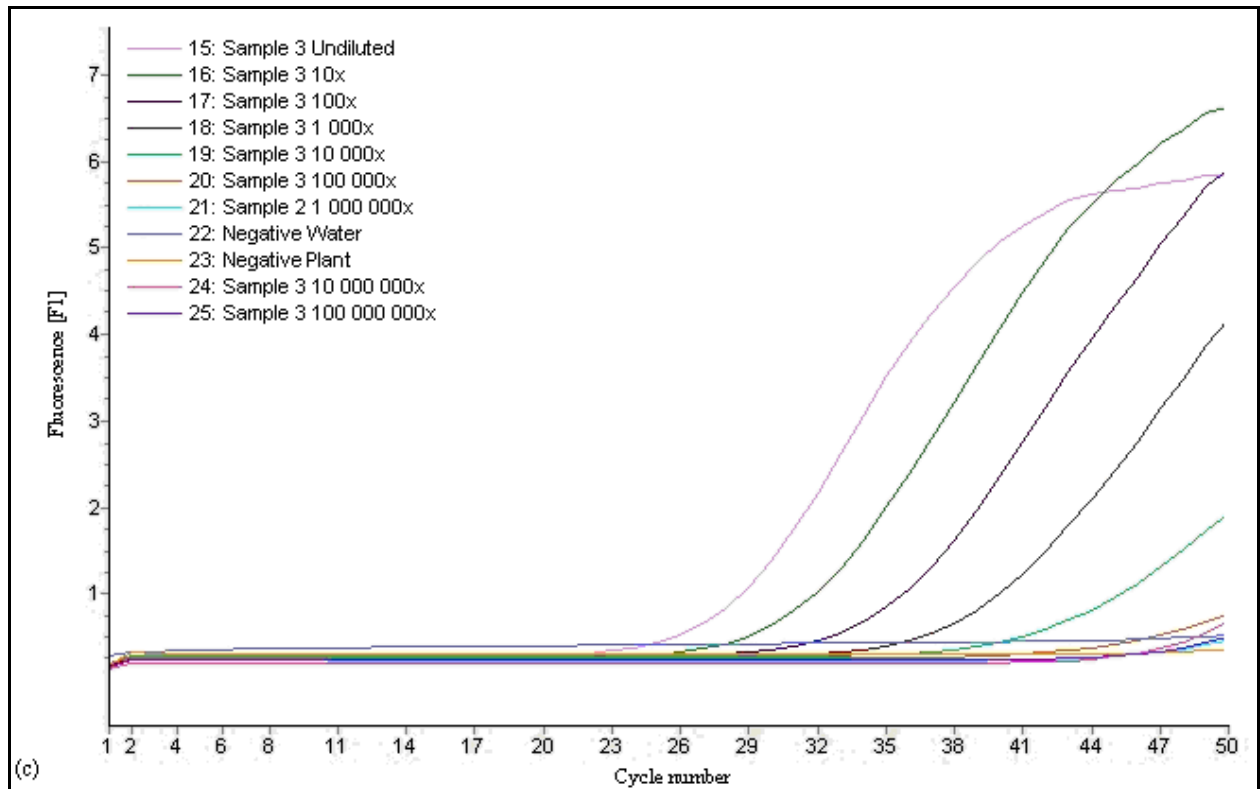
Figure 13. Comparison of SYBR® Green qRT-PCR with the BIOREBA ELISA for PVY detection. (a) Series dilution of sample 1. (b) Series dilution of sample 2. (c) Series dilution of sample 3.
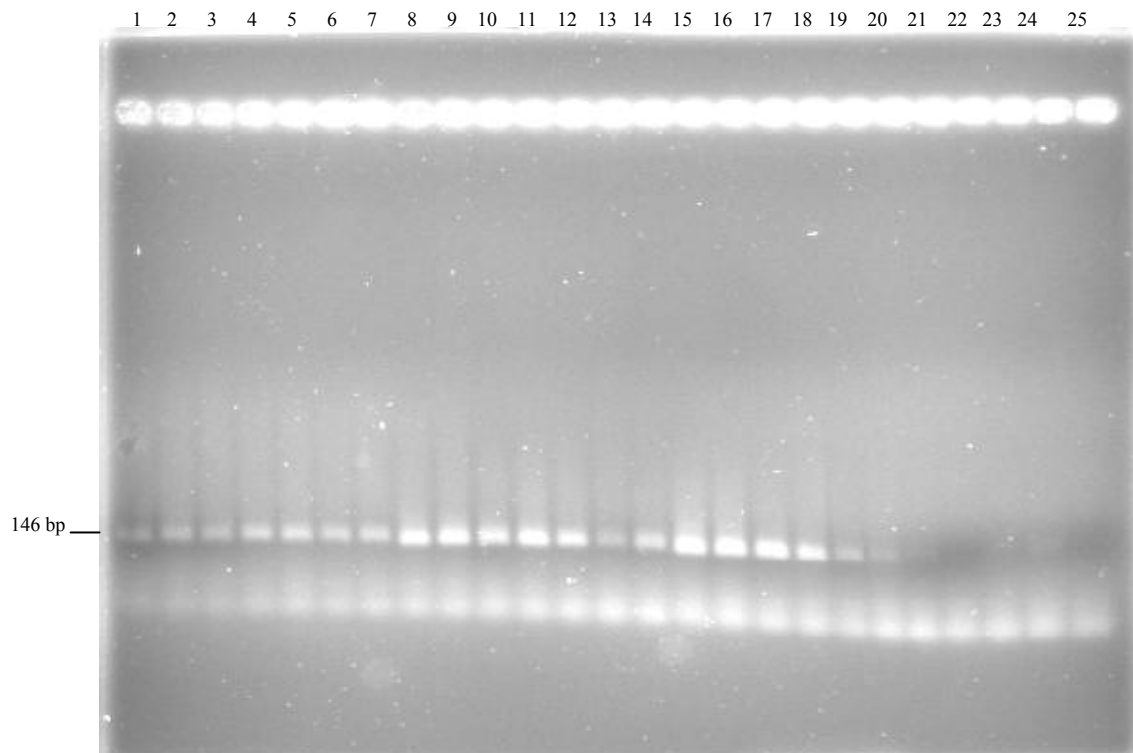
Figure 14. Analysis of the qRT-PCR products of samples 1, 2 and 3 on a 2% agarose gel. Lane 1:Sample 1, undiluted; Lane 2: Sample 1, 10^2; Lane 3: Sample 1, 10^2; Lane 4: Sample 1, 10^3; Lane 5: Sample 1, 10^4; Lane 6: Sample 1,10^5; Lane 7: Sample 1, 10^6; Lane 8: Sample 2, undiluted; Lane 9: Sample 2, 10^1; Lane 10: Sample 2, 10^2; Lane 11: Sample 2, 10^3; Lane 12: Sample 2, 10^4; Lane 13: Sample 2, 10^5; Lane 14: Sample 2, 10^6; Lane 15: Sample 3, undiluted; Lane 16: Sample 3, 10^1;Lane 17: Sample 3, 10^2; Lane 18: Sample 3, 10^3;Lane 19: Sample 3, 10^4; Lane 20: Sample 3, 10^5; Lane 21: Sample 3, 10^5; Lane 22: Negative control, H_2O; Lane 23: Negative control, PLRV; Lane 24: Sample 3, 10^7; Lane 25: Sample 3, 10^8.

Discussion

Although expression of recombinant PVY CP was achieved and it was used for antibody production, the ELISA kit developed using these antibodies was only able to detect PVY in infected samples with a very low sensitivity. The reason for the poor level of detection may be the lack of antibody recognition of the native PVY CP due to antibody production against recombinant PVY CP produced in a bacterial system. The advantage of protein expression in a bacterial system is that should bacterial proteins also be purified along with the PVY CP, it would not negatively influence the ELISA, since the ELISA is only used to detect viruses occurring in a plant system. The fact that the CP is expressed in the bacterial system may, however, also be a disadvantage. PVY CP is normally expressed and processed within plant cells and not only are there several multifunctional viral proteins involved in the processing thereof but it is thought that it is glycosylated by plant glycosylation mechanisms. Expression of the CP in a bacterial system might thus result in incorrect folding and nonglycosylation of the viral CP. Antibodies raised against the recombinant CP may therefore recognize the native CP poorly and result in lower absorbance values when used in ELISA. In future it might be better to perform traditional virus isolation of South African isolates of PVY from plant material and use these virus particles for antibody production although it could possibly result in antibodies against co-isolated plant proteins which may, in turn, lead to elevated background values if used in ELISA.

Studies have shown that plants express the so-called heat shock protein 70 (HSP70) when exposed to stressful conditions such as drought, increased average temperature and viral infection (Aparicio *et al.,* 2005). Virus particle isolation from infected plants may therefore result in co-isolation of HSP70 from leaf material. The antibodies resulting from immunization with such preparations would consequently be aimed against not only the viral CP but also against HSP70. The average temperature to which potato plants are exposed in Europe is far lower than in South Africa. This means that there would be a lower level of HSP70 expression in European plants. Resulting background due to the presence of HSP70 would thus be less noticeable. However, in South Africa potatoes are grown under much higher average temperatures which in turn would result in higher expression levels of the HSP70. This could possibly be the reason for the occasional higher background levels seen when European based ELISA kits are used for PVY detection in South African potato samples.

The designed PVY SYBR$^{®}$ Green qRT-PCR detection protocol allowed for the effective detection of PVY in potato plant material. It proved to be approximately 10^3 fold more sensitive than a commercial ELISA. The protocol also detected all major South African PVY isolates. When using the recommended 45 cycles as a standard length run (SYBR$^{®}$ Green kit recommendations by Roche) dilutions of highly infected plant samples, as high as 10^6 fold, could still be detected. It is highly unlikely that a higher number of cycles will increase the sensitivity of the protocol, in fact, the higher the number of cycles the less *Tth* is available for use (Roche, personal communication). Lower PCR efficiency due to the denaturation of *Tth* overshadows the possibility of detecting anything beyond 45 cycles. It can therefore be deduced from the Roche guidelines that qRT-PCR detection of PVY using the designed primers and

protocol be limited to a 45 cycle run since non-specific amplification is then kept to a minimum and the PCR efficiency is still acceptable. According to Roche this should be enough for the effective detection of as little as 50 viral copies.

Using the SYBR® Green protocol for qRT-PCR detection of PVY, plant samples previously classified as being PVY negative using conventional RT-PCR, were found to be positive confirming its greater sensitivity than normal RT-PCR. These results also bring into question the specificity of the commercially used ELISA. The above mentioned samples tested by qRT-PCR were classified as being PVY infected by ELISA screening. The fact that five of these samples tested negative when subjected to the much more sensitive and specific RT-PCR and qRT-PCR analyses might be indicative of background problems in the ELISA.

From dilution series studies using qRT-PCR it was found that a 10 fold dilution of the original material resulted in a higher level of fluorescence, i.e. a greater concentration of product. This has implications for qRT-PCR detection of PVY in terms of initial sample preparation. It shows that it might be of importance to make a further initial dilution to ultimately result in a higher yield of amplicon and hence greater assay sensitivity. The additional dilution step could dilute out the effect of certain inhibitory factors which may occur in the plant sap. This, however, still needs further testing and a greater number of trial runs before being accepted into standard practice.

Unlike the SYBR® Green protocol, which resulted in detection of the target amplicon in a 10^6 fold dilution, the Taqman® probe only gave a signal up to a 10^5 fold dilution of the same sample. This means that the hydrolysis probe is potentially ten fold less sensitive than SYBR® Green. SYBR® Green is an intercalating dye which binds to all double stranded DNA. This means that non-specific amplification and primer dimerization contributes to the final fluorescent signal making it important to perform a melting curve analysis after each run. Since the Taqman® probe is hydrolyzed during the reaction in the presence of a target, a melting curve analysis cannot be performed once the run is complete. This disadvantage is, however, outweighed by the fact that the Taqman® probe technology offers a greater deal of specificity than SYBR® Green. Since it is necessary for both the primer pair and Taqman® probe to bind in order to generate a fluorescent signal during a Taqman® assay, the likelihood of a non-specific signal is negligible.

The reason for the greater sensitivity of the SYBR® Green qRT-PCR may be due to the fact that the large amplicon generated allows a large amount of SYBR® Green to intercalate into it and therefore give a elevated fluorescence signal. As amplicon composition can vary between different isolates, the amount of intercalated SYBR® Green and therefore fluorescence may vary between different viral isolates. This means that the SYBR® Green qRT-PCR fluorescent signal is not necessarily directly proportional to viral load over all viral isolates. The Taqman® probe would, however, allow for determination of exact viral loads in future studies since the fluorescence is directly proportional to the amount of probe hydrolyzed.

ELISA kits currently imported for the detection of PVY may in the future fail to detect South African isolates of the virus due to genetic drift, mutations and recombination. Because of this it is important that detection assays based on South African isolates of this virus are developed and tested. This also implies

that viral drift should be monitored on an ongoing basis and detection assays should be adapted over time to accommodate for this. This project failed to deliver an ELISA assay based on a South African isolate of PVY, but did deliver a qRT-PCR assay for the detection of South African isolates of PVY. These qRT-PCR assays may therefore prove to be very valuable to screen critical higher generation and tissue culture material for PVY infection and to confirm cases with very low levels of infection in which ELISA gives very low and possibly elevated background absorbance readings.

References

Aparicio, F., Thomas, C.L., Lederer, C., Niu, Y., Wang, D. and Maule, A.J. (2005). Virus induction of heat shock protein 70 reflects a general response to protein accumulation in the plant cytosol. *Pl. Physio.*, **138**: 529-536.

Bellstedt, D.U., Human, P.A., Rowland, G.F. and Van der Merwe, K.J. (1987). Acid-treated, naked bacteria as immune carriers for protein antigens. *J. Immuno. Meth.*, **98**: 249-255.

Čeřovská, N., Moravec, T. and Velemínský, J. (2002). Polyclonal antibodies to a recombinant coat protein of potato virus A. *Acta. Virol.*, **46**: 147-151.

Héliasa, V., Jacquot, E., Guilleta, M., Le Hingrata, Y. and Giblot-Ducray, D. (2003). Production of recombinant Potato mop-top virus coat protein in Escherichia coli and generation of antisera recognizing native virus protein. *J. Virol. Meth.*, **110**: 91-97.

Mackay, I.M., Arden, K.E. and Nitsche, A. (2002). Survey and summary: Real-time PCR in virology. *Nucl. Acids Res.*, **30**: 1292-1305.

Matzopoulus, M. (2005). The development of enzyme-linked immunosorbent assays to detect potato virus Y and potato leaf roll virus using recombinant viral coat proteins as antigens. MSc thesis, University of Stellenbosch.

Ragsdale, D.W., Radcliffe, E.B. and DiFonzo, C.D. (2001). Epidemiology and field control of PVY and PLRV, pp. 237-270. In: Loebenstein, G., Berger, P.H., Brunt, A.A. and Lawson, R.H. [ed.]. Virus and virus-like diseases of potatoes and production of seed-potatoes. Kluwer Academic publishers, Dordrecht.

Rothmann, A.H. (2007). An assessment of the mutation patterns in South African strains of potato leafrll virus and the expression of recombinant viral coat protein genes in *Escherichia coli*. MSc thesis, University of Stellenbosch.

Shepardson, S., Esau, K. and McCrum, R. (1980). Ultrastructure of potato leaf phloem infected wih potato leaf roll virus. *Virol.*, **105**: 379-392.

Visser, J.C. and Bellstedt, D.U. (2007). An assessment of molecular variability and recombination patterns in the coat protein gene of South African strains of potato virus Y (PVY). Unpublished

Warren, M., Krüger, K. and Schoeman, A.S. (2005). Potato virus Y (PVY) and potato leaf roll virus (PLRV): Literature review for potatoes South Africa. Faculty of natural and agricultural sciences, Department of Zoology and Entomology, University of Pretoria.

Wittwer, C.T., Herrmann, M.G., Moss, A.A. and Rasmussen, R.P. (1997). Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques,* **22**: 130-138.

Chapter 5

Conclusion and future perspectives

In South Africa the two traditional PVY strains, i.e. PVY^N and PVY^O, as well as one distinct substrain, $PVY^{Super\ N}$, were detected. In addition, a large amount of variation in South African isolates of these strains was also found. Recombinant strains of PVY^N and PVY^O were also detected of which some exhibited the PVY^{NTN} phenotype. These results have shown that the isolates of PVY present in South Africa have their origin in a number of introductions, the most recent of which appears to be the $PVY^{Super\ N}$ substrain from China as well as evolution through mutation and recombination.

The approach to ELISA development used in this study, i.e. the production of recombinant viral coat protein followed by antibody production against it will have to be reconsidered in the light of the findings of this study. In future consideration may have to be given to the traditional isolation of viral particles of South African isolates of PVY and antibody production against them in spite of the shortcomings of this approach.

The SYBR® Green and Taqman® qRT-PCR detection techniques developed in this study show particular promise as highly sensitive and specific detection techniques which could be used to screen valuable potato material as well as confirmatory tests.

Addendum A

Aligned coat protein gene sequences of the 95 South African isolates of PVY and eleven GenBank reference sequences. A dot indicates a nucleotide identical to the top sequence.

```
                    10        20        30        40        50        60        70        80        90       100
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1     ---GGAAATGACACAATCGATGCAGGAGGAAACAACAAGAAAGATGTAAAACCAGAGCAAGGCAGCATCCAGTTAAACCCGAACAAAGGAAAAGATAAGG
PVY_O1_X14136.1     CAA.C......T....T..............................C..........A.....A....C.....T..GT.G..........A.
PVY_US1_X68222.1    ---.C........TA........G............AC...........A........................C...T..T.............A.
PVY_JC1_U25672.1    ATG.C...........T......T..A..G.........ATC................C....T.........T.......
PVY_CH1_X68221.1    ---.C...............G..G..G......G..CAC...G.....A....T....CC...T..T....G....G..C....
CC73_18             .................................................................................G.A...GT......
SS121_166 56        ATG...............G..G..........C.G..................C...........................
CC55_8 146          ATG.C......T......G..G..........C.G..................C...........................
DD037F_9 154        ATG.C......T......G..G..........C.G..................C...........................
PVYWilga CP AJ889867 CAA.C.............G..G..........C.G..................C...........................
WW154A_62 86        ATG.C......T......G..G..........C.G..................C...........................
CC66_91 47          ATG.......T.......G..G..........C.G..................C.................G..........
SS121_154 10        ATG............G..G..G..........C.G..................C...........................
DD019_141 141       ATG.C..........G..G..G..........C.G..................C...........................
WW282E_3            ATG.C.............G..G..........C.G..................C..................T......
WW010_146_164       ATG.C.............G..G..........C.G.........T.......C...........................
DD103A_101_190      ATG...............G..G..........C.G..................C..G.....T................
DD103A_80_180       ATG.......T......G..G..G..........C.G..................C...........................
PVYNTN1             ATG.C......T......G..G..........C.G..................C...........................
PP014_18            ----------------------------------------------------------------------------G..A....
PP014_21            ----------------------------------------------------.............C.............
CC24_7              ----------------------------------------------------.............C.............
DD122A_34           ATG.......T.......G..G..........C.G..................C...........................
TT138E_113 106      ATG...............G..G..........C.G..................C...........................
WW154_175 62        ATG.......T.......G..G..........C.G..................C...........................
TT141A_76 73        ATG.C......T......G..G..........C.G..................C...........................
TT138D_111 79       ATG.C.............G..G..........C.G..................C...........................
GG517_128_162       ATG.C......T......G..G..........C.G..................C...........................
CC9_48_178          ATG.C.............G..G..G........C.G..................C...........................
WW010_147_166       ATG.C.............G..G..........C.G..................C...........................
GG517_170_168       ATG...............G..G..........C.G..................C...........................
WW010_70_158        ATG...............G..G..........C.G..................C...........................
TV115_3             ----------------------------------------------------.............C..........A...G....
NN459_14            ATG.......T.......G..G..........C.G..................C...........................
TT138E_102 96       ATG...............G..G..........C.G..................C...........................
CC9_30_175          ATG.C.............G..G..........C.G..................C...........................
DD122A_25           ATG.......T.......G..G..........C.G..................C...........................
TT026B_88 115       ATG.......T.......G..G..........C.G..................C...........................
TT026B_86 128       ATG.C......T......G..G..........C.G..................C...........................
DD122A_25           ATG.......T.......G..G..........C.G..................C...........................
NN333B_28 149       ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
Z26 38              ATG...............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
CC9_12_171          ATG...............G..CT....GG...C....A.....T....T.ACC...T.TC....G.A...G.A....
CC62_20 156         ATG.......T.......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
PVY_NTN2_X79305.1   ---.......T.......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN300_76 118        ATG.......T.......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
WW073B_18           ------------------------------------------T....T.ACC...T.TC....G.A...G.A....
NN98A_35            -------------------------------------------T....T.ACC...TTTC....G.A...G.A....
PVYNTN_4_2          -------------------------------------------T....T.ACC...T.TC....G.A...G.A....
DD122A_36           ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN333B_87 152       ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
DD020_92 30         ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
SS121_82 1          ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
WW002_22 147        ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
WW002_74 150        ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
WW002_82 151        ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
SS121_197 16        ATG...............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
PVYNTN3_3           ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
PVYNTN_4_1          -------------------------------------------T....T.ACC...T.TC....G.A...G.A....
SS121_53 42         ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
SS082A_194 14       ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
TT138D_13 68        ATG.......T.......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
PP003_119 85        ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
PP003_119 133       ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
PVY_H1_X54611.1     ---...............G..CT....G....C....A.....T....T.ACC...T.TC....T...G.A...
PP026B_184 111      ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
TT014_184 135       ATG.......T.......G..CT....G....C....A.....T....T.ACC...T.AC....G.A...G.A....
SS147_144 144       ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
DD037F_96_138       ATG.......TT......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
CC9_47_177          ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
WW202B_21_173       ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A.G.G.A....
DD037F_35_188       ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC.G...G.A...G.A....
WW202B_24_184       ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
TT042A_3            -----------------------------.....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
WW655_11            -----------------------------------.C....A.....T....T.ACC...T.TC....G.A...G.A....
CC73_12             -----------------------------------------.T....T.ACCC.T.TC....G.A...G.A....
WW672_35            -----------------------------------.C....A.....T....T.ACC...T.TC....G.A...G.A....
DD037F_31_186       ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
TV120_6             -------------------------------------------T....T.ACC...T.TC....G.A...G.A....
NN470A_20           ------------------------------------------------------------------------A....
NN470A_39           ..............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN98A_22            .............................................................................
NN300_156           ---------------------------.G..C....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN305F_9            -----------------------------.C....A.....T....T.ACC...T.TC....G.A...G.A....
NN297_20            -------------------------------------------T....T.ACC...T.TC....G.A...G.A....
DD103A_184_191      ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN71_111 153        ATG.C......A......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN445_22            -------------------------------------------T....T.ACC...T.TC....G.A...G.A....
SS082A_88 7         ATG...............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
CC24_5              -------------------------G....C....A.....T....T.ACC...T.TC....G.A...G.A....
SS082A_171 4        ATG.......T.......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN300_155 19        ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN300_155 22        ATG.......T.......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN300_98 31         ATG.C......T......G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN300_99 34         ATG...............G...T....G....C....A.....T....T.ACC...T.TC....A...G.G....
NN300_60 23         ATG.C.............G...T....G....C....A.....T....T.ACC...T.TC....A...G.G....
PV026_3             -----------------G..CT.......C....A.....T....T.ACC...T.TC....A...G.G....
TT138E_111 104      ATG.C......T......G...T....G....C....A.....T....T.ACC...T.TC....A...G.G....
NN459_25            ATG...............G..CT....G....C....A.....T....T.ACC...T.TC....A...G.A....
TT019A_107 52       ATG.C.............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
TT026B_195 58       ATG...............G..CT....G....C....A.....T....T.ACC...T.TC....G.A...G.A....
NN300_41 123        ATG.......T.......G...T.......C....A.....T....T.ACC...T.TC....G.A...G.A....
GG517_93 160        ATG.......T.......G...T.......C....A.....T....T.ACC...T.TC....G.A...G.A....
PVY_NTN3_AB042812.1 ----...............GT.CT.......C....A.....T....T.ACC...T.TC....G.A...G.A....
PVY_N3_AB025415.1   ----...............GT.CT.......C....A.....T....T.ACC..GT.TC....G.A...G.A....
PVY_N2_AF126258.1   CAG............T..........G..CT.......C....A.....T....T.ACC...T.TC....G.A...G.A....
```

```
                        110       120       130       140       150       160       170       180       190       200
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1         ATGTGAATGCTGGTACATCTGGGACACATACTGTGCCGAGAATCAAGGCTATTACGTCCAAAATGAGAATGCCCAAAAGCAAGGGAGCAACCGTGCTAAA
PVY_O1_X14136.1         ........T...C.....................................C......................G...........T.G...C...C..
PVY_US1_X68222.1        ..........C.C........C............................C.......................G......G..
PVY_JC1_U25672.1        ...........G..C..A................................C..
PVY_CH1_X68221.1        .C......T......A.G....A..........A.A.....C......A............C.....A..........A.G..
CC73_18                 ..................................................C..
SS121_166 56            ..................................................C..
CC55_8 146              ..................................................C..
DD037F_9_154            ..................................................C..
PVYWilga CP AJ889867    ..................................................C..
WW154A_62 86            ...........................T......C...........C..
CC66_91 47              ..................................................C...........C..
SS121_154 10            ..................................................C..
DD019_141 141           ..................................................C..
WW282E_3                ................................C.C.........G..
WW010_146_164           ..........C...........................C..........G..
DD103A_101_190          ..................................................C...........G..
DD103A_80_180           ..................................................C..
PVYNTN1                 ..................................................C.....................T..
PP014_18                .C......T..A.......A.T.C......AC....T.A.....C.......G..
PP014_21                ..................................................C..
CC24_7                  ..................................................C..
DD122A_34               ..................................................C..
TT138E_113 106          ..................................................C..
WW154_175 62            ..................................................C..
TT141A_76 73            ..................................................C..
TT138D_111 79           ...A..............................................C..
GG517_128_162           ..................................................C..
CC9_48_178              ..................................................C..
WW010_147_166           ..................................................C..
GG517_170_168           ..................................................C..
WW010_70_158            ..................................................C..
TV115_3                 ..................................................C..
NN459_14                ..................................................C..
TT138E_102 96           ..................................................C..
CC9_30_175              ..................................................C..
DD122A_25               ..................................................C..
TT026B_88 115           ..................................................C..
TT026B_86 128           ..................................................C..
DD122A_25               ..................................................C..
NN333B_28 149           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T...
Z26 38                  .C......T..A.......A.T......AC....T.A.....C.......G...A..T.....T.A...
CC9_12_171              .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
CC62_20_156             .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
PVY_NTN2_X79305.1       .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN300_76 118            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW073B_18               .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN98A_35                .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
PVYNTN_4_2              .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
DD122A_36               .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN333B_87 152           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
DD020_92 30             .C......T..A.......A.T......AC...TCA.....C.......G..T..A..T.....T.A...
SS121_82 1              .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW002_22 147            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW002_74 150            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW002_82 151            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
SS121_197 16            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
PVYNTN3_3               .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
PVYNTN_4_1              .C......T..A.......A.T......AC....T.A.....C.......G..T..A..C...T.A...
SS121_53 42             .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
SS082A_194 14           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
TT138D_13 68            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
PP003_119 85            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
PP003_119 133           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
PVY_H1_X54611.1         .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T....G.T.A...
PP026B_184 111          .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
TT014_184 135           .C...G..T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
SS147_144 144           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
DD037F_96_138           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
CC9_47_177              .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW202B_21_173           .C......T..A.......A.T......AC....T.A.....C.......G.G..T..A..T.....T.A...
DD037F_35_188           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW202B_24_184           .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
TT042A_3                .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW655_11                .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
CC73_12                 .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
WW672_35                .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
DD037F_31_186           .C......T..A.......A.TT.....AC....T.A.....C.......G..T..A..T.....T.A...
TV120_6                 .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T....G.T.A...
NN470A_20               .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN470A_39               .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN98A_22                .C......T..A....A.T.C......AC....T.ACG.....C.......G..T..A..T.....T.A...
NN300_156               .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN305F_9                .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN297_20                .C......T..A....A.T.C......AC....T.A.....C.......G..T..A..T.....T.A..G..
DD103A_184_191          .C......T..A.......A.T......AC....T.A.....C.......GG..T..A..T.....T.A...
NN71_111 153            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN445_22                .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
SS082A_88 7             .C......T..A.......A.T......AC....T.A.....C.......GG..T..A..C.....T.A...
CC24_5                  .C......T..A.......A.T......AC....T.A.....C.......GG..T..A..T.....T.A...
SS082A_171 4            .C......T..A.......A.T......AC....T.A.....C.......GG..T..T....T.A...
NN300_155 19            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN300_155 22            .C......T..A.......A.T......AC....T.A.....C.......G..T..A..T.....T.A...
NN300_98 31             .C......T..A.......A.T......AC....T.A.....C.......G..T....T....T.A...
NN300_99 34             .C......T..A.......A.T......AC....T.A..C..C...T....G..T....T....T.A..G..
NN300_60 23             .C......T..A.......A.T......AC....T.A..C.C...T....G..T....T....T.A..G..
PV026_3                 .C......T..A.......A.T......AC....T.A..C.C...T....G..T....T....T.A..G..
TT138E_111 104          .C......T..A.......A.T......AC....T.A..C.C...T....G..T....T....T.A..G..
NN459_25                .C......T..A.......A.T......AC....T.A.....C.......A......G..T..A..T.....T.A...
TT019A_107 52           .C......T..A.......A.T......AC....T.A.....C.......GG..T..A..T.....T.A...
TT026B_195 58           ..C......T..A.......A.T......AC....T.A.....C.......T......G..T..A..T.....T.A...
NN300_41 123            .C......T..A.......A.T.C......AC....T.A.....C.......G..T....T....T.A...
GG517_93 160            .C......T..A.......A.T.C......AC....T.A.....C.......G..T....T....T.A...
PVY_NTN3_AB042812.1     .C......T..A.......A.T.C......AC....T.A.....C.......G......G..T....T....T.A...
PVY_N3_AB025415.1       .C......T..A.......A.T.C....A.AC....T.A.....C.......G..T....T....T.A...
PVY_N2_AF126258.1       .C......T..A.......A.T.C......AC....T.A.....C.......A......G..T....T....T.A.....
```

```
                    210       220       230       240       250       260       270       280       290       300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1     CTTAGAACACTTACTTGAGTATGCTCCACAACAAATTGATATTTCAAATACTCGGGCAACTCAATCACAGTTTGATACGTGGTATGAGGCAGTGCGGATG
PVY_O1_X14136.1     ..........G..C...................................C..C............................................G........
PVY_US1_X68222.1    T.........G....................................................C.............................
PVY_JC1_U25672.1    T.........G.....C...................................................................
PVY_CH1_X68221.1    ...........C......C....G.G.......C...C....A...............................A.......AG.T
CC73_18             .........G..........................................................
SS121_166 56        .........G..........................................................
CC55_8 146          .........G..........................................................
DD037F_9_154        .........G.....................C....................................
PVYWilga_CP_AJ889867 .........G..........................................................
WW154A_62 86        .........G..........................................................
CC66_91 47          .........G..........................................................
SS121_154 10        .........G..........................................................
DD019_141 141       .........G..........................................................
WW282E_3            .........G..........................................................
WW010_146_164       .........G.............................................C............
DD103A_101_190      .........G..........................................................
DD103A_80_180       .........G..........................................................
PVYNTN1             .........G..........................................................
PP014_18            .........G.............................................C............
PP014_21            .........G..........................................................
CC24_7              .........G..........................................................
DD122A_34           .........G..........................................................
TT138E_113 106      .........G..........................................................
WW154_175 62        .........G..........................................................
TT141A_76 73        .........G..........................................................
TT138D_111 79       .........G..........................................................
GG517_128_162       .........G..........................................................
CC9_48_178          .........G..........................................................
WW010_147_166       .........G..........................................................
GG517_170_168       .........G..........................................................
WW010_70_158        .........G..........................................................
TV115_3             .........G..................C.......................................
NN459_14            .........G..........................................................
TT138E_102 96       .........G..........................................................
CC9_30_175          .........G..........................................................
DD122A_25           .........G..........................................................
TT026B_88 115       .........G..........................................................
TT026B_86 128       .........G..........................................................
DD122A_25           .........G..........................................................
NN333B_28 149       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
Z26 38              T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
CC9_12_171          T..G.........C..........G......C.CC.......A.............A..G..A.AAC.T
CC62_20_156         T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
PVY_NTN2_X79305.1   T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
NN300_76 118        T..G.........C...............C..C.......A.............A..G..A.AAC.T
WW073B_18           T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
NN98A_35            T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
PVYNTN_4_2          T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
DD122A_36           T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
NN333B_87 152       T..G.........C..........G......C..C.......A.............A.....A.AAC.T
DD020_92 30         T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
SS121_82 1          T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
WW002_22 147        T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
WW002_74 150        T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
WW002_82 151        T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
SS121_197 16        T..G.........C..........G......C..C.......A.............A.....A.AAC.T
PVYNTN3_3           T..G.........C..........G......C..C.......A.............A.....A.AAC.T
PVYNTN_4_1          T..G.........C..........G......C..C.......A.............A.....A.AAC.T
SS121_53 42         T..G.........C..........G......C..C.......A.............A.....A.AAC.T
SS082A_194 14       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
TT138D_13 68        T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
PP003_119 85        T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
PP003_119 133       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
PVY_H1_X54611.1     T..GA.....G..C..........G......C..C.......A.............A..G..A.AAC.T
PP026B_184 111      T..G.........C..........G......C..C.......A.......C.....A..G..A.AAC.T
TT014_184 135       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
SS147_144 144       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
DD037F_96_138       T..G.........C........G..G......C..C.......A.............A..G..A.AAC.T
CC9_47_177          T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
WW202B_21_173       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
DD037F_35_188       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
WW202B_24_184       T..G.........C..........G......C..C...................A..G..A.AAC.T
TT042A_3            T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
WW655_11            T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
CC73_12             T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
WW672_35            T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
DD037F_31_186       T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
TV120_6             T..G.........C.......C..G......C..C.......A.............A..G..A.AAC.T
NN470A_20           T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
NN470A_39           T..G.........C..........G......C..C.......A.............A..G..A.AAC.T
NN98A_22            T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN300_156           T..G.G.......C..........G......C..C.......A.............A.....A.AAC.T
NN305F_9            T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN297_20            T..G.........C..........G......C..C.......A.............A.....A.AAC.T
DD103A_184_191      T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN71_111 153        T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN445_22            T..G.........C..........G......C..C.......A.............A.....A.AAC.T
SS082A_88 7         T..G.........C..........G......C..C.......A.............A.....A.AAC.T
CC24_5              T..G.........C..........G......C..C.......A.............A.....A.AAC.T
SS082A_171 4        T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN300_155 19        T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN300_155 22        T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN300_98 31         T..G.G.......C..........G......C..C.......A.............A.....A.AAC.T
NN300_99 34         ......T...C..........G......C..C.......A.............A.....A.A.C.T
NN300_60 23         ......T...C..........G......C..C.......A.............A.....A.A.C.T
PV026_3             ......T...C..........G......C..C.......A.............A.....A.A.C.T
TT138E_111 104      ......T...C....C.....G......C..C.......A.............A.....A.A.C.T
NN459_25            T..G.........C..........G......C..C.......A.............A.....A.AAC.T
TT019A_107 52       T..G.........C.....C...G......C..C.......A.............A.....A.AAC.T
TT026B_195 58       T..G.........C..........G......C..C.......A.............A.....A.AAC.T
NN300_41 123        T..G.........C..........G......C..C.......A.............A.....A.AAC.T
GG517_93 160        T..G.........C..........G......C..C.......A.............A.....A.AAC.T
PVY_NTN3_AB042812.1 T..G.........C..........G......C..C.......A.............A.....A.AAC.T
PVY_N3_AB025415.1   T..G.........C..........G......C..C.......A.............A...A.A.....A.AAC.T
PVY_N2_AF126258.1   T..G.........C..........G......C..C.......TA.............A.......A.....A.AAC.T
```

73

```
                        310       320       330       340       350       360       370       380       390       400
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1         GCATACGACATAGGAGAAACTGAGATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATTGAAAATGGAACCTCGCCAAATGTCAACGGAGTTTGGG
PVY_O1_X14136.1         .............C..................................................................................A......
PVY_US1_X68222.1        .....T..............................................................................................
PVY_JC1_U25672.1        ....................................................................................................
PVY_CH1_X68221.1        ...........G..T....A................................................................A................
CC73_18                 ....................................................................................................
SS121_166 56            ....................................................................................................
CC55_8 146              ....................................................................................................
DD037F_9_154            .................................................................G..................................
PVYWilga CP AJ889867     ....................................................................................................
WW154A_62 86            ....................................................................................................
CC66_91 47              ....................................................................................................
SS121_154 10            ....................................................................................................
DD019_141 141           ....................................................................................................
WW282E_3                ....................................................................................................
WW010_146_164           ..........C.........................................................................................
DD103A_101_190          ...........................................................C........................................
DD103A_80_180           ....................................................................................................
PVYNTN1                 ....................................................................................................
PP014_18                ....................................................................................................
PP014_21                ..C............................................................................T....................
CC24_7                  ....................................................................................................
DD122A_34               ...........................................................................................A........
TT138E_113 106          ....................................................................................................
WW154_175 62            ....................................................................................................
TT141A_76 73            ....................................................................................................
TT138D_111 79           ....................................................................................................
GG517_128_162           ....................................................................................................
CC9_48_178              ....................................................................................................
WW010_147_166           ....................................................................................................
GG517_170_168           ....................................................................................................
WW010_70_158            ....................................................................................................
TV115_3                 ....................................................................................................
NN459_14                .............A......................................................................................
TT138E_102 96           ....................................................................................................
CC9_30_175              ....................................................................................................
DD122A_25               .......A............................................................................................
TT026B_88 115           ....................................................................................................
TT026B_86 128           ....................................................................................................
DD122A_25               .......A............................................................................................
NN333B_28 149           .......................A....................................................CA......................
Z26 38                  .......................A....................................................CA......................
CC9_12_171              .......................A....................................................CA......................
CC62_20_156             .......................A....................................................CA......................
PVY_NTN2_X79305.1        .......................A....................................................CA......................
NN300_76 118            .......................A.............................T......................CA......................
WW073B_18               .......................A....................................................CA......................
NN98A_35                .......................A....................................................CA......................
PVYNTN_4_2              .......................A....................................................CA......................
DD122A_36               .......................A....................................................CA............C.........
NN333B_87 152           .......................A....................................................CA......................
DD020_92 30             .......................A....................................................CA......................
SS121_82 1              .......................A....................................................CA......................
WW002_22 147            .......................A....................................................CA......................
WW002_74 150            .......................A....................................................CA......................
WW002_82 151            .......................A....................................................CA......................
SS121_197 16            .......................A....................................................CA......................
PVYNTN3_3               .......................A....................................................CA......................
PVYNTN_4_1              .......................A....................................................CA......................
SS121_53 42             .........G.............A....................................................CA......................
SS082A_194 14           .......................A....................................................CA......................
TT138D_13 68            .......................A....................................................CA......................
PP003_119 85            .......................A....................................................CA......................
PP003_119 133           .......................A....................................................CA......................
PVY_H1_X54611.1          .......................A....................................................CA......................
PP026B_184 111          .......................A....................................................CA......................
TT014_184 135           .......................A....................................................CA......................
SS147_144 144           .......................A....................................................CA......................
DD037F_96_138           .......................A....................................................CA......................
CC9_47_177              .......................A....................................................CA......................
WW202B_21_173           ....G..................A....................................................CA......................
DD037F_35_188           .......................A....................................................CA......................
WW202B_24_184           .......................A....................................................CA......................
TT042A_3                .......................A....................................................CA......................
WW655_11                .......................A....................................................CA......................
CC73_12                 .......................A....................................................CA......................
WW672_35                .......................A....................................................CA......................
DD037F_31_186           .......................A....................................................CA......................
TV120_6                 .......................A....................................................CA......................
NN470A_20               .......................A....................................................CA......................
NN470A_39               .......................A............................................G.......CA......................
NN98A_22                .......................A.....................................................A......................
NN300_156               .......................A....................................................CA......................
NN305F_9                .......................A....................................................CA......................
NN297_20                .......................A....................................................CA......................
DD103A_184_191          .......................A....................................................CA......................
NN71_111 153            .......................A....................................................CA......................
NN445_22                .......................A........A...........................................CA......................
SS082A_88 7             .......................A....................................................CA......................
CC24_5                  .......................A....................................................CA......................
SS082A_171 4            .......................A....................................................CA......................
NN300_155 19            .......................A....................................................CA......................
NN300_155 22            .......................A....................................................CA......................
NN300_98 31             .......................A....................................................CA......................
NN300_99 34             ..T.....G..............A................................G...................CA......T..C....
NN300_60 23             ..T.....G..............A................................G...................CA......T..C....
PV026_3                 ..T.....G..............A................................G...................CA......T..C....
TT138E_111 104          ..T.....G..............A................................G...................CA......T..C....
NN459_25                .......................A....................................................CA......................
TT019A_107 52           .......................A....................................................CA......................
TT026B_195 58           .......................A....................................................CA......................
NN300_41 123            .......................A.....................................................A....T.................
GG517_93 160            .......................A.....................................................A....T.................
PVY_NTN3_AB042812.1      .......................A.....................................................A....T.................
PVY_N3_AB025415.1        .......................A.....................................................A....T.................
PVY_N2_AF126258.1        .......................A.....................................................A....T.................
```

```
                           410       420       430       440       450       460       470       480       490       500
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1       TTATGATGGATGGGAATGAACAAGTTGAGTACCCGTTGAAACCAATCGTTGAGAATGCAAAACCAACCCTTAGGCAAATCATGGCACATTTCTCAGATGT
PVY_O1_X14136.1       ...................A.....................T..........................................................
PVY_US1_X68222.1      ....................................................................................................
PVY_JC1_U25672.1      ....................................................................................................
PVY_CH1_X68221.1      ...................A.................A.......G.................G.....................................
CC73_18               ....................................................................................................
SS121_166 56          ....................C...............................................................................
CC55_8 146            ....................C...............................................................................
DD037F_9_154          ....................C...............................................................................
PVYWilga CP AJ889867  .......................C............................................................................
WW154A_62 86          ....................C...............................................................................
CC66_91 47            ....................C...............................................................................
SS121_154 10          ....................C...............................................................................
DD019_141 141         ....................C...............................................................................
WW282E_3              ....................C...............................................................................
WW010_146_164         ....................C...............................................................................
DD103A_101_190        ....................C.........................................C.....................................
DD103A_80_180         ....................C...............................................................................
PVYNTN1               ....................C...............................................................................
PP014_18              ....................C............................................T..................................
PP014_21              ....................C...............................................................................
CC24_7                ....................C...............................................................................
DD122A_34             ...............G....C...............................................................................
TT138E_113 106        ...............G....C...............................................................................
WW154_175 62          ...............G....C...............................................................................
TT141A_76 73          ...............G....C...............................................................................
TT138D_111 79         ...............G....C...............................................................................
GG517_128_162         ...............G....C...............................................................................
CC9_48_178            ...............G....C...............................................................................
WW010_147_166         ...............G....C...............................................................................
GG517_170_168         ...............G....C...............................................................................
WW010_70_158          ...............G....C...............................................................................
TV115_3               ...............G....C...............................................................................
NN459_14              ...............G....C...............................................................................
TT138E_102 96         ...............G....C...............................................................................
CC9_30_175            ...............G....C................................................C..............................
DD122A_25             ...............G....C...............................................................................
TT026B_88 115         ...............G....C...............................................................................
TT026B_86 128         ...............G....C...............................................................................
DD122A_25             ...............G....C...............................................................................
NN333B_28 149         ..............AG....C..A.....AC...........................A.........................................
Z26 38                ..............AG....C..A.....AC...........................A.........................................
CC9_12_171            ..............AG....C..A.....AC...........................A.........................................
CC62_20_156           ..............AG....C..A.....AC...........................A.........................................
PVY_NTN2_X79305.1     ..............AG....C..A.....AC...........................A.........................................
NN300_76 118          ..............AG....C..A.....AC...........................A.........................................
WW073B_18             ..............AG....C..A.....AC...........................A.........................................
NN98A_35              ..............AG....C..A.....AC...........................A.........................................
PVYNTN_4_2            ..............AG....C..A.....AC...........................A.........................................
DD122A_36             ..............AG....A.C..A.....AC..........................A.........................................
NN333B_87 152         ..............AG....C..A.....AC...........................A.........................................
DD020_92 30           ..............AG....C..A.....AC...........................A.........................................
SS121_82 1            ..............AG....C..A.....AC...........................A.........................................
WW002_22 147          ..............AG....C..A.....AC...........................A.........................................
WW002_74 150          ..............AG....C..A.....AC...........................A.........................................
WW002_82 151          ..............AG....C..A.....AC...........................A.........................................
SS121_197 16          ..............AG....C..A.....AC...........................A.........................................
PVYNTN3_3             ..............AG....C..A.....AC...........................A.........................................
PVYNTN_4_1            ..............AG....C..A.....AC...........................A.........................................
SS121_53 42           ..............AG....C..A......C...........................A.........................................
SS082A_194 14         ..............AG....C..A.....AC...........................A.........................................
TT138D_13 68          ..............AG....C..A.....AC...........................A.........................................
PP003_119 85          ..............AG....C..A.....AC...........................A.........................................
PP003_119 133         ..............AG....C..A.....AC...........................A.........................................
PVY_H1_X54611.1       ..............AG....C..A.....AC...........................A.........................................
PP026B_184 111        ..............AG....C..A.....AC...........................A........................................C
TT014_184 135         ..............AG....C..A.....AC...........................A.........................................
SS147_144 144         ..............AG....C..A.....AC...........................A.........................................
DD037F_96_138         ..............AG....C..A.....AC...........................A.........................................
CC9_47_177            ..............AG....C..A.....AC...........................A.........................................
WW202B_21_173         ..............AG....C..A.....AC...........................A.........................................
DD037F_35_188         .C............AG....C..A.....AC...........................A.........................................
WW202B_24_184         ..............AG....C..A.....AC...........................A.........................................
TT042A_3              ..............AG....C..A.....AC...........................A....................................T.....
WW655_11              ..............AG....C..A.....AC...........................A.........................................
CC73_12               ..............AG....C..A.....AC...........................A.........................................
WW672_35              ..............AG....C..A.....AC...........................A.........................................
DD037F_31_186         ..............AG....C..A.....AC...........................A.........................................
TV120_6               ..............AG....C..A.....AC...........................A.........................................
NN470A_20             ..............AG....C..A.....AC...........................A.........................................
NN470A_39             ..............AG....C..A.....AC...........................A.........................................
NN98A_22              ..............AG....C..A.....AC...........................A.........................................
NN300_156             ..............AG....C..A.....AC...........................A.........................................
NN305F_9              ..............AG....C..A.....AC...........................A.........................................
NN297_20              ..............AG....C..A.....AC...........................A.........................................
DD103A_184_191        ..............AG....C..A.....AC...........................A.........................................
NN71_111 153          ..............AG....C..A.....AC...........................A.........................................
NN445_22              ..............AG....C..A.....AC..........T................A.........................................
SS082A_88 7           ..............AG....C..A.....AC...........................A.........................................
CC24_5                ..............AG....C..A.....AC...........................A.........................................
SS082A_171 4          ...............G....C..A.....AC...........................A.........................................
NN300_155 19          ..............AG....C..A.....AC...........................A.........................................
NN300_155 22          ..............AG....C..A.....AC...........................A.........................................
NN300_98 31           ..............AG....C..A.....AC...........................A.........................................
NN300_99 34           ..............A.....A.....AC.C..G..G..T....A........................................................
NN300_60 23           ..............A.....A.....AC.C..G..G..T....A........................................................
PV026_3               ..............A.....A.....AC.C..G..G..T....A........................................................
TT138E_111 104        ..............A.....A.....AC.C..G..G..T....A........................................................
NN459_25              .........A.AG....C..A.....AC...........................A.........................................
TT019A_107 52         ..............AG....C..A.....AC...........................A.........................................
TT026B_195 58         ..............AG....C..A.....AC...........................A.........................................
NN300_41 123          ..............AG....C..A.....AC...........................A.........................................
GG517_93 160          ..............AG....C..A.....AC...........................A.........................................
PVY_NTN3_AB042812.1   ..............AG....C..A.....AC...........................A.........................................
PVY_N3_AB025415.1     ..............AG....C..A.....AC...........................A.........................................
PVY_N2_AF126258.1     ..........C..A..........C..A.....AC.....................A..........A................................
```

```
                    510       520       530       540       550       560       570       580       590       600
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1     TGCAGAAGCGTATATAGAAATGCGCAACAAAAAGGAACCATATATGCCACGATATGGTTTAATTCGAAATCTGCGGGATGTGGGTTTAGCGCGTTATGCC
PVY_O1_X14136.1     ...........C.........G............................................A.AA..C.....C.....
PVY_US1_X68222.1    .................................................................A..................T
PVY_JC1_U25672.1    ..................................................................
PVY_CH1_X68221.1    ................................................................A...A.AA.........C.....
CC73_18             ..................................................................
SS121_166 56        ..................................................................
CC55_8 146          ..................................................................
DD037F_9_154        ...............................................................................A.....
PVYWilga CP AJ889867 ...................................................C..............
WW154A_62 86        ...............................................................A.....
CC66_91 47          ...............................................................A.....
SS121_154 10        ..................................................................
DD019_141 141       ..................................................................
WW282E_3            ............................................................C.....
WW010_146_164       ..................................................................
DD103A_101_190      ..................................................................
DD103A_80_180       ..................................................................
PVYNTN1             ..................................................................
PP014_18            .............................................................C.....
PP014_21            ..................................................................
CC24_7              ..................................................................
DD122A_34           ..................................................................
TT138E_113 106      ..................................................................
WW154_175 62        .............................................................G.....
TT141A_76 73        ..................................................................
TT138D_111 79       ..................................................................
GG517_128_162       ..................................................................
CC9_48_178          ..................................................................
WW010_147_166       ..................................................................
GG517_170_168       ..................................................................
WW010_70_158        .......................................................T.....
TV115_3             ..................................................................
NN459_14            ...........................................................A.....
TT138E_102 96       ...................T..............................................
CC9_30_175          ...................T..............................................
DD122A_25           ..................................................................
TT026B_88 115       ...........................................C.....
TT026B_86 128       ..................................................................
DD122A_25           ..................................................................
NN333B_28 149       .................................G....T......C....GAA....G..T..C.....T
Z26 38              .................................G....T......C....GAA....G..T..C.....T
CC9_12_171          .................................G....T......C.G..GAA....G..T..C.....T
CC62_20_156         .................................G....T......C....GAA....G..T..C.....T
PVY_NTN2_X79305.1   .................................G....T......C....GAA....G..T..C.....T
NN300_76 118        .................................G....T......C....GAA....G..T..C.....T
WW073B_18           .................................G....T......C....GAA....G..T..C.....T
NN98A_35            .................................G....T......C....GAA....G..T..C.....T
PVYNTN_4_2          .................................G....T......C....GAA....G..T..C.....T
DD122A_36           .................................G....T......C....GAA....G..T..C.....T
NN333B_87 152       .................................G....T......C....GAA....G..T..C.....T
DD020_92 30         .................................G....T......C....GAA....G..T..C.....T
SS121_82 1          .................................G....T......C....GAA....G..T..C.....T
WW002_22 147        .................................G....T......C....GAA....G..T..C.....T
WW002_74 150        .................................G....T......C....GAA....G..T..C.....T
WW002_82 151        .................................G....T......C....GAA....G..T..C.....T
SS121_197 16        .................................G....T..T....C....GAA....G..T..C.....T
PVYNTN3_3           .................................G....T......C....GAA....G..T..C.....T
PVYNTN_4_1          .................................G....T......C....GAA....G..T..C.....T
SS121_53 42         .................................G....T......C....GAA....G..T..C.....T
SS082A_194 14       .................................G....T......C....GAA....G..T..C.....T
TT138D_13 68        .................................G....T......C....GAA....G..T..C.....T
PP003_119 85        .................................G....T......C....GAA....G..T..C.....T
PP003_119 133       .................................G....T......C....GAA....G..T..C.....T
PVY_H1_X54611.1     .................................G....T......C....GAA....G..T..C.....T
PP026B_184 111      .................................G....T......C....GAA....G..T..C.....T
TT014_184 135       .................................G....T......C....GAA....G..T..C.....T
SS147_144 144       .................................G....T......C....GAA....G..T..C.....T
DD037F_96_138       .............C...................G....T......C....GAA....G..T..C.....T
CC9_47_177          .................................G....T......C....GAA....G..T..C.....T
WW202B_21_173       .................................G....T......C....GAA....G..T..C.....T
DD037F_35_188       .................................G....T......C....GAA....G..T..C...A.T
WW202B_24_184       .................................G....T......C....GAA....G..T..C.....T
TT042A_3            ...................A.............G..T.T....C....GAA....G..T..C.....T
WW655_11            .................................G....T......C....GAA....G..T..C.....T
CC73_12             .................................G....T......C....GAA....G..T..C.....T
WW672_35            .................................G....T......C....GAA....G..T..C.....T
DD037F_31_186       .................................G....T......C....GAA....G..T..C.....T
TV120_6             .................................G....T......C....GAA....G..T..C.....T
NN470A_20           .................................G....T......C....GAA....G..T..C.....T
NN470A_39           .................................G....T......C....GAA....G..T..C.....T
NN98A_22            .................................G....T......C....GAA....G..T..C.....T
NN300_156           .................................G....T......C....GAA....G..T..C.....T
NN305F_9            .................................G....T......C....GAA....G..T..C.....T
NN297_20            .................................G....T......C....GAA....G..T..C.....T
DD103A_184_191      .................................G....T......C....GAA....G..T..C.....T
NN71_111 153        .................................G....T......C....GAA....G..T..C.....T
NN445_22            ..................................... T......C....GAA....G..T..C.....T
SS082A_88 7         .................................G....T......C....GAA....G..T..C.....T
CC24_5              .................................G....T......C....GAA....G..T..C.....T
SS082A_171 4        .................................G....T......C....GAA....G..T..C.....T
NN300_155 19        .................................G....T......C....GAA....G..T..C.....T
NN300_155 22        .................................G....T......C....GAA....G..T..C.....T
NN300_98 31         .................................G....T......C....GAA....G..T..C.....T
NN300_99 34         ...................T.............G..........T..GAA....G..T.........
NN300_60 23         ...................T.............G..........T..GAA....G..T.........
PV026_3             ...................T.............G..........T..GAA....G..T.........
TT138E_111 104      .............C...................G..........T..GAA....G..T.........
NN459_25            .................................G....T......C....GAA....G..T..C.....T
TT019A_107 52       .................................G....T......C....GAA....G..T..C.....T
TT026B_195 58       .................................G....T......C....GAA....G..T..C.....T
NN300_41 123        ..................................... T......C....GAA....G..T..C.....T
GG517_93 160        ..................................... T......C....GAA....G..T..C.....T
PVY_NTN3_AB042812.1 .................................G....T......C....GAA....G..T..C.....T
PVY_N3_AB025415.1   .................................G....T......C....GAA....G..T..C.....T
PVY_N2_AF126258.1   .................................G....T......C....GAA....G..T..C.....T
```

```
                        610       620       630       640       650       660       670       680       690       700
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1    TTTGACTTTTATGAGGTCACATCACGAACACCAGTGAGGGCTAGGGAAGCGCACATTCAAATGAAGGCCGCAGCATTGAAATCAGCCCAACCTCGACTTT
PVY_O1_X14136.1    .............A...........G...........................C...............................T............
PVY_US1_X68222.1   ....................................................................................................
PVY_JC1_U25672.1   ....................................................................................................
PVY_CH1_X68221.1   ..C..T..C......T....C.........C.........T..C......A....G.......G....T.................................
CC73_18            ..............................................................................................G.....
SS121_166 56       .......................................................................T............................
CC55_8 146         .......................................................................T............................
DD037F_9_154       .......................................................................T............................
PVYWilga CP AJ889867 .....................................................................T............................
WW154A_62 86       ....................................................................................................
CC66_91 47         ....................................................................................................
SS121_154 10       ....................................................................................................
DD019_141 141      ....................................................................................................
WW282E_3           ....................................................................................................
WW010_146_164      ....................................................................................................
DD103A_101_190     ....................................................................................................
DD103A_80_180      ....................................................................................................
PVYNTN1            ...........................................................................T........................
PP014_18           ..............................................G.....................................................
PP014_21           ....................................................................................................
CC24_7             ....................................................................................................
DD122A_34          ....................................................................................................
TT138E_113 106     ....................................................................................................
WW154_175 62       ............................................................................G.......................
TT141A_76 73       ....................................................................................................
TT138D_111 79      ....................................................................................................
GG517_128_162      ....................................................................................................
CC9_48_178         ....................................................................................................
WW010_147_166      ....................................................................................................
GG517_170_168      ....................................................................................................
WW010_70_158       ...............................................................................................A....
TV115_3            ....................................................................................................
NN459_14           .......................................A............................................................
TT138E_102 96      ....................................................................................................
CC9_30_175         ....................................................................................................
DD122A_25          ....................................................................................................
TT026B_88 115      ................AA..................................................................................
TT026B_86 128      ....................................................................................................
DD122A_25          .......................................................................CAT..........................
NN333B_28 149      ....................................................................................................
Z26 38             ....................................................................................................
CC9_12_171         ....................................................................................................
CC62_20_156        C...................................................................................................
PVY_NTN2_X79305.1  ....................................................................................................
NN300_76 118       ....................................................................................................
WW073B_18          ....................................................................................................
NN98A_35           .......................................................................T............................
PVYNTN_4_2         .......................................................................T............................
DD122A_36          .......................................................................T............................
NN333B_87 152      .......................................................................T............................
DD020_92 30        .......................................................................T............................
SS121_82 1         .......................................................................T............................
WW002_22 147       .......................................................................T............................
WW002_74 150       .......................................................................T............................
WW002_82 151       .......................................................................T............................
SS121_197 16       .......................................................................T............................
PVYNTN3_3          .......................................................................T............................
PVYNTN_4_1         .......................................................................T............................
SS121_53 42        .......................................................................T............................
SS082A_194 14      .......................................................................T............................
TT138D_13 68       .......................................................................T............................
PP003_119 85       .......................................................................T............................
PP003_119 133      .......................................................................T............................
PVY_H1_X54611.1    .......................................................................T............................
PP026B_184 111     .......................................................................T............................
TT014_184 135      .......................................................................T............................
SS147_144 144      .......................................................................T............................
DD037F_96_138      .......................................................................T............................
CC9_47_177         .......................................................................T............................
WW202B_21_173      .......................................................................T............................
DD037F_35_188      .......................................................................T............................
WW202B_24_184      .......................................................................T............................
TT042A_3           .......................................................................T............................
WW655_11           .......................................................................T............................
CC73_12            .......................................................................T............................
WW672_35           .......................................................................T............................
DD037F_31_186      .......................................................................T............................
TV120_6            .............................................................T.........T............................
NN470A_20          .......................................................................T............................
NN470A_39          .......................................................................T............................
NN98A_22           .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN300_156          .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN305F_9           .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN297_20           .........A..T........G.............A..G..A..............T..A.......T..T..............................
DD103A_184_191     .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN71_111 153       .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN445_22           .........A..T........G.............A..G..A..............T..A.......T..T..............................
SS082A_88 7        .........A..T........G.............A..G..A..............T..A.......T..T..............................
CC24_5             .........A..T........G.............A..G..A..............T..A.......T..T..............................
SS082A_171 4       .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN300_155 19       .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN300_155 22       .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN300_98 31        .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN300_99 34        .........A..T.......T....A.........A..G..A.............A....TC.........T....T.C......................
NN300_60 23        .........A..T.......T....A.........A..G..A.............A....TC.........T....T.C......................
PV026_3            .........A..T.......T....A.........A..G..A.............A....TC.........T....T.C......................
TT138E_111 104     .........A..T.......T...A.A........A..G..A.............A....TC.........T....T.C......................
NN459_25           .........A..T........G.............A..G..A..............T..A.......T..T..............................
TT019A_107 52      .........A..T........G.............A..G..A..............T..A.......T..T..............................
TT026B_195 58      .........A..T........G.............A..G..A..............T..A.......T..T..............................
NN300_41 123       .........A..T........G.............A..G..A..............T..A.......T..T..............................
GG517_93 160       .........A..T........G.............A..G..A..............T..A.......T..T..............................
PVY_NTN3_AB042812.1 .........A..T........G.............A..G..A..............T..A.......T..T..-.........................
PVY_N3_AB025415.1  .........A..T........G.............A..G..A..............T..A.......T..T..............................
PVY_N2_AF126258.1  ........C...A..T........G.............A..G..TA.............T..A.......T..T........................
```

```
              710       720       730       740       750       760       770       780       790       800
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PVY_O2_X68226.1      TCGGGTTGGACGGTGGCATTAGTACACAAGAGGAGAACACAGAGAGGCACACCACCGAGGATGTCTCTCCAAGTATGCATACTCTGCTTGGAGTCAAAAA
PVY_O1_X14136.1      ........T.......C...................................................T..........................G.....
PVY_US1_X68222.1     ................C...........................................................................G..
PVY_JC1_U25672.1     ................C.......................................................G.....A...........G..
PVY_CH1_X68221.1     ................C.......................................................A.....C....G..
CC73_18             ................C.............................................----------------------------
SS121_166 56        ................C..........................................................A...........G..
CC55_8 146          ................C..........................................................A...........G..
DD037F_9_154        ................C...................................................G......A...........G..
PVYWilga CP AJ889867 ................C..........................................................A...........G..
WW154A_62 86        ................C...............A..........................................A..........
CC66_91 47          ................C..........................................................A..........
SS121_154 10        ................C...............................................G..........A...........G..
DD019_141 141       ................C..........................................................A...........G..
WW282E_3            ................C..........................................................A...........G..
WW010_146_164       ................C..........................................................A...........G..
DD103A_101_190      ................C..........................................................A...........G..
DD103A_80_180       ................C..........................................................A...........G..
PVYNTN1             ................C..........................................................A...........G..
PP014_18            ................C.............................-----------------------------------------
PP014_21            ................C.............................-----------------------------------------
CC24_7              ................C.............................-----------------------------------------
DD122A_34           ................C..........................................................A..........
TT138E_113 106      ................C.........G.................................................A...........G..
WW154_175 62        ................C..........................................................A..........
TT141A_76 73        ................C..........................................................A..........
TT138D_111 79       ................C..........................................................A..........
GG517_128_162       ................C..........................................................A..........
CC9_48_178          ................C..........................................................A...........G..
WW010_147_166       ................C..........................................................A...........G..
GG517_170_168       ................C..........................................................A..........
WW010_70_158        ................C..........................................................A...........G..
TV115_3             ................C.............................----------------------------------------
NN459_14            ................C..........................................................A..........
TT138E_102 96       ................C..........................................................A..........
CC9_30_175          ................C..........................................................A...........G..
DD122A_25           .T..............C..........................................................A..........
TT026B_88 115       .T..............C..........................................................A..........
TT026B_86 128       .T..............C..........................................................A...........G..
DD122A_25           .T..............C..........................................................A..........
NN333B_28 149       ................C..........................................................A..........
Z26 38              ................C..........................................................A...........G..
CC9_12_171          ................C..........................................................A..........
CC62_20_156         ................C..........................................................A..........
PVY_NTN2_X79305.1   ................C..........................................................A...........G..
NN300_76 118        ................C..........................................................A...........G..
WW073B_18           C...............C..........................................................A...........G..
NN98A_35            ................C.............................----------------------------------------
PVYNTN_4_2          ................C.............................----------------------------------------
DD122A_36           ................C..........................................................A...........G..
NN333B_87 152       ................C..........................................................A..........
DD020_92 30         ................C..........................................................A...........G..
SS121_82 1          ..............T.C.......................A..................................A..........
WW002_22 147        ...............G.C.........................................................A..........
WW002_74 150        ................C.......................A..................................A..........
WW002_82 151        ................C..........................................................A..........
SS121_197 16        ..............T.C.......................A..................................A...........G..
PVYNTN3_3           ................C..........................................................A...........G..
PVYNTN_4_1          ................C.............................----------------------------------------
SS121_53 42         ..............T.C.......................A..................................A..........
SS082A_194 14       ................C..........................................................A..........
TT138D_13 68        ................C.......................A..................................A...........G..
PP003_119 85        ................C..........................................................A..........
PP003_119 133       ................C..........................................................A..........
PVY_H1_X54611.1     ................C..........................................................A...........G..
PP026B_184 111      ................C..........................................................A...........G..
TT014_184 135       ................C..........................................................A..........
SS147_144 144       ................C..........................................................A..........
DD037F_96_138       ................C..........................................................A..........
CC9_47_177          ................C..........................................................A...........G..
WW202B_21_173       ................C..........................................................A...........G..
DD037F_35_188       ................C..........................................................A..........
WW202B_24_184       ................C..........................................................A..........
TT042A_3            ................C.............................----------------------------------------
WW655_11            ................C.............................----------------------------------------
CC73_12             ................C.............................----------------------------------------
WW672_35            ................C.............................----------------------------------------
DD037F_31_186       ................C..........................................................A...........G..
TV120_6             ................C.............................----------------------------------------
NN470A_20           ................C.............................----------------------------------------
NN470A_39           ................C.......................A.....----------------------------------------
NN98A_22            ...A.....T...............A...................----------------------------------------
NN300_156           ...A.....T...............A.........................T..........A...........-----------
NN305F_9            ...A.....T...............A.........................T..........A...........-----------
NN297_20            ...A.....T...............A...................----------------------------------------
DD103A_184_191      ...A.....T...............A.........................T..........A...........G..
NN71_111 153        ...A.....T...............A..................................A..........
NN445_22            ...A.....T...............A...................----------------------------------------
SS082A_88 7         ...A.....T...............A.........................T..........A............G..
CC24_5              ...A.....T...............A...................----------------------------------------
SS082A_171 4        ...A.....T...............A.........................T..........A..........
NN300_155 19        ...A.....T...............A.........................T..........A..........
NN300_155 22        ...A.....T...............A.........................T..........A..........
NN300_98 31         ...A.....T...............A.........................T..........A..........
NN300_99 34         ...A.....T...............A.........................T..........A..........
NN300_60 23         ...A.....T...............A.........................T..........A...........G..
PV026_3             ...A.....T...............A.........................T..........----------
TT138E_111 104      ...A.....T...............A.........................T..........A..........
NN459_25            ...A.....T...............A..................................A..........
TT019A_107 52       ...A.....T...............A.........................T..........A..........
TT026B_195 58       ...A.....T...............A.........................T..........A..........
NN300_41 123        ...A.....T...............A.........................T..........A..........
GG517_93_160        ...A.....T...............A.........................T..........A..........
PVY_NTN3_AB042812.1 ...A.....T...............A.........................T..........A.........G..G..
PVY_N3_AB025415.1   ...A.....T.......C.......A.........................T..........A.........G..G..
PVY_N2_AF126258.1   ...A.....T...............A.........................T...T................A...........G..
```

```
                          ....|..
PVY_O2_X68226.1           CATGTGA
PVY_O1_X14136.1           .......
PVY_US1_X68222.1          .......
PVY_JC1_U25672.1          .......
PVY_CH1_X68221.1          .......
CC73_18                   -------
SS121_166 56              .......
CC55_8 146                .......
DD037F_9_154              .......
PVYWilga CP AJ889867      .......
WW154A_62 86              .......
CC66_91 47                .......
SS121_154 10              .......
DD019_141 141             .......
WW282E_3                  .......
WW010_146_164             .......
DD103A_101_190            .......
DD103A_80_180             .......
PVYNTN1                   .......
PP014_18                  -------
PP014_21                  -------
CC24_7                    -------
DD122A_34                 .......
TT138E_113 106            .......
WW154_175 62              .......
TT141A_76 73              .......
TT138D_111 79             .......
GG517_128_162             .......
CC9_48_178                .......
WW010_147_166             .......
GG517_170_168             .......
WW010_70_158              .......
TV115_3                   -------
NN459_14                  .......
TT138E_102 96             .......
CC9_30_175                .......
DD122A_25                 .......
TT026B_88 115             .......
TT026B_86 128             .......
DD122A_25                 .......
NN333B_28 149             .......
Z26 38                    .......
CC9_12_171                .......
CC62_20_156               .......
PVY_NTN2_X79305.1         ....---
NN300_76 118              .......
WW073B_18                 .......
NN98A_35                  -------
PVYNTN_4_2                -------
DD122A_36                 .......
NN333B_87 152             .......
DD020_92 30               .......
SS121_82 1                .......
WW002_22 147              .......
WW002_74 150              .......
WW002_82 151              .......
SS121_197 16              .......
PVYNTN3_3                 .......
PVYNTN_4_1                -------
SS121_53 42               .......
SS082A_194 14             .......
TT138D_13 68              .......
PP003_119 85              .......
PP003_119 133             .......
PVY_H1_X54611.1           ....---
PP026B_184 111            .......
TT014_184 135             .......
SS147_144 144             .......
DD037F_96_138             .......
CC9_47_177                .......
WW202B_21_173             .......
DD037F_35_188             .......
WW202B_24_184             .......
TT042A_3                  -------
WW655_11                  -------
CC73_12                   -------
WW672_35                  -------
DD037F_31_186             .......
TV120_6                   -------
NN470A_20                 -------
NN470A_39                 -------
NN98A_22                  -------
NN300_156                 -------
NN305F_9                  -------
NN297_20                  -------
DD103A_184_191            .......
NN71_111 153              .......
NN445_22                  -------
SS082A_88 7               .......
CC24_5                    -------
SS082A_171 4              .......
NN300_155 19              .......
NN300_155 22              .......
NN300_98 31               .......
NN300_99 34               .......
NN300_60 23               .......
PV026_3                   -------
TT138E_111 104            .......
NN459_25                  .......
TT019A_107 52             .......
TT026B_195 58             .......
NN300_41 123              .......
GG517_93_160              .......
PVY_NTN3_AB042812.1       ....---
PVY_N3_AB025415.1         .......
PVY_N2_AF126258.1         .......
```