An investigation into the *Potato leafroll virus* problem in the Sandveld region, South Africa

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Declaration

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Summary

Potato leafroll virus (PLRV) is responsible for significant yield losses in the South African (SA) potato industry. PLRV incidence in the Sandveld region has increased dramatically over the past 15 years. Enzyme-linked immunosorbent assay (ELISA) is used for routine testing by the SA Seed Potato Certification Scheme to diagnose PLRV infection, but many countries have changed to reverse transcription polymerase chain reaction (RT-PCR) for detection of PLRV because of its greater sensitivity. This project aimed to develop and validate a probe-based quantitative real-time reverse transcription PCR (RT-qPCR) to detect PLRV in potatoes and obtain an assessment of PLRV incidence in the Sandveld region, SA. This project also aimed to confirm infection in aphids and characterise aphid transmitted PLRV isolates by sequencing. Finally, this project aimed to apply a next-generation sequencing (NGS) technology to identify and characterise isolates, to compare non-coding 5' and 3' regions of the genome and lastly, to identify unknown viruses and other pathogens that possibly occur in potatoes in the Sandveld region.

Suitable primers and a TaqMan probe were designed to develop a highly sensitive RT-qPCR detection method for PLRV. An amplified complementary DNA (cDNA) was cloned into a plasmid and used for assay quantification and validation. Thereafter, potato leaves were tested over a full calendar year and results were compared to vector pressure. Overall high infection levels were found, but in certain times of the year low infection levels were found due to low vector pressure. SA tubers were also tested with this method. This study indicates that the SA Potato Certification Scheme should reconsider the use of ELISA as the method for PLRV detection and replace it with the described RT-qPCR method.

Secondly, the presence of PLRV in aphids was confirmed with RT-qPCR. A whole PLRV genome was amplified and sequenced after extraction from an infectious aphid. This generated whole PLRV genome was aligned in a data matrix with other whole genome sequences. Phylogenetic analysis of the whole genomes revealed that the aphid extracted PLRV isolate grouped with eight other SA isolates from the Sandveld region.

Lastly, Ion Torrent was used to obtain information about further PLRV isolates present in the Sandveld region. Samples with low Cq values corresponded to a high number mapping, coverage and sequencing depth of small interfering RNAs (siRNAs). Three complete genomes were obtained by mapping siRNAs to the reference sequence, as *de novo* assembly could not obtain contigs longer than 700 nucleotides. Phylogenetic analysis of the whole genomes revealed that three of the samples grouped with an Australian isolate and seven SA isolates. The remaining isolate grouped with nine other SA isolates. Minor variation between upstream and downstream non-coding regions was seen. No other potato or unknown viruses were identified, but an unknown fungus was identified in all samples which needs further investigation.

Opsomming

Aartappelrolbladvirus (PLRV) is verantwoordelik vir beduidende opbrengsverliese in die Suid-Afrikaanse (SA) aartappelbedryf. Voorkoms van PLRV in die Sandveld streek het oor die afgelope 15 jaar drasties vermeerder. “Enzyme-linked immunosorbent assay” (ELISA) word gebruik vir roetine toetsing deur die SA moersertifiseringskema om PLRV infeksies te diagnoseer, maar ander lande het verander na “reverse” transkripsie polimerase kettingreaksie (RT-PCR) vir die opsporing van PLRV weens sy beter sensitiwiteit. Die doel van hierdie projek was om ‘n peiler-gebaseerde kwantitatiewe “real-time reverse” transkripsie polimerase kettingreaksie (RT-qPCR) te ontwikkel en te valideer om PLRV in aartappels op te spoor en ‘n oorsig van PLRV voorkoms in die Sandveld streek, SA te kry. Hierdie projek het ook gemik om infeksie in plantluise op te spoor en die plantluis-oordraagbare-PLRV isolate te karakteriseer deur middel van volgordebepaling. Ten slotte het die projek “next generation sequencing” (NGS) aangewend om isolate te identifiseer en karakteriseer, nie-koderende genoom streke stroomopwaarts en stroomafwaarts te vergelyk en onbekende virusse en ander patogene te identifiseer wat moontlik in aartappels van die Sandveld streek voorkom.

Geskikte inleiers en ‘n TaqMan peiler is ontwerp om ‘n hoogs-sensitiewe RT-qPCR deteksiemetode vir PLRV te ontwikkel. Geamplifiseerde komplementêre DNA (cDNA) is in ‘n plasmied gekloneer vir toets kwantifisering en validering. Aartappelblare getoets oor ‘n volle kalender jaar en die resultate was vergelyk met vektordruk. Oor die algemeen was hoë infeksievlakke gevind, maar daar was sekere tye van die jaar wat lae infeksievlakke gehad het weens lae vektordruk. SA moere is ook getoets met die metode. Die studie dui dus dat die SA moersertifiseringskema die keuse om ELISA te gebruik as metode vir PLRV deteksie moet heroorweeg en vervang met die beskryfde RT-qPCR metode.

Tweedens is die teenwoordigheid van PLRV in plantluise bepaal met RT-qPCR. ‘n Heel PLRV genoom is geamplifiseer en sy volgorde bepaal na ekstraksie vanuit ‘n geinfekteerde plantluis. Die genereerde heel PLRV genoom is in ‘n datamatriks opgelyn met ander heelgenoom volgordes. Filogenetiese ontleding van die heel genome het getoon dat die PLRV isolaat wat uit die plantluis geekstrateer is, groepeer het met agt ander SA isolate van die Sandveld streek.

Laastens is Ion Torrent gebruik om inligting oor verdere PLRV isolate in die Sandveld streek te verkry. Monsters met lae Cq-waardes het ooreengestem met hoë getalle klein interferende RNAs (siRNAs) kartering, volgordedekking en volgordediepte. Drie volledige genome is verkry deur verwysingsgenoom siRNA kartering omdat *de novo* samestelling nie kontigs langer as 700 nukleotides kon verkry nie. Filogenetiese ontleding van die volledige genome het bepaal dat drie isolate gegroepeer het met ‘n Australiaanse isolaat en sewe SA isolate. Die oorblywende isolaat het gegroepeer met nege ander SA isolate. Min betekenisvolle stroomop- en stroomafwaartse variasies in nie-koderende streke is gesien. Geen ander aartappel of onbekende virusse geïdentifiseer nie, behalwe ‘n onbekende swam wat geidentifiseer is in al die monsters wat verdere ondersoek benodig.

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Abbreviations

ASG	accessory salivary glands
BHQ	Black Hole Quencher
BWYV	beet western yellows virus
BYDV	barley yellow dwarf virus
bp	base pairs
cDNA	complementary or copy DNA
CIP	International Potato Center
R^2	correlation coefficient
CP	coat protein
CYDV-RPV	cereal yellow dwarf virus
dsDNA	double stranded DNA
dsRNA	double stranded RNA
E	efficiency
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation
GES	glycine-EDTA-TritonX-100
GM	genetically modified
gRNA	genomic RNA
ISFET	ion-sensitive field-effect transistor
LOD	limit of detection
MP	movement protein
miRNA	micro RNA
NGS	next generation sequencing
nt/nts	nucleotide/nucleotides
ORF	open reading frame
PCR	polymerase chain reaction
PLRV	potato leafroll virus
PPV	plum pox virus
PSTVd	potato spindle tuber viroid
PVA	potato virus A
PVM	potato virus M
PVS	potato virus S
PVX	potato virus X
PVY	potato virus Y
qPCR	quantitative real-time PCR
Rap1	replication associated protein 1

RdRp	RNA-dependent RNA polymerase
RTP	readthrough protein
RT-qPCR	quantitative real-time reverse transcription PCR
RNAi	RNA silencing or interference
RT-PCR	reverse transcriptase PCR
SA	South Africa
sgRNA	subgenomic RNA
m	slope
sRNA	small RNA
siRNA	small interfering RNA
ssDNA	single stranded DNA
ssRNA	single stranded RNA
TVDV	tobacco vein distorting virus
VPg	viral genome linked protein

Chapter 1: Introduction

Seen in a global context, South Africa (SA) produces only 0.6% of the world's potatoes, but it grosses about R6.29 billion per year from this crop which is 3% of SA's total agriculture gross value (*National & International Information*, 2013). Importantly, potatoes represent 57% of vegetable production in SA (*National & International Information*, 2013). This is an important sector in South African agriculture as it generates significant employment in the rural areas of SA. The potato processing industry (frozen and potato chips) generates further employment opportunities. Over the last 26 years the area of potato production has decreased from 64 000 ha to 53 000 ha, but an increase in production from 1 320 million to 2 150 million tons was seen (*Production Information*, 2016). This illustrates that potato production and potato yield was increased per hectare due to the practice of centre-pivot irrigation and the introduction of new cultivars since 1994. By 2016, 43 000 ha of the 53 000 ha were under irrigation (*Production Information*, 2016). However, South African agriculturists are concerned about the potato industry's competitiveness in the globalising market, especially in the frozen potato market in which imports have often replaced South African produced potatoes. Research indicated that from 2011 to 2013 it was less cost effective to produce potatoes in SA than in Germany, Netherlands, Argentina, Belgium, United Kingdom and the United States of America due to higher production costs (Van der Waals *et al.*, 2016). This then renders potato growers less competitive on a global scale, even though local demand for potatoes remains high.

Potatoes are subject to numerous abiotic (e.g. temperature and moisture) and biotic (plant diseases and pests) risks that affect the crop's production sustainability and yield (Adams *et al.*, 1998). Besides direct yield reduction, pests indirectly influence yield by the transmission of viral diseases (McKinlay *et al.*, 1992). Viral diseases that infect cultivated potatoes such as potato virus Y (PVY) (family *Potyviridae*, genus *Potyvirus*, species *Potato virus Y)* and potato leafroll virus (PLRV) (family *Luteoviridae*, genus *Polerovirus*, species *Potato leafroll virus*) are both major causes of low yield (Salazar, 1996). PLRV is known as the most economically significant and devastating potato virus (Mayo and D'Arcy, 1999), second to PVY which is known to be the most important potato virus in the rest of the world (Lacomme *et al.*, 2017). PVY and PLRV are also the most significant potato viruses in SA (Denner *et al.*, 2012).

In a similar way to PVY, management of PLRV infections includes the control of vectors by regular systemic insecticide spraying, the planting of resistant potato cultivars and the planting of virus free certified seed (Van der Want, 1972) that entails testing the tubers beforehand with an accurate, rapid, sensitive and specific detection method (Salazar, 1994). Enzyme-linked immunosorbent assay (ELISA) is used for routine potato testing to detect PLRV infection by the South African Seed Potato Certification Scheme. Reverse transcription polymerase chain reaction (RT-PCR) has however proven to be a more rapid, versatile and sensitive method for virus detection (Omrani *et al.*, 2009; Kumar *et al.*, 2010). Switzerland, a country which has played a major role in the development and application

of ELISA technology for PVY and PLRV detection, has also changed in 2016 to real-time reverse transcription PCR (RT-qPCR) for the detection of these viruses because of its greater sensitivity (Schumpp, Agroscope, Switzerland, pers.comm.).

Potatoes are produced in 16 regions of SA (Figure 1.1) with different soils and climatic conditions, leading to different planting times (Haverkort *et al.*, 2013). In 2010, the Limpopo, Eastern Free State and Sandveld regions of SA produced 398 000, 256 000 and 317 000 tons of ware potatoes respectively (Van der Waals *et al.*, 2013).

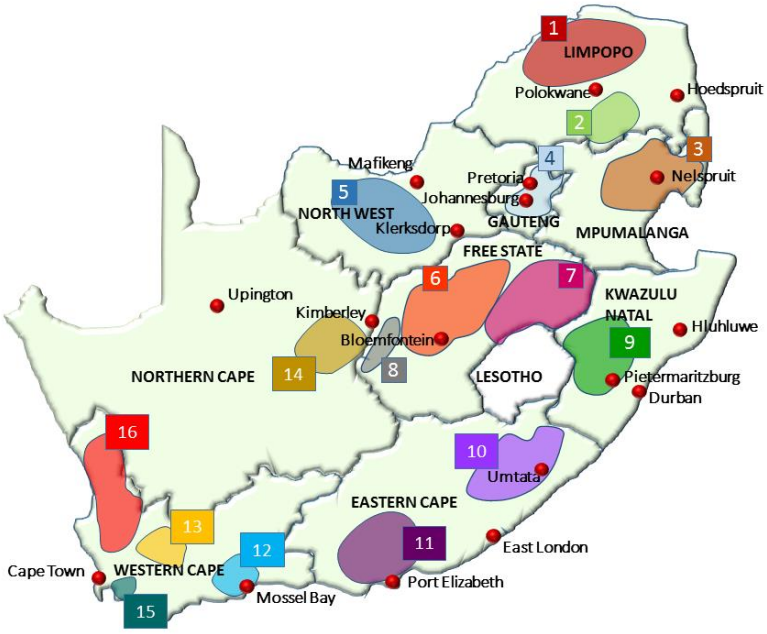

Figure 1.1: A map of SA showing the 16 potato production areas in the country (*Map of Regions*, 2017).

In the Sandveld region the main agricultural and therefore economic activity is potato production and processing, as potatoes are produced throughout the year. Potato production in this region occurs under temperature conditions close to 35˚C on average between November and March, in which many days have temperatures above 40˚C (Figure 1.2). These temperatures are significantly higher than the temperate conditions in most potato producing regions of the world in which temperatures range between 20˚C and 25˚C. The Sandveld region is perceived to have the lowest risk for growing potatoes in SA, as only high summer temperatures may pose a threat to plantings (Van der Waals *et al.*, 2016), but the Sandveld region still has to apply a five year rotation per field to prevent the accumulation of pests and diseases (Franke *et al.*, 2012) and to combat nutrient depletion. However, PLRV incidence in the Sandveld region has increased (Coetsee, 2004, 2005) dramatically from 1999 until 2005 (Figure 1.3).

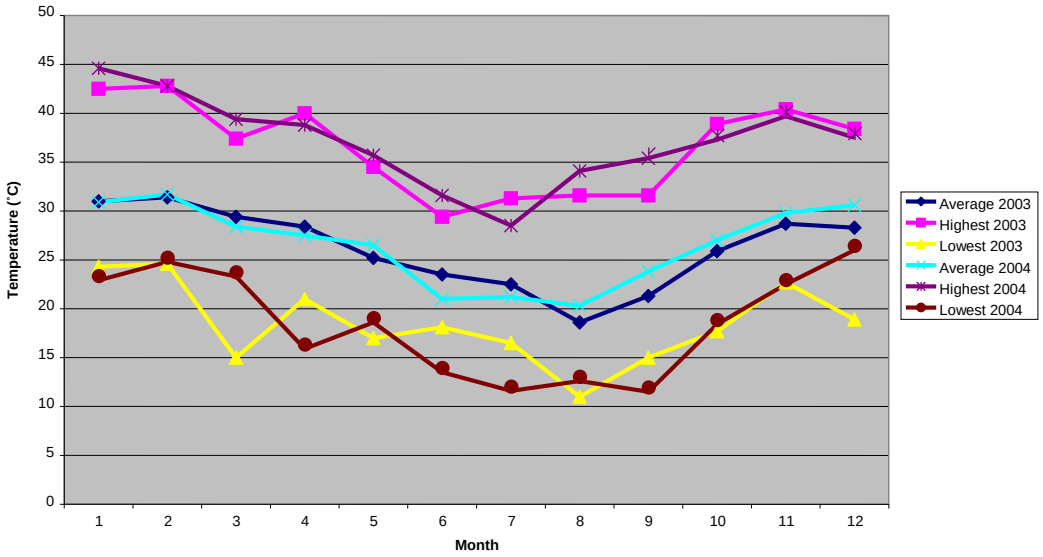

Figure 1.2: Maximum temperatures of Sandberg, in the Sandveld region, SA (Pieterse, PSA, pers. comm.).

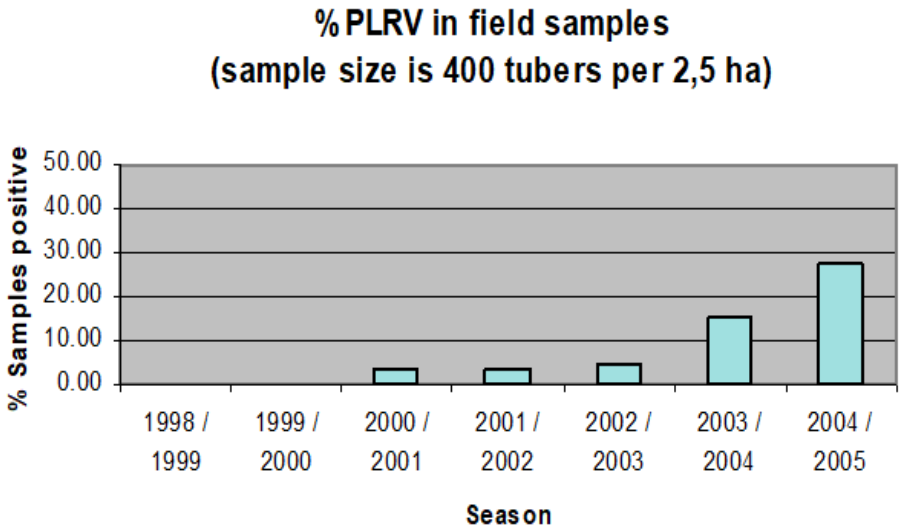

Figure 1.3: Percentage positive PLRV samples from 1998 until 2005 from the Sandveld region, SA (Pieterse, PSA, pers. comm.).

From 1997 until 2014 total registered plantings in the Sandveld region decreased about 81.8% (*National Statistics*, 2017). Cultivated seed potato hectares decreased annually by about 91% (3400 ha to 300 ha) from 2001 until 2014. The production of certified seed potatoes in 25 kg bags also decreased about ten fold (1 496 479 to 149 052) in only 13 years (*Production*, 2017).

Tuber certification as conducted by the Potato Certification Scheme in SA is based on the determination of the percentage of tubers that are infected with PLRV (and PVY) based on a tuber sample that is drawn at harvest. These tubers are then treated with gibberellic acid to induce sprouting and the sprouts that appear after about six weeks later are used for ELISA testing. This then generates a so-called “land monster” or tubers-at-harvest sample (see explanation p29) value used for

certification purposes. However, a second tuber sample is also taken at harvest, allowed to sprout naturally, and is tested for PLRV infection percentage (referred to as the "na-oes kontrole" or post-harvest-control sample (see explanation p29)). If this exceeds the first percentage infection rate, the tubers are downgraded and the tuber producer has to pay compensation to the tuber buyer for production losses thereby incurred, as by the time the "na-oes kontrole" or post-harvest-control sample result is made available, these tubers have already been planted to produce another crop. There was a steady increase in this tuber downgrading between 1999 and 2014, leading to a reduction in confidence by buyers of seed tubers originating from the Sandveld region and this also contributed significantly to the reduced tuber production in the area. As a result, the production of ware potatoes has grown in the region, whereas the traditional primary production of seed potatoes has become less important (Franke *et al.*, 2012).

The possible reasons that have been proposed for this increase in PLRV infection rates in the Sandveld region are: 1) year-round plantings facilitated by the installation of pivot irrigation systems; 2) a general intensification of the industry i.e. an increase of the total number of hectares planted with potatoes at any given time; 3) a gradual change in production from tuber production to ware potatoes with an associated reduction in general pest and pathogen control, specifically a reduction in the application of insecticides (as pest and pathogen control contributes significantly to production costs, this was economically motivated); and 4) the practice by farmers of using retained seed which was not tested for pathogens included in the tuber certification scheme i.e. bacterial wilt (*Ralstonia solanacearum*), PVY and PLRV.

These reasons could have contributed to a significant increase in PLRV inoculum for aphids, the vectors for PLRV infection, and a greater number of infected aphids causing further infections year round.

Due to the continued high levels of PLRV infections in the Sandveld region, the objectives of this study are:

(i) to develop and validate a probe-based RT-qPCR to detect PLRV in potato leaves and tubers and then use this method to test and obtain an accurate assessment of PLRV incidence in the region;

(ii) to confirm infection of aphids with PLRV by RT-qPCR and characterise aphid transmitted PLRV isolates by sequencing their whole genomes; and

(iii) to apply the next-generation sequencing (NGS) system:

a) to identify and characterise isolates occurring in the region with a view to identifying potentially more virulent strains;
b) to compare non-coding upstream and downstream (5' and 3') regions from coding genes, that may influence the expression of viral coding genes and thereby possibly pathogenicity; and
c) to identify unknown viruses that possibly occur in potatoes in the Sandveld region that may be responsible for production problems (besides PLRV) in the area.

This thesis is introduced with a literature review (Chapter 2) to potato production worldwide and in SA and the risks affecting yield, as a result of PLRV infection. The genes that PLRV encodes, its spread, RNA silencing or interference (RNAi) and the triangular infection relationship between virus, aphid and plant is described. The management of viral infections, importantly testing beforehand with methods such as ELISA, polymerase chain reaction (PCR), RT-PCR, quantitative real-time PCR (qPCR), RT-qPCR, multiplex PCR and others, and NGS, some of which were utilised for PLRV detection in this project, is outlined. In Chapter 3 the methodology used to generate a standard curve for assay validation, sample preparation for probe-based RT-qPCR and the use of this optimised RT-qPCR method to test potato leaves and tubers in the Sandveld region is described. In Chapter 4, aphids obtained from the Sandveld region were tested for PLRV infection and a whole PLRV genome was generated from an infected aphid. In Chapter 5 small interfering RNAs (siRNAs) and micro RNAs (miRNAs) were isolated and sequenced in an Ion Proton system to identify and characterise isolates to sequence non-coding upstream and downstream regions and to identify other possibly unknown potato viruses and pathogens. This is followed by a conclusion and future perspectives chapter and appendices containing the nucleotide (nt) sequence information generated in this study. Chapters 3 to 5 were written in publication format to facilitate future publication of this data. As a result, a certain amount of replication of the literature review in the introductions to these chapters was unavoidable.

Chapter 2: Literature Review

2.1 Introduction

Studies have estimated that by 2050 the world population will be about 9.7 billion (United Nations, Department of Economic and Social Affairs, 2015). This implies that agriculture production has to be dramatically increased and sustainable production will have to be ensured. Sustainable production is ensured through, amongst others, what is termed plant biosecurity. Plant biosecurity is defined as a set of measures designed to protect crops from emerging plant pests at the national, regional and individual farm levels (Garth, 2005). It will aid farmers to meet the challenges of reliable food production without draining natural and agricultural resources. Other factors such as unpredictable climatic changes including a global increase of 4.6˚C by the year 2100 (*Intergovernmental Panel on Climate Change 5th Assessment Report*, 2014), depleting water resources and a limited arable land for crop production will only worsen the situation.

Due to these uncertainties of food supply, an increase in demand for food and fixed hunger rates, the Food and Agriculture Organisation (FAO) recommends that the potato could serve as a food security crop (FAO, 2009). In the past, the crop provided cheap and plentiful food for labourers (Reader, 2008) and today it is an important part of many developing countries' diet (Dale and Mackay, 1994). Countries such as China, Bangladesh, India and the International Potato Center (CIP) are already making a major effort to establish potatoes as a source of food security (Frederick and Lei, 2015; Singh and Rana, 2013; Azimuddin *et al.*, 2009; Devaux *et al.*, 2014) and has shown that a potato is able to grow under conditions that simulate that at the surface of the planet Mars (CIP, 2017). These potato-based systems are available throughout the year as they are harvested somewhere in the world each month. Potatoes also present important opportunities for food security, poverty alleviation and improved health for the rural poor (Devaux *et al.*, 2014). Furthermore, the crop may be used for biofuel development (Gerbens-Leenes *et al.*, 2009).

2.2 The potato, *Solanum tuberosum*

2.2.1 *Background, classification and production*

The edible tuber and main cultivated species, *Solanum tuberosum*, was first domesticated at tropical latitudes in the Andes mountain region of South America (Beukema and Van der Zaag, 1990). Due to its cultivation at these high altitudes it became the staple food of the Inca nation (Ugent and Peterson, 1988). Today, this region still contains the largest amount of genetic diversity of potatoes and is also considered as the crop's center of origin (Navarre *et al.*, 2009). After Spain conquered Peru, potatoes were introduced to Europe in 1570, mostly Ireland (Drake, 1854 as referred to by Srivastava *et al.*, 2016) but it then took some time for the potato to spread across the rest of Europe (Hawkes, 1992). As many people in Ireland relied on potatoes as their only food source, the Irish Potato famine in 1845

led to the death of at least one million people due to the crop becoming diseased with late blight, *Phytophthora infestans*. As only one potato cultivar, called "Lumpers" was cultivated at the time in Ireland, this illustrates how the narrow genetic base of a single cultivar can have devastating effects on the production of a crop. Questions about *P. infestans* led to the birth of modern plant pathology (Large, 1940; Carefoot and Sprott, 1967).

Potatoes belong to the Solanaceae family whose members include tomatoes, eggplants, peppers and tomatillos. There are about 200 potato species, but only eight are currently cultivated (Smith, 1977). These eight are the diploids *S. ajanhuiri*, *S. goniocalyx*, *S. phureja*, and *S. stenotomum*, the triploids *S. chaucha* and *S. juzepczukii*, the tetraploid *S. tuberosum* and the pentaploid *S. curtilobum* (Hawkes, 1978). Today, *S. tuberosum* is the third most important food crop consumed by humans in the world after wheat and rice, and the number one vegetable (FAOSTAT, 2016). One hectare of potato can yield two to four times the food quantity of a grain crop and produces more food per unit of water than any major crop. Potatoes are capable of using water more efficiently (CIP, 2016) and provide twice as many calories per unit area of land in a shorter period of time than cereals (Ahmed and Kamal, 1984; Rodríguez Galdón *et al.*, 2010). In 2014, the FAO estimated that 400 million tons of potatoes were harvested (FAOSTAT, 2016). The largest potato production traditionally came from developed countries, but over the past ten years this has been overtaken by developing countries. The countries currently producing the most potatoes are China, India, the Russian Federation, Ukraine and the United States (FAOSTAT, 2013). Worldwide average potato production is roughly 17 tons per hectare (t/ha) and direct consumption is 31.3 kg per capita (kg/year). Africa accounts for only 5% of worldwide potato production, with a 10 t/ha average (FAOSTAT, 2014). SA is third of the top ten African potato producing countries (Muthoni *et al.*, 2011).

For human nutrition, potatoes contain substantial amounts of proteins and amino acids, dietary fibre, carbohydrates, micronutrients (iron and zinc), minerals (magnesium, potassium and phosphorus) and vitamins (B1, B6 and C) (Thompson and Kelly, 1957; Fernie and Willmitzer, 2001; Dale *et al.*, 2003; Buckenhüskes, 2005). The tuber protein provides a good source of the essential amino acids lysine, leucine, phenylalanine, threonine, isoleucine and valine (Van Gelder and Vonk, 1980). Within the tubers accumulation of beneficial secondary metabolites such as plant phenols (Friedman, 1997) and anthocyanins (Brown *et al.*, 2003) that possess antioxidant properties (Ezekiel *et al.*, 2013) occurs. Additional phytosterols could reduce intestinal cholesterol absorption (Piironen *et al.*, 2003; Tierno *et al.*, 2016) and serum LDL-cholesterol levels (Racette *et al.*, 2010). Moreover, the potato is rich in starch, the main contributor to the dietary glycemic index (Jansen *et al.*, 2001). This nutrient dense crop could therefore increase the dietary diversity for a rural household. Potatoes can be cooked, served as whole or mashed and ground to flour, playing their part in the vegetable market and processing industry.

For reproduction of the crop, a part of the potato production is set aside for reuse in the following year. A new plant then grows from the potato tuber or a piece thereof, meaning that potatoes are vegetatively reproduced and genetic clones of the mother seed (CIP, 2016). Potatoes can be grown under a wide range of climatic conditions including different altitudes, day length, latitudes, and temperatures, but main production usually occurs in an area where water is abundant (Haverkort, 1990) or where water can be supplied by the use of centre-pivot or circle irrigation (Zarzynska and Goliszewski, 2016). Although potatoes can be grown almost anywhere, the crop is very sensitive to frost leading to severe damage when temperatures are below 0˚C (Hijmans, 2003). Some studies revealed that an increase in CO_2 concentration in the atmosphere may benefit potato growth, since the tubers are made early in the plant's life and can quickly absorb the higher amount of photosynthetic products produced by increased CO_2 conditions (Franke *et al.*, 2012). Even though the area on which potatoes is grown worldwide has decreased, yield and production have increased (FAOSTAT, 2016). This illustrates how production methods and breeding efforts have aided potatoes' resilience against pests, weeds and pathogens such as bacteria, fungi and viruses, but this does not mean potatoes are immune to them.

There are some 950 plant viruses documented throughout the world (King *et al.*, 2012) that cause around $60 billion in economic losses (Xie *et al.*, 2009) to crops. Globally, some 40 plant viruses also reduce potato yield significantly (Valkonen, 2007). In potatoes, low yield can also be caused by pests and bacterial diseases; poor agronomic practices (inappropriate use of chemicals, no crop rotation, lack of proper sanitation); a lack of knowledge of farmers; but indirectly by the lack or availability of certified clean seed (seed produced from infected tubers); and high prices of certified seed; (Schulte-Geldermann, 2013). Without renewing seed and using saved seed for several plantings, seed-borne diseases and particularly viruses, can build up, causing severe yield and quality losses (Gildemacher *et al.*, 2009). Farmers from developing countries store their own seed tubers, whereas in developed countries, they are more likely to buy disease free certified seed. The International Potato Center (CIP) shows how the use of tissue culture, aeroponics technology for mini-tuber production and the use of screen houses can be used to quickly multiply virus free seed (Wang and Hu, 1982; Farran and Mingo-Castel, 2006; Gildemacher *et al.*, 2009).

2.2.2 *Abiotic and biotic risks of potatoes*

Potato crop production is affected by numerous abiotic and biotic risks in nature (Adams *et al.*, 1998). Abiotic risks include climate change such as temperature, precipitation, severe events such as droughts and poor agronomic practices, whereas biotic risks involve the spread of plant diseases and pests (Kaukoranta, 1996; Boland *et al.*, 2004; Kapsa, 2008; Van der Waals *et al.*, 2013).

In SA, abiotic risks include temperature and precipitation (Steyn *et al.*, 1998; Franke *et al.*, 2013; Haverkort *et al.*, 2013). SA is perceived as being a dry country, but surprisingly, the most common abiotic risk factor is too much rain (precipitation) that may cause water-logging and rotting of tubers in soil (Wale *et al.*, 2008; Denner *et al.*, 2012; *Irrigation and Water Use [Best Practice Guide for*

Potatoes], 2013). Late frosts can occur early in the planting season after winter, due to low temperatures that damage new emerging plants and early frosts may destroy the plant haulm completely (Haverkort *et al.*, 2013). Heat waves can strain the crop or reduce the quality of tubers if it occurs during the growing season or at the time of tuber bulking (UNECE, 2014). When normal temperatures follow, secondary growth symptoms may occur, leaving the tuber malformed. With the low risk for growing potatoes in the Sandveld region and without abiotic risks, yield may by 4.5% higher than the current average of 37 t/ha (Van der Waals *et al.*, 2016). However, the greatest risk to potato production in SA comes from biotic influences (Van der Waals *et al.*, 2016).

Preventatively, a five year rotation per field can dramatically reduce the accumulation of pests and diseases (Franke *et al.*, 2012) and is recommended as standard cultivation practice. Pests either directly influence yield via feeding or indirectly by the transmission of viral diseases, root attack, sap removal or destroying plants through defoliation (McKinlay *et al.*, 1992). In SA destructive economic losses caused from pests and pathogens include late blight (*P. infestans*), early blight and brown spot (*Alternaria solani* and *Alternaria alternata*), soft rot and blackleg (*Pectobacterium carotovorum* spp. *brasiliensis*), root-knot nematode (*Meloidogyne javanica* and *Meloidogyne incognita*), PVY and PLRV (Van der Waals *et al.*, 2013).

Late blight's major agent *P. infestans*, a fungus, is seen as the main potato disease worldwide. In 2007 Hannukkala *et al.* (2007) reinforced previous work showing the increase of *P. infestans* infection globally over the past decades (Fry *et al.*, 1993; Drenth *et al.*, 1994; Kaukoranta, 1996; Hannukkala *et al.*, 2007). This increase could be caused by the development of new populations, due to changes in their genetic diversity that allowed the pathogen to adapt and survive longer in the soil (Van der Waals *et al.*, 2013). The most important air-borne pathogens of potatoes in SA are the fungi *Alternaria solani* and *A. alternata*; they cause early blight and brown spot respectively, which accelerates the ageing process of plant leaves (Van der Waals *et al.*, 2003, 2011). The bacterium, *Pectobacterium carotovorum* spp. *brasiliensis* has significantly increased soft rot or blackleg disease in SA (Van der Merwe *et al.*, 2010; Van der Waals *et al.*, 2013). Disease development of this agent is dependent on soil water levels that aids soft rotting facultative anaerobes (Perombelon, 2002). In SA, the root-knot nematode is seen as the most common and damaging nematodes on crop plants (Fourie *et al.*, 2001), with *Meloidogyne javanica* and *M. incognita* being the most destructive species of the potato (Coetzee, 1968).

The tuber- and soil-borne fungi causing black scurf (*Rhizoctonia solani*), silver scurf (*Helminthosporium solani*), powdery scab (*Spongospora subterranea* spp. *subterranea*), *Fusarium* spp. (fusarium wilt), *Verticillium dahlia* and *V. albo-atrum* (verticillium wilt) (Denner *et al.*, 2012) can also destroy a potato field. Lastly, pests such as the green peach aphid (*Myzus persicae*), tuber moth (*Phthorimaea operculella*) and leaf miners (*Liriomyza* spp. and more recently, *Tuta absoluta*) are also found to attack potato plants in SA (Denner *et al.*, 2012; Niederwieser, 2016).

It is known that there are more than 40 viral diseases that infect cultivated potatoes (Valkonen, 2007) and present to be one of the major causes of low yield (Salazar, 1994). Highly pathogenic and new viral strains have recently emerged (Ryazantsev and Zavriev, 2009). The most economically important potato viruses worldwide include, PVY, PLRV, potato virus X (PVX) (order *Tymovirales*, family *Alphaflexiviridae*, genus *Potexvirus*, species *Potato virus X*), potato virus S (PVS) (order *Tymovirales*, family *Betaflexiviridae*, genus *Carlavirus*, species *Potato virus S*), potato virus A (PVA) (family *Potyviridae*, genus *Potyvirus*, species *Potato virus A*) and potato virus M (PVM) (order *Tymovirales*, family *Betaflexiviridae*, genus *Carlavirus*, species *Potato virus S*) (Van der Want, 1972; Srivastava *et al.*, 2016). The most important potato virus disease worldwide is PVY (Radcliffe and Ragsdale, 2002; Lacomme *et al.*, 2017). PVY and PLRV can cause major yield losses and degeneration of planting fields (Nascimento *et al.*, 2003), whilst single infections of PVX and PVS only result in minor problems (Reestman, 1972). PVY is transmitted in a non-persistent manner by potato colonising, *M. persicae* (Boquel *et al.*, 2011a) and non-colonising winged aphids (Radcliffe, 1982).

PLRV, the second most important potato virus worldwide (Mayo and D'Arcy, 1999), was assumed to reduce global annual potato yield by 20 x 10^6 tons in 1988 (Kojima and Lapierre, 1988). As mentioned in the introductory chapter, PLRV is a major limiting factor to potato production in SA, especially in the Sandveld region (Pieterse, PSA, pers. comm.).

2.3 **Potato leafroll virus**

2.3.1 *Classification and viral structure*

Luteoviruses have been documented to be present on potato fields from early in the 19th century (Oswald and Houston, 1951; McKee, 1964; Harrison, 1999). However, during the last decade researchers have grouped them into the family Luteoviridae (D'Arcy and Mayo, 1997). This family has been divided into three different genera, *Luteovirus*, *Polerovirus* and *Enamovirus* due to differences in the RNA-dependent RNA polymerase (RdRp) and structural proteins (Mayo and Ziegler-Graff, 1996). The 5' region of the viral genome is vastly variable between Luteoviridae, but the 3' region encodes a preserved structural coat protein (CP) (Torres *et al.*, 2005). The evolution of members of this family and genus is complicated by a strong contribution of recombination (Stevens *et al.*, 1994; Stevens *et al.*, 2005).

In the 1760's, a condition termed the Curl was affecting potato cultivation in Lancashire, U.K. (Barker, 1992; Taliansky *et al.*, 2003). The responsible pathogen, PLRV, the longest-known plant virus (Quanjer, 1913), was first characterised in 1913 (Quanjer *et al.*, 1916; Barker, 1992; Taliansky *et al.*, 2003) as the type species of the genus *Polerovirus* in the family *Luteoviridae*, a group of phloem-limited plant viruses (Kojima *et al.*, 1968; Mayo and D'Arcy, 1999). PLRV is a positive sense, single stranded RNA (ssRNA+) virus (Taliansky *et al.*, 2003) and to complete its life cycle, it targets plant host machinery. Its genome consists of approximately 5 900 nucleotides (nts) which is 2000 kDa in

molecular weight within isometric virus particles that are 25 nm in diameter as shown in Figure 2.1 (Harrison, 1984). Figure 2.2 shows the phloem-limited distribution of PLRV in potato tubers, and it is also limited to the phloem in haulms and leaves of the potato plants.

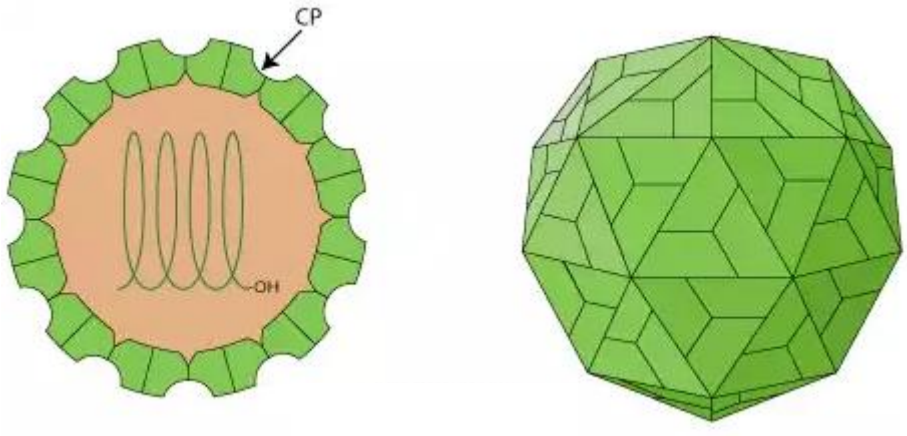

Figure 2.1: A diagram of the icosahedral PLRV viral particle (Hulo *et al.*, 2016). Coat protein (CP).

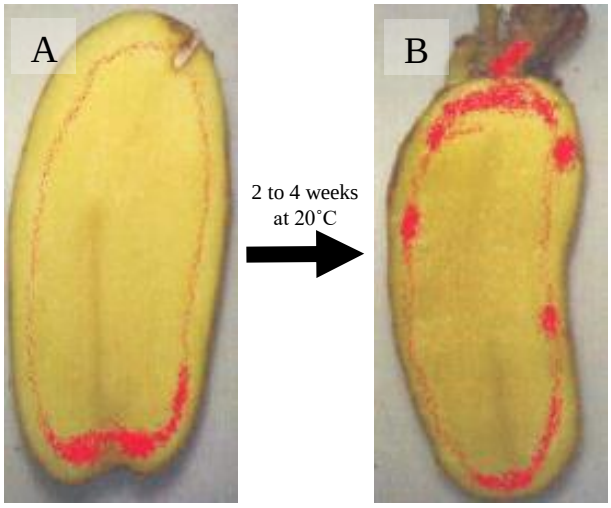

Figure 2.2: Gold label antibody that binds with PLRV which illustrates its location which is limited to the phloem (Gugerli, BIOREBA, pers. comm.). Unsprouted tuber (A) and sprouting tuber (B).

2.3.2 *Genes encoded by the PLRV genome*

The PLRV genome encodes ten open reading frames (ORFs) (Jeevalatha *et al.*, 2013; Smirnova *et al.*, 2015) that encode eleven proteins. The proteins, P0, P1, P2 and replication associated protein 1 (Rap1) are encoded from genomic RNA (gRNA) by the three 5'-proximal ORFs (ORF0-2, 8), whereas the structural proteins (P3-P7) are encoded from three subgenomic RNAs (sgRNA) by six 3'-terminal ORFs (ORF3-7). The genome is linked with a viral genome linked protein (VPg) at the 5' end, capped by an OH group at the 3'end (Figure 2.3) and contains no poly-A tail (Mayo *et al.*, 1982).

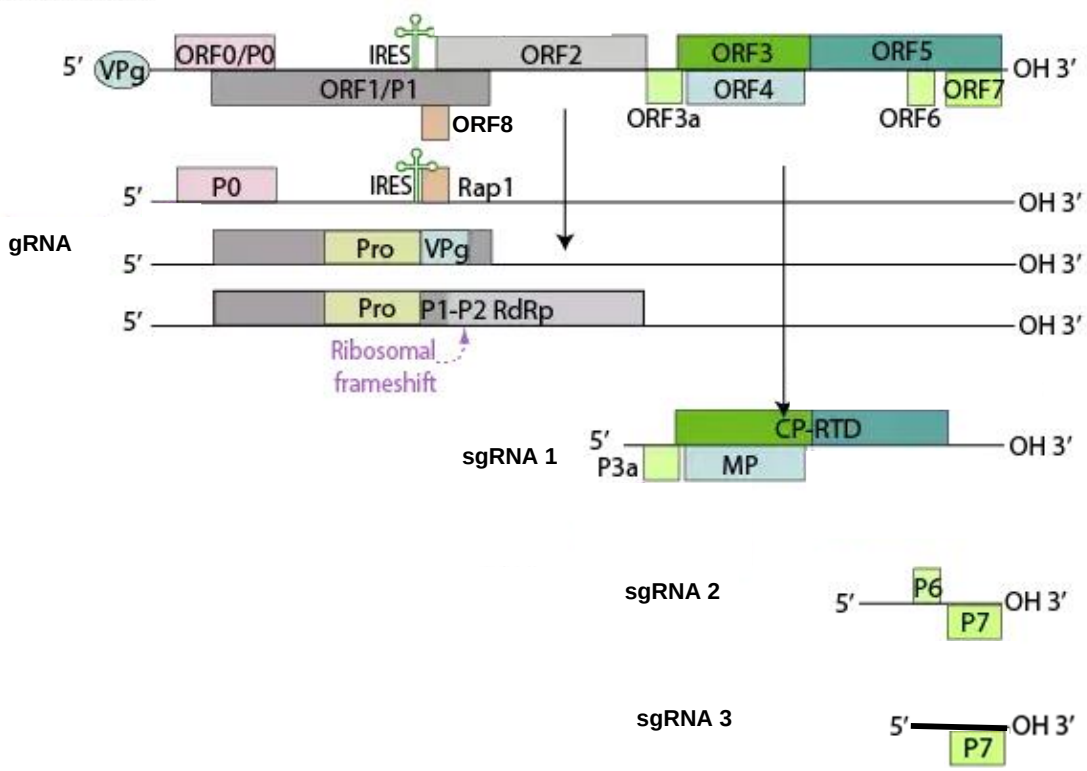

Figure 2.3: A schematic of the PLRV genome with the proteins it encodes (P0-P7), the 5' protein part occupied by the viral genome linked protein (VPg) and the free 3' OH group (Hulo *et al.*, 2016). Open reading frame (ORF).

P3a, P3, P4 and P5 are encoded by sgRNA1, whereas P6 and P7 are encoded by sgRNA2 (Van der Wilk *et al.*, 1989). In several members of genus *Luteovirus* a third sgRNA (sgRNA3) has been detected which is located at the 3'-terminus of the viral genome (Koev and Miller, 2000; Domier *et al.*, 2002). Small RNA (sRNA) sequencing profiles also detected a sgRNA3 for PLRV which also encodes P7 (Hwang *et al.*, 2013).

ORF0 encodes a protein P0 of 28 kDa that plays a role in suppressing RNA silencing by the plant host (Hauser *et al.*, 2000), symptom development and/or expression (Pfeffer *et al.*, 2002; Bortolamiol *et al.*, 2007). The consensus F-box-like motif inside ORF0 is required for the suppressor activity. Only *Polerovirus* and not *Luteovirus* expresses P0 (Mayo and Ziegler-Graff, 1996; Taliansky *et al.*, 2003).

ORF1 encodes the polyprotein, P1, of 70 kDa which is cleaved into a proteinase (Prüfer *et al.*, 1999) and the VPg protein of 7 kDa by self-proteolysis (Van der Wilk *et al.*, 1997; Prüfer *et al.*, 1999; Sadowy *et al.*, 2001). ORF8 located inside ORF1 associates with an internal ribosome entry site (IRES) and encodes a 5 kDa Rap1 that is required for viral replication (Jaag *et al.*, 2003; Jeevalatha *et al.*, 2013).

Ribosomal frame shift within ORF1/ORF2 translates the protein, P2, of 118 kDa (Prüfer *et al.*, 1992) that contains the conserved motifs typical of a RdRp (Kamer and Argos, 1984).

ORF3a, arranged upstream of ORF3, translates into the recently discovered protein P3a of 6.8 kDa that is essential for long-distance movement. A non-AUG start codon enables its translation (Smirnova *et al.*, 2015). Smirnova *et al.* (2015) also showed that P3a is targeted to the Golgi apparatus and the plasmodesmata.

ORF3 encodes the CP of 23 kDa (Jeevalatha *et al.*, 2013). It is considered that the CP consists of two main domains. Situated at the N-terminus of the protein is the R domain and the structure's major framework, known as the S domain, is situated towards the C-terminus (Terradot *et al.*, 2001). The CP is responsible for viral encapsulation and serological properties (Massalski and Harrison, 1987). It interacts with cell receptors in the salivary glands of the aphid vector (Gray and Gildow, 2003) which directly connects it to specificity, translocation through the aphid and rate of viral transmission to the host plant (Van den Heuvel, 1990; Torres *et al.*, 2005).

ORF4 encodes the recognised viral movement protein (MP), P4, of 17 kDa (Miller and Mayo, 1991; Jeevalatha *et al.*, 2013). Studies suggest that P4 is host dependent and for PLRV movement a P4-independent mechanism is functional in some plants (Ziegler-Graff *et al.*, 1996; Lee *et al.*, 2002). The MP mediates the spread of PLRV particles from cell-to-cell, so-called short-distance movement (Taliansky *et al.*, 2003).

Mayo *et al.* (1993) proposed that the readthrough protein (RTP), P5, of 76 kDa encoded from ORF5 also plays a substantial role in viral-aphid transmission. Studies showed that the P5 of luteoviruses may influence the interaction between the virus particles and aphid vector receptors (Guilley *et al.*, 1994). This protein can also be seen as an extension of the CP, enabling transmission by aphids (Tacke, Prüfer *et al.*, 1990; Brault *et al.*, 1995). Without the RTP, infection of the host can still occur but transmission via aphids was found to be unsuccessful (Peter *et al.*, 2008). The RTP aids to retain the virus in the phloem (circulation fluid providing nutrients to all parts of the plant, consisting of sieve elements that are connected to companion cells) for accessibility by aphids (Peter *et al.*, 2009) and movement of virions across the accessory salivary glands (ASG) of the vector (Mayo and Ziegler-Graff, 1996).

Even though no functions are known for these proteins, it is speculated that the P6 of 7.1 kDa encoded from ORF6 has a minor supportive role in virus replication (Mohan *et al.*, 1995) and P7 of 14 kDa encoded from ORF7 has nucleic acid binding properties (Ashoub *et al.*, 1998; Taliansky *et al.*, 2003). P7 is thought to have a regulatory transcription role within the genus *Polerovirus* (Hwang *et al.*, 2013).

There are three non-coding regions in the PLRV RNA genome: a 5'-terminal of 174 nts (Mayo *et al.*, 1989) or 70 nts (Keese *et al.*, 1990) and a 3'-terminal of 141 nts, but no obvious similarities were detected between PLRV, beet western yellows virus (BWYV) (family *Luteoviridae*, genus *Polerovirus*, species *Beet western yellows virus*) and barley yellow dwarf virus (BYDV) (family *Luteoviridae*, genus *Luteovirus*, species *Barley yellow dwarf virus*) sequences (Mayo *et al.*, 1989).

Most PLRV isolates' 5' non-coding regions appear to be 70 nts long, while the additional 104 nts from the Scottish PLRV isolate (Mayo *et al.*, 1989) could be the result of faulty amplifications and/or sequencing.

Symbionin, a bacterial endosymbiont's protein, binds the PLRV particle to protect it from proteolysis (Van den Heuvel *et al.*, 1994; Hogenhout *et al.*, 1998; Taliansky *et al.*, 2003) and determines its persistent manner (Syller, 1996).

ORF3 and ORF4 are known to be the most conserved regions in the genome (Guyader and Ducray, 2002; Plchova *et al.*, 2009). Guyader and Ducray (2002) proposed that PLRV's apparent lack in sequence variation could be due to a recent divergence from an ancestral virus or very strong selection constraints of the narrow genetic base from cultivated potatoes, even though mutations and variable sites do occur. PLRV accumulation was, for example, eliminated when mutations occurred in P0 thereby preventing its expression *in vitro* (Sadowy *et al.*, 2001). Consequently, the ORF3 region has been used for PLRV detection in potato leaves and tubers, due to its gene homology of 94 to 97% to other PLRV-CP sequences found on GenBank (Hossain *et al.*, 2013). ORF1 presently has the most variable sites, especially in the non-overlapping parts of ORF1 and ORF2 of the Australian PLRV genome (Guyader and Ducray, 2002). Sequence changes in the minor capsid RTP have also been shown to prevent PLRV transmissibility by aphid inoculation (Jolly and Mayo, 1994; Rouze-Jouan *et al.*, 2001).

2.3.3 *Host, replication and symptom expression*

Although PLRV mostly targets the Solanaceae family (Harrison, 1984; Syller, 1996; Taliansky *et al.*, 2003), about 20 other plant species have also been reported to be PLRV hosts. They include members of the following plant families: *Chenopodiaceae*, *Brassicaceae*, *Malvaceae*, *Asteraceae*, *Cucurbitaceae*, *Lamiaceae* and *Portulacaceae* (Tamada *et al.*, 1984). *Physalis floridana* and *Datura stramonium* L., both Solanaceae, are seen as good diagnostic and propagative hosts for PLRV (Harrison, 1984).

Viral particles, consisting of nucleic acids that are encapsulated by a CP, disassemble inside the cell and initiate the infectious cycle consisting of replication, cell-to-cell movement, long-distance movement and vector-mediated transmission to new hosts (Calil and Fontes, 2016). Local and long-distance movement of the virus occurs simultaneously inside the plant. Long-distance movement takes place inside the phloem sieve elements (Taliansky and Barker, 1999; Rhee *et al.*, 2000), whereas local movement occurs from cell-to-cell through the plasmodesmata (Taliansky *et al.*, 2003). Only once it is inside the cell, probably the phloem companion cells or phloem parenchyma, does the virus start to replicate. First, early gene products are expressed from the ssRNA+ that lead the formation of a viral replicase complex (VRC) to synthesise complementary ssRNA-. These ssRNA- are used to synthesise three sgRNAs and replicate new full length ssRNA+. The sgRNAs then express genes that synthesise

structural proteins, such as the CP (Figure 2.4). After assembly of the new ssRNA+ with the structural proteins it is known as a virion and can be acquired by an aphid to infect other plants (Ali *et al.*, 2014). Prüfer *et al.* (1997) and Franco-Lara *et al.* (1999) induced virus replication by transforming a full-length complementary DNA (cDNA) of PLRV into the genome of potato plants. Studies have demonstrated that limited movement of PLRV occurs in mesophyll protoplasts and inoculated epidermal or mesophyll cells (Barker, 1987; Van den Heuvel *et al.*, 1995; Derrick and Barker, 1997; Taliansky *et al.*, 2003), but it could leave the phloem and move into the mesophyll cells when the plant is infected with other viruses (Barker, 1987).

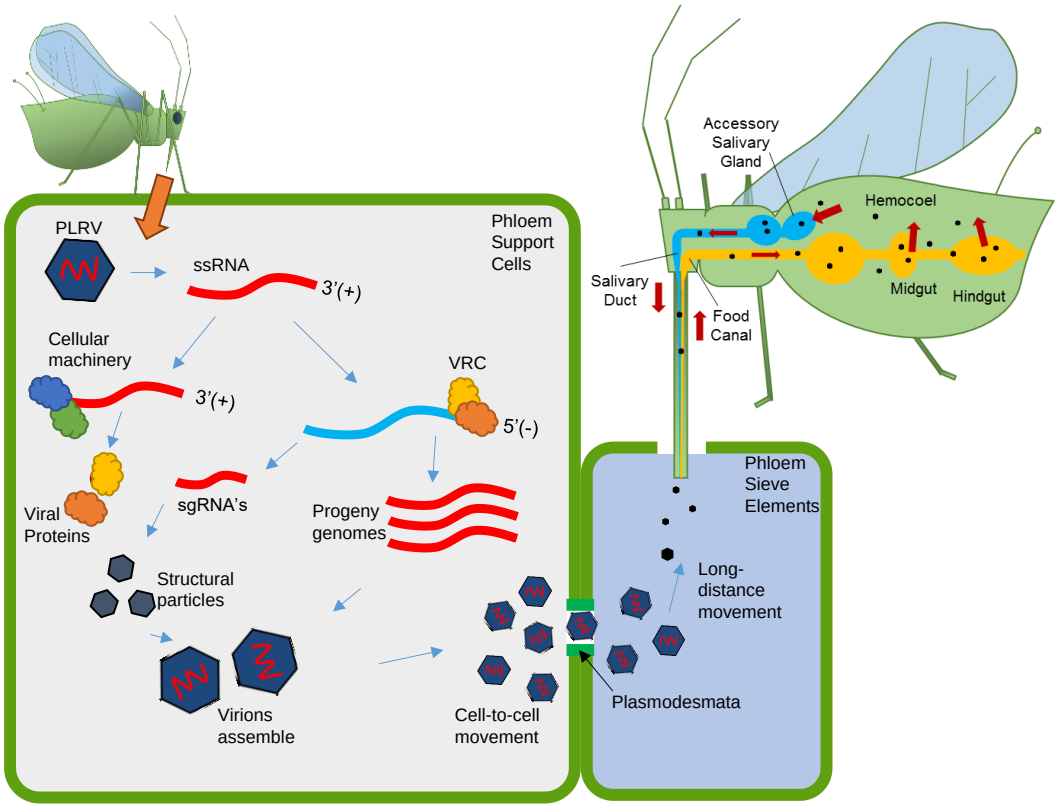

Figure 2.4: The PLRV replication cycle inside the plant host and movement through the aphid vector. Single stranded RNA (ssRNA); Subgenomic RNA (sgRNA); Viral replicase complex (VRC). (Roos, unpublished).

PLRV was shown to reach low concentrations in leaves affected by aphid feeding, whereas leaves developed after the attack, had higher values (Tamada and Harrison, 1980b). It takes PLRV one week to move from the leaves to developing tubers (Bradley and Ganong, 1952; Knutson and Bishop, 1964) and can reach maximum infection levels after 21 days but this is strongly dependent on climatic conditions (Flanders *et al.*, 1990) and can be significantly delayed depending on temperature. Certain potato cultivars have phloem-restricted resistance which limit acquisition of PLRV. This phloem resistance is seen in young apical leaves but declines in mature and senescent leaves (Alvarez *et al.*, 2006).

Growing conditions, host species, cultivar, virus variant and the age of the plant influence symptom expression. PLRV presence leads to important structural and metabolic changes in the host (Alvarez *et al.*, 2007). Dry matter production depends on the respiration rate, size and longevity of green foliage and photosynthesis rate per unit green leaf surface which determines tuber yield. Studies have shown that high virus titre in plants has a substantially negative effect on plant yield and disease severity is positively correlated to yield reduction (Jedlinski *et al.*, 1977; Bosque-Olojede and Buddenhagen, 1998). PLRV causes stunting of the potato plant (De Bokx and Van der Want, 1987) and blocks the transport of starch from leaves to tubers, because the distorted plasmodesmata within an infected plant's phloem tissue alter carbohydrate allocation patterns causing an accumulation of soluble sugars and starch, impaired sucrose loading and reduced photosynthetic capacity in the leaves (Herbers *et al.*, 1997). A reduction in tuber number and size of the diseased plant (Figure 2.5) (Harrison, 1984; Radcliffe and Ragsdale, 2002; Rahman *et al.*, 2010) and total tuber yield can amount up to 90% loss (Jayasinghe, 1988; Culver and Padmanabhan, 2007; Rahman and Abdul-Mannan, 2010) as a result of this change in distribution of carbohydrates and starch content between the leaves and tubers. It can also cause tuber net necrosis (Harrison, 1984; Radcliffe and Ragsdale, 2002) that makes tubers undesirable for the market and processing (Kuhl *et al.*, 2016) leading to crop rejection with major economic consequences (Mayo and D'Arcy, 1999). Thickening of cell walls occurs in the primary phloem cells of stems and petioles and irregular callus accumulation in sieve elements (Thomas, 1996; Hossain *et al.*, 2013). Most infected potato plants were found to have an upward rolling (Figure 2.6A), a purple edge or reddening on leaflets, leaf chlorosis (Figure 2.6B) (Taliansky *et al.*, 2003) or yellowing (Abbas *et al.*, 2016) and leathering or drying of leaflets (Alani *et al.*, 2002; Abbas *et al.*, 2016) due to a decrease in photosynthetic activity (Kogovšek and Ravnikar, 2013). Water scarcity (Beukema and Van der Zaag, 1990) and uneven mineral delivery demands (Bélanger *et al.*, 2001) may additionally inhibit growth and strain tuber yield, quality and size distribution (Borówczk, 2012).

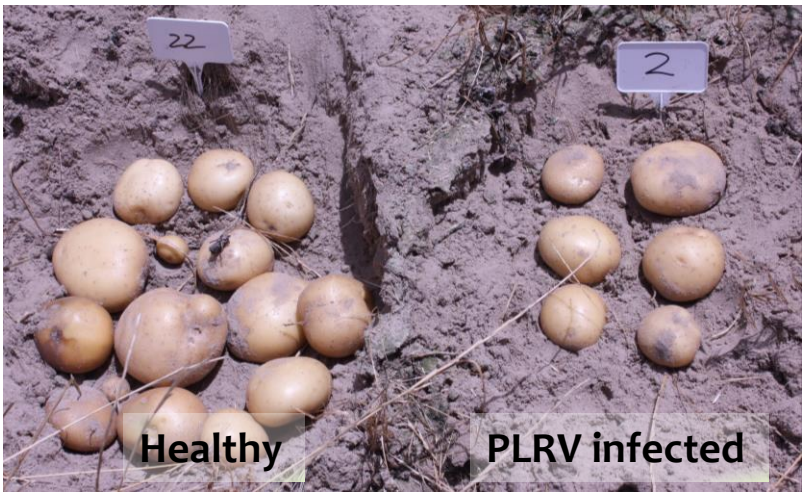

Figure 2.5: Illustration of yield reduction in the cultivar Sifra due to PLRV infection in the Sandveld region, SA (Roos, pers. comm.), (Photograph, D. U. Bellstedt).

Figure 2.6: PLRV infection symptoms leaf roll (A) and leaf chlorosis (B) in the Sandveld region, SA. (Photographs, L. van Wyk).

Harrison (1984) reported that primary infection occurring in the current season via aphid transmission of the virus, has less of an effect on yield and results in a variable amount of daughter tubers that are infected and symptom expression, than secondary infection which developed from previously infected tubers which results in all tubers being viruliferous. Symptoms such as the upward rolling, a purple edge or reddening on leaflets are less severe in primary infections than in secondary infection. It was reported that primary infected plants produce a higher yield than previously infected (secondary infection) plants and yield loss was the result of diminished growth by initiated tubers rather than fewer tubers being formed (Van der Zaag, 1987). Killick (1979) found that PLRV infection produced fewer stems per plant but more tubers per stem. Yield loss was seen as insignificant when plants were infected late in the growing season (Van der Zaag, 1987). Thus, yield loss appears to be strongly related to viral load. In some geographic regions infection levels can be high and economic losses can be serious in potato cultivars that lack resistance to the infection. Although contradictory to the

generally held belief that increased CO_2 levels are a negative consequence of climate change, elevated CO_2 levels increase plant resistance to virus titre (Trebicki *et al.*, 2015).

2.3.4 *Virus transmission by aphids*

More than 60% of plant viruses need insect vectors for transmission (Radcliffe and Ragsdale, 2002) and PLRV is known as one of the most damaging aphid-transmitted viruses (De Bokx and Van der Want, 1987). Due to the inability of a *Polerovirus* to be mechanically transmitted, aphids are only known contributors to PLRV spread (Miguel *et al.*, 2016), even though it is experimentally transmissible by grafting (Harrison, 1984). In the 1920s the discovery was made that aphids naturally transmit PLRV to potatoes in a persistent circulative (throughout the aphid) non-propagative manner by a species that colonises the potato (Ragsdale *et al.*, 2001)

When a virus free aphid feeds on the infected potato plant, its stylet should penetrate deep enough to reach the phloem tissue to become infected. It has been reported that only about 5 to 10 min are needed for an aphid to acquire PLRV (Tanaka and Shiota 1970; Singh *et al.* 1995). Thereafter, the aphid holds the ability to infect healthy plants for the rest of its life, which is about ten days long. After the virus is taken up in the food canal, the viral particles accumulate in the gut lumen (midgut or hindgut), move into the hemocoel and concentrate in the ASG (Van den Heuvel *et al.*, 1994; Gray and Gildow, 2003; De Oliveira *et al.*, 2016). The viral particles then need to cross the ASG basal lamina and plasmalemma membrane to be released into the salivary duct for inoculation into a new plant's sieve elements (Figure 2.4). PLRV does not multiply inside the aphid (Taliansky *et al.*, 2003). The gut membrane, ASG basal lamina and ASG basal plasmalemma may serve as barriers for viral transmission (Gildow, 1999). The aphid is then infective after a latent period of 8 to 72 hours (Radcliffe, 1982; Radcliffe and Ragsdale, 2002). When the infected aphid feeds with its stylet for about 2.5 min, PLRV is transmitted (Leonard and Holbrook, 1978) into the phloem tissue of the plant. The length of aphid feeding increases transmission efficiency (Wale *et al.*, 2008).

Most plant viruses have high specificity for the insects that transmit them (Maramorosch, 1963; Katis *et al.*, 2007; Bragard *et al.*, 2013; Hull, 2013). *M. persicae* is the most efficient vector of PLRV (Radcliffe, 1982; Spooner *et al.*, 2005). *M. persicae* rarely causes direct feeding damage (Marsh, Huffaker and Long, 2000). It hops or makes short flights inside the field (Harrewijn, 1986) and by favouring middle or lower leaves it infects each plant as it feeds (Taylor, 1955). Aphid density is therefore a reliable indicator of the risk of PLRV infection across a region. Entomologists have expressed aphid density as vector pressure. Vector pressure is defined as the sum of all of the species of aphids capable of transmitting PLRV as a percentage of the total aphid population. Aphid probing, inserting its mouthparts into the plant tissue, is needed because plant viruses are unable to penetrate the plant cell walls unassisted (Carbonell *et al.*, 2016). The amount of PLRV accumulation is always lower than that of PVY as PLRV is confined to the phloem tissue and therefore aphids require a longer

time to acquire PLRV (Ragsdale *et al.*, 2001). Each progeny aphid has to attain the virus by feeding on an infected plant, because PLRV cannot be passed though the egg (Johnson and Pappu, 2006).

Researchers demonstrated that *M. persicae* transmits PLRV more efficiently from solanaceous weed to potato than from potato to potato (Alvarez and Srinivasan 2005; Srinivasan *et al.* 2008; Srinivasan and Alvarez 2008). Interestingly, *M. persicae* performance such as growth rate, reproduction and longevity is actually improved when they multiply on PLRV-infected plants as compared to virus free plants, PVY-infected plants and PVX-infected plants (Castle and Berger 1993). *M. persicae* settles first on a PLRV-infected plant and then travels to uninfected (Singh 2016), PVY-infected or PVX-infected plants (Castle and Mowry, 1998). PLRV-infected *M. persicae* showed improved performance, weight gain, higher longevity and fecundity than their PLRV free counterparts (De Oliveira *et al.*, 2016).

The common potato aphid (*Macrosiphum euphorbiae*) and eleven other species (Spooner *et al.*, 2005) such as the bird cherry-oat aphid (*Rhopalosiphum padi*) or the foxglove aphid (*Aulacorthum solani*) (Kennedy *et al.*, 1962; Syller, 1996) can also transmit PLRV to potato plants. *M. persicae* and *Macrosiphum euphorbiae* have a broad range of plants that overlap with hosts of PLRV (Hameed *et al.*, 2014). *M. persicae* has been extensively recorded from the Sandveld region in SA (Krüger *et al.*, 2014).

The virus is therefore carried over to the next generation of the potato host because infected seed tubers are vegetatively multiplied (Van der Want, 1972).

2.3.5 *The effect of temperature on PLRV infection and aphid transmission*

Temperature is an important abiotic factor that may potentially have an effect on vector-borne virus incidence; aphid population growth (Barlow, 1962; Bale *et al.*, 2002; Murray *et al.*, 2013; Chung *et al.*, 2016); and plant pathogen interactions impacting on virus transmission, establishment, replication, accumulation, translocation, susceptibility and symptom expression of the host plant (Jensen, 1973; Tamada and Harrison, 1981; Matthews, 1991; Suzuki *et al.*, 2014; Ashoub *et al.*, 2015). Higher temperatures may change disease and pest intensity (Harvell *et al.*, 2002; Scaap *et al.*, 2011) by reducing the survival rate of the potato and reducing the initial disease and pest inoculum population (Van der Waals *et al.*, 2016).

The effect of temperature on PLRV levels in the host has been documented extensively (Kassanis, 1949, 1950; Roland, 1952; Rozendaal, 1952; Thirumalachar, 1954; Hamid and Lockke, 1961; Fernow *et al.*, 1962; Upreti and Nagaich, 1968; Quak, 1972; Gomez and Corzo, 1977; Kaiser, 1980; Duriat, 1989; Syller, 1991; Hanafi *et al.*, 1995; Loebenstein, 2001).

Rek (1987) investigated PLRV infection of potato plants by infected *M. persicae* and the rate of PLRV accumulation after infection. These trials were performed in summer under field conditions with

diurnal temperature variations in 1986 at the Agroscope Research Station at Reckenholz in Switzerland. Temperature data for June, July and August averaged between 16.5˚C and 19˚C with daily maxima and minima of 25˚C and 11˚C respectively. Under these temperature conditions, he found that high levels of PLRV could be detected by ELISA two to three weeks after infection. Flanders *et al.* (1990) found that PLRV could be detected within 14 days after inoculation by aphids under similar temperate conditions in Rosemont, Minnesota, USA.

Syller (1991) studied the effect of temperature on PLRV infectivity and accumulation in potatoes under controlled temperature conditions in a glasshouse. He found that potato plants pre-incubated at 15˚C were more susceptible to PLRV infection than when kept at 27˚C. PLRV accumulation increased at temperature combinations of 27 (pre-incubated)/27˚C (kept at), 15/15˚C or 27/15˚C, but the 27/27˚C combination gave significantly higher infection levels than 15/15˚C, indicating that after infection at 15˚C far less virus accumulated than at 27˚C. This led to a general perception that higher temperatures were more conducive to PLRV infection and led to a faster accumulation of the virus in infected potato plants.

However, under constant, even higher temperature conditions, PLRV levels were found to decrease in tubers at 36°C of dry heat for 40 days (Duriat, 1989). Others also demonstrated that heat therapy, dependent on temperature and duration, rids potato tubers of PLRV (Kassanis, 1949) at 37˚C (Kassanis, 1950; Loebenstein, 2001) for three to four weeks (Kaiser, 1980), illustrating PLRV titre is reduced or eliminated at higher temperatures. Field experiments conducted in a research project in Morocco concluded that seed potatoes should be produced as the first batch following a high temperature summer season (above 40˚C with low rainfall) (Hanafi *et al.*, 1995) to ensure lower PLRV infection rates. Chung *et al.* (2016) studied PLRV accumulation in *Physalis floridana*. They found that the temperature optimum for PLRV infection and accumulation is between 20˚C and 25˚C with a reduction in viral accumulation at temperatures below 20˚C and above 25˚C. In contrast to most other viruses, PLRV is sensitive to higher temperatures and can even be eliminated by constant incubation at 37˚C for three to four weeks (Kaiser, 1980). BYDV, which belongs to the family Luteoviridae, shows the same high temperature sensitivity (D'Arcy and Domier, 2000).

Furthermore, higher temperatures above 30˚C decrease the reproduction of *M. persicae* (Barlow, 1962) and at 38.5˚C the aphid dies (Broadbent and Hollings, 1951; Davis *et al.*, 2006). Thus warmer regions with higher temperatures may diminish virus transmission rates by exceeding the optimal temperature for aphid vectors to acquire viruses, for example PLRV's transmission efficiency decreases at 26˚C (Jayasinghe and Salazar, 1998) or when *M. persicae* acquired the virus at 10˚C or 30˚C (Chung *et al.*, 2016).

However, others detected a higher transmission rate of PLRV at higher temperatures by *Alternaria gossypi* and *M. persicae* (Singh *et al.*, 1988) after virus acquisition at higher temperatures between

25˚C and 30˚C than lower temperatures of 15˚C to 22˚C (Webb, 1956; Tamada and Harrison, 1981; Syller, 1987). This higher transmission rate may be due to a greater amount of viral accumulation in *M. persicae* at 27˚C than 15˚C (Syller, 1994), although some found virus content decreased in aphids as temperatures rose from 15˚C to 30˚C (Tamada and Harrison, 1981).

Thus, high temperatures (>30˚C) delay PLRV accumulation in the plant host and reduce PLRV transmission by aphids. This is extremely important in the context of this study. As indicated in the introductory chapter average daily temperatures in the Sandveld region are 35˚C for five months of the year in summer during which daily maxima often reach 45˚C. Thus, temperatures in the Sandveld region are often as high as temperatures that have been documented to eliminate PLRV completely. Furthermore, it has been found that higher temperature regimes (20˚C min/30˚C max vs 15˚C min/25˚C max) led to lower reproduction rates of *Macrosiphum euphorbiae* in SA (Krüger *et al.*, 2014). This study did not include *M. persicae*, but Chung *et al.* (2016) have shown that at temperatures above 30˚C virus acquisition is significantly lowered. However, PLRV infectivity and transmission will be high during the cooler winter months in the Sandveld region when more temperate conditions prevail.

2.3.6 *RNA silencing or interference*

To limit invading pathogens, the plant host defence machinery recognises viral presence and induces defence mechanisms that include RNAi, stress-response protein accumulation (Lu *et al.*, 2012; Fang *et al.*, 2015) or degradation, immune receptor signalling and hormone-mediated defences (Incarbone and Dunoyer, 2013). RNAi is a cytoplasmic cell surveillance system and can be subdivided into three parts: sensing and processing of viral RNA into siRNAs; amplifying these virus-derived siRNAs and assembling antiviral RNA-induced silencing complex (RISC); and targeting viral RNA for degradation (Burgyán and Havelda, 2011) as shown in Figure 2.7. Firstly, it recognises viral double stranded RNAs (dsRNAs) produced by the amplification of ssRNA via RdRp (see 1 in Figure 2.7) or ssRNAs produced during the replication of a viral genome. RNases (such as DICER) cleave these viral RNAs into siRNAs (Kreuze *et al.*, 2009) (see 2 in Figure 2.7). These virus-derived siRNAs direct targeted RNA degradation by guiding an assembled RISC nuclease complex or ARGONAUTE proteins (see 3 in Figure 2.7) to silence and destroy the infected viral RNAs through cleavage of the double stranded viral RNA molecules into sRNA such as siRNAs, miRNA and piwi-interacting RNA (piRNA) (Ding and Lu, 2011). These siRNAs bind to ssRNA that cause translational inhibition (Waterhouse *et al.*, 1998; Waterhouse *et al.*, 2001; Kreuze *et al.*, 2009; Rogers and Chen, 2013; De Vries, 2016) (see 4 in Figure 2.7) and direct antiviral immunity by a systemic signal (see 5 in Figure 2.7).

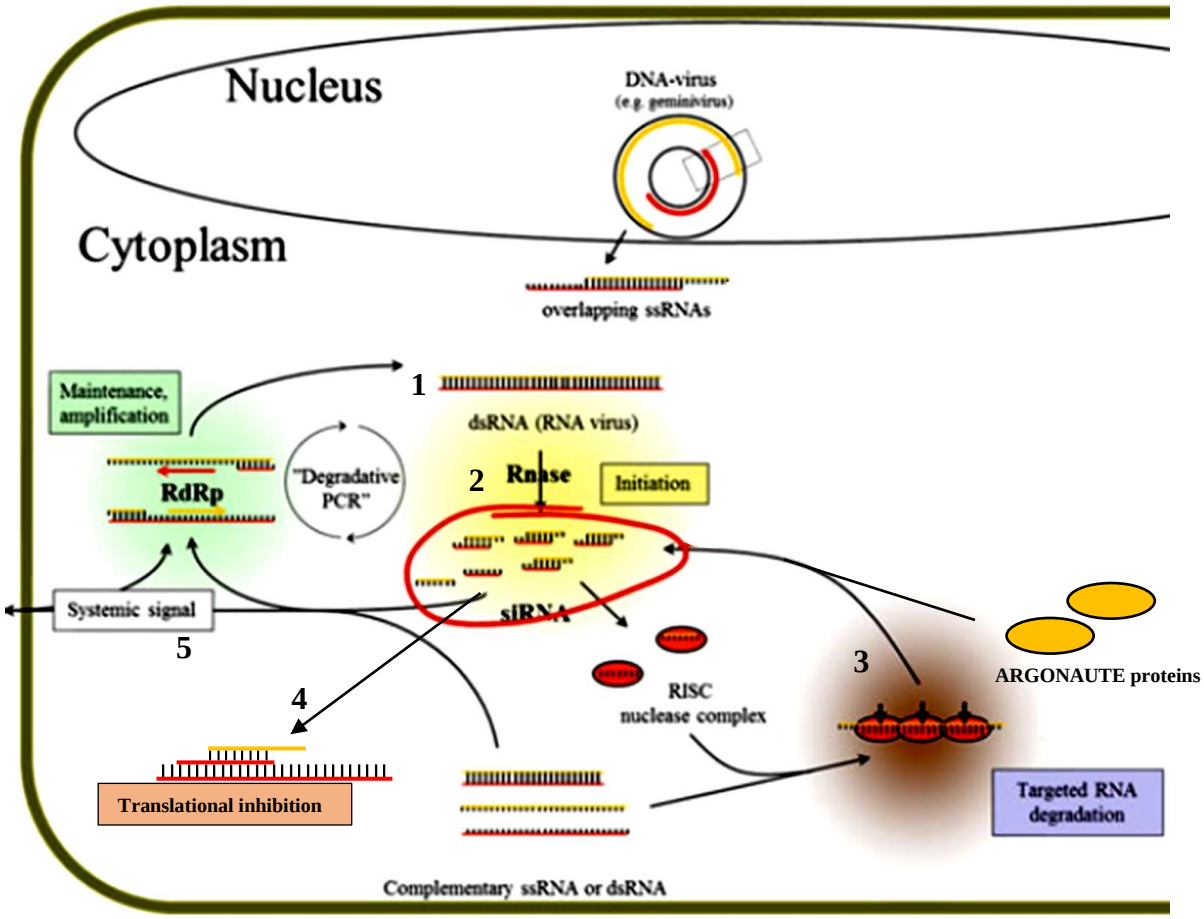

Figure 2.7: A diagram of RNAi, the viral surveillance system, which is activated to destroy invading pathogens (steps 1 to 5). This leads to virus-derived siRNA accumulation (Kreuze, 2014). Double stranded RNA (dsRNA); RNA-dependent RNA polymerase (RdRp); RNA-induced silencing complex (RISC); Single stranded RNA (ssRNA); Small interfering RNA (siRNA).

siRNAs are 21 to 24 nts in length and in uninfected cells, they are involved in heterochromatin modification, help maintain genome integrity by silencing transgenes and transposons to defend against viruses, and regulate gene expression (Chen, 2009). siRNAs occur at higher levels during virus infection (Yoo *et al.*, 2004). The profiles generated from total sRNA sequencing have been used to discover and characterise viruses in infected plant hosts (Donaire *et al.*, 2009) as deep-sequencing host sRNAs detected siRNAs (Donaire *et al.*, 2008; Kreuze *et al.*, 2009; Wu *et al.*, 2010; Hwang *et al.*, 2013; Candresse *et al.*, 2014). The first deep sequencing study revealed that these pathogen-specific siRNAs overlap (Aliyari *et al.*, 2008) and their accumulation represents about 30% of total sRNAs sequenced from diseased plants (Ding and Lu, 2011).

miRNAs are 21 nts in length and are endogenous, non-coding, ssRNAs. They regulate post-transcriptional gene expression by repressing or degrading the translocation of targeted messenger RNA (Lakhotia *et al.*, 2014; Zhang *et al.*, 2014). miRNAs may have key roles is stress responses and developmental processes, such as root development (Shukla *et al.*, 2008; Chen, 2009; Rogers and Chen, 2013; Lakhotia *et al.*, 2014). As deep sequencing has revolutionised sRNA discovery, it was

used for miRNA discovery and expression analysis in potatoes (Zhang *et al.*, 2013) during drought stress (Zhang *et al.*, 2014).

Researchers have reported that the temperature of virus transmission affects the efficiency of pathogen multiplication and establishment of host infection (Chu and Volety, 1997; Feil and Purcell, 2001). Some plants infected at higher temperatures showed less symptoms due to efficient RNAi-mediated plant defences, i.e. increased siRNAs levels (Szittya *et al.*, 2003; Chellappan *et al.*, 2005). A reduction of turnip mosaic virus (family *Potyviridae*, genus *Potyvirus*, species *Turnip Mosaic Virus*) CP accumulation from 13 to 18˚C or >33˚C (Chung *et al.*, 2015) may also be attributed to an enhanced RNAi. At low temperatures RNA-silencing-mediated plant defences seem to malfunction (Chellappan *et al.*, 2005). Viruses have evolved viral suppressors to subvert or hijack these host defence responses to benefit themselves, but inactivation of these suppressors can lead to plant recovery (Weiberg *et al.*, 2013; Calil and Fontes, 2016).

2.3.7 *The triangular relationship between plant, vector and virus*

Plant viruses have evolved to ensure transmission by connecting with the vector's biology and extensively use the host's intracellular machinery for replication of their genomes, expression of their viral genes and viral establishment (Calil and Fontes, 2016). This can be represented as a disease triangle which indicates the direct relationship between the plant, the virus and the vector. Some virus infections have been shown to alter the host in such a way that plant and vector interactions are influenced to enhance their viral transmission (Alvarez *et al.*, 2007; Mauck *et al.*, 2012). Other plant-virus interactions trigger RNAi after penetrating the cell wall.

The rate and extent of virus spread are dependent on vector activity and behaviour (Jeger *et al.*, 1998, 2004). Viruses including PLRV (Eigenbrode *et al.*, 2002) increase the "attractiveness" of infected plants to vectors by modifying the plant's olfactory signals (Alvarez *et al.*, 2007; Mauck *et al.*, 2014) and changing the nutritional quality of the plant (Castle and Berger, 1993; Mauck *et al.*, 2014) to influence aphid feeding behaviours (Tamada and Harrison, 1980a; Alvarez *et al.*, 2007; Boquel *et al.*, 2011b). Aphids have developed certain intricate relationships with specific host plants, feeding from phloem sieve elements while invading and potentially manipulating host plant defences (Jiang and Miles, 1993). Temperature changes influence plant quality, phenology, defensive compounds and morphology impacting aphid feeding habits (Kaur *et al.*, 2016). Our understanding of vector-virus interactions is needed to design novel genetic strategies to manage the vectors and the viruses they transmit (Kaur *et al.*, 2016). Thus, studies are needed to compare aphid, virus incidence and host plant responses for management strategies to protect food sources against damaging aphids and virus spread.

2.3.8 *Management of PLRV infections*

In general, and taking into account the above mentioned triangular relationship, designing an effective virus management strategy includes: understanding the disease cycle of etiological agents, molecular

nature, viral genome sequences and structure, regular inspection for healthy seed, correct identification of the problem, agricultural and crop practices, biological control and, if necessary, soil fumigation. However, even with improved pest and disease management strategies, globally plant pathogens still destroy between 10% and 16% of crop production (Chakraborty and Newton, 2011). Applied to PLRV management, this entails the control of vectors by regular systemic insecticide spraying, the planting of PLRV resistant potato cultivars and the planting of virus free certified seed that entails testing the tubers beforehand with an accurate, rapid, highly sensitive and specific detection method (Salazar, 1994).

2.3.8.1 Insecticides applied for vector control

As a first management strategy for PLRV control the spraying of rapid-acting systemic insecticides in the field should be done regularly to ensure that aphid-transmitted viruses such as PLRV, with short acquisition times, are wiped out (Oosterveld, 1987). These applications of insecticides for aphid transmitted PLRV are dependent on aphid biology, arrival in season and age of the plant (Ragsdale *et al.*, 2001). However, insecticides such as Confidor (Milosevic *et al.*, 2012), Imidacloprid (Boiteau and Singh, 1999), Thiamethoxam (Mowry, 2005), Carbamate, Aldicarb (Ragsdale *et al.*, 2001) and Acetamiprid (Javed *et al.*, 2016) have been found to control aphids effectively.

Host plant resistance can be used for aphid control together with techniques such as intercropping, antixenosis (non-preference of plant to insects), antibiosis (effect on insect growth, reproduction and survival) (Saljoqi *et al.*, 2003) or biological control involving the use of natural enemies, such as parasitic wasps or ladybeetles. The application of mineral oils can be used to manage or control aphid borne virus disease such as PVY, because the oils interfere with virus retention in aphid mouth parts (Robert *et al.*, 2000). However, others demonstrated that horticultural mineral oils altered aphid feeding behaviour (Simons and Zitter, 1980; Ameline *et al.*, 2009) and transmission of plum pox virus (PPV) (family *Potyviridae*, genus *Potyvirus*, species *Plum pox virus*) by *M. persicae*, but only for one week (Samara *et al.*, 2016). The application of these mineral oils in SA causes potato leaves to burn when exposed to the intense sunlight and higher temperature conditions often present in SA (Pieterse, PSA, pers.comm.).

Control of PLRV transmission by aphids, can very importantly also be controlled by elimination of the source of infection which is very often infected potato plants. Potato tubers that have been missed during the harvest of the previous season's crop and are infected, are often such sources of infection. A simple way to therefore eliminate this source of infection is to physically remove these plants and to destroy them. This is commonly practiced in seed tuber production and is referred to as rogueing. Plant haulm destruction can also be effective but requires knowledge about aphid flights, initial virus pressure, intensity of rogueing, mature plant resistance and cultivar's susceptibility to potato viruses (Van Harten, 1983). However, it is used extensively in SA, i.e. haulms are destroyed when plants are

almost mature late in the planting season using herbicides to stop late infections caused by aphid transmission.

Aphids may still feed on a healthy plant before they die and transmit the virus (Till, 1971; Woodford *et al.*, 1994). Thus, even though insecticides decrease aphid numbers, they are not effective enough to eliminate PLRV incidence (Hanafi *et al.*, 1995) and therefore insecticide application only plays a complementary role in PLRV management (Alani *et al.*, 2002). The cultivation of resistant cultivars appears to be a more suitable strategy, as it will also result in a reduction of insecticide spraying to circumvent aphids developing resistance.

2.3.8.2 Planting PLRV resistant cultivars

A second management strategy to control PLRV infection is the cultivation of resistant potato cultivars (Beekman, 1987). The breeding of potato cultivars containing resistance genes from wild species such as *S. acaule*, *S. brevidens* (Jones, 1979), *S. etuberosum*, *S. chacoense*, *S. demissum, S. raphanifolium* (Barker and Waterhouse, 1999) or complex hybrids, is a struggle, but some have shown noteworthy resistance to PLRV replication and accumulation (Barker and Solomon, 1990; Barker and Woodford, 1992). *S. brevidens'* resistance is likely to be associated with restricting virus spread from cell-to-cell (Valkonen *et al.*, 1991). The cultivation of PLRV resistant potato cultivars is affected by difficulties such as polygenic inheritance, non-commercial characteristics, ploidy levels (Ruiz de Galarreta *et al.*, 2015) and introgressing the monogenic resistance from wild solanum (Taliansky *et al.*, 2003).

Genetically modified (GM) cross protection, the expression of mild strains in potatoes or satellite RNAs (Fitchen and Beachy, 1993) against severe strains may be a strategy to manage PLRV (Harrison, 1958; Beachy *et al.*, 1990; Nelson *et al.*, 1990; Scholthof *et al.*, 1993). Pathogen mediated resistance, the expression of a viral gene sequence in the plant, may protect potatoes against viral infection. These gene sequences typically encode a viral CP, also named CP mediated resistance. In 1990, Kawchuk *et al.* reported GM resistance to PLRV after transforming the PLRV CP gene into the potato (Kawchuk *et al.*, 1990). Others illustrated the effectiveness of host gene-mediated and transgene-mediated resistance plants with a PLRV CP gene (Barker and Harrison, 1985; Kawchuk *et al.*, 1990; Van der Wilk *et al.*, 1991; Barker *et al.*, 1992; Brown *et al.*, 1995). When the CP is expressed it interferes with the virion's disassembly, multiplication, expression and spread of infected virus (Gupta, 2016). A study demonstrated that with the use of a CP gene, immunity or resistance can be generated with RNAi against PVS, PVX and PVY (Hameed *et al.*, 2017). Host viral protection can occur if one would induce an increase of siRNA production which directs sequence specific degradation of viral gRNA and sgRNA (Waterhouse *et al.*, 1998; Waterhouse *et al.*, 2001). Replicase protein mediated resistance occurs when a viral gene sequence that encodes a related replicase protein is expressed by the host. Other pathogen mediated resistance strategies include, expression of the MP, the polymerase and the ribozyme genes (Ruth and Barker, 2001). Even though results may be promising, they are not satisfactory for use (Syller, 1996). Transgenic expression of PLRV's CP has

demonstrated that it can encapsulate and promote the transmission of potato spindle tuber viroid (PSTVd) (family *Pospiviroidae*, genus *Pospiviroid*, species *Potato spindle tuber viroid*) by *M. persicae in vivo* (Querci *et al.*, 1996; Syller and Marczewski, 1996). Thus this approach can also have unexpected negative consequences.

Monsanto laboratories genetically engineered a potato to be resistant to PLRV in 1997 (Taliansky *et al.*, 2003), but this all came to a halt in 1999 when anti-GM companies campaigned against transgenic potatoes, ceasing all research and advancing technologies (Kaniewski and Thomas, 2004). A virus-resistant potato "NewLeaf Plus" cultivar containing a transgene with approximately 90% of the P0 PLRV gene, was commercially produced (Lawson *et al.*, 2001). J.R. Simplot's company learned from previous market difficulties and developed the "Innate" cultivar with reduced bruising of potatoes and lacking any foreign DNA (*Innate™ potato receives FDA safety clearance*, 2015). From 2016, the Innate cultivar has been sold on the US and Canadian market (Pacifico and Paris, 2016). Transgenic PLRV resistant potatoes will be more beneficial for third world countries, without healthy seed tubers and affordable insecticides, than first world countries that receive certified virus free seed annually (Valkonen *et al.*, 2015). GM potato varieties, with abiotic and biotic resistance, will require fewer inputs, produce higher yields, improve food security and contribute to economic development (Mansoor *et al.*, 2006; Qu *et al.*, 2007; Carstens and Berger, 2016). However, public perception of GM will have to change before such potatoes will become acceptable. Thus, in general, to counteract the effect of PLRV infection, cultivars with quicker growth and tuber formation, lower nitrogen requirements and higher resistance against disease are desired for planting. Currently in Europe the selection of cultivars exhibiting natural higher PLRV resistance is still thought to be the best strategy to counteract PLRV yield losses.

2.3.8.3 Production of virus free seed

The third management strategy is to only plant virus free healthy seed. To obtain virus free certified seed, early rogueing of infected plants and elimination of volunteer plants and weed hosts are necessary (Abbas *et al.*, 2016), because there is no effective viricide to control viruses in potatoes (Almasi, 2012). Techniques such as tissue culture, specific growing strategies and micropropagation have been used to protect potato plants against viruses. Chemotherapy is used to obtain virus free biological materials by supressing virus replication, genome expression, cell-to-cell movement or even long distance movement in some plant viruses (Griffiths *et al.*, 1990; Faccioli, 2001). Stem-cutting culture, chemotherapy and thermotherapy have been used to eradicate PVX and PVS from meristem tips (Faccioli and Zoffoli, 1998) but it was less successful to obtain healthy material from PLRV-infected and PVY-infected stem cuttings (Faccioli and Colalongo, 2002). Others demonstrated that PVY, PVX, PVA and PVS cannot be completely eradicated from tubers by heat treatment and listed PLRV as the only virus that could be inactivated by thermotherapy from potato tubers by using hot air treatments at 37˚C for 27 days (Kassanis, 1950; Kaiser, 1980; Loebenstein, 2001). Studies in 2016

reinforced this discovery, demonstrating that tubers treated at an average temperature of 37˚C in hot water for 2.5 hours, fully or partially eliminated PLRV successfully (Abbas *et al.*, 2016). Electrotherapy at 20 mA/20 min resulted in 42.84% PLRV free sprouts and 46.72% PLRV free shoot tips (Singh and Kaur 2016). Ribavirin or Virazole is a known antiviral agent that is highly effective against DNA and RNA viral infections by hindering the duplication of viral hereditary material (Meier *et al.*, 2003). It has been used against PVX, PVY and PVS (Cassells and Long 1982; Klein and Livingstone 1983), but not for elimination of PLRV yet.

The most effective method to ensure virus free healthy potato tuber seed is however to test for PLRV infection using a highly sensitive and reliable detection method. As this forms a major part of this thesis these methods will be outlined in detail in the following section.

2.4 Methods for the detection of viruses in potatoes

2.4.1 *Symptomology*

The most basic method for virus detection of plants is field-based inspection for viral symptoms. When a plant shows these symptoms, for example the rolling of leaves or stunted growth, they are rogued out. However, the host plant, weather and virus isolate all influence symptom expression which makes it difficult to detect all infected plants, especially when the plants have a low concentration of viral copies (Wangai and Lelgut, 2001) or the virus does not induce visual symptoms. For example, infection of the plant late in the season and with a very mild strain of PLRV with no typical symptoms, such as the SymlessLS10 isolate (Hühnlein *et al.*, 2016) may result in an underestimate of PLRV infection using this method. Thus, faster and more precise methods are required to detect viruses in order to control for them in potato seed.

In the past, grow out tests were used to assess seed potato tuber PLRV infection levels. Sprouts of tuber lots were grown out in glass houses after PLRV infection levels were determined by symptomology. However, grow out tests were insensitive for the reasons mentioned above.

2.4.2 *Enzyme-linked immunosorbent assay*

In 1977, the first ELISA for the detection of the plant viruses, PPV and arabis mosaic virus (order *Picornavirales*, family *Secoviridae*, genus *Nepovirus*, species *Arabis mosaic virus*) was published by Clark and Adams (1977). ELISA simplified detection and results were obtained much faster (Torrance and Jones, 1981) than the traditional methods which entailed grow out tests in glasshouses. ELISA developed as a major tool for areas such as breeding, quarantine and certification (e.g. confirming plant tissues are virus free) and seed production schemes use it routinely on large-scale for potato indexing (Wangai and Lelgut, 2001). As a common method for virus detection, ELISA was adopted for routine testing in seed certification schemes (Spiegel and Martin, 1993). At present, ELISA is still established as the most commonly used method and applied as an international agriculture technique

for virus detection in crops. Advantages such as speed, lower potential for cross-contamination, simple implementation, procedure and analysis of results, makes ELISA an excellent tool for high-throughput testing (Boonham *et al.*, 2014). Although ELISA has these advantages and its cost effectiveness, robustness and easiness to use (Boonham *et al.*, 2014), it has some shortcomings that restrict its use for universal and relevant diagnosis of plant viruses, especially for the detection of a viroid (Grothaus *et al.*, 2006). A viroid does not possess a CP and only consists of naked dsRNA as a result of which it is very difficult, if not impossible, to raise antibodies against viroids. The assay's specificity and sensitivity is dependent on high-quality antisera and most antisera only detect the virus CP (Wangai and Lelgut, 2001). As a diagnostic tool, especially to identify different pathogens in one sample, ELISA lacks elasticity and compatibility. Another major disadvantage is that ELISA has problems in detecting viruses at low concentrations (De Bokx and Beemster, 1987), especially in dormant tubers (Spiegel and Martin, 1993; Huttinga, 1996; Mumford *et al.*, 2004). The sensitivity of the ELISA technique is estimated to be in the order of 5 ng/ml of PLRV, i.e. cut-off values therefore represent 0.5 ng in 100 µl viral particles or more (Kojima *et al.*, 1980). ELISA tuber index tests require tuber sprouting and leaf samples, which prolongs testing time to about three months (Mumford *et al.*, 2004). Sprouting causes the rapid development of phloem tissue (Weidemann and Casper, 1982) that leads to an increased virus concentration, including PLRV (Tamada and Harrison, 1980a).

After suitable antibodies against PLRV became available (Murayama and Kojima, 1974; Maat and De Bokx, 1978; Gugerli, 1979; Rowhani and Stace-Smith, 1979; Tamada and Harrison, 1980b), ELISA was used as a quantitative assay to detect PLRV in leaves, sprouts and aphids (Casper, 1977; Gugerli, 1979; Clarke *et al.*, 1980; Kojima *et al.*, 1980; Tamada and Harrison, 1980b, 1981; Clarke, 1981). Even though immunosorbent electron microscopy (ISEM) identified PLRV in potato tubers, leaves and single aphids (Roberts *et al.*, 1980), the assay has low throughput capabilities, and therefore could not be applied as a routine detection method.

Harrison (1984) associated BYDV, BWYV, beet mild yellowing virus (family *Luteoviridae*, genus *Polerovirus*, species *Beet mild yellowing virus*), pepper vein yellows virus (family *Luteoviridae*, genus *Polerovirus*, species *Pepper vein yellows virus*) and tobacco necrotic dwarf virus (family *Luteoviridae*, species *Tobacco necrotic dwarf virus*) serologically with PLRV. Common epitopes were found between PLRV, BWYV and BYDV (Ohshima *et al.*, 1988). Based on antibody cross-reactivity some hypothesised that PLRV was more closely related to BWYV than BYDV (Van der Wilk *et al.*, 1989). Even though common epitopes are shared between these viruses this does not pose a problem for PLRV detection in potatoes as BWYV, BYDV and tobacco necrotic dwarf virus do not occur in potatoes. Although Massalki and Harrison (1987) developed monoclonal antibodies against a British PLRV isolate in 1987 to overcome this problem for the abovementioned reason, polyclonal antibodies can be used equally well. The most common ELISA format which is used for PLRV detection is the double-antibody sandwich ELISA (DAS-ELISA) which works through capture of PLRV particles on ELISA-plate-immobilised-antibodies followed by the binding of an antivirus antibody enzyme

conjugate, and detection with an appropriate substrate that produces a coloured product indicating that the virus is present (Wangai and Lelgut, 2001).

Rek (1987) conducted studies in Switzerland on the detectability and reliability of DAS-ELISA testing of tubers for PLRV infection. His study showed that late primary infection resulted in less reliable tuber detection, whereas secondary infection was easily detected by ELISA. Others also demonstrated difficulty with ELISA for PLRV detection from late infected leaves (Tamada and Harrison, 1980b; Ehlers *et al.*, 1983). ELISA reliability was also questioned when plants were infected late in the season (primary) and not all tubers were infected. Rek (1987) concluded that late primary infections did not lead to clear symptoms at harvest, even though tubers were infected and that it was problematic to use results obtained from leaves as a reliable source for viral prediction in tubers. ELISA was found not to be reliable enough for routine PLRV detection in untreated tubers (Rek, 1987). He found that Rindite (or gibberellic acid) treatment of tubers in Switzerland gave 100% reliable PLRV detection in the sprouts after four weeks of treatment. Natural sprouting was found to give less reliable ELISA results and the rose end presented to be more reliable. ELISA absorbance values of untreated tubers in Switzerland ranged between 0.4 and 0.8, well above their ELISA cut-off value of 0.16. Consequently, Switzerland's test was found to be 100% reliable.

Currently, the South African Seed Potato Certification Scheme uses the same DAS-ELISA as a diagnostic tool to test potatoes for the PLRV infection as this ELISA was commercialised and is now produced by BIOREBA AG in Switzerland. However, routine use of this ELISA in SA has shown that the absorbance values of positives are often well below the values of 0.4 to 0.8 obtained in Switzerland. In an effort to increase the sensitivity and reliability of PLRV detection in SA the cut-off value was reduced from 0.16 to 0.1. Low ELISA values in SA versus Switzerland may be coupled to different climates, specifically higher summer temperatures during the growing season (as outlined in 2.3.5). Roos (unpublished) found that ELISA testing of Sandveld potatoes showed repeatedly higher infection levels in naturally sprouting potatoes ("na-oes kontrole" or post-harvest-control sample) than samples tested at harvest ("land monster" or tubers-at-harvest sample) causing frequent downgrading of seed potatoes (as outlined in the introductory chapter). Low absorbance values indicate low PLRV levels, which in all likelihood may be the result of the extremely high temperatures that prevail for the summer months in the Sandveld region (as outlined in the introductory chapter). This indicates the need for a more sensitive detection method for PLRV than ELISA, such as a PCR-based method.

2.4.3 *Polymerase chain reaction*

2.4.3.1 Introduction

PCR is an essential modern-day method for quantifying viral, bacterial or fungal titres in samples and the quantitative analysis of gene expression. This methodology has been applied extensively in this thesis and thus a more comprehensive literature overview of PCR will be given.

2.4.3.2 The history of PCR

After major advances in viral diagnostics using ELISA in the 1980's, the development of new methods for viral RNA and DNA detection continued. PCR drew the most attention for molecular applications due to its increased sensitivity as well as use in cloning, site directed mutagenesis and sequencing (Boonham *et al.*, 2014). It was developed after a breakthrough made by Kary Mullis in the 1980's at Cetus Corporation in California (Bartlett and Stirling, 2003), which led to the PCR method applied currently. In 1993 he was awarded the Nobel Prize for chemistry for this method, but further development of PCR was completed by his colleagues, primarily Henry Erlich (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Mullis and Faloona, 1987; Bartlett and Stirling, 2003).

The novel concept of PCR was a merger of several components that were already in existence at the time: synthesis of short lengths of single stranded DNA (ssDNA) and the use of these to direct the target-specific synthesis of new DNA copies using DNA polymerases. Mullis' idea consisted of using the juxtaposition of two oligonucleotides, complementary to opposite strands of DNA, to specifically amplify the region between them and achieve this in a repetitive manner so that the product of one round of polymerase activity was added to the pool of template for the next round, hence the chain reaction. Others at Cetus such as David Gelfand and colleagues, discovered that the bacterium *Thermophilus aquaticus* (Taq) tolerated very high temperatures. It also produced a DNA polymerase that did not inactivate quickly at high temperatures, as the previously isolated DNA polymerase from *Escherichia coli* did. So in 1985, they successfully purified and cloned a Taq DNA polymerase, that allowed for a complete PCR amplification of a gene without repetitive DNA polymerase addition (Saiki *et al.*, 1988; Lawyer *et al.*, 1993; Bartlett and Stirling, 2003) which made it possible to amplify a specific region of a template sequence. Factors such as template DNA or cDNA, primers, dNTPs, a polymerase enzyme and magnesium ion concentration influence the success of PCR (Saiki *et al.*, 1985). In 1990, Vunsh *et al*. (1990) published the first PCR method for virus detection, namely for detection of bean yellow mosaic virus (family *Potyviridae*, genus *Potyvirus*, species *Bean yellow mosaic virus*).

2.4.3.3 Modern PCR

Modern PCR detects small amounts of DNA in a sample via three steps: denaturation, annealing and extension. Denaturation is normally induced at 94˚C and distorts the hydrogen bonds between complementary DNA strands, making single stranded molecules. The temperature is then lowered to an efficient temperature (routinely between 45˚C and 60˚C) for the forward and reverse primers to anneal to their complementary sequences. Lastly, the extension of new strands is induced at 72˚C, which is the optimal temperature of Taq DNA polymerase. During extension, Mg^{2+} binds to deoxynucleotides (dNTPs), oligonucleotides and negatively charged ions of the DNA template buffer. The DNA polymerase enzyme requires these divalent cations for maintenance of the correct conformation of DNA and dNTPs and hence activity (Cheng *et al.*, 1995) and it adds dNTPs onto a

pre-existing 3'-OH group to form complementary double stranded DNA (dsDNA) or single stranded cDNA (Sambrook *et al.*, 1989). After each cycle of denaturation, annealing and extension, the sequence of interest will be increased by two fold (Cooper and Hausman, 2007). A small amplicon or amplified target sequence size increases PCR sensitivity (Singh 1998). Amplified DNA is detected by pre-staining an agarose or polyacrylamide gel traditionally with ethidium bromide or currently more often Gel Red Nucleic stain (1:10000) (Biotium) before electrophoresis or post-staining the polyacrylamide or agarose gel with ethidium bromide or silver nitrate after electrophoresis or via hybridisation with labelled probes (Wangai and Lelgut, 2001).

PCR is a rapid, versatile and highly sensitive method for virus detection (Kumar *et al.*, 2010; Omrani *et al.*, 2009). It ensures the safety of regulated products, foods taken up by humans or animals and the fight against bioterrorism (MacCuspie *et al.*, 2006). Although many PCR assays have been published for plant virus detection, diagnostic laboratories only use a small number of PCR techniques routinely because PCR has such high levels of sensitivity that even small amounts of environmental DNA can be detected if tubes are opened after thermal cycling (Boonham *et al.*, 2014). The problem is known as post-PCR contamination and it leads to false positive results. PCR demonstrates its ever growing role in pathogen diagnostics, due to its accurate detection and sensitivity of as few as 10 DNA or cDNA copies (Ryazantsev and Zavriev, 2009).

2.4.4 *Reverse transcriptase polymerase chain reaction*

RT-PCR is the production of single stranded cDNA of RNA through the enzyme, reverse transcriptase (RT) (Fuchs *et al.*, 1999) followed by a standard PCR amplification. It is 1000 to 10 000 fold more sensitive and can detect lower levels of virus infection than ELISA (Hossain *et al.*, 2013). RT-PCR properties include: detection of small amounts of viral RNA, high specificity for virus detection due to specific primers for target sequence and highly sensitive detection of as little as between >20 and <100 RNA copies (Hayward *et al.*, 1998; Bao *et al.*, 2008). In the case of potatoes, testing can also be performed immediately after harvest. RT-PCR was thus demonstrated to be a reliable and efficient method for the detection of potato viruses (Singh and Nie 2003). It explicitly provides narrow (specific virus) and broad (general viruses e.g. potyviruses) specificity for RNA virus detection (Singh, 1998; Singh and Singh, 1998; Klerks *et al.*, 2001). Viral RNA, such as PLRV which is a ssRNA virus, is reverse transcribed from ssRNA+ by the RT enzyme to synthesise cDNA (Ali *et al.*, 2014). RT-PCR has been used for rapid, sensitive and specific molecular detection of PLRV in dormant potato tubers or leaves (Hadidi *et al.*, 1993; Spiegel and Martin, 1993; Singh *et al.*, 1995) and viruliferous aphids (Singh and Singh, 1996; Singh *et al.*, 2000; Klerks *et al.*, 2001; Awan *et al.*, 2010). It is a gold standard for high sensitivity, reproducibility, ease of use and detecting low abundance pathogens (Hockman *et al.*, 2017). A PLRV RT-PCR assay can be a useful tool for epidemiological studies and certification schemes to detect PLRV early in potato crops (Hossain *et al.*, 2013), but analysis via agarose gel electrophoresis is time consuming and less accurate.

2.4.5 *Quantitative real-time polymerase chain reaction*

Originally qPCR was designed for quantification in gene expression studies, for greater sensitivity of assays for virus detection or if antibodies could not be developed. qPCR has demonstrated high reproducibility and less variability compared to culture based methods (Dong *et al.*, 2016). The qPCR assay is implemented for diagnostic and virus detection amplifications since it shows the correlation between the time of amplification and the inverse logarithm of the amount of target DNA (Mumford *et al.*, 2000; Boonham *et al.*, 2014). Traditional PCR requires agarose gel electrophoresis for the detection of PCR products (Bao *et al.*, 2008), but in qPCR, amplification product formation is measured by fluorescence. It has increased sensitivity levels and measures product formation in real time. The rapid detection of a fluorescent signal in the closed tube occurs during amplification (real-time) or at the end of it (end-point) (Bao *et al.*, 2008), removing any risk of pre-PCR contamination from post-PCR products (Boonham *et al.*, 2014). Low virus concentrations, uneven virus distribution within a plant, varied virus concentrations over time and plant inhibitors such as phenols, tannins and complex polysaccharides influence reliable detection of plant viruses, but qPCR overcomes some of these challenges. It reduces cost of virus detection and is suitable for high-throughput testing (Agindotan *et al.*, 2007). qPCR methods triumph ELISA based on performance criteria requirements and three key practical reasons. Firstly, the development of antibodies for new viruses requires more time than establishing a qPCR method. Secondly, qPCR is generally more applicable than ELISA and lastly, even though qPCR per-test cost is more than ELISA, the development of antibodies adds additional costs (Boonham *et al.*, 2014).

qPCR sensitivity is expressed as quantification cycle (Cq) or cycle threshold (Ct) values. These values are defined as the number of cycles required for the fluorescent signal to cross the threshold to exceed background noise (Figure 2.8) (Whitman, 2012) and ΔRn is the fluorescence at any given point minus the baseline fluorescence emission (Gibson *et al.*, 1996; Arya *et al.*, 2000). If no virus (no template DNA) is present no fluorescence will be detected, resulting in no signal or Cq value. Cq values are inversely proportional to the amount of target nucleic acid (Whitman, 2012) meaning a lower Cq value indicates a greater amount of target nucleic acid in the sample. Too high Cq values may represent environmental contamination. A negative result should be defined as having no Cq value at all, such as the negative or no template control (Whitman, 2012).

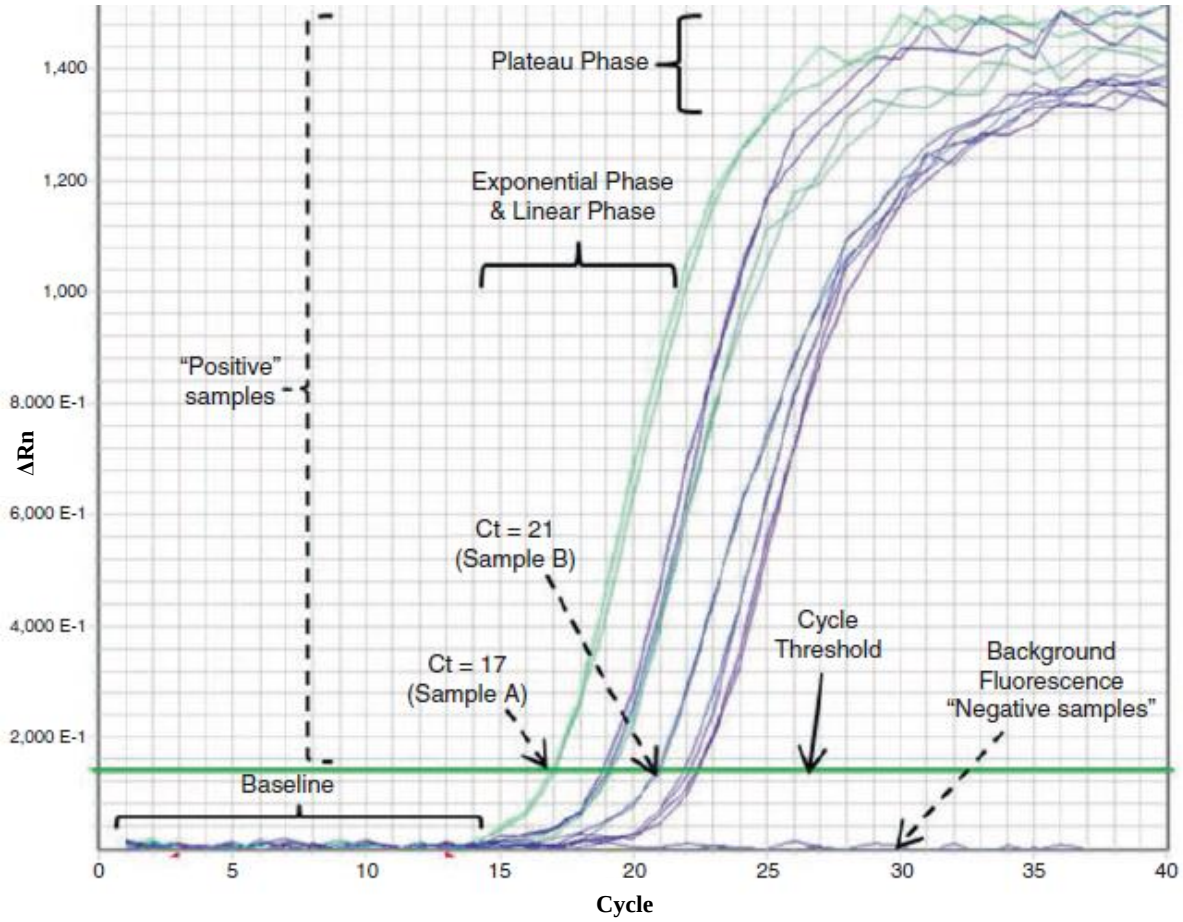

Figure 2.8: Example of a real-time amplification plot of several samples tested. Cycle threshold (Ct) values for two positive samples are given as example (Glais *et al.*, 2017).

The signal could either be detected by non-probe based or probe-based systems. Non-probe based systems use primers only and PCR product formation is indicated using dyes such as SYBR green which intercalate into the PCR products and thereby fluoresce. SYBR green is a dsDNA binding dye that distinguishes between different primer artefacts and sample amplification by analysing melting profiles (Ririe *et al.*, 1997; Boonham *et al.*, 2014). Advantages include the detection of template even though a mutation may have occurred in the target sequence and reduced cost (Papin *et al.*, 2004).

TaqMan® probes (probe-based system) were the first type of oligoprobe readily available and marketed for qPCR. These probes, otherwise known as hydrolysis probes, recognise a specific target and are applied due to the 5'-3' exonuclease activity of Taq DNA polymerase and the Fluorescent Resonance Energy Transfer (FRET) activity of sets of quenchers and reporters, known as fluorescent dyes (Boonham *et al.*, 2014). The probe sequence is complementary to the target sequence and consists of a 5' reporter or fluorophore, e.g. FAM, and a 3' fluorescent quencher, e.g. Black Hole Quencher (BHQ). During amplification the probe is cleaved by a Taq DNA polymerase during strand elongation, leaving the reporter no longer quenched and it fluoresces. The reporter molecule is excited by a specific wavelength of light and the emission spectra are detected. After each cycle of synthesis the fluorescence increases until a measurable signal is detected, which is expressed as a Cq value (Livak

et al., 1995). The fluorescent signal accumulates as the process proceeds and is proportional to the amount of product formed (James *et al.*, 2006). Effective probes are difficult to design and expensive, but specificity is high due to the fact that fluorescence is only detected when a primer hybridises completely to the complementary target sequence. A probe is less susceptible to non-specific amplification than non-probe based detection using SYBR green and increases sensitivity five to ten fold (Sharman *et al.*, 2000).

Quantification can be either be absolute or relative (Pfaffl, 2001). Absolute quantification transmits the PCR signal to input copy number using a calibration curve, whereas a relative change of, for example, mRNA expression levels is measured by relative quantification (Pfaffl, 2004). Successful qPCR relies on factors such as nucleic acid preparation including extraction, cDNA generation and quantity (from RNA), qPCR conditions, melt-curve analysis if it is utilised and well designed and optimised primers and probes (James *et al.*, 2006). Enzyme optimisation beforehand is also important as the RT step (RNA to cDNA) is a source of variability (Pfaffl, 2004).

A calibration or a standard curve is used in absolute quantification, but not in relative quantification. It is based on known concentrations of DNA standards. Serial dilutions from cDNA transcripts or recombinant plasmid DNA are designed to range in magnitude of $<10^1$ to $>10^{10}$ viral start molecules (Pfaffl, 2004). These dilutions correspond to copy numbers per reaction mixture and a standard curve is generated by linear regression, setting Cq values against cDNA concentrations. Cloned recombinant DNA is very stable and generates highly reducible results in comparison to newly amplified DNA, such as the DNA generated during the qPCR (Pfaffl, 2004).

The slope (m) of a standard curve is used to estimate the efficiency (E) of assay using the formula, $E = (10^{-1/m}-1) \times 100$. E is an important marker in real-time quantification (Liu and Saint, 2002). Equal amplification efficiencies are assumed between quantification standards and an unknown test sample when E is calculated from a standard curve (Livak and Schmittgen, 2001; Raymaekers *et al.*, 2009). A measurement of the closeness of a linear relationship between two variables is known as the correlation coefficient (R^2) and the closer R^2 is to 1, the closer the variables are to each other (Snedecor and Cochran, 1980; Raymaekers *et al.*, 2009). The limit of detection (LOD) is known as the lowest amount of analyte that is still detected by the assay in at least 95% of all reactions (Raymaekers *et al.*, 2009).

Even though qPCR is more expensive, increased sensitivity, specificity and speed (which translates into operator costs) (Coudray-Meunier *et al.*, 2016) compensates for the higher costs. qPCR allows for high-throughput testing at a fairly low cost per sample and swift setup of a new assay (Boonham *et al.*, 2014). qPCR has been readily established in testing laboratories worldwide, leading to competition between instrument manufacturers and reduced price of reagents, plastic ware and

equipment. qPCR is becoming the preferred method for plant virus detection compared to ELISA because PCR-based assays are reliable, fast and rarely fail (Boonham *et al.*, 2014).

2.4.6 *Quantitative real-time reverse transcriptase polymerase chain reaction*

With the incorporation of an RT enzyme, the qPCR method has been modified into an RT-qPCR method. RT-qPCR delivers highly reproducible results and can be applied for the detection of plant RNA viruses or quantitative measurements of RNA (Wang and Brown, 1999; Roberts *et al.*, 2000; Bester *et al.*, 2014). Even though it is generally not possible to use DNA as a standard for the absolute quantification of RNA as there is no control for the efficiency of the RT-step (*Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR*, 2003), Pfaffl and Hageleit (2001) mentioned that known concentrations of recombinant plasmid DNA can be used for absolute quantification of mRNA. An advantage of RT-qPCR over RT-PCR is that its real-time chemistries are fully automated and avoid the potential cross-contamination of samples during sample handling for post-PCR analysis (Bao *et al.*, 2008). The only disadvantage is the high cost of reagents and equipment, but these are decreasing as well.

RT-qPCR has been used to detected PLRV in dormant potato tubers (Leone *et al.*, 1998; Agindotan *et al.*, 2007; Mortimer-Jones *et al.*, 2009). An RT-qPCR method has also been developed in SA for the detection of PLRV in potato leaves and tubers (Espach, 2015).

2.4.7 *Multiplex polymerase chain reaction*

Multiplex is defined as a PCR wherein two or more loci are simultaneously amplified in the same tube (Henegariu *et al.*, 1997; Singh and Nie, 2003), reducing material costs and time. Studies have recorded the application of multiplex for various areas of DNA testing, including RT-PCR for the detection of the potato viruses, PVA, PVS, PVX, PVY, PLRV, potato virus M (order *Tymovirales*, family *Betaflexiviridae*, genus *Carlavirus*, species *Potato virus M*), potato aucuba mosaic virus (order *Tymovirales*, family *Alphaflexiviridae*, genus *Potexvirus*, species *Potato aucuba mosaic virus*) and PSTVd (Nie and Singh, 2000; Agindotan *et al.*, 2007; Kumar *et al.*, 2017; Zhang *et al.*, 2017). Simultaneous detection from aphids (Singh *et al.*, 1996; Venkateswarlu *et al.*, 2016) and potato tubers (Singh *et al.*, 2000; Klerks *et al.*, 2001) of PLRV and PVY via a duplex RT-PCR were developed. Multiplex qPCR uses fluorescent probes, such as the TaqMan probe, but this comes at a high cost. SYBR Green multiplex RT-PCR has limited multiplexing capabilities (Diawara *et al.*, 2016), meaning that no more than two targets can be simultaneously quantified because only one amplification plot is generated. In indexing of seed potatoes where the detection of multiple pathogens or viral infections is necessary, multiplex testing allows a single assay to detect more than one virus per sample and decreases the time required to obtain results. A multiplex RT-PCR could replace the routine testing by various ELISAs and it may therefore be a cost-effective assay for tuber indexing tests (Agindotan *et al.*, 2007; Mortimer-Jones *et al.*, 2009). However, due to plant viral co-infection and differences in

viral titre levels during multiplex PCR, however the underrepresented templates may not be detected, because of competition between individual amplifications (Ryazantsev and Zavriev, 2009). Other difficulties include the optimisation of primer concentrations in relation to viral template concentrations (Mumford *et al.*, 2000; Singh *et al.*, 2000).

2.4.8 *Other detection and future detection methods*

Immunostaining also highlighted PLRV restriction to the phloem tissue of potato tubers (Weidemann and Casper, 1982; Barker and Harrison, 1986), thus detection requires tuber sample to contain phloem. Staining tests for callus in tubers were also used (De Bokx, 1967; Ehlers *et al.*, 1983) and bioassays on indicator hosts proved pathogenicity (Sastry, 2013), but are too slow and labour intensive for routine testing. A multiarray on a test strip (MALTS) consisting of different viral antibodies was developed for detection of eight viral and bacterial potato pathogens, but is less sensitive than ELISA (Safenkova *et al.*, 2016).

Nucleic acid sequence-based amplification (NASBA) could be used for direct detection of PLRV in potato tubers (Leone *et al.*, 1997) by amplifying viral RNA at a temperature of 41˚C (Compton, 1991). Detection of PLRV and PVY in dormant tubers via a multiplex AmpliDet RNA, a combination of NASBA and molecular beacons or probes, was shown to be more reliable than ELISA (Klerks *et al.*, 2001). Northern blotting (Gildow *et al.*, 2000), Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) (Almasi, 2012), immunocapture (Schoen *et al.*, 1996; Almasi *et al.*, 2012) and a fluorogenic 5’ nuclease RT-PCR (Schoen *et al.*, 1996) have also been used for PLRV detection. New highly sensitive methods for viral diagnosis include biosensors, rolling circle amplification and deep sequencing (Sastry, 2013).

2.4.9 *Sample preparation for PCR, RT-PCR and RT-qPCR*

Generally, the ionic detergent hexadecyltrimethylammonium bromide (CTAB) (Doyle and Doyle, 1987) is used to release DNA from plant cells and dissociate proteins from DNA. A chloroform isoamyl alcohol mixture (24:1) is added to partition the contaminants into an organic phase, whereas the DNA is kept in the aqueous phase. Thereafter, the DNA is precipitated using cold isopropanol, washed in 70% ethanol and redissolved. The method often fails to isolate pure DNA which inhibits downstream amplification. Other procedures may take 24 hours before the DNA can be used and are expensive. RT-PCR requires minimal manipulation of the plant materials due to the lability of released RNA from macerated tissues. RNA extraction also requires inhibitors such as polysaccharides and polyphenols that co-precipitate with RNA, to be removed (Singh and Singh 1996). RNA quality has been shown to have an influence on RT-qPCR performance (Fleige and Pfaffl, 2006). However, procedures used during routine RNA isolation may decrease the RNA concentration of a sample potentially leading to false negatives in subsequent RT-PCR.

La Notte *et al.* (1997) developed a rapid and inexpensive method for direct amplification of viral RNA from plant tissue using two buffers. A grinding buffer releases the RNA from the plant tissue and during maceration in the grinding buffer the RNA is protected from oxidative and enzymatic damage. The other buffer, glycine-EDTA-TritonX-100 (GES, originally SDS now replaced with TritonX-100), leads to the release of proteins from RNA, allowing primers to bind. If this method is used no losses in viral RNA concentration occur, making subsequent RT-PCR and RT-qPCR detection more sensitive.

2.5 Metagenomics for virus identification

2.5.1 *Introduction*

Serological testing, PCR, nucleic acid hybridisation and microarrays are useful and very specific if there is prior knowledge about the pathogen, but these tests are not highly efficient to detect novel viruses that may be involved in the etiology of a particular disease (Coetzee *et al.*, 2010; Yanagisawa *et al.*, 2016; Zheng *et al.*, 2017). Traditionally, new viruses are identified and characterised using electron microscopy or biological indicator plants, but these approaches often fail to distinguish between unknown viral agents and RNA or circular DNA viruses (Balijja *et al.*, 2008). Diagnostic microarrays and mass spectrometry have also been used as generic tools to identify novel viruses (Mumford *et al.*, 2006; Boonham *et al.*, 2008).

The standard technique, Sanger sequencing, has been used to identify virus sequences since it was first described in 1977. This first generation sequencing platform has come a long way from only sequencing a few 100 nts to modern Sanger capillary sequencing systems that can determine the sequence of between 600 and 1 000 base pairs (bp) per run (Wu *et al.*, 2015). Even though it has a low error rate, it has an overall high cost per gigabase (Thomas *et al.*, 2012) and also requires prior sequence information of primer binding sites so it is unsuitable for identifying novel sequences of unknown viruses.

The direct genetic analysis of genomes within an environmental sample, is known as metagenomics (Thomas *et al.*, 2012). Metagenomics attempts to sequence the total nucleic acids including the sampled fragments of a whole genome in diseased samples without costly purification, cloning and screening techniques by using high-throughput NGS platforms for subsequent identification and diagnosis of viruses by bioinformatics tools (Kreuze *et al.*, 2009; Mokili *et al.*, 2012). NGS is a cost effective approach which generates high-throughput data (Marz *et al.*, 2014) and does not require prior knowledge about the diseased samples to identify both known and novel viruses. It can be used as an investigative tool to sequence multiple viruses (Cox-Foster *et al.*, 2007; Quan *et al.*, 2008; Wu *et al.*, 2015). NGS steps include: sampling, sample fractionation, DNA extraction, DNA sequencing, assembly, binning, annotation, statistical analysis, data storage, metadata and data sharing (Thomas *et al.*, 2012). In this process, the elimination of host nucleic acids (through purification) or host sequence

information (through software screening e.g. BLAST) is important as the pathogen's signal is enriched and this increases the probability of identifying low titre viruses. NGS has been used to evaluate viruses present in a grapevine (Al Rwahnih *et al.*, 2009), identify an unknown virus (Adams *et al.*, 2009; Coetzee *et al.*, 2010) and provide deep sequencing data of virus infected plants (Kreuze *et al.*, 2009; Lotos *et al.*, 2017).

Extraction protocols for NGS sequencing can include: total mRNA (Al Rwahnih *et al.*, 2009; Wylie and Jones, 2011), sRNAs including siRNAs (Kreuze *et al.*, 2009) and dsRNAs found in RNA virus infected material (Dodds *et al.*, 1984; Coetzee *et al.*, 2010). The most extensively utilised approach today is total sRNA sequencing (Seguin *et al.*, 2014; Wu *et al.*, 2015). The assembly of siRNAs can be used to identify both DNA and RNA viruses. However, dsRNAs can only be used to identify RNA viruses (Seguin *et al.*, 2014; Wu *et al.*, 2015). It is also less technically demanding to prepare samples for total sRNA sequencing than for dsRNA sequencing (Wu *et al.*, 2015). Hwang *et al.* (2013) reported the utility of total sRNA sequencing to detect structural features and add to the characterisation of virus regulation. It proved to be an efficient method for plant and animal viral detection by focussing on the natural antiviral defence mechanism, RNAi, which produces massive amounts of relatively short sequences, siRNAs. When a large number of copies of siRNAs are sequenced by NGS, it results in high sequence depth (how many times a nt is sequenced at a specific nt position) which is known as deep sequencing. Thus, virus discovery by deep sequencing has identified unknown viruses at an extremely low titre from the infected plant (Kreuze *et al.*, 2009). This approach uses sRNAs that are enriched from diseased cells or tissues to assemble large contiguous sequences, known as "contigs", from NGS sequencing (Wu *et al.*, 2015) with or without prior knowledge about the sequence information for novel virus discoveries. It could also be used to identify dominant variants of viruses and indicates the frequency of viruses found (Coetzee *et al.*, 2010).

However, even though siRNA sequencing is very sensitive in identifying viruses of different nucleic acid types and genome structures in low titres that are not readily detected by other methods (Kreuze, 2014; Wu *et al.*, 2015), assembly of full genome sequences or coverage (average number of reads that align to or map to or "cover" the known reference bases, as a percentage) of the viral genome may be difficult (Kreuze, 2014) as small endogenous plant RNAs may interfere with short 21 to 24 nts (Boonham *et al.*, 2014) siRNA sequences. Short read lengths produced by NGS platforms may also limit the ability to accurately characterise large repeat regions, leaving portions of the genome inaccurate (Snyder *et al.*, 2010). Sometimes classification of reads is not possible because a majority of sequences have no known reference genome (Edwards and Rohwer, 2005), but deep sequenced samples were *de novo* assembled or mapped to a reference genome for viral discovery (Kreuze *et al.*, 2009; Coetzee *et al.*, 2010; Hwang *et al.*, 2013; Maree *et al.*, 2015). These methods attempt to assemble the genomes of the major species in the sample, ignoring low-frequency variants and technical errors. Low coverage or sequencing depth is due to poor sequence similarity to reference sequences as not many reads may cover the same fragment of DNA (Thomas *et al.*, 2012) or the

genome, particularly for GC-rich regions and long homopolymer stretches (Ross *et al.*, 2013). Differences in coverage could possibly reflect relative abundance of the virus in the plant (Kreuze, 2014). siRNAs produced from RNAi target conserved sequences containing essential viral factors, e.g. structural proteins such as CP and regulatory proteins such as RdRp, for durable inhibition of virus replication (Lindbo and Dougherty, 1992; Lee *et al.*, 2002; Park *et al.*, 2002). Kreuze *et al.* (2009) indicated siRNA coverage was unevenly aligned over the viral genomes, with short regions of high coverage separated by regions with relatively low coverage. This may be due to the method of siRNA library preparation that introduce a bias towards certain sequences (Linsen *et al.*, 2009) or the need for pure, intact and high quality RNA that prevents low quality reads (Poong *et al.*, 2017), but the most likely reason is the uneven production of siRNAs by the plant host due to the need to inhibit the translation of specific viral ssRNA+. Uneven distribution of reads could potentially inhibit efficient *de novo* assembly of siRNAs into whole viral genome sequences (Kreuze, 2014).

2.5.2 *Next-generation sequencing platforms*

New sequencing technologies, with the ability to generate vast amounts of sequence data, are based on a shotgun sequencing approach. Smaller fragments, or shredded manageable DNA fragments that were sequenced in a massive parallel sequencing method, are overlapped with one another to generate contigs. Currently, the first generation of these NGS platforms have been superceeded by second and third generation sequencing platforms. The difference between the second and the third generation sequencing is that third generation sequencing determines the sequence from a single DNA strand and has no need for any DNA fragment amplification procedure or there is no halt between the reads (Schadt *et al.*, 2010). Thus it eliminates possible DNA amplification errors.

The 454-platform or pyrosequencing detects when an inorganic pyrophosphate molecule is released (a photon is emitted) upon the incorporation of one of the four nts. This is measured by means of a light reaction involving enzymes such as DNA polymerase, ATP sulfurylase, luciferase and apyrase and substrates adenosine 5'-phosphosulfate and luciferin (Droege and Hill, 2008). The incorporation of an nt via a DNA polymerase is directly proportional to the amount of pyrophosphate being released. ATP sulfurylase, luciferase and both substrates generate visible light during this incorporation that is captured, visualised and converted to the actual sequence of the template (Thomas *et al.*, 2012; Wu *et al.*, 2015). However, as of 2013, Roche Diagnostics discontinued this second generation NGS platform mainly for cost and sensitivity reasons (*Roche Shutting Down 454 Sequencing Business*, 2013).

The SOLiD platform is also known as ligation-based sequencing. In short it is based on a polymerase colony, the "polony" sequencing assay, meaning a single sheared DNA fragment attached to a magnetic bead is amplified on that bead by emulsion PCR. Sequencing occurs through various cycles of hybridisation and ligation with 16 different dinucleotide combinations to the beads attached to a glass surface (Metzker, 2010). The dye read-out from all the primers are converted to a DNA sequence (Wu *et al.*, 2015) and the NGS platform provides a low error rate (Thomas *et al.*, 2012). However,

expense and output data incompatibility have largely stopped the use of this second generation NGS platform.

Sequencing with Illumina instruments, a second generation NGS platform also known as sequencing-by-synthesis, involves the attachment of ssDNA molecules and primers to a slide where after amplification creates a cluster of original sequences in very close proximity to each other. Four dNTPs with different reversible dye terminators are used in the sequencing reaction. After the incorporation of each base the reaction is stopped to facilitate the read-out of the base with fluorescent dyes. The Illumina NGS platform was chosen by a majority of investigators for viral metagenomics studies in plants (Wu *et al.*, 2015) due to lower costs and successes in its application (Hess *et al.*, 2011). This approach determined the virome of a severely diseased vineyard with dsRNAs as starting material (Coetzee *et al.*, 2010). Main concerns of the platform include potential faulty base incorporations and the generation of short reads that may hinder contiguous sequence assembly (Kircher *et al.*, 2009).

Ion semiconductor sequencing, Ion Torrent, is relatively new and between the second and third generation sequencing platforms (Barzon *et al.*, 2011). The technique measures a change in pH (hydrogen concentration) when a proton is released during nt incorporation with a DNA polymerase (Rothberg *et al.*, 2011; Thomas *et al.*, 2012; Oulas *et al.*, 2015; Wu *et al.*, 2015). An ion-sensitive field-effect transistor (ISFET) measures this change in pH, when only a single nt is washed over the sequencing chip and is incorporated into the DNA being synthesised (Figure 2.9). Ion Torrent read lengths of 200 bp, 400 bp and 600 bp can be produced (Kchouk *et al.*, 2017). This platform has a maximal throughput after 2 to 8 hours of 10 Gb (10 000 000 000 bp) generating more than 60 000 000 to 80 000 000 reads per run (Kchouk *et al.*, 2017), and is used for whole genome and exome sequencing (Morey *et al.*, 2013).

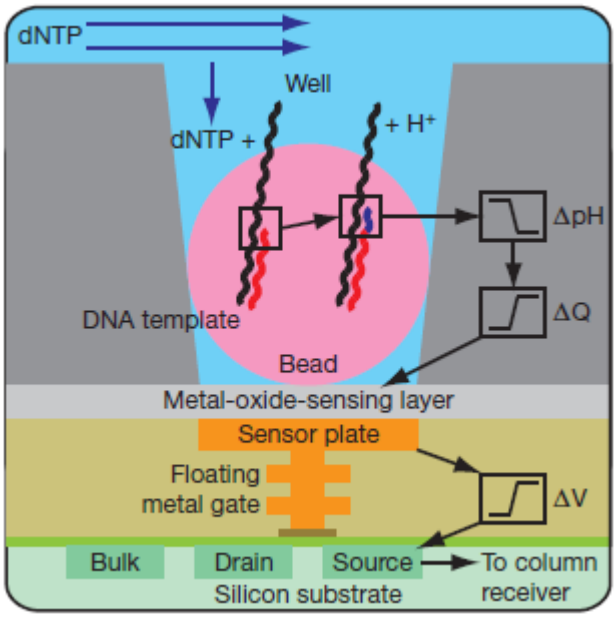

Figure 2.9: Process of an ion conductor sequencing system. A well containing a bead with an attached DNA fragment. A proton (H^+) is released after nt incorporation which changes the potential (V) in a sensing layer at bottom. This change in potential is received by a transistor (Rothberg *et al.*, 2011).

Ion Torrent library building includes using a nebuliser, sonication or enzyme-based degradation methods to shear the DNA, with overhanging or blunt ends, into appropriate lengths for sequencing. The fragments 5' ends are subsequently phosphorylated for ligation of the fragments and the blunt ends of the fragments have ligated adapter sequences. Singular fragments attached to non-paramagnetic beads are amplified by an emulsion PCR step and a single bead with attached DNA are deposited into a well, large enough for only one bead, on the ion semiconductor chip. The well is positioned above an ISFET sensor plate that is sensitive to pH changes (Rothberg *et al.*, 2011; Morey *et al.*, 2013).

Ion Torrent sequence determination consists of flooding the chip with one DNA base per time to generate a sequence. When a base is incorporated a proton is released which causes a drop in pH above the ISFET sensor plate. The number of incorporated nts is proportional to the amount of ions released (Morey *et al.*, 2013). This sequencing method can be applied to RNA samples (Barba *et al.*, 2013).

An Ion Torrent advantage is the relative low upfront costs as there is no need for an imaging system and modified nts for polymerisation (Perkel, 2011). However, the generated sequence may not be perfectly linear which causes misinterpretations in homopolymer sequences (Rothberg *et al.*, 2011; Morey *et al.*, 2013) and relatively short read lengths may hamper *de novo* assembly of genomes.

Single-molecule real-time (SMRT) DNA-sequencing or Smart sequencing does not require sample amplification, because it detects DNA polymerisation of a ssDNA isolated from all other strands in a massive parallel sequencing array. It is known as a revolutionary third generation sequencing platform. SMRT eliminates the necessity to amplify pieces of DNA to high enough concentrations in order to

detect them (Wu *et al.*, 2015). Limitations include a 14% single read error, less data generated than other NGS platforms and necessity of a very pure sample for the assay (Roberts *et al.*, 2013). Another third generation sequencing strategy, known as the Oxford Nanopore, relies on the transition of individual nts or DNA through a protein nanopore (Wang *et al.*, 2015). Sequencing occurs when nts are threaded through the pore by a molecular motor protein causing characteristic variations in the ionic current. These electric fluctuations are base-specific and can be converted into DNA sequence information (Deamer and Akeson, 2000). Library preparation methodology can be done with or without PCR amplification, but, to date, this platform has high error rates and low throughput (Ashton *et al.*, 2015). Both of the previously mentioned sequencing technologies therefore need refinement before they can be routinely applied.

By sequencing multiple samples marked with barcodes in a single lane, efficient data are provided for pathogen discovery (Wu *et al.*, 2015). Raw data generated by these NGS platforms must be processed with algorithms compacted into bioinformatics tools to remove adaptors and low quality sequences. Assembled sequences are then compared with the non-redundant nt database of GenBank using their BLAST functions, either BLASTn or BLASTx. When contigs show high similarity, more than 90% similarity and about 85% coverage, with a known virus, it is identified as the known virus (Wu *et al.*, 2010; Reyes *et al.*, 2011). NGS has become of significant interest for international certification schemes that test import and export crops to detect known and unknown pathogens in crops (Barba *et al.*, 2013; Candresse *et al.*, 2014). Hwang *et al.* (2013) have applied NGS for total sRNA sequencing from PLRV-infected potato plants.

Chapter 3: Development, validation and application of a probe-based RT-qPCR to detect PLRV in potato leaves and tubers in the Sandveld region

3.1 Introduction

PLRV, the type species of the genus Polerovirus in the family Luteoviridae, a group of phloem-limited plant viruses (Kojima *et al.*, 1968; Mayo and D'Arcy, 1999), is a ssRNA+ virus (Taliansky *et al.*, 2003) and targets the Solanaceae family (Harrison, 1984; Syller, 1996; Taliansky *et al.*, 2003). Its genome consists of approximately 5 900 nts (Mayo *et al.*, 1982) and contains ten ORFs that encode eleven proteins (Jeevalatha *et al.*, 2013; Smirnova *et al.*, 2015). The CP is responsible for viral encapsulation and serological properties (Massalski and Harrison, 1987). This region is also very conserved (Guyader and Ducray, 2002; Plchova *et al.*, 2009) and has been used for detection of PLRV by RT-PCR in potato leaves and tubers, due to its gene homology of 94 to 97% to other PLRV-CP sequences found on GenBank (Hossain *et al.*, 2013). Stunting of the potato plant (De Bokx and Van der Want, 1987), a drop in tuber number and size of the potato crop (Rahman *et al.*, 2010) and tuber net necrosis are the result of PLRV infection (Harrison, 1984; Radcliffe and Ragsdale, 2002) causing significant yield losses, up to 90% (Jayasinghe, 1988; Culver and Padmanabhan, 2007; Rahman and Abdul-Mannan, 2010), with consequent major economic losses (Mayo and D'Arcy, 1999). The virus is carried over to the next generation because infected seed tubers are vegetatively multiplied (Van der Want, 1972).

A substantial amount of South African potatoes are currently infected and thus affected by viral organisms, mostly PVY and PLRV (Van der Waals *et al.*, 2013). This can be attributed to a decrease in effectiveness of chemicals used against aphid vectors, the use of infected propagation material and incorrect farming methods (Coetsee, 2005). Combating PLRV infection includes regular systemic insecticide spraying, the planting of PLRV resistant potato cultivars and the planting of virus free certified seed which requires the testing of tubers beforehand with an accurate, rapid, sensitive and specific detection method (Salazar, 1994). Currently ELISA is used for routine potato testing by the South African Seed Potato Certification Scheme to detect viruses, including PLRV. These ELISAs have been efficient in detection South African PLRV isolates, but this ELISA fails to detect PLRV at low concentrations (De Bokx and Beemster, 1987), especially in dormant tubers (Spiegel and Martin, 1993; Huttinga, 1996; Mumford *et al.*, 2004). Tuber index tests also require tuber sprouting, which prolongs the testing time (Mumford *et al.*, 2004). Roos (unpublished) showed that this ELISA was less sensitive than RT-PCR in detecting low levels of PLRV infection. Together with time constraints caused by induced sprouting, this prompted a search for an alternative method that would allow rapid, highly sensitive, versatile and reliable detection of PLRV.

RT-PCR was demonstrated to be: (a) a reliable and efficient method for the detection of potato viruses (Singh and Nie 2003); (b) 1000 to 10 000 fold more sensitive than ELISA (Hossain *et al.*, 2013); and

(c) through primer design, have narrow (specific virus) and broad (general viruses e.g. potyviruses) specificity for RNA virus detection (Singh, 1998; Singh and Singh, 1998; Klerks *et al.*, 2001). RT-PCR has been shown to be effective for the detection of PLRV in dormant potato tubers or leaves (Hadidi *et al.*, 1993; Spiegel and Martin, 1993; Singh *et al.*, 1995), but post-run analysis using agarose gel electrophoresis proved to be very time consuming and laborious. RT-qPCR is an attractive alternative to traditional RT-PCR in which product formation is shown in real time using fluorescence. This method is more expensive than traditional RT-PCR and ELISA, but it does offer a higher level of sensitivity, is quicker than post-run agarose gel electrophoresis and less labour intensive. A full-length infectious copy of PLRV has been used for absolute quantification of virus titre (Hwang *et al.*, 2013). RT-qPCR has been used to detected PLRV in dormant tubers (Leone *et al.*, 1998; Agindotan *et al.*, 2007; Mortimer-Jones *et al.*, 2009). An RT-qPCR for PLRV detection has been developed in SA (Espach, 2015).

The objective of this study was to develop, validate and apply a probe-based RT-qPCR to detect PLRV in potato leaves and tubers to obtain an accurate assessment of PLRV incidence in the Sandveld region. To this end, all available, previously sequenced, PLRV whole genomes were aligned and from the CP region a primer pair and a fluorescent hydrolysis probe were designed for probe-based RT-qPCR detection of PLRV. To obtain a standard curve for assay quantification of virus titre and validation of the assay, a part of the PLRV genome, containing the PLRV CP region, of about 3 283 nts was amplified and cloned using the pGEM-T vector system as a standard. The method was then applied to test for PLRV infection in leaf samples collected between July 2015 and June 2016 from the Sandveld region and tuber samples received in 2016 from throughout SA. The infection levels were compared to vector pressure from the Sandveld region.

3.2 Material and methods

3.2.1 *Primer and probe design*

Whole genome sequences of PLRV (Table 3.1) were downloaded from GenBank, aligned with the ClustalW program in BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999). Forward and reverse primers were designed to bind to short stretches of the CP region which was conserved across all isolates. The forward primer, PLRVSybrF, and reverse primer, PLRVSybrR, was designed to produce an amplicon of 147 bp. The probe, PLRVP, was designed using Primer3 online to bind between the primers to the amplicon following RT-qPCR amplification. The positions and sequences of these primers and probe are shown in Appendix A. The probe reporter, a 6-carboxyfluorescein (FAM), was used as the 5’ fluorescent label and quenched by a Black Hole Quencher (BHQ), which was attached to the 3’ end.

Table 3.1: The whole genome sequences used for primer and probe design for RT-qPCR detection of PLRV. The sequences of South African isolates, which were determined by Roos (pers. comm.), have not yet been published or submitted to GenBank.

No.	Isolate	Accession number	Origin	No.	Isolate	Accession number	Origin
1	303	Unpublished	South Africa	25	NOIR	AF453390	France
2	ASL2000	JQ346190	Germany	26	OP	AF453389	Spain
3	AUS	D13953	Australia	27	OTNI2	JQ420904	India
4	AY138	AY138970	Egypt	28	PBI6	JQ420903	India
5	CAN	D13954	Canada	29	PERU	AF453392	Peru
6	CAN2W	JX855134	Canada	30	SCOT	D00530	Scotland
7	CHN1HB	KC456053	China	31	SDR4	Unpublished	South Africa
8	CHN2IM	KC456052	China	32	SDR6	Unpublished	South Africa
9	CHN3YM	KC456054	China	33	SDR7	Unpublished	South Africa
10	CUBA87	AF453393	Cuba	34	SDRM2	Unpublished	South Africa
11	CYDV-RPV	EF521839	USA	35	SifStph1	Unpublished	South Africa
12	CZECH1	EU717546	Czech Republic	36	SifStph2	Unpublished	South Africa
13	CZECH2	EU717545	Czech Republic	37	SMI5	JQ420905	India
14	CZECH3	EU313202	Czech Republic	38	SPA	Unpublished	South Africa
15	DR5	Unpublished	South Africa	39	SPC	Unpublished	South Africa
16	FvAv2	Unpublished	South Africa	40	SPF	Unpublished	South Africa
17	FRC	AF453394	France	41	SPL24	Unpublished	South Africa
18	FR1	AF453391	France	42	SymlessLS1	JQ346189	Germany
19	GAF318_13	KU586456	CIP accession	43	TVDV	EF529624	China
20	JokerMV10	JQ346191	Germany	44	X747	X74789	Poland
21	JPI1	JQ420901	India	45	Y074	Y07496	Netherland
22	KHPI1	JQ420902	India	46	Z26	Unpublished	South Africa
23	KP090	KP090166	USA	47	ZIM13	AF453388	Zimbabwe
24	MonStph	Unpublished	South Africa				

3.2.2 *Virus isolation, amplification and PCR cleanup for plasmid standard development*

RNA was isolated from a PLRV infected tuber (a PLRV infected potato plant, named FvAv2 (Table 3.1), grown in a glasshouse on the Welgevallen Experimental Farm, University of Stellenbosch) using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol.

The 3' section of the PLRV genome was amplified by RT-PCR using the KAPA LongRange HotStart DNA Polymerase system. The 25 µl reaction mixture contained 1.6 µl viral RNA, 5 µl 5X KAPA LongRange Buffer, 1.75 µl 25 mM $MgCl_2$, 0.75 µl 10 mM dNTPs, 0.625 µl 20 mM of the forward (Table 3.2, 2710) and the reverse primer (Table 3.2, SBgE), 0.25 µl 2.5 U/µl KAPA LongRange HotStart DNA Polymerase, 0.1 µl 200 U/µl SuperScriptIII, 1 µl 0.1 M DTT and 11.1 µl RNase free water. The PCR steps were: 48˚C for 30 min, 94˚C for 3 min followed by 32 cycles of denaturation, annealing and elongation (94˚C for 20 s, 56˚C for 15 s and 68˚C for 3.1 min) and with a final extension step of 72˚C for 3.1 min. Four microliters of the RT-PCR reaction were mixed with a loading dye

(glycerol, 0.5 M EDTA, 1 M Tris, pH 8.0, bromophenolblue) and loaded on a 1% agarose gel that contained Gel Red Nucleic stain (1:10000) (Biotium). The gel was immersed in a 1X TAE electrophoresis buffer (0.48% (w/v) Tris-base, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0) and electrophoresis was performed at 120 V for ±60 min using a CS 300 V Electrophoresis Power Supply. A UV transilluminator was used to verify the presence of RT-PCR products.

Table 3.2: Primers used for LongRange amplification of the 3' section of the PLRV genome and plasmid sequencing.

Name	Sequence (5' to 3')	Sense
2710[1]	CTGGTAGCCCGGGTTCTG	Forward
4190-AS[1]	ACCCCGTTTATCATCCGCG	Reverse
Cpstart2[2]	CCCACGTGCGATCAATTGTTAA	Forward
5000-AS[1]	CACTCTGTTACGCGAACCAG	Reverse
4900[2]	TGATGGGCGATTCTTTCTCG	Forward
SbgE[1]	CTACACAACCCTGTAAGAGG	Reverse

[1]Primers designed by Guyader and Ducray (2002).

[2]Primers designed by Roos (2013).

The RT-PCR product was purified using a Wizard SV Gel and PCR Clean-up System (Promega), according to the manufacturer's protocol. Four microliters of the purified cDNA samples were loaded on a 2% (w/v) agarose gel and a UV transilluminator was used to verify the presence of purified RT-PCR products. The purified samples were stored at -20˚C.

3.2.3 *Cloning into a pGEM-T vector*

The cDNA fragment was cloned into the pGEM-T Vector (Promega) according to the manufacturer's protocol. The concentration of insert was calculated following the manufacturer's protocol and ligation into pGEM-T Vector was performed in 10 µl. A molar ratio of 1:1, insert to vector, was used. The ligation reaction consisted of 5 µl 2X Rapid Ligation Buffer (30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM adenosine triphosphate (ATP), 5% polyethylene glycol (PEG)), at 4˚C overnight.

The whole ligation reaction mixture was then added to 50 µl JM109 High Efficiency Competent Cells in a sterile 17 x 100 mm polypropylene tube and flicked gently to mix. The cells were incubated on ice for 20 min, followed by a heat-shock for 45 s in a 42˚C water bath and immediately returned to ice for 2 min. LB broth (950 µl, Bacto-Tryptone, yeast extract and NaCl, pH 7.0) was added to the tube and incubated at 37˚C 150 rpm for 90 min. Cells were centrifuged at 1 000 x g for 10 min and the supernatant removed. The pellet was resuspended in the remaining 150 µl LB broth and plated out on LB plates (15 g agar per litre) containing 286 mM Ampicillin, which is the selective antibiotic for pGEM-T. The plates also contained 160 µl/100ml IPTG and 80 µl/100ml 5-bromo-4-chloro-3-

indolyl-β-D-galactoside (X-Gal), meaning blue and white colony selection was used after incubation at 37˚C for 16 to 24 hours.

White colonies were screened by PCR to confirm presence of the insert. A sterile wooden toothpick was dipped into a white colony and added to a 10 µl PCR reaction mixture containing 1 µl 10X PCR-buffer, 0.6 µl 25 mM $MgCl_2$, 0.1 µl 250 U Taq DNA polymerase (Super-Therm Polymerase), 0.5 µl of 20 mM forward (Table 3.2, 2710) and reverse primer (Table 3.2, 4190-AS), 0.4 µl 5 mM dNTPs and RNase free water. The PCR cycling conditions were 94˚C for 5 min, followed by 26 cycles of denaturation, annealing and elongation (94˚C for 30 s, 56˚C for 30 s and 72˚C for 30 s) and 72˚C for 7 min. A confirmed positive white colony was inoculated in 5 ml LB medium with 100 µg/ml Ampicillin and incubated at 37˚C 150 rpm for 16 to 24 hours.

3.2.4 *Plasmid isolation*

The NucleoSpin Plasmid/Plasmid (NoLid) (Macherey-Nagel) plasmid isolation kit was used to isolate the plasmid, according to the manufacturer's protocol. About 2 ml of the overnight culture was pelleted at 11 000 x g for 30 s and resuspended in 250 µl resuspension buffer. The tube was inverted eight times after each addition of 250 µl lysis buffer and 300 µl neutralisation buffer. Clear lysate was added to the spin column in a collection tube after centrifugation for 5 min at 11 000 x g. The flow through was discarded after centrifugation for 1 min at 11 000 x g. In order to remove the high levels of host strain (JM series) nucleases, 500 µl of pre-wash buffer was added to the column and centrifuged for 1 min at 11 000 x g. Wash buffer (600 µl) was added and centrifuged for 1 min at 11 000 x g. An additional centrifugation step at 11 000 x g for 2 min dried the column before 50 µl of elution buffer was added to the column and centrifuged for 1 min at 11 000 x g to elute the plasmid DNA. Plasmid DNA was stored at 4˚C.

3.2.5 *Plasmid sequencing*

T7, SP6 and internal primers (Table 3.2, $Cpstart_2$, 5000-AS, 4900 and SbgE) were used for sequencing of the insert at the Central Analytical Facility (CAF), University of Stellenbosch. ChromasPro version 1.7.7 (Technelysium, Pty., Ltd.) was used to produce a consensus sequence from resulting chromatograms. Sequence comparisons were performed using BLASTn (Altschul *et al.*, 1990) on the NCBI database and against PLRV sequences downloaded from GenBank (Table 3.1) utilising the software package BioEdit.

3.2.6 *Sample preparation for RT-qPCR*

The procedure for plant sample preparation of La Notte, Minafra and Saldarelli (1997) with minor modifications was used for RT-qPCR. About 0.1 g of each leaf sample or one sprout per tuber, 2 ml grinding buffer (2% (w/v) PVP40, 1% $Na_2S_2O_5$, 35 mM $NaHCO_3$, 15 mM Na_2CO_3, 0.2% (w/v) BSA, 0.05% (v/v) Tween20, pH 9.6) and one small 4 mm stainless steel ball were added to a 2 ml Eppendorf

microfuge tube. Thereafter the tube was placed in a TissueLyser Qiagen Retsch MM301 for 2 min 30 s at full speed or a Bead Ruptor 24 (OMNI) for 2 min 30 s at 4 m/s. Approximately 1.5 ml of the homogenised leaf or tuber sample was transferred to a 1.5 ml Eppendorf tube and stored at -80˚C.

Four microliters of the homogenised leaf or tuber were 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) TritonX-100) and boiled for 10 min at 95˚C in a digital dry bath (Labnet International). The samples were placed on ice immediately after this incubation step and kept on ice for no less than 5 min before use in RT-qPCR.

3.2.7 *RT-qPCR*

RT-qPCR detection was performed in a Light Cycler 96 (Roche) using the KAPA PROBE FAST One-Step qRT-PCR Universal kit (Roche Molecular Diagnostics) for rapid one-step, one tube RNA, quantification of viral RNA. The primer and probe concentrations and the annealing temperature of the assay were optimised and the final 20 µl reaction mixture contained 1 µl of boiled GES homogenised leaf sample of FvAv2 as the positive control (Table 3.1), 10 µl 2X KAPA PROBE FAST qPCR Master Mix (KAPATaq Hotstart DNA polymerase, KAPA PROBE FAST qPCR Buffer, dNTPs, $MgCl_2$ and stabilisers), 0.4 µl 50X KAPA RT Mix (M-MuLV Reverse Transcriptase and RNase Inhibitor), 0.4 µl 10 mM of each primer (PLRVSybrF and PLRVSybrR) and 0.1 µl 10 mM probe (PLRVP) and 7.7 µl RNase free water. The following thermal profile was found to give optimal results: an initial reverse transcription step at 42˚C for 5 min, followed by enzyme inactivation at 95˚C for 3 min and 40 cycles of amplification, 3 s at 95˚C, 20 s at 57˚C and 1 s at 72˚C. Negative controls to demonstrate assay specificity and no-template controls to detect reagent contamination or increased background signal were included in each RT-qPCR run. During initial runs, amplified products were loaded on a 2% agarose gel to confirm the presence of the amplicon and subsequently sent for sequencing at the Central Analytical Facility (CAF), University of Stellenbosch to confirm amplification of the correct target.

3.2.8 *Assay quantification*

Tenfold serial dilutions of uninfected potato leaf samples spiked with plasmid DNA and purified plasmid DNA, were set up for RT-qPCR quantification. The reaction mixture and thermal profile for RT-qPCR were used as described previously in section 3.2.7, except that 2 µl of plasmid DNA solution was added to the reaction mixture. Standard curves were derived from these serial dilutions. ART Barrier Specialty Pipette Tips (Thermo Fisher Scientific) were discarded after each dilution step (done in triplicate) to ensure accuracy.

The Cq values of the diluted reference samples were plotted against the logarithm of the samples' concentrations. The y-intercept, *m* and R^2 were calculated by linear regression. The LOD and *E* were calculated from the standard curves.

A potato leaf infected with the PLRV isolate, FvAv2, was diluted in ten fold dilutions up to a 10^{-11} dilution. These dilutions were analysed by RT-qPCR with the reaction mixture and thermal profile previously described in section 3.2.7. Each dilution (done in duplicate) was compared to its Cq value.

3.2.9 *Sample collection and preparation for RT-qPCR*

Potato leaves were collected from July 2015 until June 2016. In each month 80 potato leaves were randomly picked from four fields respectively in the Sandveld region. Tuber samples were sent to this laboratory mainly from the Sandveld region, but also from other areas in SA between August 2016 and February 2017. Sample preparation of potato leaves and tubers for RT-qPCR was performed as described previously in section 3.2.6.

3.2.10 *Detection of PLRV in potato plant material using the probe-based RT-qPCR*

The reaction mixture and thermal profile for RT-qPCR detection in potato leaves and tubers were used as described in section 3.2.7. The potato leaf sample infected with the isolate FvAv2 was used as a positive control in all assays.

3.2.11 *PLRV infection of potato leaves versus vector pressure*

The RT-qPCR results from the PLRV infected potato leaves were plotted against the vector pressure of the Sandveld region over the period when potato leaves were sampled (obtained from Dr J. M. Laubscher, Western Cape Department of Agriculture, Elsenburg).

3.3 **Results**

3.3.1 *Primer and probe design*

The PLRVSybrF, PLRVSybrR and PLRVP combination was found to be highly specific for PLRV detection whilst the negative and no-template controls did not result in any fluorescence. Agarose gel and sequence analyses confirmed an amplicon of 147 bp (Figure 3.1) with a sequence that was identical to the existing PLRV CP gene sequences, see Appendix B, Amplicon_147bp.

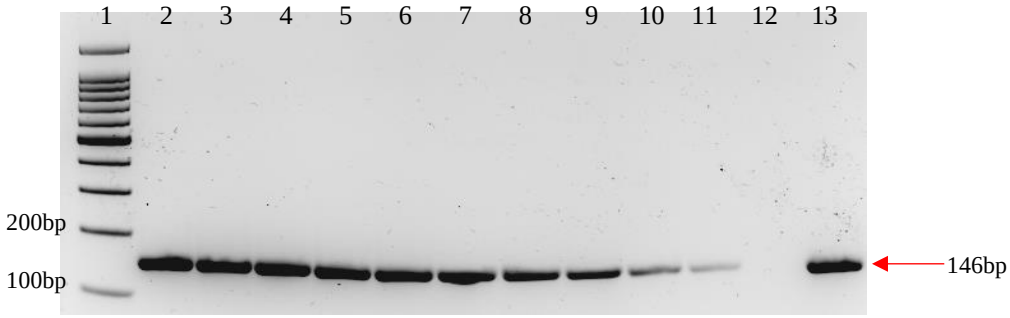

Figure 3.1: An image of a gel agarose electrophoretic separation of the 147 bp PLRV amplicon (red arrow) obtained when using primers PLRVSybrF and PLRVSybrR for the amplification of a PLRV infected potato leaf sample diluted as follows: lane 2, $1:10^0$; lane 3, $1:10^1$, lane 4, $1:10^2$, lane 5, $1:10^3$, lane 6, $1:10^4$, lane 7, $1:10^5$; lane 8, $1:10^6$; lane 9, $1:10^7$; lane 10, $1:10^8$; and lane 11, $1:10^9$. DNA molecular mass standard (lane 1), negative control (lane 12) and positive control (lane 13).

3.3.2 *Amplification of PLRV fragment through LongRange RT-PCR and cloning into pGEM T*

The LongRange RT-PCR amplified a PLRV genome fragment of about 3 283 bp in length (Figure 3.2) that was cloned into the pGEM T vector.

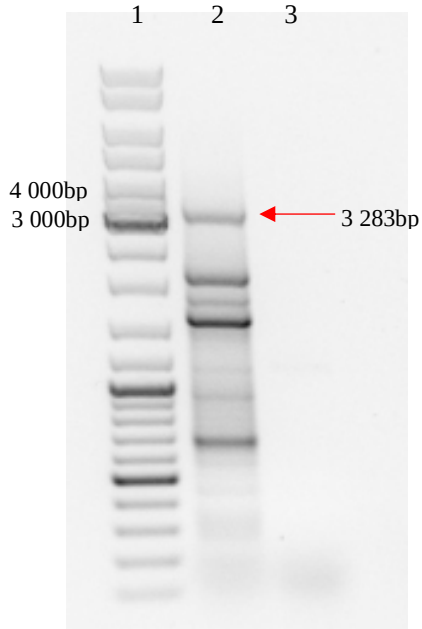

Figure 3.2: An image of a gel agarose electrophoretic separation of the 3283 bp PLRV genome fragment (red arrow) obtained when using primers 2710 and SbgE (see Table 3.2) for LongRange amplification of the 3' section of the PLRV genome. DNA molecular mass standard (lane 1), amplified 3283 bp PLRV genome fragment (lane 2) and negative control (lane 3).

3.3.3 *Plasmid isolation and sequencing*

The plasmid was successfully isolated. Sequencing analysis confirmed the presence of the 3 283 bp PLRV insert, containing the CP region with the correct orientation from genome positions 2704 to 5986 based on Guyader and Ducray (2002), see Appendix B, Plasmid_3200bp.

3.3.4 *Assay quantification*

It was calculated that the total plasmid size was 6 283 bp and a stock solution of the plasmid was prepared with a concentration of 307 ng/ul calculated by Qubit 2.0 analysis (Thermo Fisher Scientific). The mass of one plasmid molecule was calculated to be 6.89 x 10^{-18} g and the mass of the insert contained in the plasmid was calculated to be 2.1 x 10^{-12} g. These values were used to compute the plasmid DNA concentrations of serial dilutions with their corresponding viral copy numbers (Table 3.3). These serial dilutions, with specific numbers of viral copies, were then used in the RT-qPCR to compute corresponding Cq values as shown in Figure 3.3.

Table 3.3: Serial dilutions of plasmid DNA to obtain desired copy numbers.

Concentration of plasmid DNA needed to achieve copy of interest			
Copy	**Mass of plasmid DNA needed (g)**	**µl**	**Final concentration of plasmid DNA (g/µl)**
300000	2.10E-12	2	1.05E-12
30000	2.10E-13	2	1.05E-13
3000	2.10E-14	2	1.05E-14
300	2.10E-15	2	1.05E-15
30	2.10E-16	2	1.05E-16
3	2.10E-17	2	1.05E-17
0.3	2.10E-18	2	1.05E-18

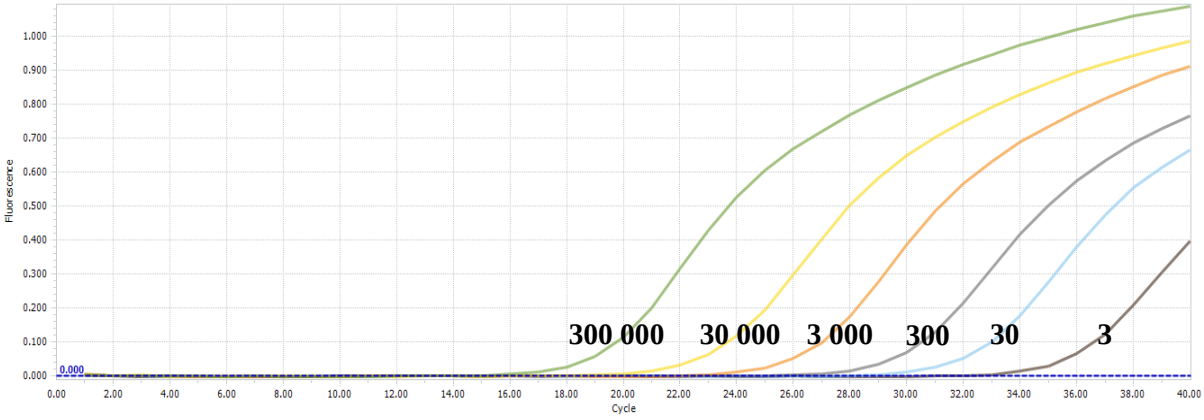

Figure 3.3: The amplification plot of a single dilution series of plasmid containing the 3 283 bp PLRV insert. The number of viral copies is shown in bold on their corresponding curves.

The copy number and corresponding average Cq values are shown in Table 3.4.

Table 3.4: The number of viral copies with corresponding RT-qPCR average Cq value per sample.

Copy	300 000	30 000	3 000	300	30	3	0.3
Average Cq (purified)	19.64	22.95	25.97	29.86	32.85	35.81	none
Average Cq (spiked)	25.3	29.6	33.4	none	none	none	none

The logarithm of plasmid concentration was plotted against Cq values (Figure 3.4). The spiked potato leaf sample's regression formula was: y = -4.03x -22.96 and a 77.06% *E*. The purified plasmid DNA's regression formula was: y = -3.2701x -19.569 and a 102.2% *E*. The LOD was found to be between 3 and 30 viral copies, i.e. 88.8% of the 3 copies per µl PLRV were detected (Cq value of 35.81, Table 3.4) and a 100% PLRV was detected in the 30 copies per µl standard dilution (Cq value of 32.85, Table 3.4). Therefore, at a Cq value of 32.85 the percentage chance of a false negative was found to be 0%, whereas at a Cq value of 35.81 there was a 12% chance of false negatives. Spiked potato leaf samples' LOD was 3000 viral PLRV copies. Thus by deciding on a cut-off Cq value of 34 the chance of a false negative would be between 0% and 12%.

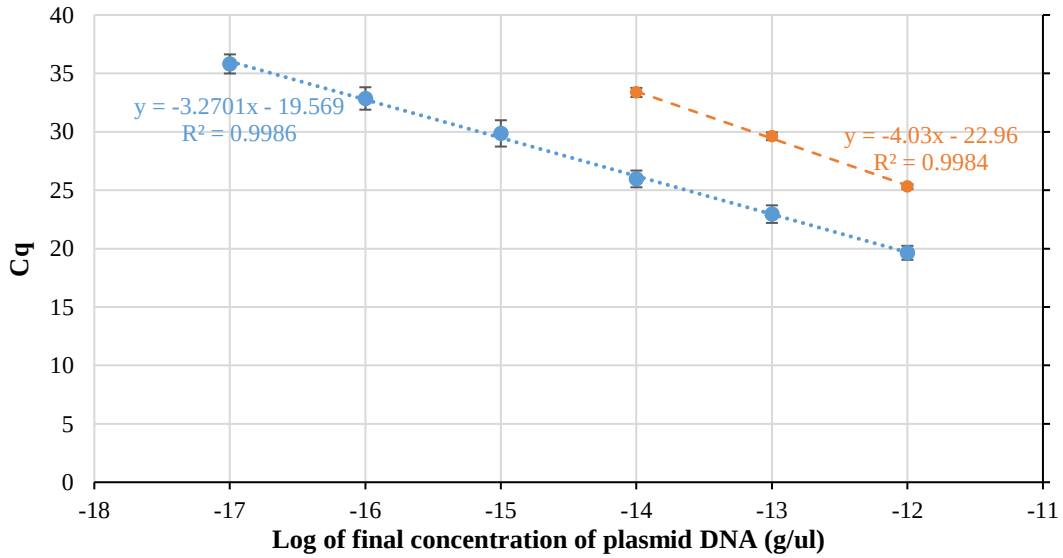

Figure 3.4: The linear regression line of quantitative cycle (Cq) values against the logarithm of the samples' plasmid DNA concentrations (Table 3.3) with error bars. Spiked potato leaf samples (blue dotted line); purified plasmid DNA with the 3 283 bp PLRV insert (orange dashed line).

Figure 3.5 and 3.6 indicate that at a Cq value of 32.7 fluorescence saturation is reached and thereafter background noise was detected. This dilution range has a cut-off Cq value at 33.

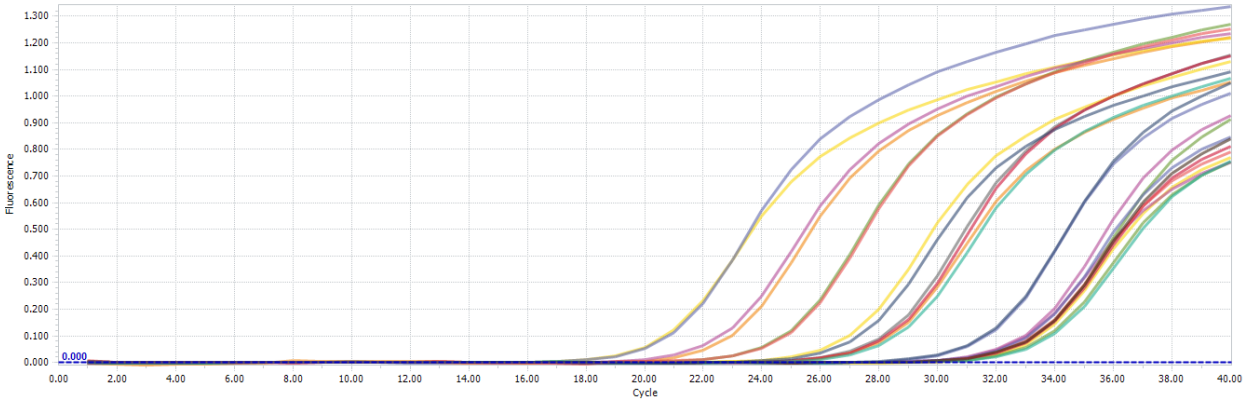

Figure 3.5: The amplification plot of a duplicate dilution series of the PLRV positive control, FvAv2, from undiluted to a 10^{11} fold dilution.

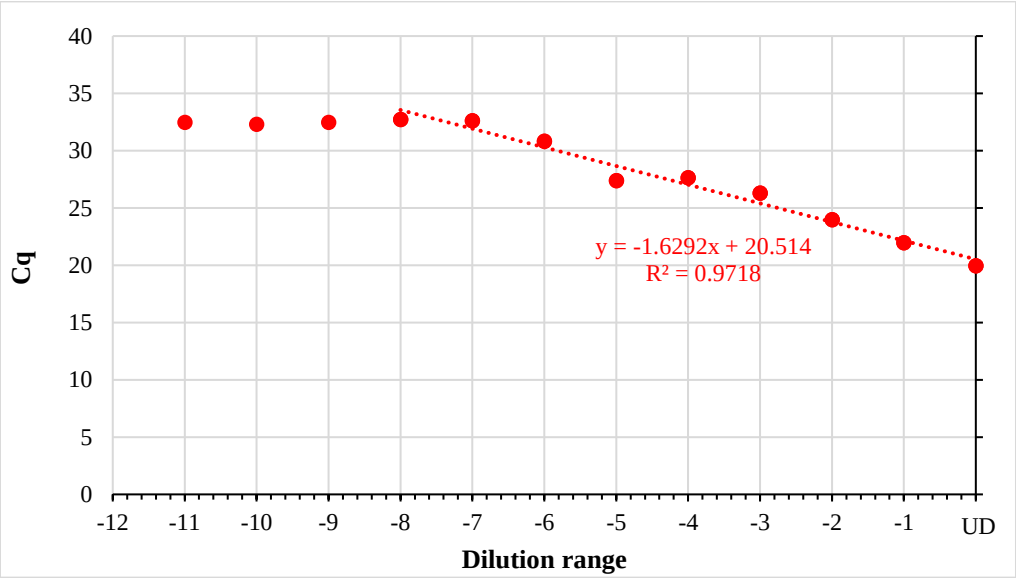

Figure 3.6: The linear regression line of quantitative cycle (Cq) values against the logarithm of the positive control's dilution range excluding the values after saturation. Undiluted sample (UD).

3.3.5 *Detection of PLRV in potato plant material using the probe-based RT-qPCR*

The results of RT-qPCR testing for PLRV in potato leaf samples collected from July 2015 until June 2016 showed two separate groupings, a group with lower Cq and another with a higher Cq as shown in Figure 3.7.

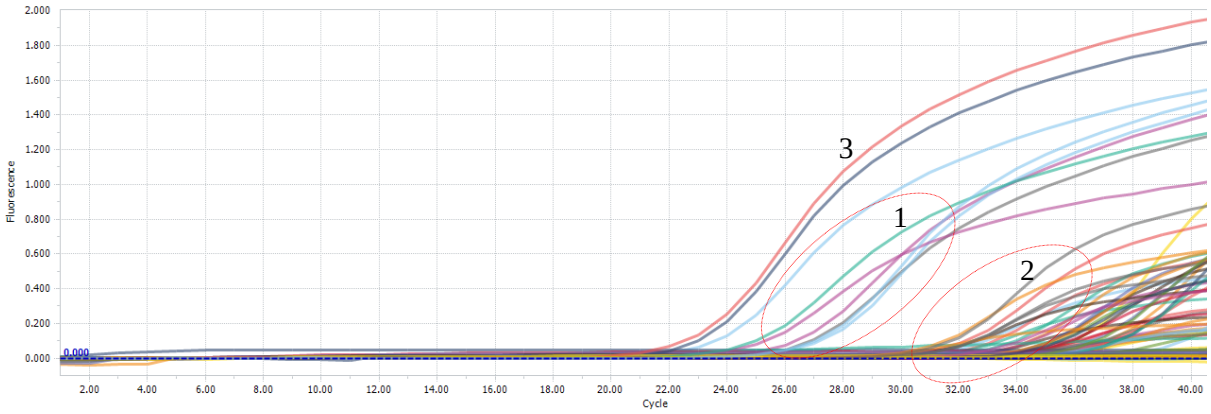

Figure 3.7: Typical RT-qPCR results from 80 potato leaf samples that segregate into two groups (1) and (2). Positive control in duplicate (3).

All the results (high, low and total percentages) of RT-qPCR testing for PLRV in potato leaf samples collected from July 2015 until June 2016 are shown in Table 3.5. The total of the infection percentages of RT-qPCR testing for PLRV in potato leaf samples collected from July 2015 until June 2016 in Table 3.5 are graphically shown in Figure 3.8. The percentage of PLRV infected leaf samples varied from as low as 0% from samples in January 2016 to as high as 96% in November 2015 with considerable variation in the infection levels throughout the whole period. No periods of the year showed higher infection levels than others. The large variation between these infection percentages

can rather be attributed to the fact that the samples of the leaves on which these determinations were performed were from different cultivars, at different locations and from retained seed with viral build up or disease free certified tubers. Consequently, it would therefore not be valid to calculate averages for each month and this would not allow the reader to assess the variation in the data.

Table 3.5: RT-qPCR results of the percentage high, low and total infected PLRV leaf samples of four fields per month collected in the Sandveld region, SA from July 2015 until June 2016.

2015																	
July			August			September			October			November			December		
High	Low	Total	High	Low	Total	High	Low	Total	High	Low	Total	High	Low	Total	High	Low	Total
8.75	66.25	75.00	13.75	28.75	42.50	11.25	43.75	55.00	7.50	2.50	10.00	6.25	90.00	96.25	20.00	30.00	50.00
28.57	28.57	57.14	18.75	23.75	42.50	27.50	36.25	63.75	3.80	1.27	5.06	3.75	50.00	53.75	16.25	32.50	48.75
16.25	38.75	55.00	56.25	13.75	70.00	12.50	26.25	38.75	3.80	1.27	5.06	66.25	2.50	68.75	2.50	17.50	20.00
10.00	31.25	41.25	26.25	53.75	80.00	10.00	6.25	16.25	18.18	0.00	18.18	1.25	31.25	32.50	0.00	23.75	23.75
2016.00																	
January			February			March			April			May			June		
High	Low	Total	High	Low	Total	High	Low	Total	High	Low	Total	High	Low	Total	High	Low	Total
2.50	0.00	2.50	83.75	0.00	83.75	42.50	2.50	45.00	70.00	16.25	86.25	48.75	35.00	83.75	15.00	42.50	57.50
2.50	0.00	2.50	62.50	13.75	76.25	32.50	11.25	43.75	36.25	23.75	60.00	86.25	10.00	96.25	23.75	20.00	43.75
6.25	0.00	6.25	13.75	6.25	20.00	8.75	20.00	28.75	81.25	0.00	81.25	50.00	33.75	83.75	75.00	0.00	75.00
0.00	0.00	0.00	47.50	5.00	52.50	1.25	21.25	22.50	22.50	55.00	77.50	36.25	28.75	65.00	25.00	20.00	45.00

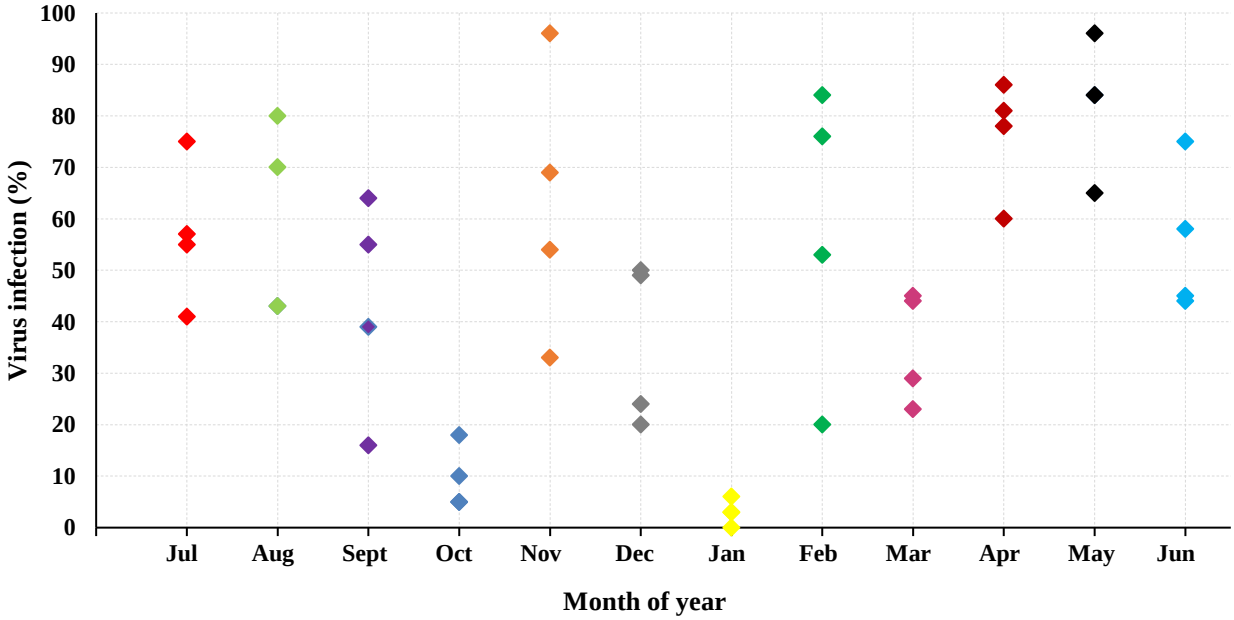

Figure 3.8: The total percentage (high and low infection level) PLRV-infection of potato leaves per field from July 2015 until June 2016 collected in the Sandveld region, SA. Some points may have overlapped.

The results of RT-qPCR testing for PLRV in potato tubers samples sent to our laboratory between August 2016 and February 2017 are shown in Table 3.6. Infection percentages ranged from 0% to 100% and no seasonal patterns could be observed. The large variation in infection percentages can

again be attributed to the fact that the tuber samples on which these determinations were performed were from different cultivars, at different locations and from retained seed with viral build up or disease free certified tubers. The average infection level of these tubers was 18.5% from 5510 seed tubers tested.

Table 3.6: RT-qPCR results of the total and percentage positive and negative PLRV tuber samples from August 2016 until February 2017. KwaZulu Natal (KZN); North Eastern Free State (NEF).

Month	Region	Total	Percentage positive
August	Sandveld	19	100.0
September	Sandveld	40	100.0
	KZN	16	18.8
	Sandveld	100	100.0
	Sandveld	20	50.0
	Sandveld	60	100.0
	Sandveld	100	0.0
	Sandveld	100	0.0
	Sandveld	100	2.0
	Sandveld	100	1.0
	Sandveld	100	1.0
	Sandveld	100	0.0
	Sandveld	100	0.0
	Sandveld	100	1.0
	Sandveld	100	1.0
	Sandveld	100	0.0
	Sandveld	100	0.0
October	Sandveld	60	3.3
	Sandveld	100	48.0
	Sandveld	100	1.0
	Sandveld	100	63.0
	Sandveld	100	16.0
	Sandveld	98	15.3
	Sandveld	97	12.4
	Sandveld	100	9.0
	Sandveld	100	9.0
	Sandveld	100	0.0
	Sandveld	100	0.0
	Sandveld	100	3.0
	Sandveld	100	2.0
	Sandveld	100	4.0
	Sandveld	100	52.0
	Sandveld	20	0.0
	Sandveld	100	26.0
	Sandveld	100	10.0
	Sandveld	100	15.0
	Sandveld	100	21.0
	Sandveld	20	10.0
November	Sandveld	50	10.0
	Sandveld	100	13.0
	Sandveld	100	18.0
	Sandveld	100	12.0
	Sandveld	100	7.0
	Sandveld	76	28.9
	Sandveld	100	7.0
	Sandveld	100	2.0
	Sandveld	100	0.0
	Sandveld	100	0.0
	Sandveld	100	15.0
	Sandveld	100	6.0
	Sandveld	25	0.0
	Sandveld	99	13.1
	Sandveld	40	2.5
	Sandveld	100	0.0
	Sandveld	100	5.0
	Sandveld	100	6.0
	Sandveld	100	13.0
	Sandveld	100	9.0
	Sandveld	100	18.0
December	Sandveld	20	10.0
	Sandveld	100	24.0
January	NEF	200	5.5
February	Sandveld	50	0.0
Total		**5510**	**18.5**

3.3.6 *PLRV infection of potato leaves versus vector pressure*

Figure 3.9 and 3.10 show the RT-qPCR results from the PLRV infected potato leaves plotted against the vector pressure from July 2015 until June 2016.

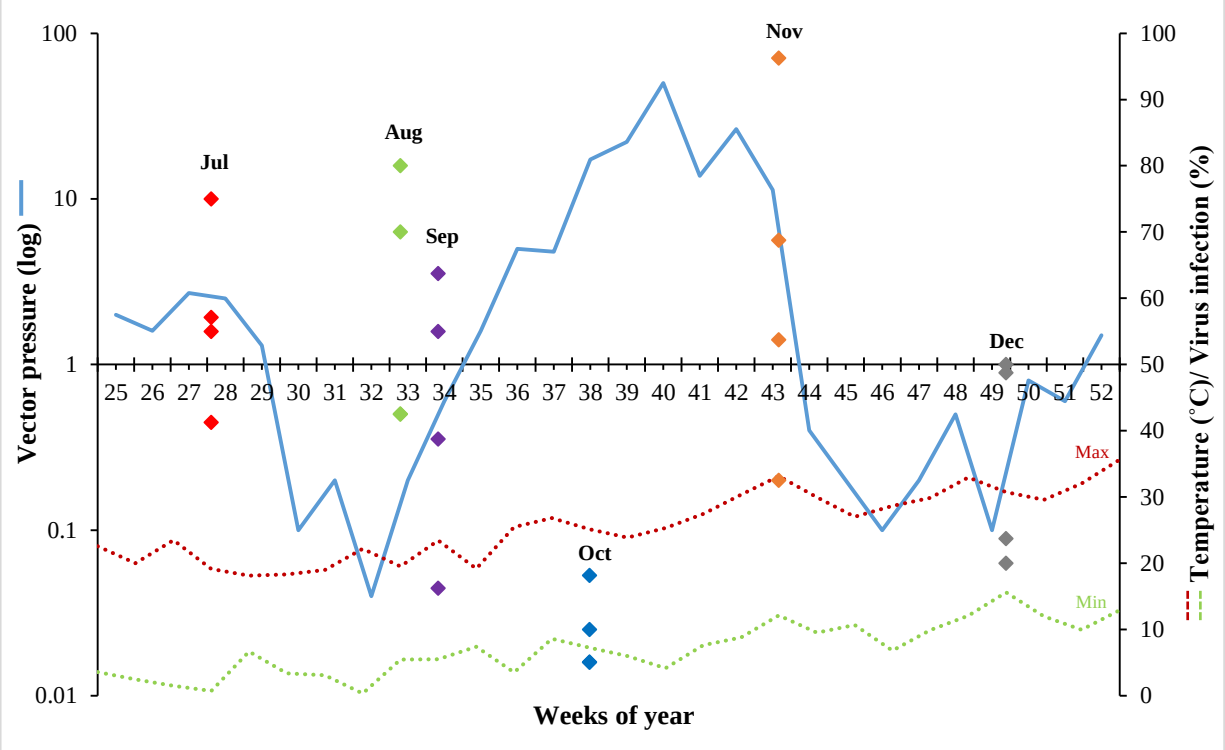

Figure 3.9: The vector pressure, number of aphids, per week of the year compared to percentage PLRV-infection from July 2015 until December 2015. Maximum and minimum temperatures are also plotted on the graph with the axis shown on the right.

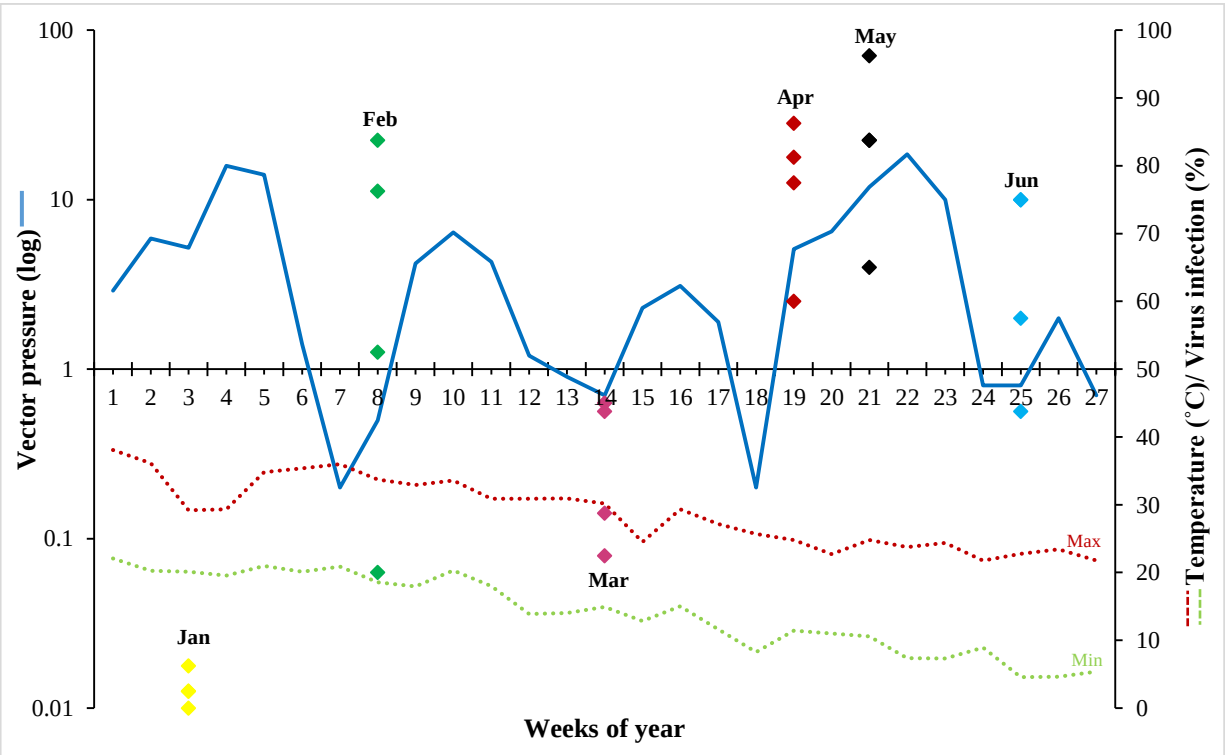

Figure 3.10: The vector pressure, number of aphids, per week of the year compared to percentage PLRV-infection from Jan 2016 until June 2016. Maximum and minimum temperatures are also plotted on the graph with the axis shown on the right.

The vector pressure as plotted in Figure 3.9 and 3.10 shows considerable variation with some periods showing very low vector pressure and other periods showing very high vector pressure. If a potato planting was exposed to a high vector pressure during its growing period from week 2 after planting up to week 14 to 18 when leaf-die-off occurs it could be expected that high infection levels could result which therefore shows that plantings were hardly grown during extended periods of low vector pressure during the whole test period. Vector pressure compared to the infection levels (Figure 3.9 and 3.10) only showed that at some points low virus infection levels followed after low vector pressure. At week 38 low virus infection was seen, due to low virus pressure from week 29 to 34 (Figure 3.9) and at week 3 (Figure 3.10), due to low vector pressure from week 44 to 52 of the previous year in 2015.

3.4 **Discussion**

The probe-based RT-qPCR assay developed and validated in this study was used to detect PLRV from South African potato leaves and tubers with high specificity. The standard consisting of only 3 200 bp of the whole genome was used for assay quantification. According to Raymaekers *et al.* (2009) an *m* value of between -3.1 and -3.6 with an *E* value between 90% and 110% are generally accepted for a qPCR assay, as 100% indicates that the number of target molecules exactly doubles after each PCR cycle. The purified plasmid DNA linear regression line's *m* value of -3.2701 and *E* of 102.2% fell inside this accepted range, but the spiked potato leaf sample linear regression line's *m* value of -4.03 and low *E* value of 77.06% did not. The latter could have been influenced by PCR inhibitors or enhancers, RNA or cDNA degradation or concentration, length of the amplicon, primer quality and secondary structures (Bustin and Nolan, 2004; Wong and Medrano, 2005; Raymaekers *et al.*, 2009). High concentrations of naturally occurring RNAs could also have influenced the RT-step and cDNA synthesis rate, altering the *E* value (Pfaffl, 2004). Both linear regression lines with R^2 values of 0.9986 and 0.9984 respectively indicate highly reproducible results. Hühnlein *et al.* (2016) detected PLRV from *in vitro* RNA transcripts of genomic and subgenomic PLRV RNA. Their standard curves values had a 94.1% *E* for both RNA transcripts, an R^2 value of 0.999 and 0.998 respectively and an *m* value of −3.472 and −3.472 respectively which are very similar to those obtained in this study. The RT-qPCR gave Cq values of between 17 and 36 in this study. Mortimer-Jones *et al.* (2009) detected PLRV at Cq values between 11 and 19 using a multiplex/uniplex RT-qPCR. The difference in Cq values could be attributed to their use of a higher probe concentration of 0.2 µM, compared to this study's probe concentration of 0.05 µM. Agindotan *et al.* (2007) detected PLRV at Cq values between 21 and 25, with a 21.5 Cq value positive control using a multiplex RT-qPCR. They also had a higher probe concentration of 0.1 µM. Espach (2015) detected PLRV at Cq value between 26 and 30 with a probe-

based RT-qPCR with a probe concentration of 0.25 µM. This study's probe optimisation indicated that increased probe concentration increases Cq values, but does not increase sensitivity. Furthermore, by using the purified plasmid DNA the LOD was found to be between 3 and 30 viral copies. On the basis of these criteria, it was concluded that the RT-qPCR that was developed in this study was highly sensitive and produced results sufficiently reliable for routine PLRV detection.

The newly designed probe-based RT-qPCR assay was used to assess PLRV infection levels monthly in the Sandveld region over a full calendar year. The PLRV infected samples with lower Cq values between 20 and 25 represented between 3 000 and 300 000 viral copies respectively and higher Cq values ranging between 30 and 35 represent between 3 and 30 viral copies respectively. The two separate groupings of amplification curves in these ranges were commonly seen in RT-qPCR results. The group of samples that had lower Cq values with higher virus titre may indicate previous season host PLRV infections (secondary infection), whereas the group of samples that had the higher Cq values with lower, yet definitely positive virus titre, may indicate in season infections (primary infection).

Total PLRV infection levels averaged 48.2% from July 2015 to June 2016 in the Sandveld region representing 21.9% low infection levels from PLRV transmission by aphids in the current season and 26.3% high infection levels from the previous season (Table 3.5 and Figure 3.8). This implies that 26.3% of seed tubers that are planted are infected. This is known to lead to greater yield losses than primary aphid infection (Harrison, 1984; Rek, 1987; Van der Zaag, 1987).

As mentioned, potato production in the Sandveld region occurs in temperatures averaging 35˚C between November and March, with daytime temperatures often above 40˚C (Figure 1.2). The low PLRV concentrations (Figure 3.9 and 3.10) may be the result of high summer temperatures in the Sandveld region that reduce PLRV in the potato plants and in aphids. According to Kassanis (1950), Kaiser (1980) and Loebenstein (2001) PLRV can be eliminated at a constant temperature of 37˚C for three to four weeks. Broadbent and Hollings (1951) and Davis *et al.* (2006) concluded that the aphid dies at 38.5˚C. Jayasinghe and Salazar (1998) found that PLRV transmission decreases at temperatures >26˚C, whereas Chung *et al.* (2016) found that temperatures <10˚C and >30˚C hinder the acquisition of PLRV by *M. persicae*. Chung *et al.* (2016) also found that temperatures >25˚C hinder PLRV multiplication. This may explain the low concentration, i.e. low copy numbers of PLRV, detected in many of the tested samples. However, during cooler winter temperatures in the Sandveld region, increased vector pressure, i.e. aphids, and increased PLRV infection rates may lead to higher rates of PLRV transmission and multiplication as these occur optimally at lower temperatures which in turn would lead to higher PLRV copy numbers during the cooler months.

This study confirms that there is a serious presently underestimated PLRV outbreak in the Sandveld region. If RT-qPCR with its higher sensitivity was used as the detection method for PLRV, it could

be expected that PLRV infection levels of seed tubers could be decreased dramatically. This study also indicates that the South African Potato Certification Scheme should reconsider the use of ELISA as the method for PLRV detection and replace it with an RT-qPCR method as described here to detect the apparently low concentration, yet high levels of PLRV infection in the Sandveld region.

Chapter 4: Detecting PLRV in aphids by RT-qPCR and whole genome sequencing of virus isolates

4.1 Introduction

Plant viruses have evolved to ensure transmission by connecting with the vector's biology (Calil and Fontes, 2016). Viruses alter the host in such a way that plant and vector interactions are influenced to enhance their transmission (Alvarez *et al.*, 2007; Mauck *et al.*, 2012). Rate and extent of virus transmission are dependent on vector activity and behaviour (Jeger *et al.*, 1998, 2004).

PLRV is one of the most damaging aphid-transmitted viruses known (De Bokx and Van der Want, 1987). Aphid vectors are responsible for PLRV transmission to uninfected fields (Miguel *et al.*, 2016) and aphid prevalence is indicativeof the risk of PLRV infection across a region. *M. persicae* is the most efficient vector of PLRV (Radcliffe, 1982; Spooner *et al.*, 2005) and has been recorded in the Sandveld region in SA (Krüger *et al.*, 2014). Singh (2016) demonstrated that *M. persicae* settles first on a PLRV-infected plant and then travels to uninfected plants. Each progeny aphid has to attain the virus by feeding on infected plants as the virus cannot be passed though the egg (Johnson and Pappu, 2006). To control PLRV transmission, aphid flight patterns should be monitored regularly in potato growing regions. Besides aphid counting and identification, this should include the testing of aphids for PLRV infection for initial virus pressure, as the results will determine the control of vectors by regular insecticide spraying and timing of plant haulm destruction (Van Harten, 1983). This assay should be accurate, rapid, sensitive and specific for PLRV (Salazar, 1994). ELISA (Clarke *et al.*, 1980), immunosorbent electron microscopy (ISEM) (Roberts *et al.*, 1980) and dot-blot hybridisation (Smith *et al.*, 1993) were used as quantitative assays in attempts to detect PLRV isolated from aphids. RT-PCR was used to detect PLRV in aphids (Hadidi *et al.*, 1993; Singh and Singh, 1996; Singh *et al.*, 2000; Klerks *et al.*, 2001; Awan *et al.*, 2010) that were freshly caught or stored at -70˚C for a year (Singh *et al.*, 1995) and is a useful tool for epidemiological studies (Hossain *et al.*, 2013). A duplex RT-PCR for simultaneous detection of PLRV and PVY from aphids has been described (Singh *et al.*, 1996; Venkateswarlu *et al.*, 2016).

RT-qPCR has not been used to detect PLRV in South African aphids. RT-PCR and Sanger sequencing has never been used to sequence a PLRV whole genome extracted from an aphid. Thus, the objective of the present study was to confirm infection of aphids with PLRV by RT-qPCR and to sequence the whole genomes of aphid transmitted PLRV isolates to determine which types of PLRV are being transmitted by aphids in the Sandveld region. Aphids were collected from the Sandveld region and tested with RT-qPCR to detect PLRV. The RNA of samples testing positive was extracted and PLRV overlapping genome segments were amplified and sequenced to generate contigs of whole genome PLRV sequences. The generated PLRV whole genome sequence was then characterised by comparing it to 47 other PLRV whole genomes with the use of phylogenetic analysis.

4.2 Materials and methods

4.2.1 *Collection of winged aphid vectors and preparation for RT-qPCR*

M. persicae and *Brevicoryne brassicae* were collected from yellow-pan traps in the Sandveld region by Dr J. M. Laubscher and stored in 70% ethanol. At arrival in the laboratory aphids were individually placed in 2 ml microfuge tubes and delicately washed with 1 ml RNase free water, where after the water was removed. To the aphid, a stainless steel ball and 250 µl grinding buffer, as used for direct RT-PCR from potato leaves, (2% (w/v) PVP40, 1% $Na_2S_2O_5$, 35 mM $NaHCO_3$, 15 mM Na_2CO_3, 0.2% (w/v) BSA, 0.05% (v/v) Tween20, pH 9.6) were added and homogenised for 20 s at 4 m/s in a Bead Ruptor 24 (OMNI) and finally, stored at -80˚C. Further sample preparation was completed as described previously in section 3.2.6, except that 4 µl of homogenised aphid sample was added to the GES buffer for boiling when aphids were tested instead of homogenised potato leaf or tuber sample.

4.2.2 *RT-qPCR*

Reaction mixture and thermal profile for RT-qPCR were used as described previously in section 3.2.7.

4.2.3 *RNA isolation and whole-genome amplification of PLRV isolates from aphids using RT-PCR*

The RNA of PLRV positive samples was extracted with RNeasy Plant Mini Kit (Qiagen). Instead of macerating the aphid in liquid nitrogen, 1 ml of the homogenised aphid in grinding buffer was added directly to the column, where after the rest of the protocol was completed according to the manufacturer's protocol. Viral RNA within the aphid material was amplified by means of RT-PCR. The primers designed previously by Guyader and Ducray (2002) and Roos (2013) for amplification of overlapping segments of the whole PLRV genome were used as shown in Table 4.1, segment 1 to 6. To this end, 1.6 µl of template RNA solution was added to 18.4 µl of a standard RT-PCR mixture. This mixture consisted of 2 µl of a 10X PCR-buffer, 1.2 µl 25 mM $MgCl_2$, 0.2 µl 250 U/µl Taq DNA polymerase (Super-Therm Polymerase), 1 µl 0.1 M DTT, 0.8 µl 5 mM dNTPs, 0.5 µl 20 mM of each forward and reverse primer, 0.1 µl 200 U/µl SuperScriptIII and 12.3 µl RNase free water. Primer binding sites are indicated in Figure 4.1. All RT-PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler using the following program: a single reverse transcription step of 48˚C for 30 min, followed by 35 cycles of DNA amplification at 94˚C for 30 s, 55˚C for 45 s and 72˚C for 60 s as well as an extension completion period at 72˚C for 10 min.

Table 4.1: Primers used for amplification of the PLRV genome. Primer combinations produced six overlapping segments covering the entire genome.

Segment	Name	Sequence (5' to 3')	Sense	Amplicon size (nt)
1	ORF0-S2[1]	GAAATTGCAGCTTTAG	Forward	933
	ORF0-AS[1]	AGGCGTTCTCTCCACTGTAC	Reverse	
2	900[2]	GCTGTGGAAGGATACAAAGGGT	Forward	1172
	1990-AS[1]	GCTTGTTCTTCCCTCCACG	Reverse	
3	1810[1]	GGCAACTCCGACATCCCC	Forward	1224
	2930-AS[1]	GCATCCAATACGCGACTGAC	Reverse	
4	2710[1]	CTGGTAGCCCGGGTTCTG	Forward	1594
	4190-AS[1]	ACCCCGTTTATCATCCGCG	Reverse	
5	Cpstart2[2]	CCCACGTGCGATCAATTGTTAA	Forward	1401
	5000-AS[1]	CACTCTGTTACGCGAACCAG	Reverse	
6	4900[2]	TGATGGGCGATTCTTTCTCG	Forward	1212
	SbgE[1]	CTACACAACCCTGTAAGAGG	Reverse	

[1]Primers designed by Guyader and Ducray (2002).

[2]Primers designed by Roos (2013).

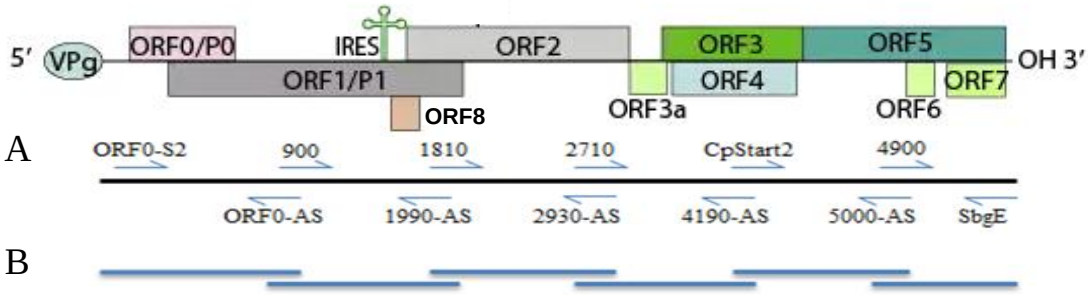

Figure 4.1: A diagram of the PLRV genome with the open reading frames (ORF0-8), the 5' protein part occupied by the viral genome linked protein (VPg) and the free 3' OH group (Hulo *et al.*, 2016). Primer binding sites of the primers used for the amplification of genome segments (Table 4.1) are shown in (A) and RT-PCR products are shown in (B).

4.2.4 *Gel electrophoresis of amplified RT-PCR products*

The total volume of each of the RT-PCR reaction mixtures was mixed with a loading dye (glycerol, 0.5 M EDTA, 1 M Tris, pH 8.0, bromophenolblue and RNase free water) and loaded on a 1% agarose gel that contained Gel Red Nucleic stain (1:10000) (Biotium). The gel was immersed in a 1X TAE electrophoresis buffer (0.48% (w/v) Tris-base, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0) and electrophoresis was performed at 120 V for ±60 min using a CS 300 V Electrophoresis Power Supply. The presence of nucleic acid bands was confirmed on a UV transilluminator.

4.2.5 *RT-PCR product purification*

RT-PCR products were purified from the abovementioned gels using a Wizard SV Gel and PCR Clean-up System (Promega), according to the protocol supplied by the manufacturer. Four microliters of the purified cDNA samples were analysed on a 2% (w/v) agarose gel to confirm amplification and to approximate DNA concentration. Purified samples were stored at -20˚C.

4.2.6 *Direct sequencing of purified RT-PCR amplification products by Sanger cycle sequencing*

The sequencing mix for a single reaction to sequence each of the amplification products consisted of 2 µl 5X sequencing dilution buffer (Applied Biosystems), 0.5 µl Terminator Dye (BigDye Terminator v3 Cycle Sequencing Kit, Applied Biosystems), 2 µl 0.8 µM primer, 2 µl of the cDNA template solution and 3.5 µl RNase free water. The same set of PLRV specific primers used in the amplification RT-PCR was used in the sequencing PCR (Table 4.1, segment 1-6). The sequencing PCR was performed in an Applied Biosystems 2720 Thermal Cycler with the following program: 35 cycles of DNA amplification at 96˚C for 10 s, 52˚C for 30 s and 60˚C for 4 min as well as an elongation step at 60˚C for 10 min. The products of Sanger cycle sequencing were sequenced on a 3 730 XL DNA Analyser (Applied Biosystems) at the Central Analytical Facility (CAF), University of Stellenbosch.

4.2.7 *Nucleotide sequence analysis and alignment*

ChromasPro version 1.7.7 (Technelysium, Pty., Ltd.) was used to produce consensus sequences from the chromatograms of each of the amplification products. These consensus sequences were used to generate a contiguous whole genome sequence. The generated PLRV whole genome sequences were compared to the available whole genome sequences of other strains of PLRV downloaded from GenBank (Table 3.1) utilising the software package BioEdit. These PLRV genome sequences and the outgroups, cereal yellow dwarf virus-RPV (CYDV-RPV) (family *Luteoviridae*, genus *Polerovirus*, species, *Cereal yellow dwarf virus-RPV*) and tobacco vein distorting virus (TVDV) (family *Luteoviridae*, genus *Polerovirus*, species *Tobacco vein distorting virus*), were aligned using a Geneious 10.2.3 MUSCLE alignment plugin with default parameters and further aligned manually.

4.2.8 *Phylogenetic analysis of the PLRV whole genome sequence*

The matrix of aligned sequences was trimmed at the 5’ and 3’ end to exclude any missing characters. Phylogenetic analysis of the aligned sequences was performed using PAUP version 4.0a (build 157) (Swofford, 2003).

4.3 **Results**

4.3.1 *RT-qPCR*

PLRV presence was detected in two (with a Cq value of 23 and 25 respectively) of the 20 homogenised *M. persicae* samples using RT-qPCR (Figure 4.2). RT-qPCR detected no PLRV in 29 other *M.*

persicae and 21 *Brevicoryne brassicae* samples. The negative and no-template controls did not result in any fluorescence.

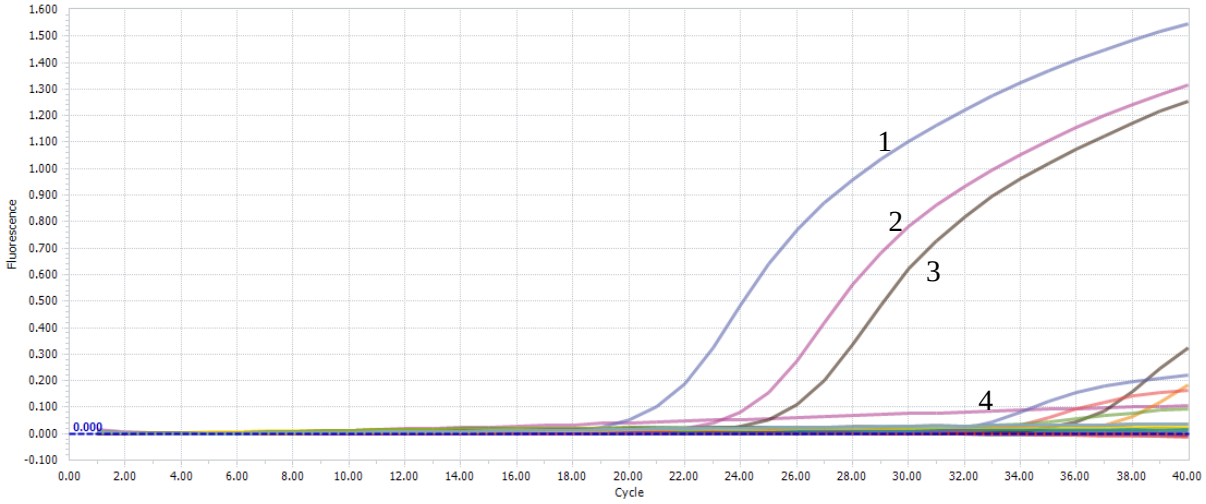

Figure 4.2: The amplification plot of 20 *M. persicae* homogenised for PLRV detection. Positive control (1); Two *M. persicae* samples (2 and 3) were thus confirmed to be infected with PLRV; Negative control (4).

4.3.2 *RNA isolation and whole-genome amplification of PLRV isolates from aphids using RT-PCR*

Six overlapping genome segments of the PLRV isolate extracted from the one aphid (aphid 2) were successfully amplified with the RT-PCR procedure. Amplification of CP in the other PLRV isolate (aphid 1) was unsuccessful and thus only the sequences of five segments were obtained. Images of the gel electrophoretic separation of the amplicons of all six regions are shown in Figure 4.3.

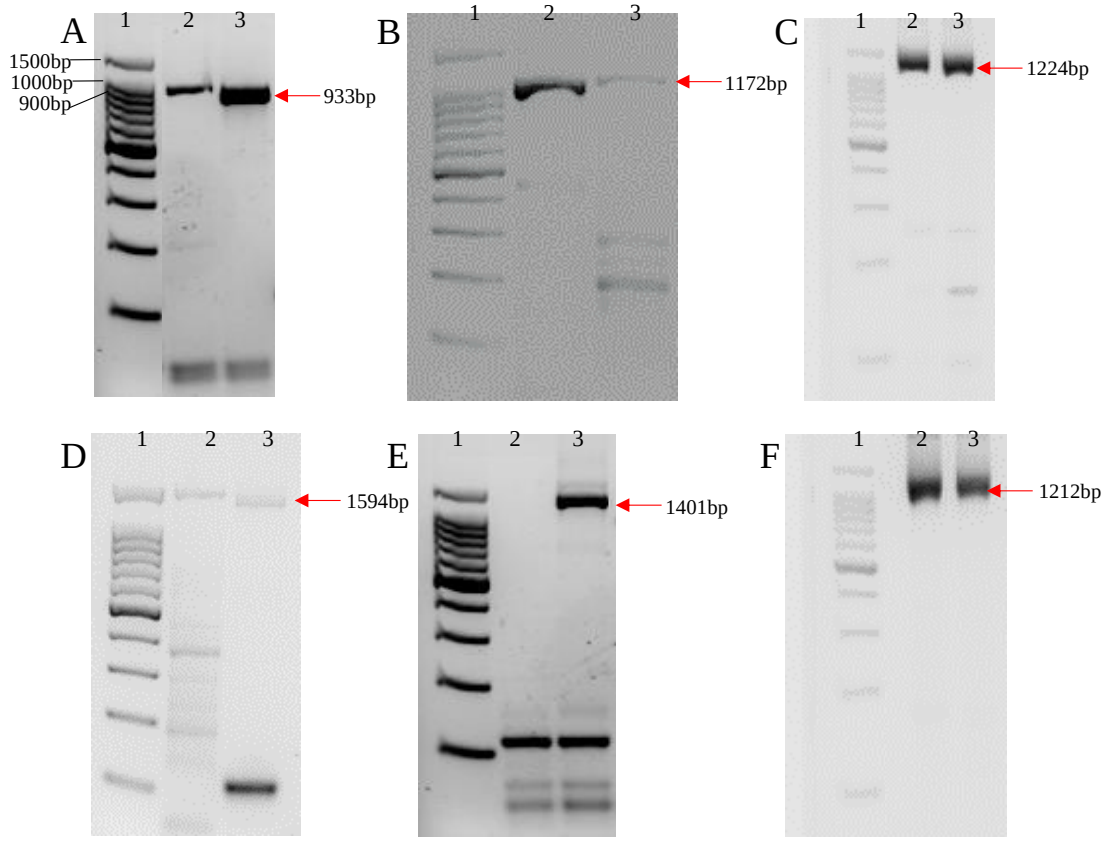

Figure 4.3: An image of a gel agarose electrophoretic separation of the six genome segments of each of the PLRV extracted from aphids obtained from amplification when using primers as follows: ORF0-S_2 and ORF0-AS (A); 900 and 1990-AS (B); 1810 and 2930-AS (C); 2710 and 4190-AS (D); Cpstart2 and 5000-AS (E); and 4900 and SbgE (F). DNA molecular mass standard (lane 1), aphid 1 (lane 2) and aphid 2 (lane 3), except in (B) in which aphid 2 was in lane 2 and aphid 1 was in lane 3. The DNA molecular mass standards used are the same on all images and sizes therefore are only indicated in (A).

4.3.3 *Nucleotide sequence analysis and alignment*

The six amplified segments were combined to generate a contiguous whole genome sequence for the PLRV isolate referred to as Aphid6_17. Due to the fact that the ORF0 fragment of the second PLRV isolate could not be sequenced the whole genome sequence could not be assembled and this sequence was therefore excluded from the subsequent phylogenetic analysis.

4.3.4 *Phylogenetic analysis of the PLRV whole genome sequence*

The isolate Aphid6_17 was 5 990 nts in length. Alignment of the 48 taxa with outgroups, CYDV-RPV and TVDV, led to the consequent total character length of 6358. Of the 6358 total characters in the matrix, 2865 characters were constant, 1306 characters were parsimoniously informative and 2187 variable characters were parsimony uninformative. The heuristic search retrieved a tree with a length of 6219 nts and is displayed in Figure 4.4 and the ingroup with greater resolution in Figure 4.5. The phylogenetic tree had a CI of 0.717 and a RI of 0.675.

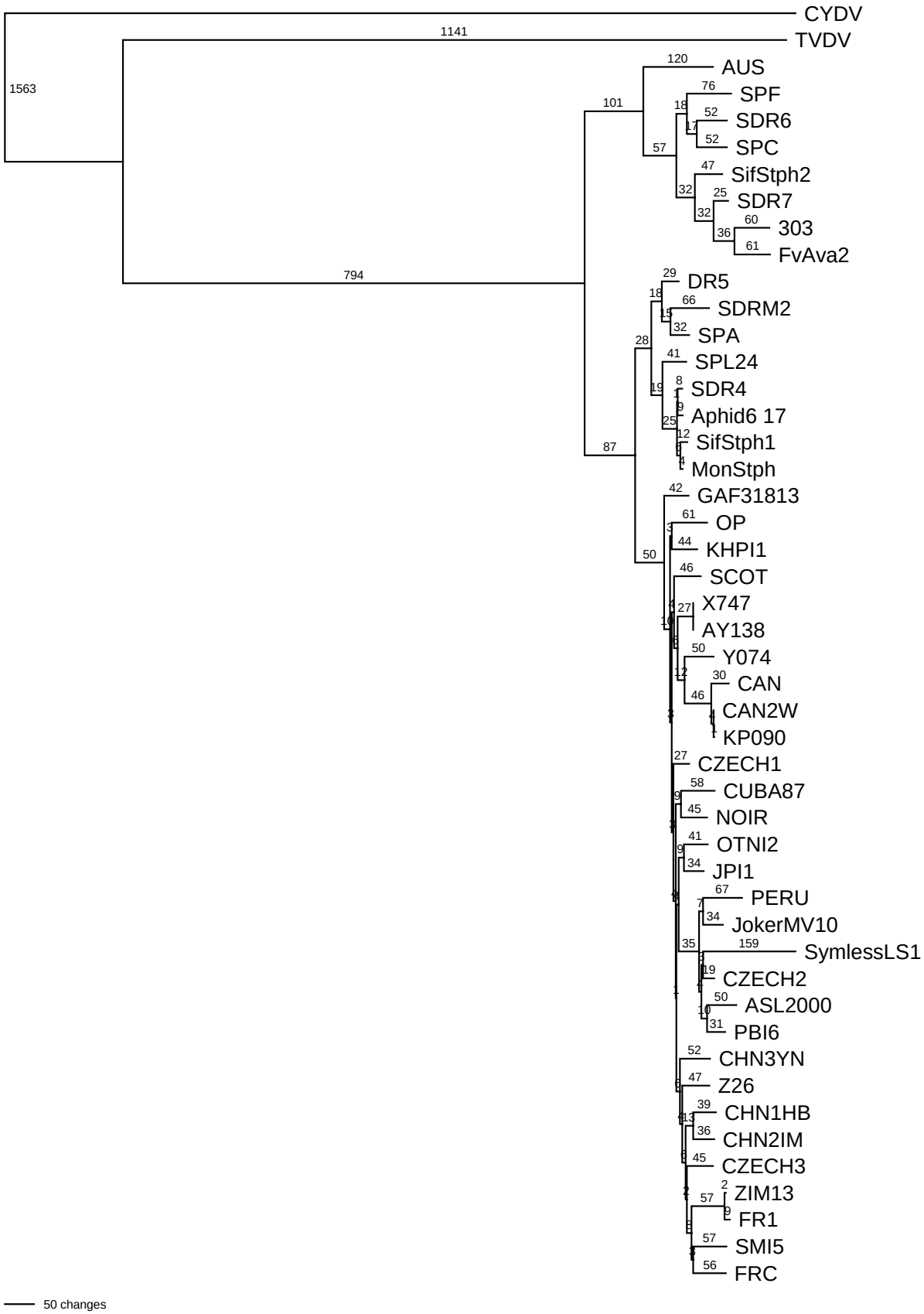

Figure 4.4: One of the shortest trees found by a heuristic search performed on the PLRV whole genome sequence matrix. Branch lengths are indicated. The ingroup, excluding the outgroups CYDV-RPV and TVDV, was expanded and is shown in more detail in Figure 4.5. Accession numbers and country of origin are shown in Table 3.1.

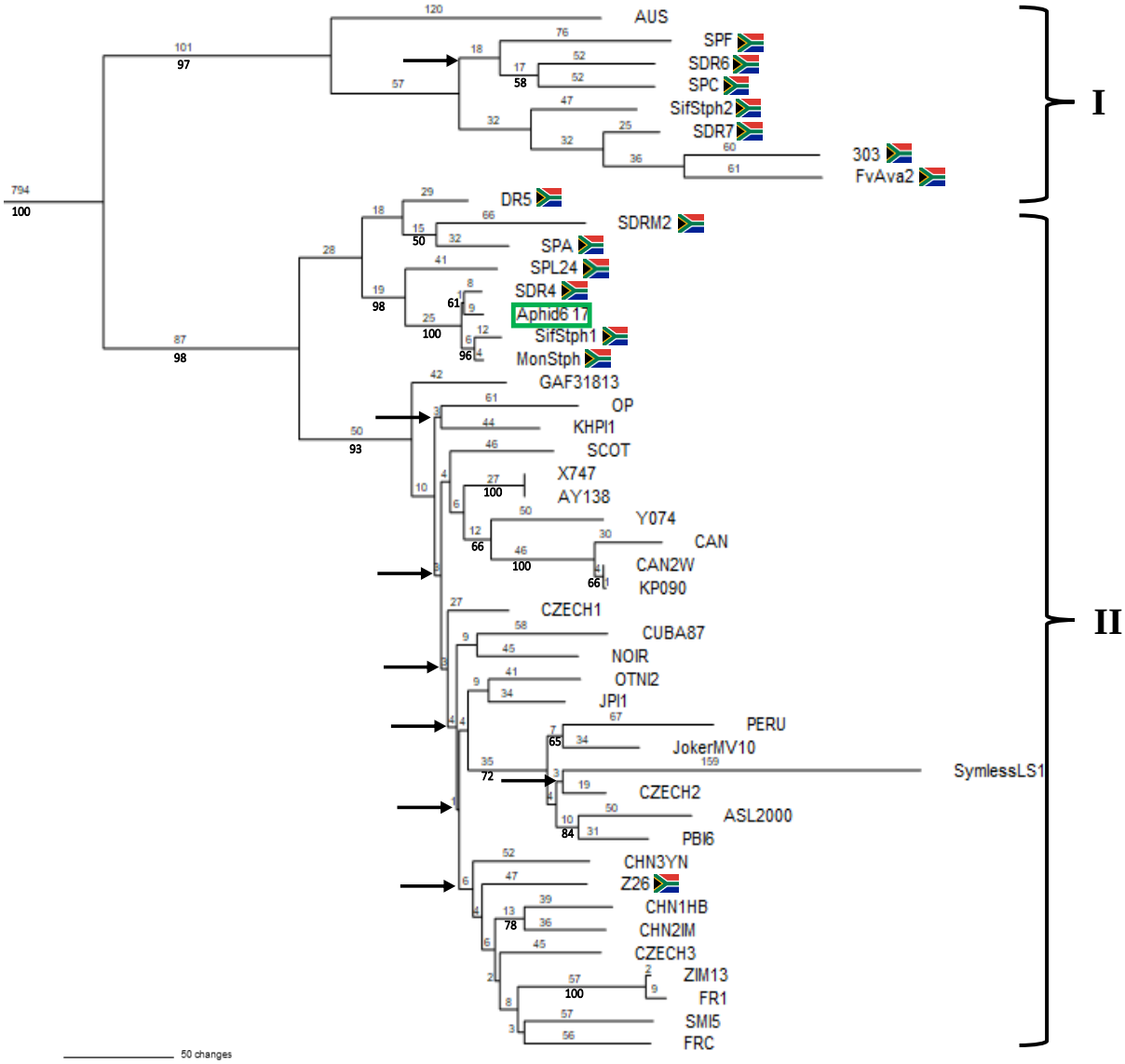

Figure 4.5: The expanded tree from Figure 4.4. A green box indicates the sequenced PLRV isolate Aphid6_17. Bootstrap values are indicated below branch lengths (branch length "794" is not drawn to scale). Black arrows illustrate nodes which collapsed in the strict consensus tree. Accession numbers and country of origin are shown in Table 3.1.

Phylogenetic analysis of the 48 whole genome sequences retrieved two major clades, i.e. clade I and clade II (Figure 4.4 and 4.5). The isolate Aphid6_17 was found to be related to the second group of eight isolates sequenced in our laboratory (Roos, unpublished) as it was located in clade II. Clade I consists of an Australian isolate and seven South African isolates.

4.4 Discussion

Two *M. persicae* aphids from the Sandveld region were found to be infected with PLRV using an RT-qPCR assay to detect the virus. No *Brevicoryne brassicae* were found to be infected with PLRV. In *M. persicae*, two positive RT-qPCR Cq values of 23 and 25 indicated high virus titres of between 3 000 to 30 000 viral copies (Figure 3.3). Only one whole PLRV genome was generated from RT-

PCR amplification and Sanger cycle sequencing, Aphid6_17. The 10 fold fewer (3 000 to 30 000) viral copies of the other PLRV isolate that were extracted from the aphid appear to have been too low for amplification of all of the whole genome segments. This was evidenced by poor bands on a 2% agarose gel after PCR segment amplification.

The phylogenetic analysis performed in this study confirms that Australian type PLRV isolates occur in SA. Rothman (2007) established the presence of Australian type PLRV isolates (Thomas, 1984) by sequencing CP genes only. Roos (2013, unpublished) sequenced whole genomes of South African isolates confirming the presence of Australian type isolates in SA. Furthermore, Roos (unpublished) found a second clade of South African isolates, into which the Aphid6_17 grouped. However, Roos (unpublished) found that European type PLRV isolates also occurred in SA, i.e. the Z26 isolate. The research in which South African isolates were characterised forms part of the PhD study of Roos which is to be submitted shortly. The method of phylogenetic analysis chosen, i.e. parsimony, is routinely used in this laboratory and is found to be equally suitable to other methods which is why it was used in this study.

The PLRV sequence of the isolate Aphid6_17 confirms that *M. persicae* is the viral vector, as the aphid extracted PLRV sequence grouped with seven South African PLRV isolates that were obtained from the Sandveld region. This also confirms that the aphids transmit South African PLRV isolates. The fact that all 21 *Brevicoryne brassicae* were found not to be infected, whilst two out of 31 *M. persicae* were found to be infected, confirms the importance of *M. persicae* as a PLRV vector in the Sandveld region.

This is the first study to sequence and characterise a whole PLRV genome (whole genome in the context of this study refers to the entire coding region) extracted from an aphid. This study determined an appropriate Cq value for whole PLRV genome sequencing of an isolate extracted from an aphid. Although this approach proved to be effective to produce a PLRV whole genome sequence from an individual *M. persicae*, NGS may be a more efficient technique to sequence multiple isolates from infected aphids.

Chapter 5: The application of next generation sequencing to obtain complete genome sequences of PLRV isolates from the Sandveld region

5.1 Introduction

RNA viruses produce considerable amounts of dsRNAs during replication which activates the host defence pathway, RNAi, to produce sRNAs, known as siRNAs. A novel strategy for virus detection and genome sequencing has been developed by sequencing siRNAs (Donaire *et al.*, 2008, 2009; Kreuze *et al.*, 2009; Wu *et al.*, 2010; Candresse *et al.*, 2014) as it is less technically demanding to prepare samples for total sRNA (including siRNAs and miRNAs) sequencing than dsRNA purification protocols (Wu *et al.*, 2015). This approach, known as virus discovery by deep sequencing, uses enriched sRNAs from diseased cells or tissues to assemble large sequence contigs from NGS sequencing (Wu *et al.*, 2015) with or without prior knowledge about the sequence information for novel virus discoveries. It is also identifies viruses in low titres that are not readily detected by other methods (Kreuze, 2014; Wu *et al.*, 2015). Kreuze *et al.* (2009) was able to detect and identify RNA and DNA viruses in low titre as well as symptomless infections from sweet potatoes with the use of high-throughput sRNA sequencing. *De novo* assembly or mapping to reference has been used for viral discovery in deep sequenced samples (Kreuze *et al.*, 2009; Coetzee *et al.*, 2010; Hwang *et al.*, 2013; Maree *et al.*, 2015).

This study aimed to apply a NGS system, the Ion Proton to identify and characterise PLRV isolates, compare non-coding upstream and downstream regions from coding genes and identify unknown viruses that possibly occur in potatoes in the Sandveld region using a similar approach. For NGS analysis, total sRNAs was first extracted and enriched by a mirVana miRNA Isolation Kit from six PLRV infected samples from the Sandveld region. Ion Proton sequencing of six PLRV isolates was performed by labelling sequence reads from different sources with specific fluorescent tags. Library preparation and quantification, template quantification and sequencing was performed at the Stellenbosch Central Analytical Facility. NGS sample data were *de novo* assembled and mapped to PLRV reference whole genomes. Whole genome sequences generated were compared by alignment and phylogenetic analysis.

5.2 Materials and Methods

5.2.1 *Sample collection and preparation for RT-qPCR*

Six visibly PLRV infected potato leaves, five from the cultivar Mondial and one from the cultivar Labadia, were collected in May and June 2017 in the Sandveld region.

Sample preparation of potato leaves for RT-qPCR was used as described previously in section 3.2.6.

5.2.2 *RT-qPCR*

Reaction mixture and thermal profile for RT-qPCR were used as described previously in section 3.2.7.

5.2.3 *sRNA extraction*

The mirVana miRNA Isolation Kit (Invitrogen) was used to extract and enrich sRNA species (siRNAs and miRNAs) 21 to 24 nts in length from fresh potato leaf material. This consisted of an organic extraction, i.e. disruption in a denaturing lysis buffer and Acid-Phenol : Chloroform extraction, followed by RNA immobilisation on glass-fibre filters, according to the manufacturer's protocol.

5.2.4 *sRNA processing, quantitative and qualitative assessment*

Enriched 21 to 24 nts sRNA samples were sent for processing and sequencing at the Central Analytical Facility (CAF), University of Stellenbosch. The quantity and quality of these sRNAs was assessed on the BioAnalyzer 2100 using the sRNA chip and reagents kit (Agilent Technologies), according to the G2939-90094 REV.C. protocol. RNA was concentrated for a starting amount larger than 1 ng of sRNA for samples 1, 2, 3 and 4. Samples 5 and 6 yielded sufficiently high quantities of sRNA obviating the additional concentration step.

5.2.5 *Library preparation*

sRNAs 21 to 24 nts in length were converted to a representative cDNA library using the Ion Total RNA-Seq Kit v2 for strand specific sequencing on the Ion Proton system according to the MAN0010654 REV.C.O. protocol. Briefly, 3 µl of these sRNAs were hybridised (an adapter consisting of a degenerate sequence bound to a defined sequence hybridise to the fragmented ssRNA) and then the adapters were ligated at 16˚C overnight. The sRNA, with adapters, was reverse transcribed to generate single stranded cDNA libraries. These cDNA products were purified and size selected using the magnetic bead cleanup module from the RNA-Seq kit. The purified cDNA products were amplified on the SimplyAmp Thermal Cycler (ThermoFisher Scientific) to prepare barcoded cDNA libraries using the Ion Xpress RNA-Seq Barcode Kit. The process of library preparation is shown in Figure 5.1.

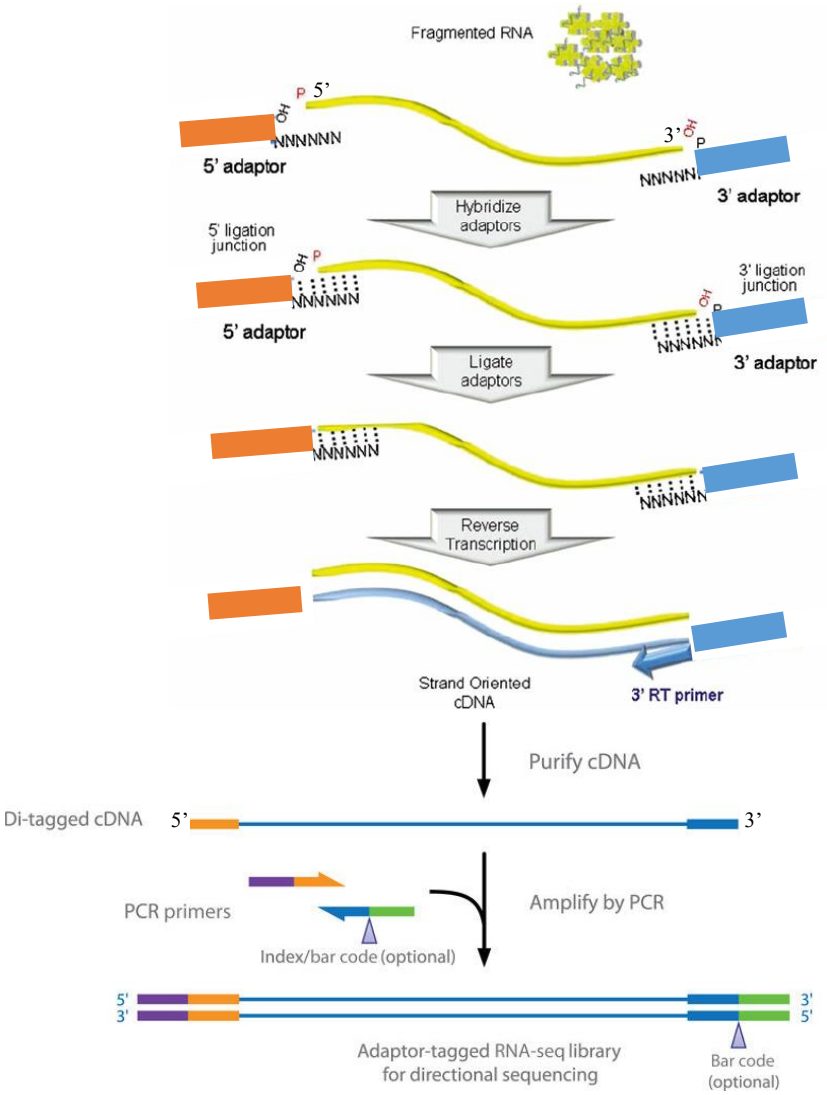

Figure 5.1: General diagram illustrating library preparation of sRNA samples for Ion Proton sequencing. This diagram was modified from Pease and Sooknanan (2012) and Chierico *et al.* (2015).

5.2.6 *Library quantification*

Libraries from each sample were purified and assessed for yield and fragment size distribution using the High Sensitivity DNA Kit and chips on the BioAnalyser 2100 (Agilent Technologies) according to the G2938-90322 REV.C. protocol.

5.2.7 *Template preparation and enrichment*

A target concentration of 80 pM was obtained by diluting the libraries. Equimolar amounts of the diluted barcoded cDNA libraries were combined for sequencing template preparation using the Ion PI Hi-Q Chef Kit. Briefly, 25 µl of the pooled diluted library was loaded on the Ion Chef liquid handler according to the protocol, MAN0010967 REVB.O.

5.2.8 *Sequencing*

The use of sequencing solutions, reagents and supply kits on the Ion Proton System leads to massive parallel sequencing according to the MAN0010850 REVD.O. protocol. Flow space calibration and basecaller were used for analysis of sequencing results using standard analysis parameters in the TorrentSuite version 5.4.0 Software.

5.2.9 *NGS data analysis*

5.2.9.1 Map to reference and de novo assembly

The sequence reads of each of the six samples were mapped to the Australian PLRV genome (GenBank Accession Number D13953, hereafter referred to as AUS), followed by mapping to a CIP accession PLRV isolate GAF318_13 (GenBank Accession Number KU586456, hereafter referred to as GAF318_13) and a potato genome that consisted of twelve chromosomes using a "Map to Reference" Geneious 10.2.3 plugin with default parameters. After potato genome mapping the unused reads were used for mapping to the AUS and GAF318_13 and *de novo* assembly was performed using a "*De novo* Assemble" Geneious 10.2.3 plugin with default parameters or a short read assembler Velvet 1.2.10 (Zerbino and Birney, 2008). The NCBI database was used for sequence similarity searches of the *de novo* assembled contigs using BLASTn.

The logarithm of the reads, which mapped to AUS, from the Mondial cultivar samples (MB2_8, MJ1_10, MJ2_11, miV1_12 and miV3_13) was compared to their RT-qPCR Cq value.

Samples were analysed with SimPlot version 3.5.1 (Ray, 2003) which showed comparisons between the sample's genome and a reference genome.

5.2.9.2 Sequence coverage of siRNAs

The distribution of each sample's siRNAs to AUS, sequence depth at different nt positions on the genome, total AUS coverage and mean sequencing depth were obtained from Geneious 10.2.3.

5.2.9.3 Nucleotide alignment for PLRV whole genomes

Samples' siRNA mapping to AUS was used to generate whole genome sequences. These sequences, outgroups CYDV-RPV and TVDV and other available PLRV whole genome sequences downloaded from GenBank (Table 3.1) were aligned using a Geneious 10.2.3 MUSCLE alignment plugin with default parameters and further aligned manually.

5.2.9.4 Phylogenetic analysis of the PLRV-derived siRNA whole genome sequences

Phylogenetic analysis of the aligned PLRV whole genome sequences was performed using PAUP.

5.2.9.5 *5' and 3' non-coding regions*

Upstream and downstream (5' and 3') non-coding regions of PLRV whole genomes were compared on Geneious 10.2.3 in order to assess possible deviations in the sequences of these regions from SA isolates in comparison to published sequences on GenBank.

5.3 Results

5.3.1 *RT-qPCR*

In Table 5.1, five of the six samples PLRV were detected with the RT-qPCR. The miV3_13 sample tested negative as its Cq value was above the cut-off Cq value of 34 (see section 3.3.4).

Table 5.1: RT-qPCR Cq values of potato leaves collected between May and June 2017 from the Sandveld region, SA.

Sample	MB2_8	LB3_9	MJ1_10	MJ2_11	miV1_12	miV3_13
Cq	28.56	28.26	31.59	22.75	33.99	38.10

5.3.2 *Total sRNA quality and quantity*

In all of the samples, the fraction 21 to 24 nts sRNAs were more than 0.5% of the total sRNAs (Table 5.2) confirming sufficient enrichment of RNA.

Table 5.2: Small RNA (sRNA) quality control assessment results from the BioAnalyzer 2100 with total sRNAs, 21 to 24 nts sRNA and fraction of 21 to 24 nts sRNA to total sRNA.

Sample	**Total sRNA (pg/µl)**	**21-24 nts sRNA (pg/µl)**	**Fraction 21-24 nts sRNAs as percentage of total sRNA**
MB2_8	1408.4	231.6	16
LB3_9	3763.5	682.6	18
MJ1_10	2959.2	566.1	19
MJ2_11	4310.4	986	23
miV1_12	20121.7	3717.8	18
miV3_13	23450.6	4321.6	18

5.3.3 *Library quantification*

Since about 50% of the fragments present were between 89 nts and 110 nts in length consisting of 2 x 40 nts adapters + (9 to 30 nts sRNA fragments) (Table 5.3), the libraries were considered sufficient for template preparation and enrichment. After cDNA synthesis new adapters with a barcode were ligated to the double stranded cDNA. These adapters are specific for the 5' and 3'ends of the template, but ligation errors might occur (template-template, 5'-template-5', 3'-template-3'). Thus, enrichment of samples occurs when Streptavidin beads C1 (Thermo Fisher Scientific) are added to the ligated

double stranded cDNA to bind and remove specific error 3' P1 adapters. During sequencing a second enrichment occurs as only specific 5' adapters with double stranded cDNA are amplified.

Table 5.3: Molarity of library quantity and percentage fragments smaller than 110 nts.

Library ID barcode	[Library] 89-110 nts (nM) representing 9 to 30 nts sRNAs	Total [library] (nM)	Percentage fragments <110 nts
MB2_8	36.62	57.24	61
LB3_9	45.35	79.51	56
MJ1_10	40.53	86.98	47
MJ2_11	60.69	121.23	48
miV1_12	59.38	109.09	49
miV3_13	64.19	111.66	53

5.3.4 *NGS data analysis*

5.3.4.1 Map to reference and de novo assembly

siRNA and miRNA profiles indicated read lengths of between 22 nts and 24 nts. The number of each sample's total reads that mapped to AUS and GAF318_13 are indicated in Table 5.4.

Table 5.4: Total reads obtained from sequencing with Ion Proton and number of reads mapped to the Australian (AUS) and GAF318_13 PLRV genomes.

Sample	Total reads	AUS	% of total reads	GAF 318_13	% of total reads	AUS - GAF
MB2_8	7 476 686	25 840	0.3456	24 925	0.3334	915
LB3_9	6 942 975	6 579	0.0948	6 086	0.0877	493
MJ1_10	3 844 501	7 561	0.1967	7 549	0.1964	12
MJ2_11	6 957 213	207 742	2.9860	206 183	2.9636	1 559
miV1_12	10 634 775	2 464	0.0232	2 791	0.0262	-327
miV3_13	11 147 630	66	0.0006	61	0.0005	5

The percentage of total reads that mapped to AUS and GAF318_13 was between 0.0005% and 2.986% (Table 5.4). More reads mapped to AUS than GAF318_13, except those of the miV1_12 sample in which this trend was reversed (Table 5.4).

After each sample's total reads were mapped to the potato genome (chromosome 1 to 12), some of the remainder mapped to AUS and GAF318_13 (Table 5.5).

Table 5.5: Total reads obtained from sequencing with Ion Proton and number of reads mapped to the potato genome and number of unused reads that mapped to the Australian (AUS) and GAF318_13 PLRV genomes.

Sample	Total reads	Potato	%	Reads left	%	AUS	% reads left	GAF 318_13	% reads left	AUS - GAF
MB2_8	7 476 686	5 603 308	74.9	1 873 378	25.1	17 440	0.9309	16 350	0.8728	1090
LB3_9	6 942 975	4 980 416	71.7	1 962 559	28.3	5 145	0.2622	4 693	0.2391	452

MJ1_10	3 844 501	2 658 776	69.2	1 185 725	30.8	5 693	0.4801	5 695	0.4803	-2
MJ2_11	6 957 213	4 922 709	70.8	2 034 504	29.2	50 479	2.4811	49 471	2.4316	1008
miV1_12	10 634 775	6 875 493	64.7	3 759 282	35.3	1 985	0.0528	2 271	0.0604	-286
miV3_13	11 147 630	6 839 123	61.4	4 308 507	38.6	37	0.0009	35	0.0008	2

The percentage of total reads that mapped to the potato genome was between 61.4% and 74.9% (Table 5.5). From the unused reads between 0.0008% and 2.4811% mapped to AUS and GAF318_13 (Table 5.5). More reads mapped to AUS than GAF318_13, except those of the miV1_12 sample (Table 5.5).

The MJ2_11 sample had the highest overall number of reads mapped to the Australian (207 742 and 50 479) and GAF318_13 (206 183 and 49 471) PLRV isolates, whereas the miV3_13 sample had the overall lowest number (35 to 66) even though the latter had the highest total reads (11 147 630) (Table 5.4 and 5.5). After the reads mapping to the potato genome were removed, all samples had a higher percentage of total reads that mapped to AUS and GAF318_13 than the starting percentage of total reads that mapped to AUS and GAF318_13, except for the MJ2_11 sample. Only about 0.0005 to 0.0009% (Table 5.5 and 5.6) of the total reads in the miV3_13 sample mapped to AUS and GAF_13.

De novo assembly of siRNAs (after removal of reads that mapped to the potato genome and PLRV genomes) using Genious 10.2.3 yielded longer contigs than those assembled using Velvet 1.2.10. The number of contigs varied between samples (97 857 for the MB2_8 sample, 91 791 for the LB3_9 sample, 44 891 for the MJ1_10 sample, 78 614 for the MJ2_11 sample, 80 443 for the miV1_12 sample and 76 000 for the miV3_13 sample). The longest *de novo* contig was 529 nts in length (Table 5.6). Blast searches identified contigs from all of the samples that mapped to the uncultured soil fungus clone Indiana (Acc. numb. KT194413) although these contigs were different in length.

Table 5.6: NCBI BLASTn results of each sample's longest *de novo* assembled contig using Geneious 10.2.3.

Sample	Length (nt)	Short description	E value	Accession number
MB2_8	529	*Keiskea australis* internal transcribed spacer	1×10^{-67}	KY552534
LB3_9	246	*Vitis coignetiae* chloroplast	6×10^{-14}	LC333363
MJ1_10	427	*Lonicera acuminata* isolate internal transcribed spacer	6×10^{-78}	FJ372925
MJ2_11	391	Uncultured soil fungus clone Indiana	1×10^{-35}	KT194413
miV1_12	389	*Tupidanthus calyptratus* internal transcribed spacer	1×10^{-54}	AF229769
miV3_13	418	*Gentiana straminea* clone internal transcribed spacer	2×10^{-71}	KM236549

Figure 5.2 shows the logarithm of the reads, that mapped to AUS from the Mondial cultivar samples (MB2_8, MJ1_10, MJ2_11, miV1_12 and miV3_13), compared to their RT-qPCR Cq value.

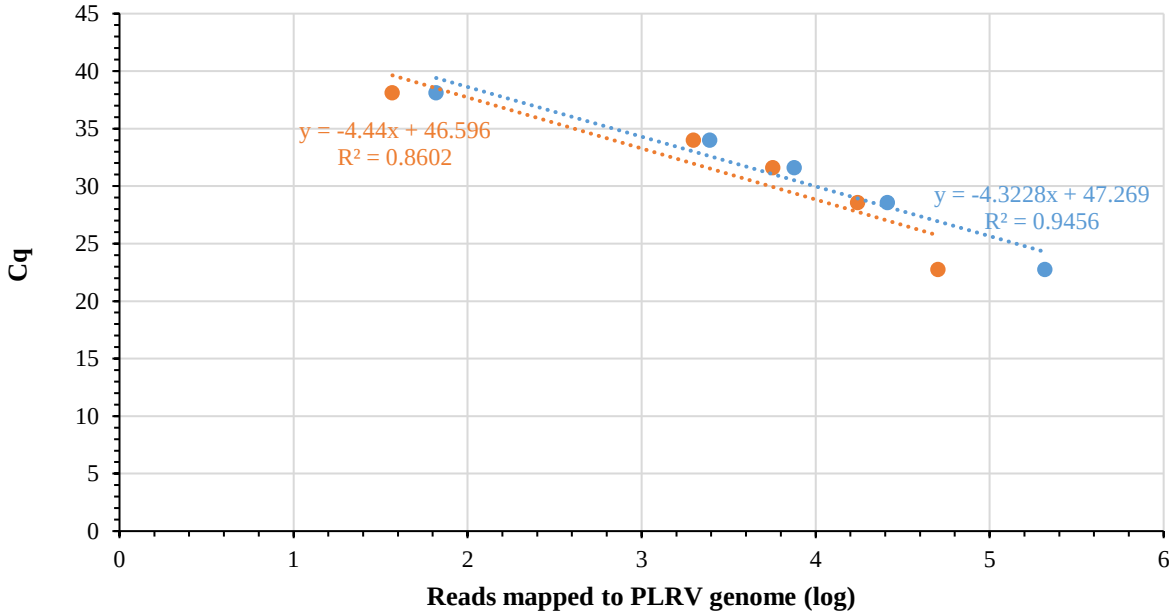

Figure 5.2: Logarithm of the five Mondial cultivar samples' reads mapped to the Australian PLRV genome (AUS), before (blue) and after (orange) mapping to the potato genome, compared to their RT-qPCR Cq value.

5.3.4.2 Sequence coverage of siRNAs

The MJ1_10 sample was found to be infected with two PLRV isolates, an Australian and a South African recombinant, as ambiguities were seen between nt positions 880 and 2000 as can been seen from alignment matrix in Appendix B. However, the Simplot analysis (Figure 5.3) showed that from positions 1 to 880 and 2000 to 6000 the MJ1_10 sequence showed no repetitive pattern of ambiguities and was very similar to South African isolates.

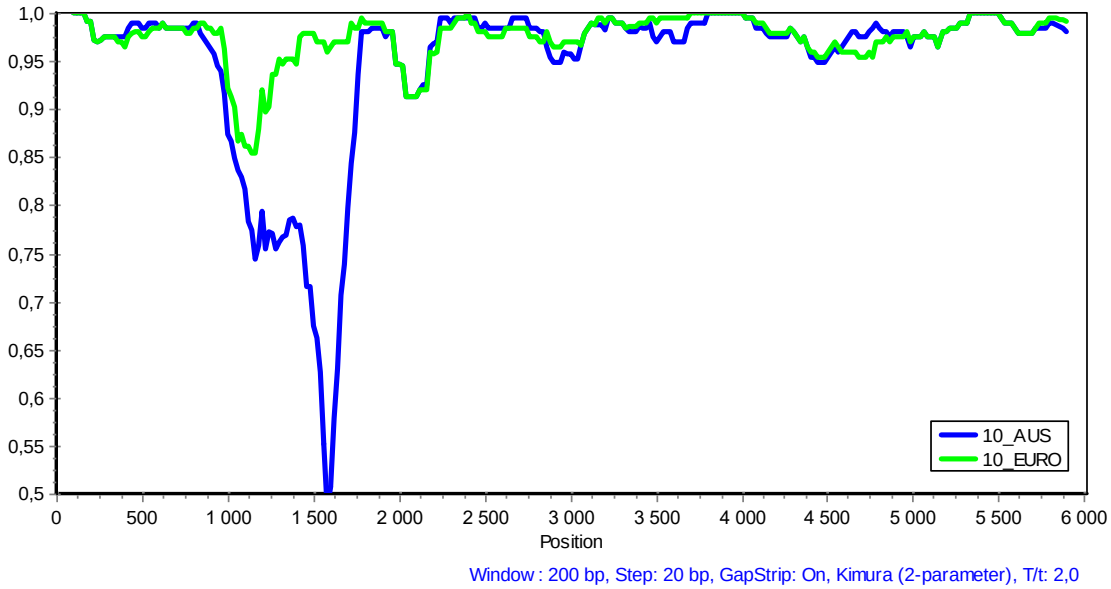

Figure 5.3: Simplot graphic view of two isolates (10_AUS and 10_EURO) represented in the MJ1_10 sample against the PLRV isolate Aphid6_17.

From the miV3_13 reads only 0.0005 to 0.0009% (Table 5.4 and 5.5) mapped to AUS and GAF318_13 as a result of which it was not sensible to generate a distribution map of the reads to AUS.

The whole genome sequences from the LB3_9 sample showed ambiguities between nt positions 1020 and 1930. The LB3_9 sample may therefore also be infected with more than one isolate, but this requires further investigation including an analysis of recombination which falls outside the scope of this thesis. However, the LB3_9 sequence was still included in the phylogenetic analysis because parsimony analysis uses the largest part of the sequence of a genome to assign it to its nearest relative.

Samples MB2_8, LB3_9, MJ2_11 and miV1_12 were used from here onwards and their siRNA distribution over AUS is shown in Figure 5.4 and 5.5.

The MB2_8 sample had a high mean sequence depth (205 to 383X) between nt positions 3336 and 5707 with the highest peak at nt position 5696 (Figure 5.4A). A high mean sequence depth (64 to 119X) was shown for the sample LB3_9 between nt positions 3360 and 5739 with the highest peak at nt position 5141 (Figure 5.4B). The MJ2_11 sample had a high mean sequence depth (906 to 2153X) between nt positions 115 and 142; 3498 and 5401 with the highest peak at nt position 4032 (Figure 5.5A). A high mean sequence depth (26 to 60X) was shown for the miV1_12 sample between nt positions 3406 and 5869 with the highest peak at nt position 5140 (Figure 5.5B). Overall a high number of siRNAs accumulated at the 3’ region of the viral genome between positions 3400 and 5400 or 3400 and 5800 which corresponds to the overlapping CP, MP, RTP, P6 and P7 genes (ORF3, ORF4, ORF5, ORF6 and ORF7 respectively).

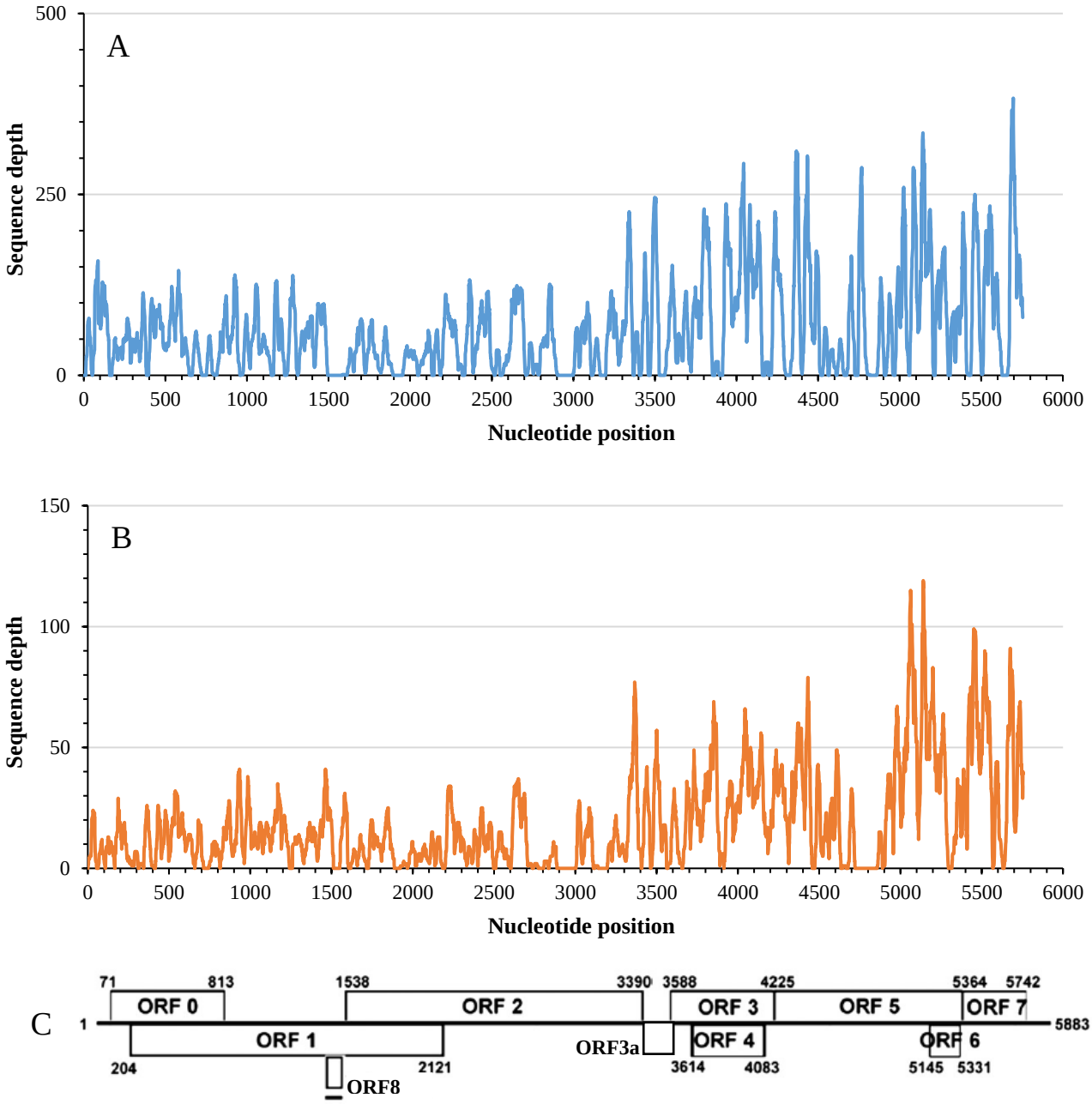

Figure 5.4: Distribution of siRNAs mapped to the Australian PLRV genome (AUS) as sequence depth at specific nt positions. MB2_8 (A) and LB3_9 (B). The y-axis scale of each sample differs. Schematic diagram of genomic organisation of PLRV with ten open reading frames (ORF) that encode 11 proteins, obtained from Hwang *et al.* (2013) (C).

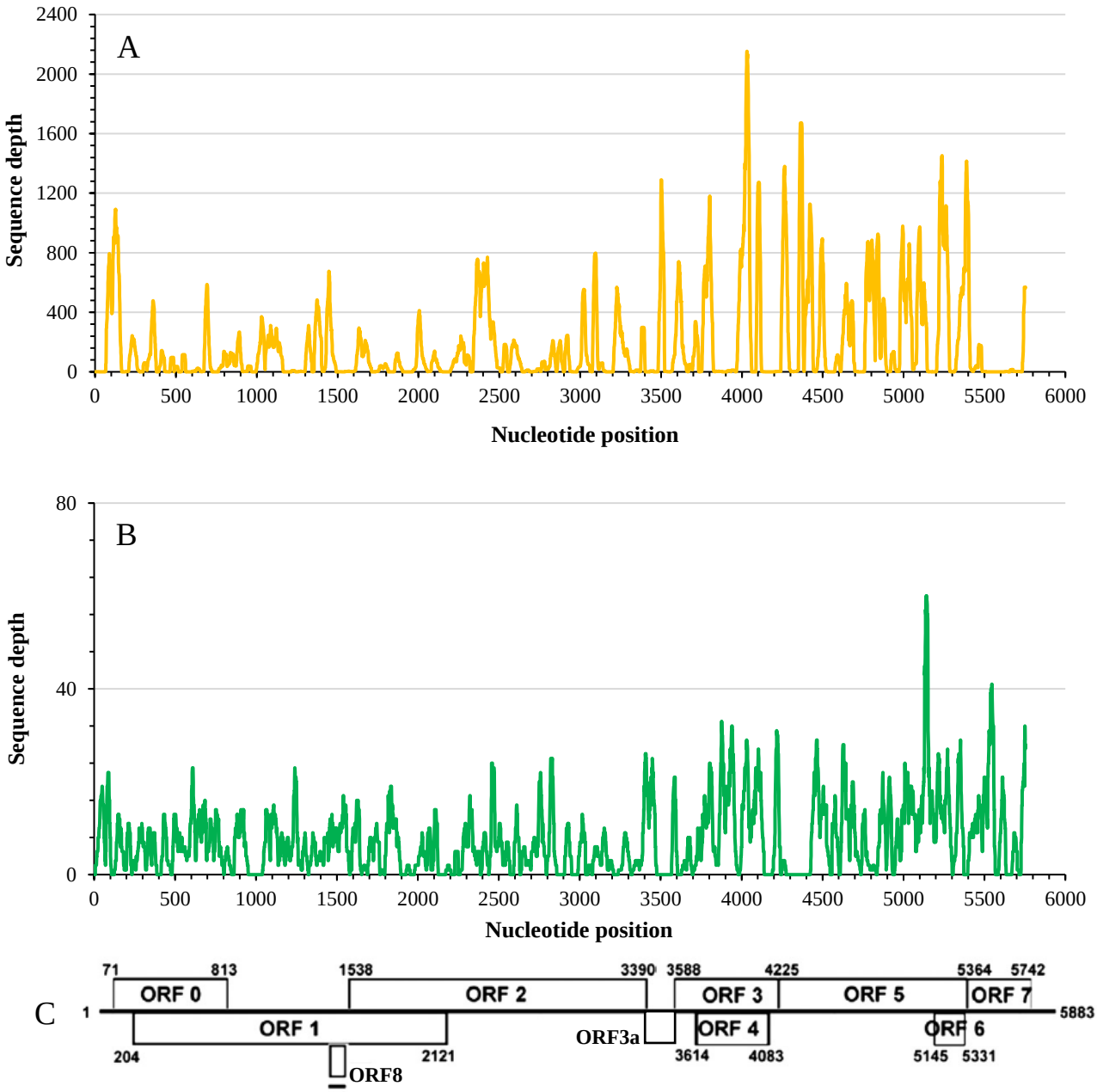

Figure 5.5: Distribution of siRNAs mapped to the Australian PLRV genome (AUS) as sequence depth at specific nt positions. MJ2_11 (A) and miV1_12 (B). The y-axis scale of each sample differs. Schematic diagram of genomic organisation of PLRV with ten open reading frames (ORF) that encode 11 proteins, obtained from Hwang *et al.* (2013) (C).

siRNA distribution of each sample, independent of sizes, along the PLRV genome is presented as coverage and overall mean sequencing depth in Table 5.7.

Table 5.7: Whole genome coverage and mean sequencing depth of each sample against the Australian PLRV genome (AUS).

Sample	Total reads mapped		Reads left mapped	
	Coverage	Mean sequencing depth	Coverage	Mean sequencing depth
MB2_8	98.3	95.6	86.3	65.1
LB3_9	95.9	24.3	88.2	19
MJ1_10	96.1	28	87.7	21.2
MJ2_11	100	801.2	83	201.4
miV1_12	82.2	9.2	77	7.4
miV3_13	13.9	0.2	11.7	0.1

The siRNA distribution of all the samples was not uniform and had varying gaps, except for the MJ2_11 sample. It covered the whole AUS when reads were mapped to the reference. Reads that mapped to AUS after the potato genome reads had been removed, only covered 83% of the reference genome. Coverage and mean sequencing depth of all the samples' siRNAs/reads were decreased when potato genome reads were removed. Table 5.7 shows the miV3_13 sample only covers 11.7% and 13.7% of AUS with a sequence mean depth between 0.1 and 0.2. This is, however, in agreement with the high Cq value and low number of reads that mapped to AUS and GAF318_13.

5.3.4.3 Phylogenetic analysis of the PLRV-derived siRNA whole genome sequences

Samples of PLRV-derived siRNA whole genome sequences varied in consensus sequence length. MB2_8 was 5 933 nts, LB3_9 was 5 861 nts, MJ2_11 was 6 775 nts and miV1_12 was 5 779 nts in length. These sequences were aligned with the 50 whole genome sequences of PLRV with outgroups, CYDV-RPV and TVDV, and used to generate a nexus file. This was analysed using parsimony in PAUP. The total character length of 6 899 consisted of 3 363 constant characters, 1229 parsimony informative characters and 2 307 parsimony uninformative variable characters (Figure 5.6). A heuristic search retrieved a tree with a length of 6 371 nts with a CI of 0.701 and a RI of 0.693.

Alignment and phylogenetic analysis of the 52 whole genome sequences retrieved two major clades, clade I and clade II (Figure 5.6). The sequenced MB2_8, LB3_9 and MJ2_11 samples were placed in clade I together with an Australian and seven other South African isolates, whereas the sequenced miV1_12 sample was placed in clade II together with nine other South African isolates. Phylogenetic analysis therefore confirmed that PLRV isolates similar to those identified by Roos (unpublished) occur in the Sandveld region.

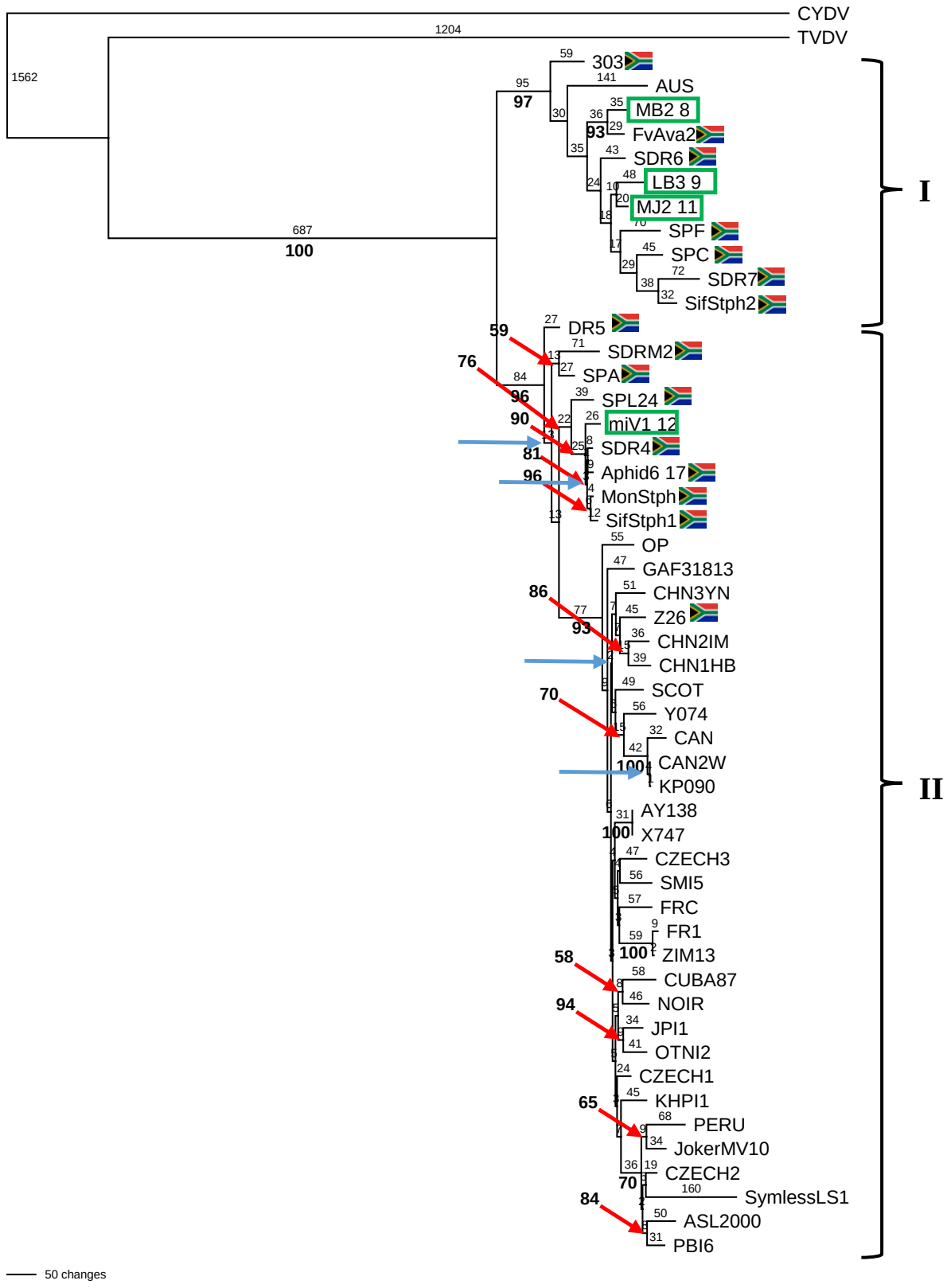

Figure 5.6: One of the trees retrieved from a heuristic search performed on the PLRV whole genome sequence matrix. Green boxes indicate the sequenced PLRV isolates MB2_8, LB3_9, MJ2_11 and miV1_12. Branch lengths are indicated above and bootstrap values are indicated in bold below each line or with a red arrow. Blue arrows indicate nodes which collapsed in the strict consensus tree. Accession numbers and country of origin are shown in Table 3.1.

5.3.4.4 *5' and 3' non-coding regions*

An alignment of the 5' non-coding regions is shown in Figure 5.7.

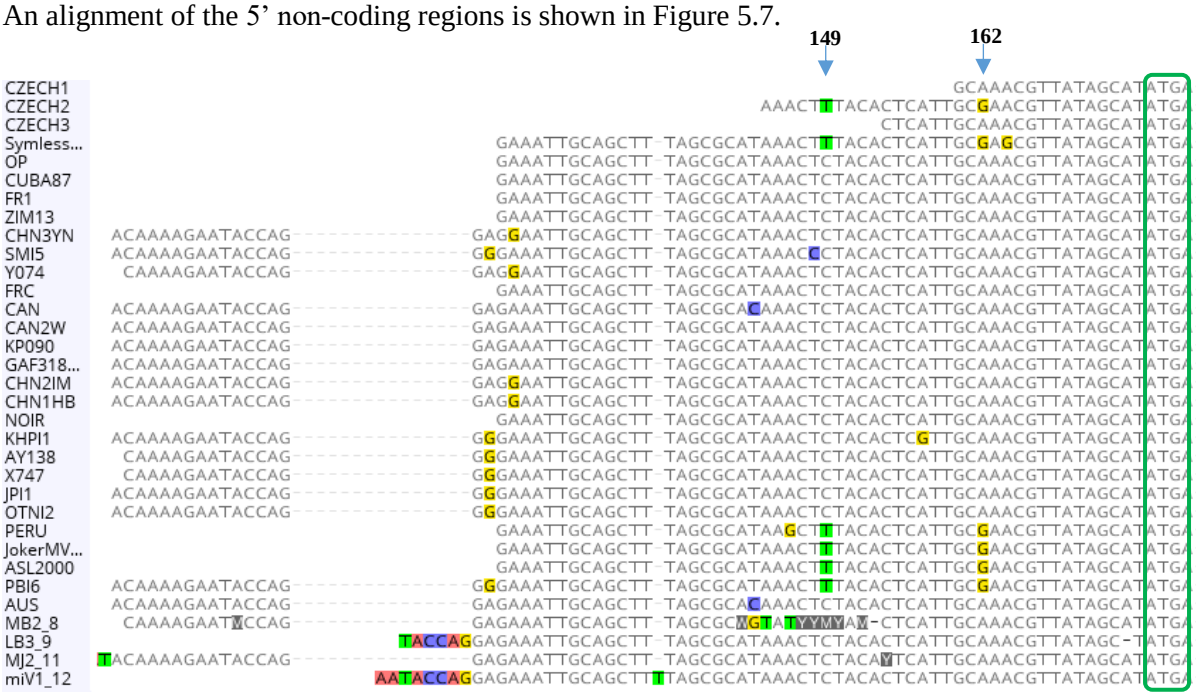

Figure 5.7: Upstream (5') non-coding region from nt position 90 to 179 of PLRV whole genomes obtained from GenBank and sequenced in this study. Green box represents P0 start codon.

Differences between PLRV isolates are only seen at nt position 149 showing a C or T and nt position 162 showing a G or A (Figure 5.7). With the exception of isolated bases no major upstream variation was seen between the isolates sequenced in this study.

An alignment of the 3' non-coding region is shown in Figure 5.8.

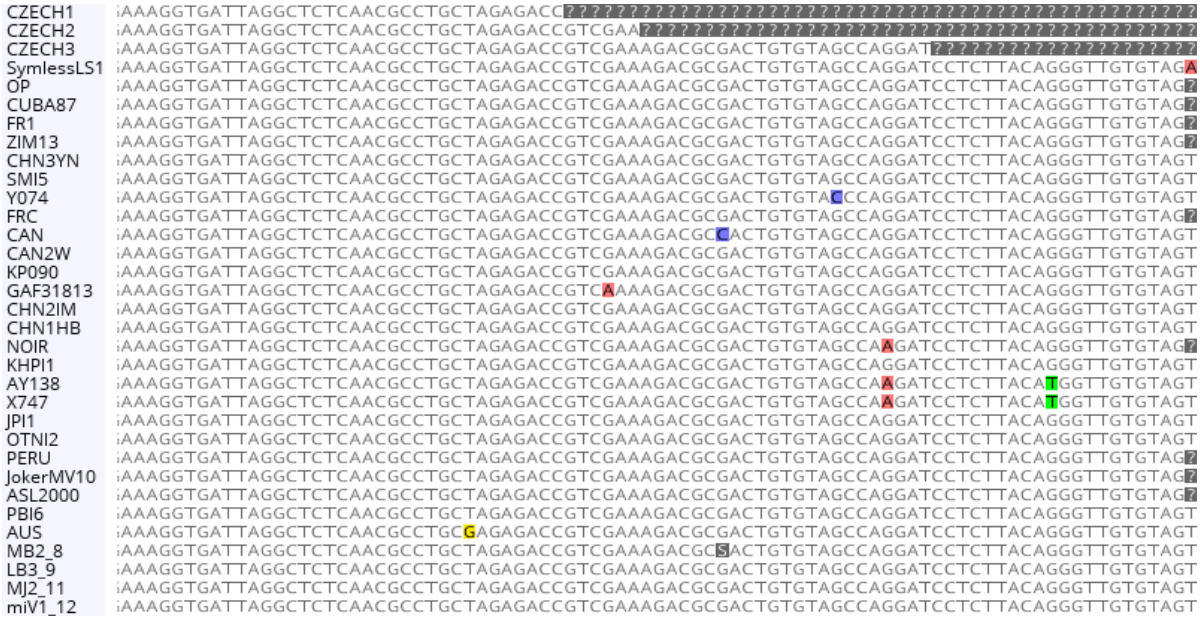

Figure 5.8: Downstream (3') non-coding region from nt positon 6560 to 6646 of PLRV whole genomes obtained from GenBank and sequenced in this study.

Downstream non-coding regions showed no major variation patterns, except for a few different nts (Figure 5.8).

Both the upstream and downstream non-coding regions therefore did not differ significantly from those of other PLRV isolates published on GenBank.

5.4 Discussion

Next generation sequencing was found to be an efficient technique to sequence enriched sRNA from six visually PLRV infected samples from the Sandveld region as previously used to sequence viruses from sweet potatoes by Kreuze *et al.* (2009). Three complete PLRV genomes were generated by mapping their siRNAs to a PLRV whole genome as previously applied by Hwang *et al.* (2013). This was completed in a single NGS run which is much less labour intensive than the Sanger sequencing approaches applied in the study reported in Chapter 4 of this study.

When the Mondial cultivar samples (MB2_8, MJ1_10, MJ2_11, miV1_12 and miV3_13) RT-qPCR Cq values were plotted against their corresponding logarithm of "reads mapped to AUS", before and after potato genome mapping, an inversely proportional correlation was observed. A lower Cq value (below 29) gave a higher number of reads mapped to the Australian PLRV genome (AUS) (between 17 440 and 207 742 siRNAs). For example the MJ2_11 sample (Cq value of 22.75) mapped between 50 479 and 207 742 siRNAs, whereas a higher Cq value gave lower coverage, with the miV3_13 sample (Cq value of 38.10) mapped only between 37 and 61 siRNAs. A higher R^2 was seen for reads mapped to AUS before removal of the miRNAs from the analysis that mapped to the potato genome than seen thereafter (Figure 5.2).

All samples had roughly the same percentage of plant miRNAs, with about 60 to 75% of the miRNAs mapping to the potato genome (Table 5.5). A decrease in number of reads or siRNAs, coverage and mean sequencing depth was seen when samples mapped to AUS after potato genome miRNAs were removed than siRNAs previously mapping to AUS. This may be due to miRNAs that are mostly 22 to 24 nts in length which is consistent with the production of different size sRNAs by DICER-like ribonuclease protein induced by positive-strand RNA viruses (Waterhouse *et al.*, 1998; Donaire *et al.*, 2009) that could bind almost anywhere on the potato genome. This means that some of the reads that mapped to AUS and GAF318_13 in the complete sequence data, also mapped to the potato genome and were consequently removed when screening reads for potato genome mapping. The medium-low sensitivity default Geneious setting for mapping, may have mapped siRNAs or miRNAs that are not 100% identical to AUS, GAF318_13 and the potato genome. An increase, however, was seen in the percentage siRNAs mapped relative to total number of siRNAs. This is essential because it could not be expected that reads would be identical to the reference genome.

The NCBI BLASTn results of most of the samples *de novo* assembled contigs showed no other viruses were found with *de novo* assembly or that mapped to the known potato viruses PVA, PVM, PVS, PVX, PVY, PSTVd or TSWV, but did map to the uncultured soil fungus clone Indiana. Although this is not a virus, it may be an unidentified potato disease in SA causing symptoms to be overlooked or coupled to another disease.

The siRNA distribution found not to be homogenous over the viral genome, which is similar to that found for PLRV (Hwang *et al.*, 2013) and was also found for sweet potato viruses (Kreuze *et al.*, 2009). This may be due to the synthesis of two sgRNAs (sgRNA1 and sgRNA2) between 3400 and 5800 (Hwang *et al.*, 2013). These sgRNAs encode CP, MP, RTP, P6 and P7 at the 3' end of the PLRV genome which caused the greater availability of dsRNA intermediate templates during virus infection (Tacke *et al.*, 1990; Miller and Mayo, 1991; Ashoub *et al.*, 1998). The MJ2_11 sample, with the highest Cq value and siRNAs mapped to AUS and GAF318_13, siRNAs accumulated between nt positions 3400 and 5400, but interestingly enough also between nt positions 115 and 142. This region is also known for encoding P0 that plays a role in suppressing RNA silencing (Hauser *et al.*, 2000), symptom development and/or expression (Pfeffer *et al.*, 2002; Bortolamiol *et al.*, 2007). Even though all the samples clearly showed PLRV symptoms, the MJ2_11 sample was the only one with high siRNA accumulation at this region. Szittya *et al.* (2003) and Chellappan *et al.* (2005) reported that some plants infected at higher temperatures showed fewer symptoms due to efficient RNAi-mediated plant defences, increased siRNA levels. Although the MJ2_11 sample had a high number of siRNAs mapped to the PLRV genome, symptoms were still visible, lending support to the notion that the high production of the MJ2_11 sample's P0 could have suppressed the plant's RNAi mechanism (Hauser *et al.*, 2000). siRNA distribution over the viral genome provided important information about the mechanism of viral defence of the plant against PLRV as the plant siRNAs target important regions of the PLRV genome. It has been found that siRNAs produced from RNAi target conserved sequences containing essential viral factors, e.g. structural proteins such as CP and regulatory proteins such as RdRp, for durable inhibition of virus replication (Lindbo and Dougherty, 1992; Lee *et al.*, 2002; Park *et al.*, 2002).

During these PLRV infections not all sections of the viral genome had siRNAs made against them which resulted in gaps between the siRNAs that mapped to the PLRV's whole genome. These gaps were not the same as minor gaps at nt positions 1800, 3500, 5000 and 5300 found previously by Hwang *et al.* (2013). The sequence of one of the samples, miV1_12, had a gap between 5313 and 5319 nts, but the others had none at these positions. There was an inverse correlation between coverage of the whole genome (total reads mapped) and the Cq values. A lower Cq value (below 29) gave higher coverage (95%), for example the MJ2_11 sample's Cq value was 22.75 giving a 100% coverage, whereas a higher Cq value gave lower coverage, for example the miV3_13 sample's Cq value was 38.10 with only a 13.9% coverage (Table 5.1 and 5.7) and even though it had the highest number of total reads (11 147 630) (Table 5.4). This low Cq values of the MB2_8, LB3_9 and MJ2_11 samples

also indicate high virus titre (high number of dsRNA intermediates), which causes more siRNAs to be produced by the plant to combat viral infection. More siRNAs means more siRNAs mapped to AUS and GAF318_13 leading to higher coverage of AUS and mean depth of sequencing.

The MB2_8 and LB3_9 samples came from the same field and had almost the same Cq value of ±28 which indicates the same level of infection or number of viral copies. The samples were however different potato cultivars: the MB2_8 sample was from the cultivar Mondial and the LB3_9 sample was from the cultivar Labadia. The MB2_8 sample had a higher genome coverage of total reads, 98.3%, which represents more siRNAs produced per plant than the LB3_9 sample with a genome coverage of total reads, 95.9%. The mean sequencing depth of the MB2_8 sample (95.9X) was also higher than the LB3_9 sample (24.3X). This may indicate that the Mondial cultivar has better resistance than the Labadia cultivar to PLRV because it produces more siRNAs to target and destroy invading PLRV RNA.

From the six samples that were sequenced, five could be used to generate complete or almost complete PLRV genome sequences, with three samples missing a few nts. The MJ1_10 sample appeared to be infected with two PLRV isolates illustrated by a high number of ambiguities early in the complete genome sequence. Based on similar criteria, the LB3_9 sample may also be infected with more than one isolate but this requires further analysis before a clear separation of the two isolates contained in the sample can be made. As mentioned before, the miV3_13 sample was found to give a high Cq value in RT-qPCR (above cut-off) a low coverage and very low mapping percentage (0.0005%) to AUS. Due to the low coverage it was not meaningful for it to be included in the phylogenetic analysis. This can be interpreted to either indicate that this plant was in the very early stages of PLRV infection or not infected. If the plant was not infected yet it produced these siRNAs, then it may be that these siRNAs are made to prevent an infection. However, further investigation is needed to confirm this hypothesis. Due to the fact that the other samples (MB2_8, MJ2_11 and miV1_12) had high coverage of total reads that mapped to AUS, their complete genomes could be generated.

The upstream, 5', and downstream, 3', non-coding regions of the sequenced PLRV isolates appeared to have no sequence variation, except at 5' nt positions 149 and 161 (Figure 5.7). The six samples that differ JokerMV10, PBI6, ASL2000, CZECH2, PERU and SymlessLS1 are a unique group previously identified as recombinants by Hühnlein *et al.* (2016), but none of the South African isolates possessed these mutations. This study showed that there is no variations in the 5'and 3' non-coding regions of these four South African isolates in comparison to 5'and 3' non-coding regions of complete PLRV isolate sequences on GenBank. It can therefore be concluded that the pathogenicity of South African PLRV infections cannot be attributed to differences in the promoters of the 5' and 3' non-coding regions that may influence the expression of viral coding genes in the South African isolates.

This study found that the application of NGS technology using an Ion Torrent platform and RNA enriched from potato tissue, can be used to obtain and characterise complete genome sequences of PLRV isolates successfully. It also suggests that this approach may be valuable for identifying other potato viruses or pathogens.

Chapter 6: Conclusions and future perspectives

The first objective of this study was to develop and validate a probe-based RT-qPCR to detect PLRV in potato leaves and tubers. This objective was achieved and a highly sensitive probe-based RT-qPCR was developed. Using the plant material preparation described by La Notte, Minafra and Saldarelli (1997) (with a few adjustments), PLRV infection levels in the Sandveld region were determined. PLRV incidence in the Sandveld region has increased dramatically (Coetsee, 2004, 2005) from 1999 until 2005 (Figure 1.3). This study confirms the high incidence of PLRV in potato leaves and tubers across the Sandveld region. The pathology of PLRV infections in the Sandveld region needs to be considered in the context of plant viral infections in regions with much higher temperatures than the prevailing temperatures in Europe and the USA. In Morocco, high summer temperatures were found to suppress PLRV multiplication in potatoes after primary infection due to higher temperatures (Hanafi *et al.*,1995). The Sandveld region's climate is similar to that of Morocco's and therefore similar climatological effects may occur. Similar to the findings of Hanafi *et al.* (1995) potatoes grown during the warmest months of the year, were found to have low PLRV levels as the conditions are not optimal for viral multiplication and aphid transmission. The high incidence of PLRV infection levels in potato leaves and tubers across the Sandveld region occurred whilst PLRV detection was performed by Potato Certification Services using the BIOREBA ELISA. The use of the much more sensitive detection method for PLRV, RT-qPCR, therefore clearly indicates that the ELISA method used by the South African Potato Certification Scheme is not sufficiently sensitive to control PLRV infections in the Sandveld region. This study also has implications for other potato growing regions in SA. The Limpopo province's potato growing areas experience similar high summer temperatures and it can be expected that PLRV replication may be lower and therefore underestimated. Furthermore, the problem may become far more generalised in SA if global warming increases ambient temperatures.

The second objective of this study was to confirm infection of aphids with PLRV by RT-qPCR and characterise aphid transmitted PLRV isolates by sequencing their whole genomes. *M. persicae* was confirmed as an aphid vector in the Sandveld region by direct RT-qPCR. The PLRV isolate sequenced after extraction from the aphid, when compared with whole genome sequences of other isolates by phylogenetic analysis, groups with isolates extracted from potato leaves in the Sandveld region. This confirms that these aphids are the viral vector and that they transmit PLRV isolates in the region.

The third objective of this study was to apply the next-generation sequencing (NGS) system to identify and characterise a larger number of isolates, to compare 5' and 3' non-coding regions and to identify possibly unknown viruses or other pathogens occurring in potatoes in the Sandveld region. Ion Torrent sequencing was successfully applied to sequence siRNAs and miRNAs that mapped to the PLRV and potato genome. Complete genome sequences could be generated from five of the PLRV infected leaf samples. The cultivar, e.g. Mondial, appears to be more resistant to PLRV as more siRNAs were

produced during viral infection in the Mondial cultivar than in the Labadia cultivar, but this would have to be confirmed by a larger, statistically more robust study. The NGS data also verified that the samples tested were not infected with other potato viruses such as PVA, PVM, PVS, PVX, PVY, PSTVd or TSWV. It can therefore be concluded that the threat to potato production by these viruses is lower in the Sandveld region compared to other parts of the world (Van der Want, 1972; Lacomme *et al.*, 2017; Zhang *et al.*, 2017) and confirms the findings of Roos (2013). However, a fungal pathogen was found in all samples, and needs further investigation to determine whether it is a hitherto unknown pathogen.

The results obtained in this study indicate that urgent measures need to be taken to decrease PLRV infection levels in potatoes in the Sandveld region by applying sufficiently sensitive detection methods such as the RT-qPCR developed in this study to ensure sustainable potato production in the region.

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