DETECTION, DIFFERENTIATION AND GENOME ANALYSIS OF *POTATO VIRUS Y* ISOLATES INFECTING POTATO (*Solanum tuberosum* L.) IN THE MSINGA AREA IN KWAZULU-NATAL, REPUBLIC OF SOUTH AFRICA

by

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Submitted in fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

in the

Discipline of Plant Pathology

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Republic of South Africa

June 2016

Dissertation Summary

Potato (*Solanum tuberosum* L.) is one of the most important food crops grown and consumed worldwide. *Potato Virus Y* (PVY), the type member of the genus *Potyvirus* in the Family *Potyviridae* causes losses as high as 100% in the field. The objective of this study was to determine the incidence of PVY strains infecting potatoes grown in Msinga, a community that largely relies on farming as source of food and income generation in the KwaZulu-Natal province, Republic of South Africa.

The first part of the study focused on detecting PVY on potatoes grown in Msinga. This was successfully achieved by testing 57 potato samples (leaves) exhibiting virus-like symptoms using enzyme-linked immunosorbent assay (ELISA). PVY positive samples were subsequently inoculated onto *Nicotiana tabacum* cv Samsun, a propagation host. Total plant RNA extracted from *N. tabacum* displaying typical PVY symptoms was subjected to reverse-transcription polymerase chain reaction using primers specific to the coat protein gene. The expected 512 bp product was amplified. Samples were further differentiated into serotypes. PVY^{N} and PVY^{O} were the serotypes identified. Symptomatic tobacco leaves were harvested and PVY standard purification procedures were performed. Flexuous rod particles of about 730 nm long and 12 nm wide were observed upon visualization of the purified PVY under the transmission electron microscope. A viral 30 kDa protein band, which corresponds to the PVY coat protein was observed after sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis.

The second part of the study focused on analysing the full genome sequences of four randomly selected isolates. This was done to assess any recombination patterns in the genomes of the selected isolates. Total RNA was extracted from *N. tabacum* plants that had been mechanically inoculated with these isolates and used as a template for next generation sequencing. The resulting contigs, generated by *de novo* assembly, were subjected to BLAST on Genbank database in order to identify PVY genomes. Recombination was

detected using RDP4 software. The results revealed that recombinants PVY^{NTN} and PVY^{N} Wilga strains exist in potatoes grown in Msinga. The results of this study will influence the management/control strategies to be employed to control PVY disease in Msinga.

Declaration

I, Sinethemba Patience Fanelwe Ximba, declare that:

i. The research reported in this thesis except otherwise indicated is my original work;
ii. This dissertation has not been submitted for any degree or examination at any other university
iii. This dissertation does not contain other person's data, pictures or graphs or other information, unless specifically acknowledged as being sourced from other persons
iv. This dissertation does not contain other person's writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, their words have been re-written but the general information attributed to them has been referenced.
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Signed…… **Date:……**

SPF Ximba (Candidate)

Signed…… **Date:……..**

Dr A.Gubba (Supervisor)

Acknowledgements

- To everyone who supported me through this darkest yet best Chapter of my life I say thank you.
- A big thank you to Dr Augustine Gubba my supervisor for constructive criticism, open door policy and for always asking me “what’s the rush” everytime I got too agitated.
- To my mentor Dr Kwasi Sackey Yobo, for always reminding me about the importance of earrings on a lady, for always saying “WEEENAAAA” when I started going off tracks; for always listening to me through all the drama I went through while doing this degree and for providing valuable guidance. Thank you for never being too busy to listen when I needed to talk.
- To my mentor Dr Jacques Davy Ibaba for never ever running out of patience with me, for always assisting me whenever I asked for his help and never complaining at all. A big thank you to his wife Nicole for never having issues with all the time her hubby spent assisting me with experiments, reading and editing my work.
- To my mother Phumelele a big thank you for being a Prayer Warrior, without your prayers I wouldn’t be where I am.
- To the Khoza family especially my maternal grandmother maNene, thank you and thank you and many more thank you’s for the love, guidance and support
- To my siblings Mzwa, Simtho “Fahla”, Thandaza “Imma” and Mzo “Phakethe” Ximba for being the best siblings one could ever ask for. You guys are just awesome
- To Noluthando Ximba, my very close cousin who knows me very much, thanks for showing interest in my work even though you never had the slightest idea of what I was really doing in the Labs till the wee hours of the morning.
- A big thank you to Tumisho Mapheto for the conversations about current affairs, travel and life as a whole.
- To Nonduduzo Ncwane for keeping me sane when I thought I was about to lose my mind, for being an inspiration and for just being there.

- To Joey Moodley, thanks for the guidance, motivation, for always sharing your knowledge pertaining to the Virus in this study and for simply being a great Lab mate.
- To Charles Karavina, the first to get to the Office and the last to leave, thanks for assisting with switching off those machines when time was against me and I had to leave things hanging. Another thank you for editing this manuscript.
- To my cousin Thulisile Ngidi and her hubby Lucky, thank you for all the emotional support. May God bless you abundantly.
- To Ncane Zama Khoza, thank you for the medical advice and emotional support.
- To the room 340 family, Ncesh, Cleopas, Sizwe, Thembani, Mxo, Jean Pierre, Thembeka… Africa has produced another graduate.
- To Richard Burgdorf for all technical guidance, going the extra mile to make sure this project was a success I thank you.
- To Thandinkosi Majikijela, thanks for the support, for sticking around and for believing in me.
- To Zethembiso…Because of you my sweet daughter I discovered that indeed I am a strong woman.
- To God…only You who can see the depth of my heart know how grateful I am for closing this Chapter. All that happened, You planned and I thank You for emerging more victorious.
- To my mentor and scholarship fund manager Phumelele Tshabalala, thank you for the Campus Visits, the swift response to emails and lastly the Gala Dinners, you made being a WK Fellow a very wonderful experience.
- To WK Kelloggs Foundation Scholarship thank you for the funding. I can't even imagine where I would be without the financial support. It really made a HUGE difference in my life and that of other people from my community.
- To Yakesh Jagesar and Nicole Pitout from Life Science Library, thank you for the experience and the wonderful environment you provided me.

Dedication

{To Zethembiso, the apple of my eye}

Table of Contents

Introduction to Dissertation

Plant pathogens are a serious threat to global food security, especially in less developed countries. Damage caused by plant pathogens has been estimated to reduce the global food production by 10% (Strange and Scott, 2005). Separating crop species from their wild environment into agro-ecosystems has resulted in the emergence of new pathogens and the rapid evolution of the pathogen populations already existing in the wild ancestor of the cultivated crop (Stukenbrock and McDonald, 2008). Viruses and many important plant diseases are responsible for huge losses in crop production and quality in all parts of the world. (Adams and Antoniw, 2007).

Potato virus Y (PVY), the type member of the genus *Potyvirus*, is one of the most damaging potato viruses affecting crop yields and tuber quality. PVY also affects other economically important solanaceous crops and non-solanaceous weeds (Kerlan, 2006). It is transmitted in a non-persistent manner by many aphid species (Shukla *et al.*, 1994). Several distinct PVY strains have been recognised worldwide. Those infecting potatoes were historically classified as ordinary or common strains (PVY^{O}) and C strains (PVY^{C}) based on the hypersensitive response they cause on potatoes (Mascia *et al*., 2010). Other strains were classified as necrotic strains (PVY^{N}) although they do not elicit hypersensitive resistance response in potato, but cause vein necrosis in tobacco. Recombination events in field-grown potatoes have led to the identification of some new pathotypes of strains PVY^{N} and PVY^{O}, denoted PVY^{Z}, PVY^{N} Wilga and PVY^{NTN} (Mascia *et al*., 2010).

The study site

Msinga, a poor rural community which largely relies on agriculture for food security and income generation, is located in the Midlands of KwaZulu-Natal Province. The underdeveloped and deep rural nature of Msinga has resulted in a lack of infrastructure with only an estimated ten percent of the population having access to potable water (Stats SA, 2006). There are also high levels of

unemployment, and the lack of infrastructure makes it difficult to attract investors. Therefore, most of the employment is either in the social sector, in Provincial or National government or in the informal sector which consists mostly of subsistence farming and micro-enterprises (Msinga IDP, 2011). Farming plays a huge role in this community thus alleviating poverty.

Significance of the Research

Very little work has been done in Msinga district regarding the prevalence and importance of viral diseases in crop production. Given that potato is one of the crops being grown in the area and that PVY is a major limiting factor to potato production globally, Msinga was selected as an ideal study site. This research aims at generating information that will be useful in developing effective and sustainable PVY management strategies in Msinga.

Research Objectives

The objectives of this study were therefore to:

a) Assess the existence of the PVY strains infecting potatoes in the Msinga district;
b) Isolate and characterise the PVY strains infecting potatoes in the Msinga district using molecular techniques;
c) Sequence the whole genomes of selected PVY isolates from the Msinga district;
d) Perform recombination and phylogenetic studies of selected PVY infecting potatoes in Msinga.

Dissertation Structure

This dissertation is organised into three chapters. Chapter 1 is a review of literature which highlights the importance of farming in the Msinga community, the characteristics and distribution of PVY as well as control measures against PVY. Chapter 2 concentrates on the detection and identification of PVY infecting potatoes in Msinga. Chapter 3 focuses on genome sequencing, recombination,

and phylogenetic analysis of four randomly selected PVY isolates from the various location where sampling took place. The dissertation ends with Chapter 4 that outlines the major findings of this study as well as suggestions for future research.

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Chapter 1

Literature Review

1.1 Introduction

According to FAOSTAT (2014), potato (*Solanum tuberosum* L.) is one of the four most important crops in the world; with a world production estimated at 364 million tons in 2012. Potatoes are grown throughout South Africa in different climatic regions all year round owing to the country's unique geography and climate (DAFF, 2013). Since potatoes are vegetatively propagated this creates unique opportunities to propagate and spread many diseases (Khurana, 2004). Potatoes are affected by multiple key pests, including several viruses which contribute to major yield losses.

Viruses are responsible for huge losses in crop production and quality all over the world. Infected plants may show a range of symptoms depending on the disease, with leaf yellowing (either of the whole leaf or in a mosaic pattern), leaf distortion such as curling, and other growth abnormalities such as plant stunting being common (Adams and Antoniw, 2007). A number of re-emerging viruses and newly emerging viruses have been threatening potato cultivation. These viruses have the potential of severely limiting potato production in the future if their control is not addressed promptly (Salazar, 2003).

Potato virus Y (PVY) is the type member of the genus *Potyvirus* under the family *Potyviridae* (Nie *et al*., 2004). It infects many crop species in the *Solanaceae* family including potato , tomato (S. *lycopersicum* Mill.), tobacco (*Nicotiana* sp.) and pepper (*Capsicum annuum L*.) (Shukla *et al*., 1994). Its host range also includes non-solanaceous weeds in the families *Amaranthaceae*, *Leguminosae*, *Chenopodiaceae* and *Compositae* (Edwardson and Christie, 1997).

1.2 PVY Genome Organization

PVY has a single-stranded positive-sense RNA genome of about 10000 bp (Chare and Holmes, 2006). The 5'-end of the viral genome is covalently linked to the viral protein genome-linked encoded protein (VPg). The 3'-end is constituted by a poly-A sequence. A single Open Reading Frame (ORF) is present on the PVY genome. This ORF encodes a polyprotein cleaved by three viral protease (P1, HC-Pro and Nib) to produce ten functional proteins namely: P1, HC-Pro, P3, VPg, 6k1, CI, 6k2, NIa, NIb, and CP (Figure 1.1). An additional peptide P3N-PIPO translated from an overlapping ORF +2 shifting of the P3 cistron was discovered by Chung *et al*., (2008). These viral proteins are involved in different steps of the viral cycle.

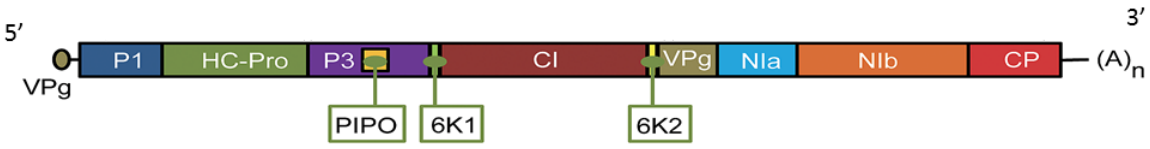

Figure 1.1: Schematic representation of the PVY genome (Cuevas *et al*., 2012).

1.3 Functions of PVY encoded proteins

1.3.1 P1

This protein is found at the N-terminus of the PVY polyprotein and is suspected to be involved in RNA binding and suppression of host defence mechanisms (Verchot and Carrington, 1995). According to Klein *et al*. (1994), even though P1 is not needed by the virus to infect a plant, however, it affects infectivity and movement of PVY in the host.

1.3.2 P3

The P3 protein gene shows little sequence homology among the *Potyvirus* species and few functional motifs and structures have been identified in the resulting protein (Aleman-Verdaguer *et al*., 1997). Studies of mutation by Riechmann *et al*. (1995) showed that the lack of cleavage between P3 and the downstream 6K1 peptide in *Plum pox virus* (PPV) had no effect on virus viability but resulted in symptomless infections. Further analyses of the effect of mutations

in the cleavage site between P3 and 6K1, which only slightly affected *in vitro* cleavage, led to variations in the appearance and strength of the symptoms during infection. Sáenz *et al*. (2000), discovered that the C-terminal region of the P3-6K1 complex carries a pathogenicity determinant of PPV.

1.3.3 Helper component proteinase (HC-Pro)

Research shows that potyviruses need a component other than the virus particle to be successfully transmitted by their aphid vectors (Govier and Kassanis, 1974). The potyviral HC-Pro has three essential regions that have been recognised. The N-terminal part allows virus transmission by aphid vectors and genome amplification (Atreya and Pirone, 1993; Kasschau and Carrington, 1995). The central region of HC-Pro assists in long-distance movement and replication maintenance (Kasschau *et al*., 1997; Klein *et al*., 1994). The C-terminal is a cysteine-type proteinase (Carrington *et al*., 1989) that assists in cell to cell movement. Studies have shown that HC-Pro acts as a suppressor of post-transcriptional gene silencing (Brigneti *et al*., 1998; Kasschau and Carrington, 1998).

1.3.4 P3N-PIPO

A small ORF named “pipo” (Pretty Interesting Potyviridae ORF) was discovered to overlap with the P3 coding region in all members of the *Potyviridae* (Chung *et al*., 2008). Experiments found that immunodetection of the pipo-encoded protein in *Turnip mosaic virus* (TuMV)-infected cells revealed an approximately 25kDa consistent with the expression of “pipo” as a translational fusion with the N-terminus of P3 (Chung *et al*., 2008). This protein is therefore called P3N-PIPO**.** Studies have shown that P3N-PIPO, the frameshift resulting protein, is plasmodesmata (PD)-located protein (Hoda,2015) and involved in potyviral cell-to-cell movement by mediating the targeting of the potyviral CI protein to PD to form canonical structures for potyviral cell-to-cell movement. In a study by Hoda (2015), a stop codon or point mutations was introduced in a full-length cDNA infectious clone of TuMV into P3N-PIPO without affecting the amino acid sequence of other

viral proteins including P3. The effects of these mutations on TuMV were then evaluated. It was discovered that elimination of PIPO or substitution of the positive-charged amino acid lysine with the negatively charged amino acid glutamic acid within PIPO compromises TuMV cell-to-cell spreading in *Nicotiana benthamiana* plants. PEG-mediated transfection assay revealed that virus replication of these mutants is not affected in *Nicotiana benthamiana* protoplasts. Moreover, transient co-expression of CI and P3N-PIPO mutants showed that the PIPO mutants lose ability to target the TuMV CI protein to plasmodesmata. Subcellular localization of these PIPO mutants indicated that the substitution mutants retain their PD-targeting. These results strongly suggested that the potyviral P3N-PIPO protein is likely a protein for potyviral intercellular movement via PD (Hoda, 2015).

1.3.5 Viral genome-linked protein (VPg)

Most of the potyviral proteins have multiple functions with the VPg being one of the most versatile proteins. It has a role in translation and RNA synthesis and assists in potyviral movement. It also functions as an avirulence determinant in certain resistant hosts in which the virus fails to achieve systemic infection (Keller *et al*., 1998; Lellis *et al*., 2002).

1.3.6 6K1

The 6K1 is a 6 kDa protein which usually associates with P3. The exact function of 6K1 is yet to be determined, but deletion and insertion studies have resulted in non-infectious mutants (Kekarainen *et al*., 2002, Merits *et al*., 2002). Since this protein is generally found bound to P3, it is believed that it plays a supporting role to P3.

1.3.7 Cytoplasmic inclusion (CI)

The CI protein has been implicated in cell-to-cell PVY movement. Recombinant CI injected into mesophyll cells were found to form aggregates in the cytoplasm rather than moving to neighbouring cells (Rojas *et al*., 1997). The CI of *Tobacco vein mottling virus* (TVMV) was found to aggregate close to the plasmodesmatal connections during the initial phase of infection (Rojas *et al*., 1997).

1.3.8 6k2

Chu *et al*., (1997) proposed that this protein is required for genome amplification and that it anchors the replication apparatus to endoplasmic reticulum membranes.

1.3.9 NIa (Nuclear inclusion a)

The NIa is a trypsin-like serine protease which cleaves peptide bonds following a positively charged amino acid and is the primary protease for post-transcriptional cleavage of the viral polyprotein and cleaves the polyprotein both in a *cis* and *trans* manner (Riechmann *et al*., 1992). The NIa is responsible for cleavage at all the proteolytic sites within the polyprotein with the exception of the P1/HC-Pro site (Riechmann *et al*., 1992).

1.3.10 NIb (Nuclear inclusion b)

This is an RNA-dependent RNA polymerase that plays an essential role in virus replication. Nib interacts with 6k2-VPg and is recruited in the process for viral replication complexes (Li *et al*., 1997).

1.3.11 Coat protein (CP)

The potyviral CP plays a major role in the cell-to-cell transport of viruses (Mảikinen and Hafrẻn, 2014). According to Anindya and Savithri (2003), the N-terminal plays a role in the assembly of the *Pepper vein banding virus* (PVBV). Ivanov *et al*. (2001, 2003) found that phosphorylation of *Potato Virus A* CP

regulates its RNA binding function and viral spread in infected plants. These findings suggest that the CP capacity to assemble is a crucial factor in the cell to cell movement of potyviruses (Måikinen and Hafrẻn, 2014). Potyviral CP has also an essential role in the regulation of infection. In addition to its role in encapsidation, the potyviral CP can regulate viral gene expression (Hafrén *et al*., 2010) and movement (Dolja *et al*., 1994, 1995; Ivanov *et al*., 2003).

1.4 Diversity of PVY infecting potato

The genetic diversity of positive RNA plant viruses is driven by three main factors: mutation, recombination and reassortment (Roossinck, 1997; Simon and Bujarski, 1994). All three factors are believed to produce a vast pool of virus genomes required for virus adaptation to new evolutionary niches (Roossinck, 1997). PVY is a good example of an RNA virus using high mutation rates and numerous recombinants that generate large genetic diversity and, therefore, survive and succeed in infecting multiple hosts and in various environments (Blanchard *et al*., 2008; Hu *et al*., 2009). PVY infecting potatoes is a group that displays a huge variability of strains. Many strains exist and are grouped into three parental strains namely O, N and C (Kerlan and Moury, 2008). Strains that are of major interest lately are Wilga, NTN, Z and E. With continued research and constant mutation there are possibilities of more strains being discovered.

1.4.1 PVY ^{O}

PVY^{O}, the common or ordinary strain, induces systemic mottle or mosaic in most potato cultivars (Figure 1.2). It has been the most predominant strain throughout the world (Lorenzen *et al*., 2006) hence the name ‘common’ (Crosslin, 2013).

1.4.2 PVY ^{N}

According to Riggoti and Gugerli (2007), this strain also known as tobacco veinal necrosis strain, induces mottling and characteristic vein necrosis in tobacco but mild mottling in almost all potato cultivars (Figure 1.2). It has been reportedly

found in potato growing areas of North America, (Crosslin *et al*., 2002) Africa and New Zealand (Ibaba, 2009).

1.4.2.1 PVY^{NTN}

PVY^{NTN} (N-tuber necrotic) is an isolate of PVY^{N} and it incites Potato Tuber Necrotic Ringspot Disease or PTNRD (Beczner *et al*.,1984) that is characterised by raised external rings on tuber (Figure 1.2). It causes internal tuber symptoms and this results in huge damage to the economy as such tubers are not suitable for the market (Crosslin, 2013).

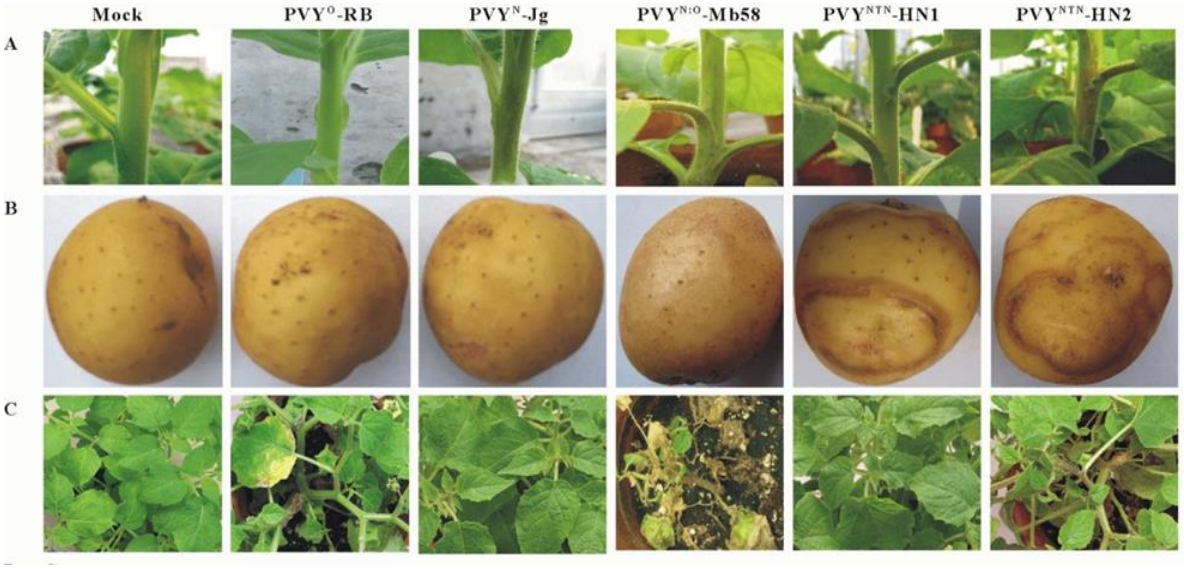

Figure 1.2: Symptoms induced by different isolates of Potato virus Y (PVY) on tobacco, potato and *Phyalis floridana*. A. Symptoms on tobacco (*Nicotiana tabacum* cv. 'Samsun') plants 15 days after inoculation. B. Symptoms on potato (*Solanum tuberosum* cv. 'Yukon Gold') tubers harvested from plants 90 days after inoculation. C. Symptoms on *P. floridana* 25 days after inoculation. (Hu *et al*., 2011)

1.4.2.2 PVY N Wilga

This strain of PVY^{N} sometimes referred to as PVY Wilga was first described in Europe after being found in the cultivar ‘Wilga’ (Crosslin, 2013). This isolate causes tobacco veinal necrosis but has the PVY^{O} strain CP sequence.

1.4.2.3 PVY$^{N:O}$

PVY$^{N:O}$ has mixed properties of PVY^{O} and PVY^{N}, and it was found in North America (Crosslin *et al.,* 2005; Piche *et al*., 2004; Singh *et al*., 2003).

1.4.3 PVY^{C}

This strain causes stipple streak symptoms in potato cultivars containing N_C resistant genes to PVY (Kerlan, 2006). This strain's occurrence and distribution has not been greatly studied as the other parental strains. Biological differences between pepper-type and potato-type PVY^{C} isolates, supported by differences in their genomic sequences (Moury *et al*., 2002), led to the description of two distinct phylogenetic subgroups, PVYC1 and PVYC2, for isolates able to infect specifically pepper or potato, respectively (Kerlan and Moury, 2008).

1.4.4 PVY^{U}

In a study conducted by Janzac *et al*. (2015), PVY isolates sampled in different locations in Brazil were analysed and most of these Brazilian PVY isolates were assigned to a newly described serogroup: PVY^{U}. Specific amino acid residues were identified in the N-terminal of the PVY^{U} CP isolates, and phylogenetic analyses supported a division into a new PVY clade (Janzac *et al*., 2015). PVY^{U} also differed from the known PVY potato strains by its biological properties (Janzac *et al*., 2015).

1.5 Methods used in the detection and characterization of PVY

Numerous methods can be used for PVY detection. Among them are nucleic acid-based, serological and biological assays, electron microscopy, and phylogenetic analysis.

1.5.1 Nucleic acid-based methods

Reverse transcription-polymerase chain reaction (RT-PCR) is a common method for detecting and characterising virus isolates (Lorenzen *et al*, 2006). This

procedure involves synthesizing complementary DNA (cDNA) from viral RNA with a reverse transcriptase whereafter the resulting product is amplified using gene-specific primers The generated amplicon can be detected by agarose gel electrophoresis (Giovanna *et al*., 2003). Some of these assays have been developed to differentiate between PVY strains (Crosslin *et al*., 2002; Crosslin *et al*., 2005; Nie and Singh, 2002) while others are aimed at evaluating the CP region of the PVY genome (Blanco-Urgoiti *et al*.,1996; Blanco-Urgoiti *et al*.,1998; Boonham *et al*., 2002, Ellis *et al.,*1996 and Singh *et al*.,1996).

1.5.2 Serological methods

Serology has been indispensable in the detection and identification of plant viruses. Serological techniques used to detect viruses are based on the reaction between viral nucleoprotein or viral protein and its specific antibody (Hsu, 1996). Two methods are commonly used namely enzyme-linked immunosorbent assay (ELISA) and Dot immunobinding assay. PVY detection and tracking has been facilitated by the production of PVY-specific polyclonal antibodies and the adoption of ELISA (Lorenzen *et al*., 2006). This has been advantageous as immunoassays are sensitive, fast and more amenable to high-throughput use than bioassays. ELISA is normally used as it is rapid, cheap and easy to automate (Wangai and Lelgut, 2004). Dot-immunobinding assay or Dot-Iba, as it is commonly known, involves antibodies or antigens that are dotted directly onto solids such as nitrocellulose membranes or filter paper. This assay is best suited for use in laboratories where there are existing constraints in laboratory resources (Sumi *et al*., 2009).

1.5.3 Biological assays

Bioassays have been in use for a very long time in virus characterization. They can be used to investigate biological properties of viruses that include host range, symptoms and mode of transmission (Ibaba, 2009). This can be achieved in numerous ways such as mechanical inoculation, vegetative production, grafting or by the virus vector.

1.5.4. Electron microscopy (EM)

Virus particles are easily viewed under the electron microscope because of their minute size. Immunosorbent electron microscopy (IEM) which focuses on the specific trapping of viral particles on grids that have been pre-treated with antiserum has increased the sensitivity and specificity of transmission electron microscopy by up to 10,000 times (Bos,1999). A major constraint in EM is the expense involved in purchasing and maintaining the facility. Pinwheel, bundle-like structures found in PVY-infected tissue can be seen under the electron microscope (Figure 1.3).

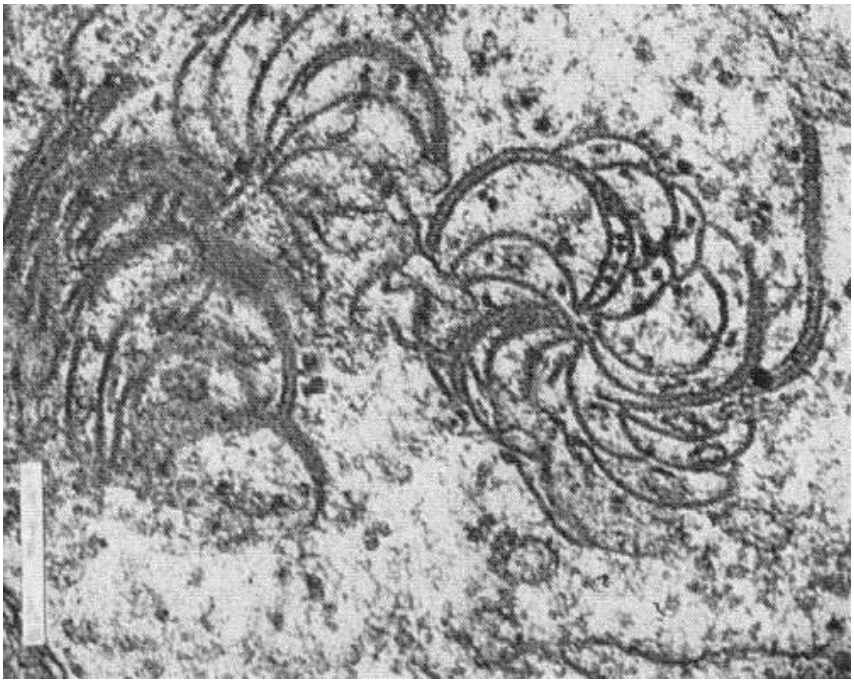

Figure 1.3: Pinwheel structures observed in PVY-infected tissue under an electron microscope (Image courtesy of Edwardson JR, University of Florida, and Gainesville, USA,1997).

1.5.5 Phylogenetic Analysis

Phylogenetic analysis is an important tool for studying the evolutionary history of organisms from bacteria and viruses to humans (Dayhoff, 1972). It is also important for the clarification of the evolutionary pattern of multigene families

as well as for understanding the adaptive evolution at the molecular level (Nei, 1996). Phylogenetic analysis contributed enormously in understanding the diversity of PVY. Partial and whole genome sequencing has been performed in several studies (Hu *et al*., 2009; Lorenzen *et al*., 2006; Moodley *et al*., 2014).

1.6 Genome sequencing technologies

1.6.1 Sanger sequencing

Since the early 1990s, DNA sequence production has almost exclusively been carried out with capillary-based, semi-automated implementations of the Sanger biochemistry. In high-throughput production pipelines, DNA to be sequenced is prepared by one of two approaches: first, for shotgun *de novo* sequencing, randomly fragmented DNA is cloned into a high-copy-number plasmid, which is then used to transform *Escherichia coli;* or second, for targeted resequencing, PCR amplification is carried out with primers that flank the target (Sanger *et al*., 1977; Swerdlow *et al*., 1990). The output of both approaches is an amplified template, either as many 'clonal' copies of a single plasmid insert present within a spatially isolated bacterial colony that can be picked, or as many PCR amplicons present within a single reaction volume. The sequencing biochemistry takes place in a 'cycle sequencing' reaction, in which cycles of template denaturation, primer annealing and primer extension are performed (Hunkapiller *et al*., 1991; Swerdlow *et al*., 1990). The primer is complementary to known sequence immediately flanking the region of interest. Each round of primer extension is stochastically terminated by the incorporation of fluorescently labelled dideoxynucleotides (ddNTPs). In the resulting mixture of end-labeled extension products, the label on the terminating ddNTP of any given fragment corresponds to the nucleotide identity of its terminal position (Hunkapiller *et al*., 1991). The sequence is determined by high-resolution electrophoretic separation of the single-stranded, end-labeled extension products in a capillary-based polymer gel. Laser excitation of fluorescent labels as fragments of discreet lengths exits the capillary, coupled to four-color detection of emission spectra, provides the readout that is represented in a Sanger sequencing 'trace' (Ewing and Green, 1998). Software

translates these traces into DNA sequence, while also generating error probabilities for each base-call. The approach that is taken for subsequent analysis—for example, genome assembly or variant identification—depends on precisely what is being sequenced and why. Simultaneous electrophoresis in 96 or 384 independent capillaries provides a limited level of parallelization (Ewing *et al*., 1998).

1.6.2 Next Generation Sequencing (NGS)

With the limitations of Sanger sequencing such as restricted scalability, cost when applied to large genomes and frequent reliance on prior and specific template amplification by PCR, a new method of sequencing was developed namely NGS (Margulies *et al*., 2005). In NGS, vast numbers of short reads are sequenced in a single stroke. NGS has diverse platforms however their workflows are conceptually similar. This section focuses on the three mostly used NGS platforms.

1.6.2.1 454 Sequencing

The 454 system was the first NGS platform available as a commercial product (Margulies *et al*., 2005). In this approach, libraries may be constructed by any method that gives rise to a mixture of short, adaptor-flanked fragments. Clonal sequencing features are generated by emulsion PCR, with amplicons captured to the surface of 28-μm beads (Dressman *et al*., 2003). After breaking the emulsion, beads are treated with denaturant to remove untethered strands, and then subjected to a hybridization-based enrichment for amplicon-bearing beads (that is, those that were present in an emulsion compartment supporting a productive PCR reaction). A sequencing primer is hybridized to the universal adaptor at the appropriate position and orientation, that is, immediately adjacent to the start of unknown sequence. During the sequencing, one side of the semi-ordered array functions as a flow cell for introducing and removing sequencing reagents, whereas the other side is bonded to a fiber-optic bundle for CCD (charge coupled device)-based signal detection. At each of several hundred cycles, a single species

of unlabelled nucleotide is introduced. On templates where this results in an incorporation event, pyrophosphate is released (Dressman *et al.,* 2003).

1.6.2.2 Illumina

In Illumina, sequencing also known as Solexa, 100-150 bp reads are used. Longer fragments are ligated to generic adaptors and annealed to a slide using adaptors. PCR is carried out to amplify each read, creating a spot with many copies of the same read. They are then separated into single strands to be sequenced. The slide is flooded with nucleotides and DNA polymerase (Fedurco *et al*., 2006). These nucleotides are fluorescently labelled, with the colour corresponding to the base. They also have a terminator, so that only one base is added at a time. An image is taken of the slide. In each read location, there will be a fluorescent signal indicating the base that has been added. The terminators are then removed, allowing the next base to be added, and the fluorescent signal is removed, preventing the signal from contaminating the next image. The process is repeated, adding one nucleotide at a time and imaging in between. Computers are then used to detect the base at each site in each image and these are used to construct a sequence. All of the sequence reads will be the same length as the read length depends on the number of cycles carried out (Turcatti *et al*., 2008).

1.6.2.3 ION Torrent

Unlike Illumina and 454, Ion torrent and Ion proton sequencing do not make use of optical signals. Instead, they exploit the fact that addition of a dNTP to a DNA polymer releases an H+ ion. As in other kinds of NGS, the input DNA or RNA is fragmented to ~200 bp (Dressman *et al.,* 2003). Adaptors are added and one molecule is placed onto a bead. The molecules are amplified on the bead by emulsion PCR. Each bead is placed into a single well of a slide. Like 454, the slide is flooded with a single species of dNTP, along with buffers and polymerase, one NTP at a time. Each H+ ion released will decrease the pH. The changes in pH allow us to determine if that base, and how many thereof, was added to the sequence read (Fedurco *et al*., 2006). The dNTPs are washed away, and the

process is repeated cycling through the different dNTP species. The pH change, if any, is used to determine how many bases (if any) were added with each cycle (Turcatti *et al*., 2008).

1.7 Transmission

In order to ensure survival in nature, plant viruses must be efficiently transmitted among plants and gain new hosts. Transmission from one host plant to another is, therefore, an important phase in the virus life cycle (Syller, 2005). PVY can be transmitted by sap inoculation, grafting and by aphids.

PVY is transmitted in a non-persistent manner which means that the virus particles are carried on the stylets of the vector, and acquisition and transmission of the virus do not require contact of the aphid's stylets with the phloem; time required for virus acquisition and transmission is in the range of a few seconds (Martín *et al*., 1997), but the capability of the vector to transmit the virus is of limited duration. In contrast, persistently transmitted viruses (e.g. *Potato leafroll virus*, PLRV) are taken up from the phloem, so that the time necessary for virus acquisition and transmission is longer than in non-persistently transmitted viruses, but the ability of the vector for virus transmission lasts longer.

Aphididae family makes up the largest and most versatile group of plant virus vectors (D'Arcy and Nault, 1982; Gray and Banerjee, 1999). More than 50 aphid species are reported to transmit PVY although some species are more efficient than others (Ragsdale *et al*., 2001). *Myzus persicae* (Sulzer), commonly known as the green peach or peach-potato aphid, is reportedly the most efficient PVY vector (de Bokx and Huttinga, 1981). The virus can be maintained for up to 24 hours if feeding does not occur.

1.8 Control strategies

Virus infections can have crippling effects on any economy, this debacle, however, can be limited if proper control measures such as minimising virus infection or suppression of virus are put into place. Individual measures used alone are not very beneficial and eventually become ineffective over long terms (Jones,

2004). A better approach is when different control measures are combined thus resulting in more effective control. According to Jones (2001), selecting such a combination requires vast knowledge of the epidemiology of the causal virus and the mode of action of each control measure.

Once PVY has been introduced into a field, complete eradication of the virus is virtually impossible due to the intimacy of the virus-host interaction as the virus hijacks the host's biosynthetic machinery. The main objective should, therefore, be to prevent the introduction of the virus in a given ecosystem. Recommended strategies for controlling virus transmission include control by chemical, physical and biological means. These methods aimed at controlling the vector and reducing virus inoculum.

1.8.1. Vector control

Reducing the spread of PVY by controlling its vectors can be achieved in three ways namely chemical, physical and biological methods.

1.8.1.1. Chemical methods

PVY spread cannot be limited by aphicides because the time for virus acquisition and transmission is too short for the aphicides to kill the vector (Radcliffe, 1982). Insecticides are not recommended either because they cause the vectors to get into a feeding frenzy which results in increased virus dissemination (Stapathy, 1998). If it is really essential to use chemicals, then insecticide classes need to be rotated to avoid accumulation of resistance.

1.8.1.2. Physical methods

Using fine mesh screens on doors and windows is a reliable solution for greenhouse crops. This practice produces a considerable decline in the problem by avoiding early crop contamination (Roselló *et al.,* 1996). Aphids are attracted to the colour yellow therefore using sticky traps can also limit their activity.

1.8.1.3. Biological methods

Control of pests by disrupting their ecological status, as through the use of organisms that are natural predators, parasites, or pathogens is known as biocontrol. A study was conducted by Al-Ani *et al*. (2013) to test the activity *of Pseudomonas fluorescens*, *Rhodotorula* sp and fermented neem extract to protect potato plants against PVY disease development under field conditions. The results confirmed that the use of bioagents to induce systemic resistance provides an efficient tool, as insecticide alternative to manage PVY in potato.

1.8.2 Cultural methods

Planting early-maturing potato cultivars such as Norgold Russet assists in avoiding peaks in aphid migration. Barrier plants such as maize; taller than the primary crop, act as a physical barrier between the vector and the primary crop (Hooks and Fereres, 2006). DiFonzo *et al*. (1996), using soybean as the preferred barrier crop, noted that PVY incidence was less in plots with borders compared to borderless plots.

1.8.3 Use of resistant cultivars

These can be developed using natural or engineered resistance. Both have their advantages and disadvantages.

1.8.3.1 Natural resistance

Natural breeding is a very old way to breed for resistance. The most common mechanism associated with active defence is known as hypersensitive response (HR). Hypersensitivity, expressed as a necrotic reaction to infection, is conferred by resistant genes whereby the cells surrounding the primary infection site die rapidly resulting in visible necrotic local lesions (Goldbach *et al*., 2013). The occurrence of this response is followed by a specific recognition of the intruding pathogen eg. a virus and is usually based on the matching dominant gene products of the plant which are produced from dominant resistance genes, *R* genes and the virus (avirulence genes) (Goldbach *et al*., 2013). *R* genes that have

been identified encode proteins which can be placed into 5 classes (Dangl and Jones, 2001). To date, all *R* genes that have been isolated conferring resistance to viruses belong to the largest class of R genes. This class encodes for a nucleotide-binding site plus leucine-rich repeat (NB-LRR) type of protein. Extreme resistance (ER), elicited by *Ry,* is considered to be durable and effective against a wide range of known PVY strains. Plants with ER to the virus show no symptoms or limited necrosis when inoculated with PVY. ER genes introduced into the potato gene pool have originated from a few sources. The genes conferring extreme resistance to PVY are known in *S. tuberosum* subsp. *andigena*, *S. hougasii* and *S. stoloniferum* and were used in different breeding programmes (Swieżynski, 1994). Resistance from *S. chacoense* is inherited in a dominant, monogenic way. Two varieties with resistance to PVY from *Solanum chacoense* were released from a Japanese breeding programme (Sato *et al*., 2006). Also, the extreme resistance to PVY from *S. stoloniferum* was introgressed into *S. tuberosum* and about 20 varieties in Europe were listed to carry the Ry_{sto} gene (Ross, 1986).

1.8.3.2 Engineered resistance

The advancement of studies has allowed for plants to be transformed using transgenic methods based on concepts such as pathogen-derived resistance. The viral coat protein gene has been the preferred viral component used in pathogen-derived resistance (Goldbach *et al*., 2013). However, concerns were raised of the possibility of heterologous recombination with other viruses in nature that could lead to unusually dangerous viruses. RNA-derived resistance appears to be safer in this regard since it only involves viral sequences. Moreover, it allows the production of transgenic plants with multiple virus resistance (Zhu *et al.*, 2009).

1.9 References

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Chapter 2

Detection and Differentiation of *Potato virus Y* infecting potato in the Msinga district in KwaZulu-Natal, South Africa

Abstract

Accurate identification of plant pathogens is crucial towards initiating sustainable control strategies to ensure economic agricultural production. The aim of this study was to detect and differentiate *Potato Virus Y* (PVY) isolates infecting potato (*Solanum tuberosum L*.) in the Msinga district in the Province of KwaZulu-Natal, South Africa. Potato leaf samples exhibiting virus-like symptoms were collected from four different farms in the district. Initial detection of PVY in the leaf samples was done using triple antibody sandwich enzyme-linked immunosorbent assay. PVY-positive samples were further tested using antibodies specific to PVY serotypes O and N. *Nicotiana tabacum* cv Samsun plants were individually mechanically inoculated with all 32 PVY-ELISA samples. Symptoms on inoculated tobacco were monitored over a 4 week period. They consisted of vein clearing, faint mosaic patterns, yellowing, chlorotic spots and the veinal necrosis symptom, characteristic of *PVY*^{N}, *PVY*^{N}Wilga and *PVY*^{NTN} strains. Reverse transcription-polymerase chain reaction, using primers specific to the coat protein gene of PVY, was performed as a confirmation test on total RNA of four PVY-ELISA positive samples. Following virus purification, samples were viewed under an electron microscope and flexuous rod particles that were approximately 730nm long and 12nm wide were observed. Such particles are typical of PVY. Sodium dodecyl sulphate polyacrylamide gel electrophoresis confirmed the presence of PVY by yielding a 30kDa band characteristic of PVY CP. Strains PVY^{N} and PVY^{O} were identified with PVY^{N} being the most prevalent at incidence levels of about 96%. This study showed the widespread occurrence of PVY on potatoes in Msinga.

2.1 Introduction

Viruses are known to cause serious economic crop losses worldwide. Virus infections cause a variable range of symptoms in most cultivated plants, thus adversely affecting crop productivity (Lopez *et al*., 2003). Viruses cause plant diseases that are difficult to control because of the lack of suitable products for chemical treatment under field conditions. *Potato virus Y* (PVY) has a wide host range that includes many genera in the family *Solanaceae*, including important crop plants such as potato (*Solanum tuberosum* L), pepper (*Capsicum annuum* L, tomato (*S.lycopersicon* Mill), and tobacco(*Nicotiana* spp.) (Karasev and Gray, 2013). Accurate virus detection is crucial if effective and sustainable disease control strategies are to be devised.

PVY is a type member of the genus *Potyvirus* in the family *Potyviridae* with a single-stranded, positive-sense, RNA genome of approximately 9.7 kb (Cai *et al*., 2011). The PVY genome has a poly (A) tail at the 3' terminus and a covalently linked VPg protein at the 5' terminus; both terminal structures are involved in genome protection and genome replication as well as regulation of the genome expression (Kerlan and Moury, 2008). Expression of the PVY genome occurs through synthesis of a single, large polyprotein, which is later cleaved into 10 mature proteins by three virus-specific proteases. A small ORF named "pipo" (Pretty Interesting Potyviridae ORF) was discovered to overlap with the P3 coding region in all members of the *Potyviridae* (Chung *et al*., 2008). A study by Hoda (2015) has shown that P3N-PIPO, is a plasmodesmata (PD)-located protein which is involved in potyviral cell-to-cell movement by mediating the targeting of the potyviral CI protein to PD to form canonical structures for potyviral cell-to-cell movement.

PVY has three parental strains namely $PVY^{O,}$ PVY^{C} and $PVY^{N.}$ Several recombinant strains such as $PVY^{NTN,}$ PVY^{Z} and PVY^{N} Wilga have been reported amongst others (Nie *et al*., 2004; Ogawa *et al*., 2008; Visser *et al*., 2012). PVY is transmitted by at least 60 aphid species in a non-persistent manner. It is also transmitted mechanically and vegetatively through potato tubers (Kerlan and Moury, 2008).

There are different techniques used to detect PVY in infected plant material. Commonly used techniques include enzyme-linked immunosorbent assay (ELISA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). ELISA is an immunological technique used to diagnose various infectious diseases of human, animals as well as plants. It is more than 99% sensitive and specific compared to other serological tests (Kemeny and Challacombe, 1989). Several primers that target different parts of the PVY genome have been designed to make PVY detection simpler.

According to the Department of Agriculture and Fisheries (2013), PVY is one of the most devastating plant pathogens in South Africa (SA). PVY has previously been reported in KZN (Budnik *et al.*, 1996; Ibaba and Gubba, 2011; Visser and Dirk, 2009).

Msinga is a poor rural community in the province of KwaZulu-Natal (KZN), in SA. Due to the underdeveloped nature of this area, farming plays a major role in providing food and is an important source of household income (Machethe *et al*., 2004). Potato is an important food staple and the number one vegetable crop in the world. Potatoes are available year-round as they are harvested every month of the year. Potato is grown as a major income generating crop by a majority of the small-scale farmers in Msinga.

To date, no studies have been done on the PVY affecting potatoes in Msinga. Against this background, the aim of this project was to check for the presence of PVY and any of its strains in the Msinga district of KZN.

2.2 Materials and Methods

2.2.1 Virus sampling

The virus isolates were obtained from potato leaf samples exhibiting PVY-like symptoms such as light to dark green mosaic patterns. Samples was done in small-scale farms in Msinga. Sampling sites are listed in Table 2.1 and shown in Figure 2.1. Sampling was done between March and May 2013. Potato leaves showing such as were collected and stored in ice boxes to preserve them during

the trip from the field to the laboratory where they were kept in plastic bags and stored at -80°C until analysis.

Table 2.1: Number of samples collected from various locations in Msinga.

Location	GPS co-ordinates	Sample description	Number of samples
Tugela Ferry	-28.741499, 30.461213	Leaves	24
Nxamalala	-28.836295, 30.453915	Leaves	10
Muden	-28.946990, 30.380757	Leaves	11
Keates Drift	-28.842955, 30.509758	Leaves	12

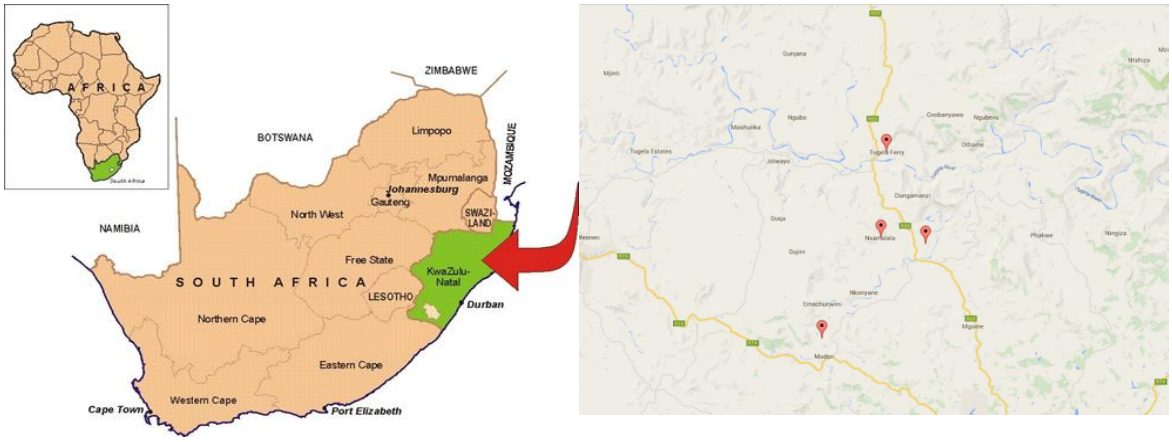

Figure 2.1: Geographical locations where sampling took place in Msinga, KwaZulu-Natal

2.2.2 Detection of PVY

The presence of PVY in the collected field samples was detected using triple antibody enzyme-linked Immunosorbent assay (TAS-ELISA). The Monoclonal antibody (Mab) TAS-ELISA reagent set for *PVY*$^{O/C/N}$ (Neogen Corporation, UK) was used according to the manufacturer's instructions. Approximately 1 g of plant tissue was collected from the actively growing parts of the individual plants. The leaf material was transferred to 2 ml microcentrifuge tube containing four to five plastic beads. To each microcentrifuge tube, 1 ml of

extraction buffer [PBS-Tween 20 with 2% (w/v) PVP-40 pH 7.4] was added and placed in a bead-beater for 1 min to homogenize the samples. The homogenate was then centrifuged for 5 min at 13000 x g at room temperature. The supernatant was transferred to the respective wells of antibody-coated ELISA plates. Each sample was tested in duplicate. A positive control, which was a PVY infected pepper plant was included. The sap from mock-inoculated disease free, control plants was used as the negative controls. The plates were incubated at 4°C overnight. 100µl of antibody probe was loaded onto the wells and the plates were incubated for 2 hours. Conjugate antibody was added and the plates were incubated for 1 hr before the final addition of 100 µl of 1mg/ml of the enzyme substrate 4-Nitrophenyl phosphate disodium salt hexahydrate (SIGMA, USA) solution (pNPP). The plates were incubated at 37°C for 30 min in the absence of light and results were assessed visually. A yellow colour was recorded as a positive reaction and no colour change was interpreted as a negative reaction. Negative samples were subsequently discarded.

2.2.3 Differentiation of PVY isolates

All PVY isolates identified with TAS-ELISA were differentiated into strains using ELISA. To this end, PVY isolates were tested for the serotypes O and N using *PVY*^{N} Mab double antibody sandwich (DAS) ELISA and *PVY*^{O} Pab + Mab TAS-ELISA reagents according to manufacturer's instructions (Neogen Corporation, UK).

2.2.4 Biological assays

All PVY isolates detected with the TAS-ELISA test were mechanically inoculated onto PVY-susceptible five-leaf stage *Nicotiana tabacum* cv Samsun plants. Infected leaf tissue was homogenized with mortar and pestle in phosphate buffer (0.1M phosphate buffer pH 7.4) using liquid Nitrogen and carborundum abrasives to aid with grinding of the inoculum and cell lysis. Inoculated *N. tabacum* plants were maintained in an insect proof glasshouse at the University of KwaZulu-Natal facility (Discipline of Plant Pathology, UKZN PMB Campus) under natural

sunlight conditions. The plants were watered every 2 days and monitored for symptom development over a 4 week period.

2.2.5 Reverse transcription – polymerase chain reaction (RT-PCR)

A total of four PVY-inoculated tobacco plants one from each sampling site, were randomly selected. RT-PCR was performed on them to confirm the presence of PVY. Total plant RNA was extracted from these leaf samples using the Quick RNA Mini-Prep kit (ZymoResearch, USA) according to the manufacturer's instructions. The first strand complementary DNA (cDNA) was synthesized using 3'NTRC primer (5'-GTCTCCTGATTGAAGTTTAC-3') by Glais *et al.,* (2005) according to the manufacturer's instructions using a RevertAid RT Reverse Transcription kit (ThermoFisher Scientific, USA). The primer pair, *PVY* 2F (5'-ACG TCM AAA ATG AGA ATG CC-3') and *PVY* 2R (CATTTGWATGTGCGCTTCC-3'), designed by Aramburu *et al.* (2006) from the conserved region of the coding sequences of the coat protein (CP) of PVY was used for PCR. This primer pair amplifies a 512bp product in all PVY strains (Aramburu *et al.,* 2006).

Kapa2G™ Fast HotStart ReadyMix (Kapa Biosystems, RSA) was used according to the manufacturer's instructions for PCR. Thermocycler conditions consisted of an initial denaturation of 2 min at 95°C, 40 cycles of 15 s at 95°C, 15 s at 55°C and 10 s at 72°C with a final extension at 72°C for 1 min. The product was analyzed by electrophoresis on a 1.5% (w/v) agarose gel in Tris-acetate, EDTA (TAE) buffer pre-stained with SYBRSAFE (ThermoFisher Scientific, USA) and viewed using the G:Box (Syngene version 7.09.06).

2.2.6 Virus Purification and Electron Microscopy

Four PVY-infected tobacco leaf samples, previously confirmed by ELISA and RT-PCR to be PVY-positive, were purified according to Van Oosten (1972) with some modifications. Infected tobacco leaves (100g) were homogenized in 0.1 M sodium citrate buffer (pH 8.3) containing 0.02 M sodium diethyldithiocarbamate (NaDIECA) and 0.002 M EDTA (1:2 w/v), the homogenate was filtered through cheesecloth and centrifuged at 6 000x g for 10 min using a Beckman Coulter,

Avanti J-26 XP1 centrifuge. To the supernatant; 3% Triton X-100 was added, mixed on ice for 1 hr and centrifuged at 75 000 x g for 2 hrs. The pellet was re-suspended overnight in 0.01 M sodium citrate buffer pH 8.3 containing 0.5 M urea and 0.1% (v/v) 2-mercaptoethanol. The suspension was layered onto a 30% (w/v) sucrose cushion in 0.01 M sodium citrate buffer pH 8.3 and centrifuged at 50 000 x g for 2 hrs. The final pellet was re-suspended in 0.01 M sodium citrate buffer pH 8.3.

For electron microscopy studies, a droplet of the suspension of the purified virus was placed on Parafilm where after a copper grid coated with Formvar was inverted onto the droplet. A drop of 2% uranyl acetate was placed on the Parafilm and the grid was inverted onto the stain. After 30 s the grid was wicked dry using filter paper. The grid was allowed to dry completely before being viewed under JEOL JEM-1400 Transmission Electron Microscope (TEM).

2.2.7 SDS-PAGE

In order to determine the size of the coat protein of the purified PVY sample, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with some modifications. The final polyacrylamide concentrations were 6.5% w/v for the running gel and 3 % w/v for the stacking gel. A detailed description of the solutions used in gel preparation and electrophoresis are presented in Appendix A. The volumes of the different solutions used are in Table 2.2. A volume of 4 µl of the purified virus samples were added to 6 µl reducing treatment buffer and denatured at 100˚C for 3 min before loading it onto the gel. They were allowed to migrate at 200 V for 120 min in a Hoefer mini vertical electrophoresis system. The virus CP molecular mass was determined by comparing their mobility with that of a PageRuler prestained protein ladder (10-170 kDa; Thermo Scientific, USA). The gel was submerged in Aqua Stain (Aquascience, UK) for 2 hrs on an orbital shaker at 40 rpm and photographed using a G: Box Genesnap documentation system (Syngene, UK).

Table 2.2: Solutions used for the SDS running and stacking gels.

Solution	6.5% Running gel (ml)	3% Stacking gel
A	3	0.625
B	3.75	0
C	0	0.630
D	0.1	0.050
E	0.050	0.0015
Distilled Water	3	3.75
TEMED	0.001	0.0007

2.3 Results

2.3.1 Detection of PVY

A total of 32 out of 57 samples collected from four different locations tested positive for PVY using antibodies specific to all PVY strains (Table 2.3). A very bright yellow colour indicative of a positive reaction was observed (Figure 2.1).

Table 2.3: ELISA results for the detection of PVY in the samples collected

Location	**Number of samples tested**	**Number of positive samples**
Tugela Ferry	24	12
Nxamalala	10	6
Muden	11	7
Keates Drift	12	7
Total	57	32

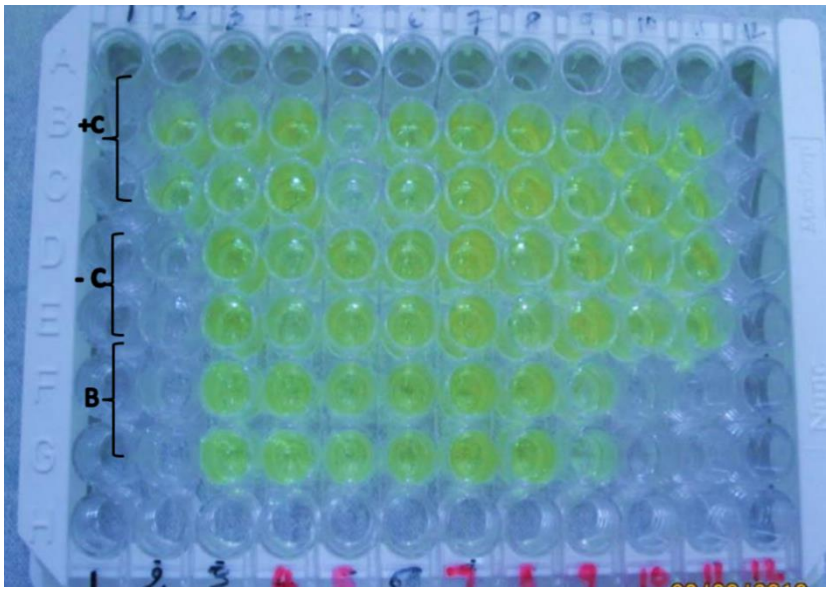

Figure 2.2: Typical ELISA plate showing positive and negative results. Positive results are identified by a distinct yellow colour similar to that of the positive control (+C). A negative reaction is observed by lack of colour change similar to that of the buffer (B) and negative control (-C).

2.3.2 Differentiation of PVY isolates

Of the 32 PVY infected samples identified using TAS-ELISA, 31 samples reacted positively with Mab specific to PVY^N. Only one sample reacted with Mab specific to PVY^O. All ELISA results for strain differentiation are shown in Table 2.4.

Table 2.4: ELISA results for strain differentiation of PVY isolates infecting potato in Msinga

Location	PVY^O	PVY^N
Tugela Ferry	0	12
Nxamalala	0	6
Muden	1	6
Keates Drift	0	7

2.3.3 Biological assays

In response to PVY infection, the susceptible indicator host plant *Nicotiana tabacum* cv Samsun showed different symptoms (Figure 2.3). These symptoms started appearing 7 days post inoculation (dpi). Symptoms such as vein clearing, mosaic patterns (Figure 2.3D), yellowing (Figure 2.3B), chlorotic spots (Figure 2.3A) and vein necrosis (Figure 2.3C) were observed on the inoculated tobacco plants.

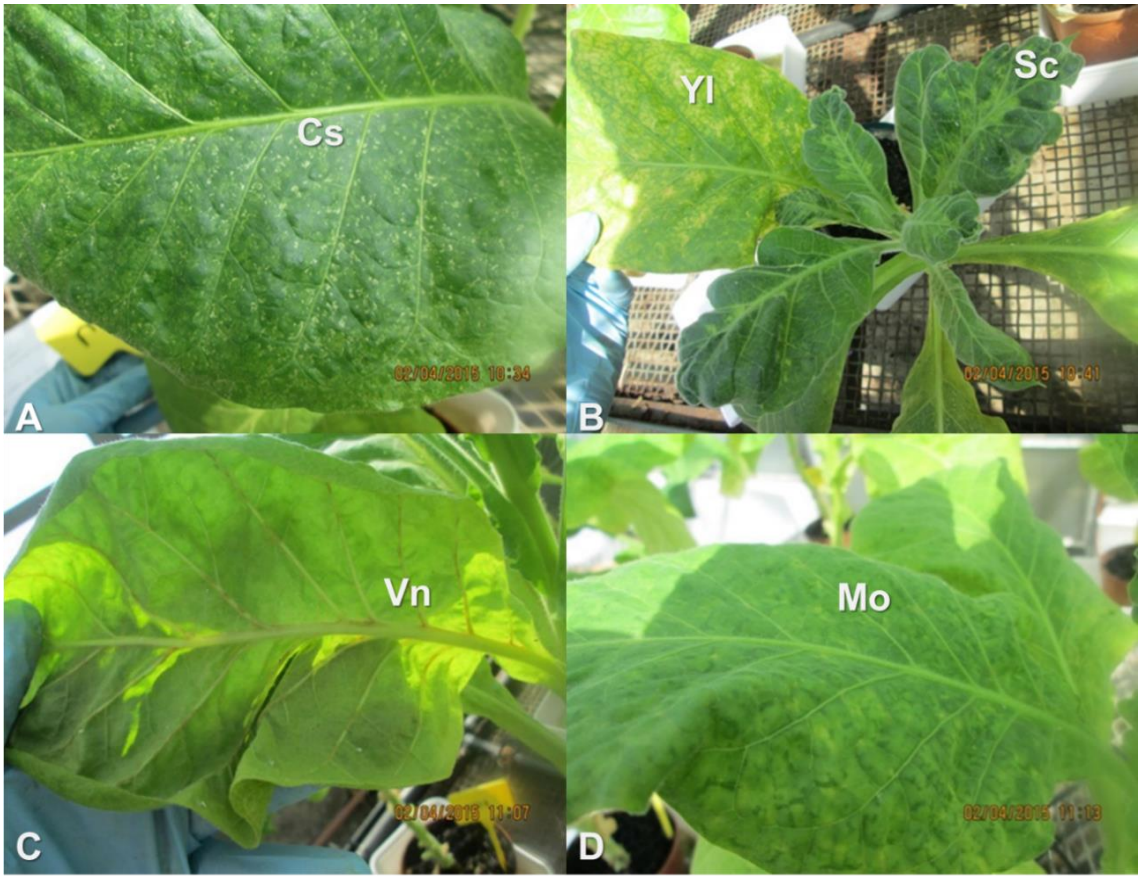

Figure 2.3: Symptoms on *Nicotiana tabacum* cv. Samsun plants. A: *N. tabacum* plant mechanically inoculated with PVY isolate obtained from Keates Drift. B: *N. tabacum* plant mechanically inoculated with PVY isolate obtained from Nxamalala. C: *N. tabacum* plant exhibiting veinal necrosis on the underside of the leaf. D: Mosaic patterns are easily observed on this *N. tabacum* plant inoculated with a PVY isolate from Muden. Cs: Chlorotic spots; Yl: yellowing; Sc: Shrivelled and curled leaves; Vn: vein necrosis; Mo: mosaic patterns.

2.3.4 RT-PCR

The expected amplicon of 512 bp observed from the four samples tested with RT-PCR (Figure 2.4) confirmed the presence of PVY in all four of the tested potato samples.

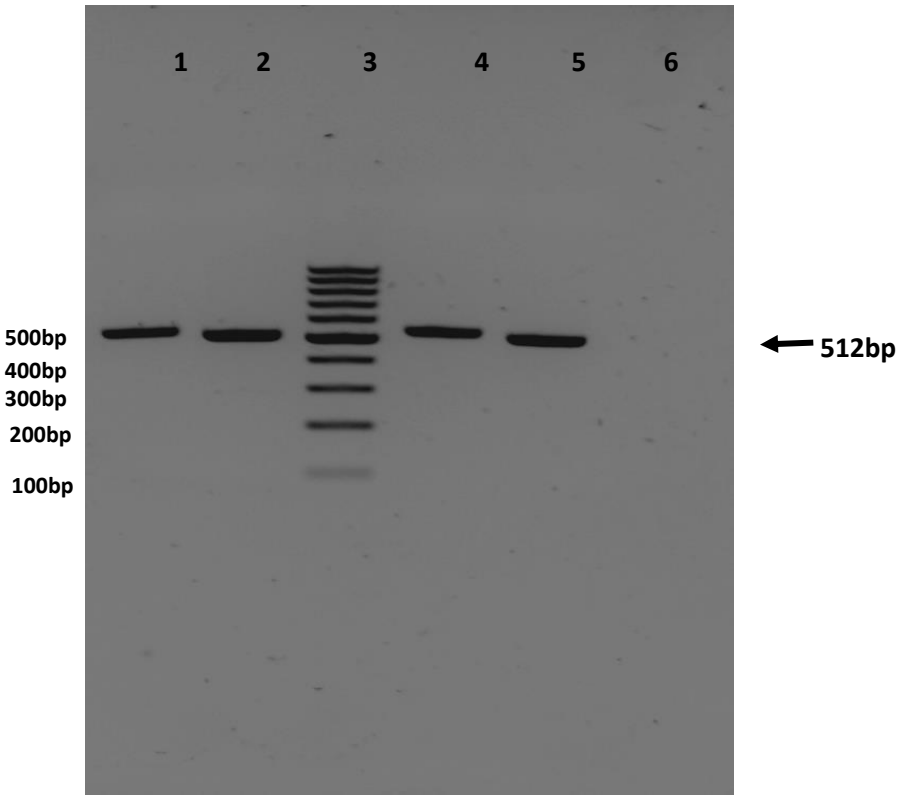

Figure 2.4: Agarose gel electrophoresis of RT-PCR products of four PVY isolates from Msinga using primers designed by Aramburu *et al* (2006). Lane 1 Isolate MOD1 from Muden; Lane 2: Isolate SneP3 from Tugela Ferry; Lane 3: 100 bp DNA ladder (ThermoFisher Scientific, USA); Lane 4: Isolate A4 from Nxamalala; Lane 5: Isolate KD3 from Keates Drift. Lane 6: Water Control.

2.3.5 Electron Microscopy

Potyvirus-like particles were observed in a purified sample prepared from *N. tabacum* cv Samsun plants inoculated with one of the four randomly selected samples showing symptoms. The particles that were approximately 720-730 nm in length with a diameter of about 11 nm were observed under the TEM (Figure 2.5).

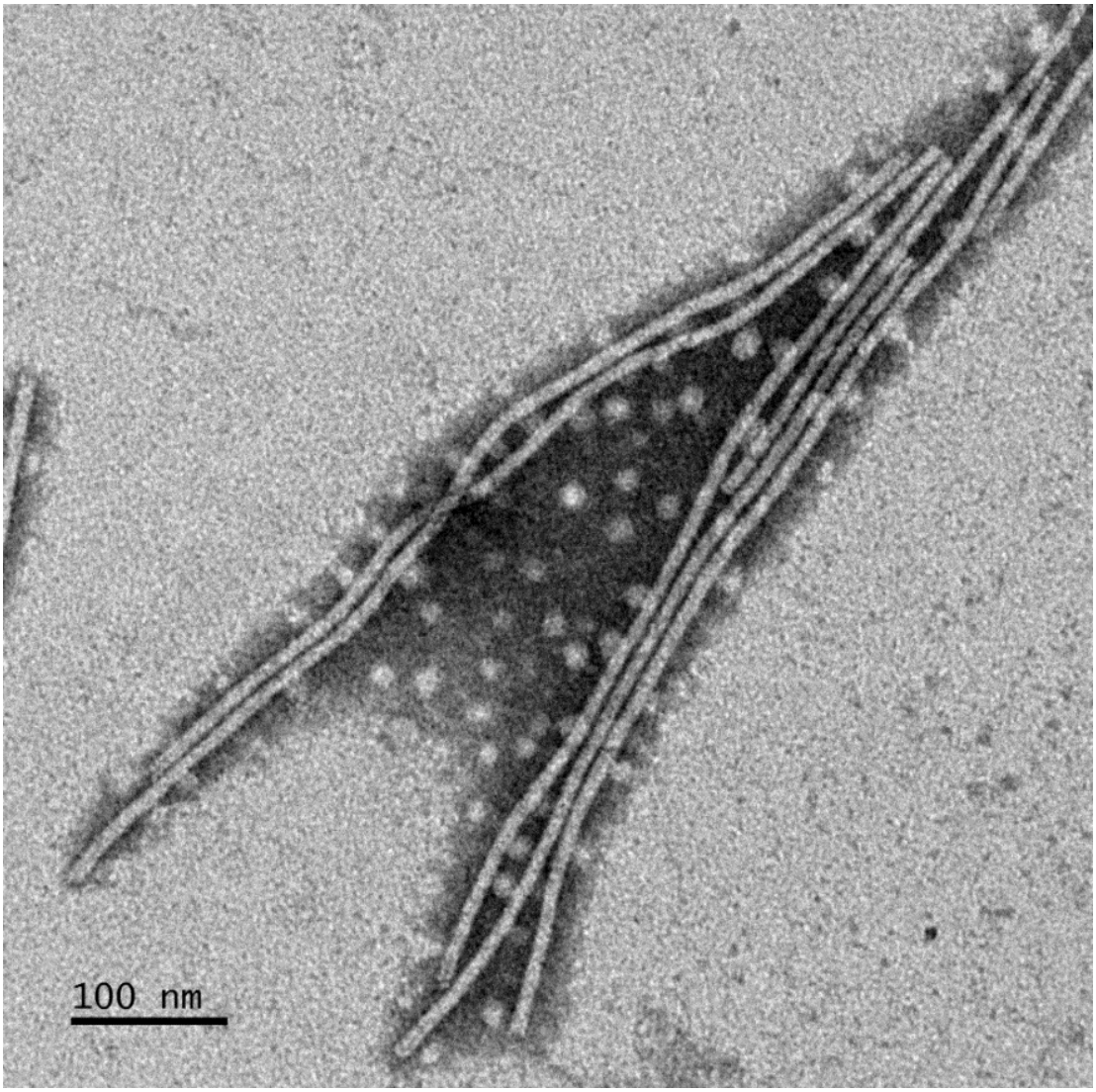

Figure 2.5: Potyviral particles as seen under an electron microscope. The particles roughly 720-730 nm long and about 11 nm in width**.**

2.3.6 SDS-PAGE

A band of roughly 30 kDa was observed on a SDS-PAGE gel for all the four randomly selected samples (Figure 2.6). This was the expected size of the potyviral CP which plays a major role in the cell-to-cell transport of viruses (Mảikinen and Hafrẻn, 2014).

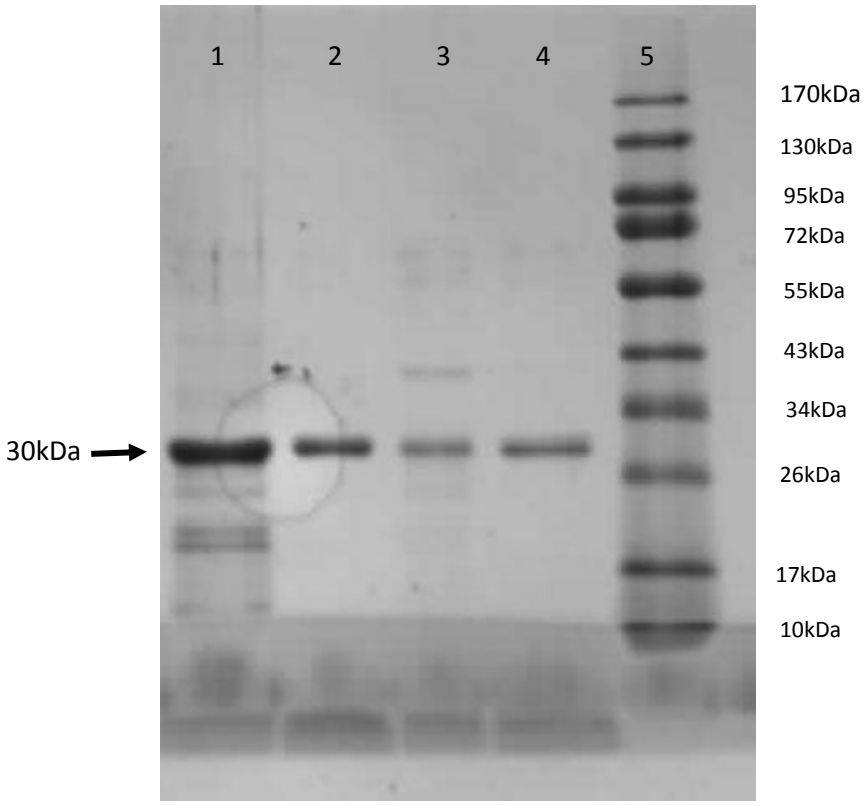

Figure 2.6: SDS-PAGE analysis of purified PVY samples. Lane 1-4: Purified PVY samples showing a single 30 kDa band; Lane 5 : Protein ladder (ThermoFisher Scientific, USA).

2.4 Discussion

PVY was successfully detected using ELISA. PVY was found in all locations where samples were collected. This was confirmed with Monoclonal antibodies specific to PVY^{O} and $PVY^{O/C/N}$ (Table 2.2 and Table 2.3). The PCR results obtained in Figure 2.4 are in agreement with the ELISA results. This confirms that either test can be successfully used for PVY detection. This study along with other PVY studies in SA (Budnik *et al*., 1996; Ibaba and Gubba, 2011; Visser and Dirk, 2009) confirms the existence of PVY and the threat it poses to vegetable production in SA. In a study by Abbas and Hameed (2012), potato samples were examined serologically and it was found that ELISA technique is mostly reliable with higher concentration of virus.

Mechanical inoculation was done to propagate the virus to have enough infected material for the study. Symptoms such as mosaic patterns were observed on *N. tabacum* plants (Figure 2.3). According to Schubert *et al.* (2007), symptoms of PVY^{O} and PVY^{C} include mild to severe leaf mosaic, rugosity, crinkling, severe systemic necrosis, dropping of leaves (leaf drop streak) and dwarfing. PVY^{N} and $PVYN^{W}$ isolates, however, cause only mild leaf symptoms and frequently these may not always be obvious in infected plants. Symptom appearance depends on potato cultivar, strain of PVY as well as environmental conditions (Karasev and Gray, 2013). In foliage, PVY may induce mosaic or rugose mosaic which can cause growth retardation and the stunting of the plant (Karasev and Gray, 2013). PVY^{NTN} may cause severe leaf symptoms in addition to severe tuber necrosis on the surface of the tubers (Schubert *et al*., 2007).

The high proportion of samples in which PVY^{N} was detected (96%) indicates that this strain is now endemic to potato-growing regions of Msinga. This is likely to have serious implications on potato production in Msinga. PVY^{NTN}, a recombinant of PVY^{N} and $PVY^{O,}$ has the ability to cause potato tuber ringspot disease (PTRND). This disease of potatoes drastically reduces tuber quality and yield (Karasev and Gray, 2013). The existence of PVY^{C} in South Africa and worldwide has not been well documented, with only two reports of its occurrence in South Africa. (Brunt, 2001; de Bokx and Huttinga, 1981). None of the recent studies of PVY in South Africa reported the existence of this strain in potatoes. This study did not detect PVY^{C} either.

The high PVY incidence requires a closer look at control strategies and assessment of their effectiveness. PVY can be managed by controlling aphids. However, this is challenging because these vectors transmit the virus in a non-persistent manner. Use of insecticides would worsen the problem as the aphids, when sprayed, become agitated and probe many healthy plants before dying (de Bokx and Huttinga, 1981). Use of resistant cultivars is a good management strategy. Some farmers, however, tend to plant retained tubers. This then serves as inoculum for the virus and can create serious losses in crop production. Thus

controlling PVY in potatoes will always remain a challenge for resource-poor farmers like those found in Msinga.

In conclusion, various virus detection methods confirmed the high prevalence of PVY in potatoes in Msinga. It also confirmed that PVY^N is the most prevalent strain in Msinga with over 96% incidence. There is no plausible explanation as to why PVY^O strain was only found at Muden area. According to Navas-Castillo *et al*. (2011) numerous factors that contribute to the emergence of plant virus diseases include genetic changes in the virus genome [mutation, recombination, or reassortment (pseudorecombination)], long distance traffic of plant material or of vector insects due to trade of vegetables and ornamental plants, and changes in the vector populations.

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Chapter 3

Genome analysis of selected *Potato virus Y* isolates infecting potatoes in Msinga, KwaZulu-Natal.

Abstract

Potato virus Y (PVY), a single-stranded positive sense RNA virus is an important pathogen belonging to the family *Potyviridae*. Recombination is prevalent in PVY and its impact on the virulence of the virus may lead to great economic losses. The aim of this study was to analyse the full genome sequences of four randomly selected PVY isolates infecting potatoes in the Msinga district in the province of KwaZulu-Natal, Republic of South Africa in order to understand the evolution of the virus in this area. To achieve this, total RNA extracted from PVY isolates (A4, KD2, MOD1 and SneP3) which were propagated in tobacco (*N. tabacum cv* Samsun) was used as a template for next generation sequencing (NGS). NGS was run on Illumina HiSeq using paired-end chemistry 125×125bp reads. *de novo* assembly of the generated reads was performed. The resulting contigs were blasted to the GenBank database in order to identify PVY genomes. Isolates SneP3, KD2 and A4 had the same genome so only the sequences of isolates SneP3 and MOD1 were used in the subsequent analyses. Phylogenetic analysis was conducted using 39 closely related PVY sequences including the following strains: PVY^{N}, PVY^{O}, PVY^{NTN}, $PVY^{N:O}$ and PVY^{C} . *Sunflower chlorotic mottle virus* was used as an outgroup. Recombination events were assessed using RDP4 software. Phylogenetic results revealed that PVY Isolate SneP3 belonged to the PVY^{NTN} strain group while isolate PVYMOD1 belonged to the PVY^{N} Wilga strain group. Recombinant analyses confirmed the occurrence of PVY recombinant strains in the Msinga district. This has serious implications on the management of PVY diseases by small-scale farmers growing potato for a livelihood.

3.1 Introduction

Potato virus Y (PVY) is an important pathogen that infects several solanaceous crop species including potato (*Solanum tuberosum* L.), tobacco (*Nicotiana* spp), pepper (*Capsicum* spp.), eggplant (*S. melongena*) and tomato (*S. lycopersicum* Mill). Infection leads to significant yield losses and tuber quality degradation (Shukla *et al*., 1994). According to Visser *et al*. (2012), PVY has evolved into a major crop disease within the last 80 years. All PVY infections reduce yield, but under warmer growing conditions (such as in the potato growing regions of southern Europe and South Africa) the most devastating strains can entirely compromise the economic viability of a crop (Visser *et al*., 2012). PVY is the type species of the genus *Potyvirus* in the family *Potyviridae* (Hollings and Brunt, 1981). The single-stranded positive sense RNA genome is ~9.7 kb in size and is translated into a polyprotein consisting of 3,061 amino acids and then cleaved into ten multifunctional proteins by P1 serine, HC-Pro cysteine and NIa-Pro cysteine proteinases**.** In addition, a short polypeptide (PIPO) is translated by +2 nucleotide frame shifting from the P3 region, resulting in a P3-PIPO fusion product (Chung *et al*., 2008; Gao *et al*., 2014). The length of pipo is highly variable among PVY isolates, and this intraspecific variation in length may be maintained by host-driven adaptation (Gao *et al*., 2014; Hillung *et al*., 2013).

PVY is known for displaying a high degree of genetic variability and exists as multiple crop dependent strains (Glais *et al.,* 2002; Le Romancer *et al*., 1994). Different PVY strains are associated with differing degrees of pathogenicity, of which the most common and economically important are known to be recombinant (Visser *et al*., 2012). Three classical strains of PVY are known: PVY^{C}, PVY^{O} and PVY^{N}. These strains were named according to the symptoms they cause in potato and tobacco plants, which are the main indicators of infection with these strains. When molecularly characterized, several PVY^{NTN} isolates have been described as recombinants of PVY^{N} and PVY^{O} because they present three or four recombination junctions (RJs) in different regions of the genome (Kerlan *et al*., 2006; Singh *et al*., 2008). Currently PVY^{NTN} is described in several potato fields around the world (Galvino-Costa *et al*., 2012). In Poland, in 1991, a recombinant was detected for

the first time in the Wilga cultivar and was named PVY "Wilga" (PVYN-Wi) (Chrzanowska, 1991). PVYN-Wi may or may not cause symptoms in tubers. This recombinant also represents a combination of PVY^{O} and PVY^{N} genomic segments organized between two RJs with the major part of its genome belonging to the PVY^{O} strain group, which explains the O serology found for these isolates. Although the PVY^{O} exerts a serological reaction, biologically it is able to induce vein necrosis in tobacco plants, which is a typical reaction reported for necrotic isolates. PVY isolates that share these same features have been reported in North American countries. However, the genomes of these PVY isolates have only one RJ and are named $PVY^{N:O}$ (Gray *et al*., 2010; Singh *et al*., 2008).

According to Marston *et al*. (2007), RNA viruses have small, simple genomes, which have a high level of diversity, due to the low-fidelity viral polymerase used for replication. Traditionally, due to their small genomes, 'genome-walking' was used to obtain a reference sequence, from which primer pairs can be designed for down-stream use on similar viruses (Delmas *et al*., 2008). This methodology can however be time-consuming, laborious and expensive because of the variation within virus species, which results in the need to redesign primer pairs and constant re-optimization of conditions. These issues can be overcome by using Next Generation Sequencing (NGS), a high-throughput sequencing methodology which generates millions of sequences simultaneously from one sample (Schuster, 2008). There are numerous advantages of NGS technologies in virology, and its use is becoming more commonplace (Radford *et al*., 2012) particularly to detect and characterize viruses without the requirement of specific primers (Wu *et al*., 2012). In this study, the genome sequences of four randomly selected PVY isolates infecting potato in Msinga was recovered using NGS before being analysed for recombination events.

3.2 Materials and Methods

3.2.1 Virus Sampling

As outlined in Chapter 2.

3.2.2 RNA extraction, quantity and quality assessment

Due to financial constraints, only the four PVY isolates (A4, KD2, MOD1 and SneP3) analysed in Chapter 2 were analysed in this Chapter. Total RNA was extracted from PVY-infected fresh tobacco leaves using Quick RNA Mini-Prep (ZymoResearch, USA) according to the instructions of manufacturers. RNA concentration and quality were assessed using a NanoDrop 2000c (Thermo Scientific, USA).

3.2.3 NGS sequencing and data analysis

Total RNA was used as a template for NGS performed at the Agricultural Research Council's Biotechnology Platform (ARC-BTP) in Pretoria (RSA). Samples were treated with Ribo-Zero (New England Biolabs, United Kingdom) to remove ribosomal RNA prior to library preparation and sequencing on the Illumina HiSeq using the paired-end chemistry 125×125bp reads.

Sample demultiplexing was done using CASAVA pipeline software, an optional analysis tool for Illumina sequencing experiments, which enables the ability to understand INDEL detection, SNP information, and allele calling **(**Hosseini *et al*., 2010). Quality analysis of raw DNA reads was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before and after trimming on CLC-Bio WorkBench version 7.5.1 according to the following settings: trimming 3' bases with qualities lower than 25 and discarding reads shorter than 15 nt or longer than 200 nt. The data was imported back into CLC-Bio Workbench and paired-end as well as single reads libraries were generated. The paired and single reads were assembled into contigs using CLC-Bio Workbench with a kmer of 63. Paired-end reads were used for scaffolding to increase library size. All sequences shorter than 200 bp were discarded from the final assembly. *De novo* assembly was run according to the CLC-Bio (version 7.5.1) default settings; NGS host genome data which consisted of the whole genomes of potato and tobacco was removed during *de novo*. Contigs, generated from *de novo* assembly, were subjected to BLAST on the GenBank database and those matching PVY were selected for further analysis.

3.2.4 PVY sequence analysis and phylogeny

All contigs that matched PVY were selected and imported onto MEGA6 software (Tamura *et al*., 2013). Phylogenetic analysis was conducted using 39 closely related PVY sequences including the following strains: PVY^{N} , PVY^{O} , PVY^{NTN}, $PVY^{N:O}$ and PVY^{C} . *Sunflower chlorotic mosaic Virus* (SuCMoV) was used as an outgroup (Table 3.1). Sequences were downloaded from National Center for Biotechnology Information (NCBI) and aligned with the selected PVY sequences using the Muscle program installed on MEGA6 software. A phylogeny tree was then constructed on MEGA6 software. The evolutionary history was inferred by using the Maximum Likelihood method based on the general time reversible model (Nei and Kumar, 2000) with a Gamma distribution and invariable sites. The bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 70% bootstrap values were collapsed. Nucleotide and amino acid sequence identity comparisons were performed using Sequence Identity and Similarity (SIAS) online tools (http://imed.med.ucm.es/Tools/sias.html).

3.2.5 Recombination analysis of isolates

Sequences of the isolates under study together with those in Table 3.1 were assessed for evidence of recombination using RDP4 software. Each identified recombination was examined individually. Recombination analysis was conducted with the RDP suite of recombination programs (Martin *et al*., 2015). The analysis utilized the embedded programs namely RDP (Martin and Rybicki, 2000), BootScan (Martin *et al*., 2005), MaxChi (Smith, 1992), SiScan (Gibbs *et al*., 2000); Chimaera (Posada and Crandall, 2001), GENECONV (Padidam *et al*., 1999) and 3Seq (Boni *et al*., 2007). Recombination events detected by more than two methods at a p-Value < 0.05 were accepted.

Table 3.1. Full length PVY isolates used for phylogenetic analysis

Strain	Isolate	Country	Genbank Accession Code	References
N_Wilga	PVYMOD1	South Africa	KX710153	This Study
NTN	SneP3	South Africa	KX710154	This study
N	SS082A 88	South Africa	JN936433	Visser *et al*.,2012
N	NN300_41	South Africa	JN936422	Visser *et al*.,2012
N	34/01	Poland	AJ890342	Schubert *et al*.,2007
N	SYR-NB-16	Syria	AB270705	Chikh *et al*.,2010
N	SASA-61	UK	AJ585198	Gao et al.,2014
N	Mont	USA	AY884983	Lorenzen *et al*.,2006
C	NNP	Italy	AF237963	Fanigluilo *et al*.,2005
C	NC57	USA	DQ309028	Gao *et al*.,2014
C	Adgen	France	AJ890348	Schubert *et al*.,2007
O	Oz	USA	EF026074	Gao *et al*.,2014
O	SASA-110	UK	AJ585195	Gao *et al*.,2014
SYR-II	SYR-II-2	Syria	AB461451	Chikh *et al*.,2010
SYR-II	SYR-II-DrH	Syria	AB461453	Chikh *et al*.,2010
SYR-II	SYR-II-Be1	Syria	AB461452	Chikh *et al*.,2010
NTN	Gr99	Poland	AJ890343	Schubert *et al*.,2007
NTN	12-94	Poland	AJ889866	Schubert *et al*.,2007
NTN	HN2	China	GQ200836	Hu *et al*.,2009
NTN	NTN	USA	EF026075	Gao *et al*.,2014
NTN	PVY-AST	Brazil	JF928460	Galvino-Costa *et al*.,2012
NTN	RRA-1	USA	AY884984	Lorenzen *et al*.,2006
NTN	NTN24 1	South Africa	JN936431	Visser *et al*.,2012
NTN	Z14	South Africa	JN936440	Visser *et al*.,2012
NTN	Z16	South Africa	JN936441	Visser *et al*.,2012
NTN	Z26	South Africa	JN936442	Visser *et al*.,2012
NTN	NTN16 1	South Africa	JN936428	Visser *et al*.,2012
NTN	NTN17 1	South Africa	JN936429	Visser *et al*.,2012
NTN	NTN23 1	South Africa	JN936430	Visser *et al*.,2012
NTN	CC9_12	South Africa	JN936416	Visser *et al*.,2012
E	PVY-MON	Brazil	JF928458	Galvino-Costa *et al*.,2012
E	PVY-AGA	Brazil	JF928459	Galvino-Costa *et al*.,2012
N:O	GF_YL20	China	KJ634023	Gao *et al*.,2014
N_Wilga	PN_10A	USA	DQ008213	Gao *et al*.,2014
N_Wilga	N1	USA	HQ912863	Karasev *et al*.,2011
N_Wilga	5	Germany	AJ890350	Schubert *et al*.,2007
N : O	PB209	USA	EF026076	Gao *et al*.,2014
N : O	N:O-Mb112	Canada	AY745491	Nie *et al*.,2004
N : O	N:O-L56	Canada	AY745492	Nie *et al*.,2004
SuCMoV	-	Argentina	NC014038	Bejerman *et al*.,2010

3.3 Results

3.3.1 RNA Quality and quantity assessment

An OD 260/280 ratio greater than 1.8 is considered pure RNA as was found in this study (Table 3.2). There was a direct relationship between the RNA concentration of the samples and the absorbance. Isolate A4 had the lowest concentration and so was its absorbance value. Furthermore, PVYMOD1 had a concentration of 327.7ng/µl and the highest absorbance of 2.08

Table 3.2: RNA Quantification of samples.

Sample	ng/µl	A260	A280	260/280
A4	88.1	2.203	1.042	2.01
KD3	206.0	5.15	2.5	2.03
PVYMOD1	327.7	8.192	3.93	2.08
SneP3	314.1	7.853	3.83	2.05

The total data generated amounted to 11 gigabytes. The number of raw reads ranged from ~19.7 million to just over 23 million. Trimming the reads had an effect on the average length of the nucleotides as it reduced it from 125 bp to approximately 102 bp (Table 3.3). Over 80% of the data was host genome data and was therefore discarded. The percentage of the reads mapping to the virus ranged from 0.04% to 47%. Amongst the four isolates there were only 6-17 contigs matching PVY that were generated by de *novo* assembly (Table 3.3).

3.3.2 Phylogenetic analyses

Isolate A4, SneP3 and KD3 had the same genome upon inspection so only isolate SneP3 was use for phylogeny analysis to avoid repetition. PVY MOD1 had a completely different genome and it too was considered for phylogenetic and recombination analyses. Isolate SneP3 genome was 9705 bp and PVY MOD1 9658 bp. The maximum likelihood tree generated split PVY isolates into 10 major clusters (Figure 3.1). Isolate SneP3 clusters within the PVY NTN-A cluster as identified by Gao *et al* (2014). The most noticeable fact is that Isolate SneP3 did

not cluster with the previously reported South African isolates (Figure 3.1). Isolate SneP3 clustered with PVY^{NTN} (EF026075) and PVY AST (JF928460) of which they share 99.24% nucleotide sequence identity and 99.7% amino acids sequence identity. Sequence comparison of the Isolate SneP3 with the South African NTN-A previously indicated that they shared about 95% nucleotide sequence identity and about 75% amino acid sequence identity.

Table 3.3: Assembly statistics of PVY genomes

	Sample			
Statistic	A4	SneP3	KD3	PVYMOD1
Number of raw reads	23 322960	20 446210	19 715784	21 040982
Average length	125	125	125	125
Number of reads after trim	23 298806	20 427351	19 684845	20 994846
Average length	102.3	103.1	101.5	100.3
Number of reads after host removal	1 0077928	12 766226	15 51086	11 497841
Reads mapping to PVY	10 622	8 310519	9 290689	4 883025
Contigs generated	76 643	19001	8462	5217
Contigs matching PVY	6	17	9	7

Isolate PVYMOD1 formed a cluster with Isolates PVY PN_10A (DQ008213) and PVY N1 (HQ912863) as shown in Figure 3.1. It shares a nucleotide sequence identity of 99.1% with both of these isolates and 98% nucleotide sequence identity with PVY Wilga (AJ890350). This cluster was identified by Gao *et al* (2014) as a PVY^{N} Wilga cluster. In terms of amino acid sequence, Isolate PVYMOD1 shares a 99.8% sequence identity with Isolates PVY PN_10A and PVY N1 and 99% sequence identity with PVY Wilga. Both PVY isolates from Msinga described in this study shares 91.9% and 96.2% nucleotide and amino acid sequence identity respectively.

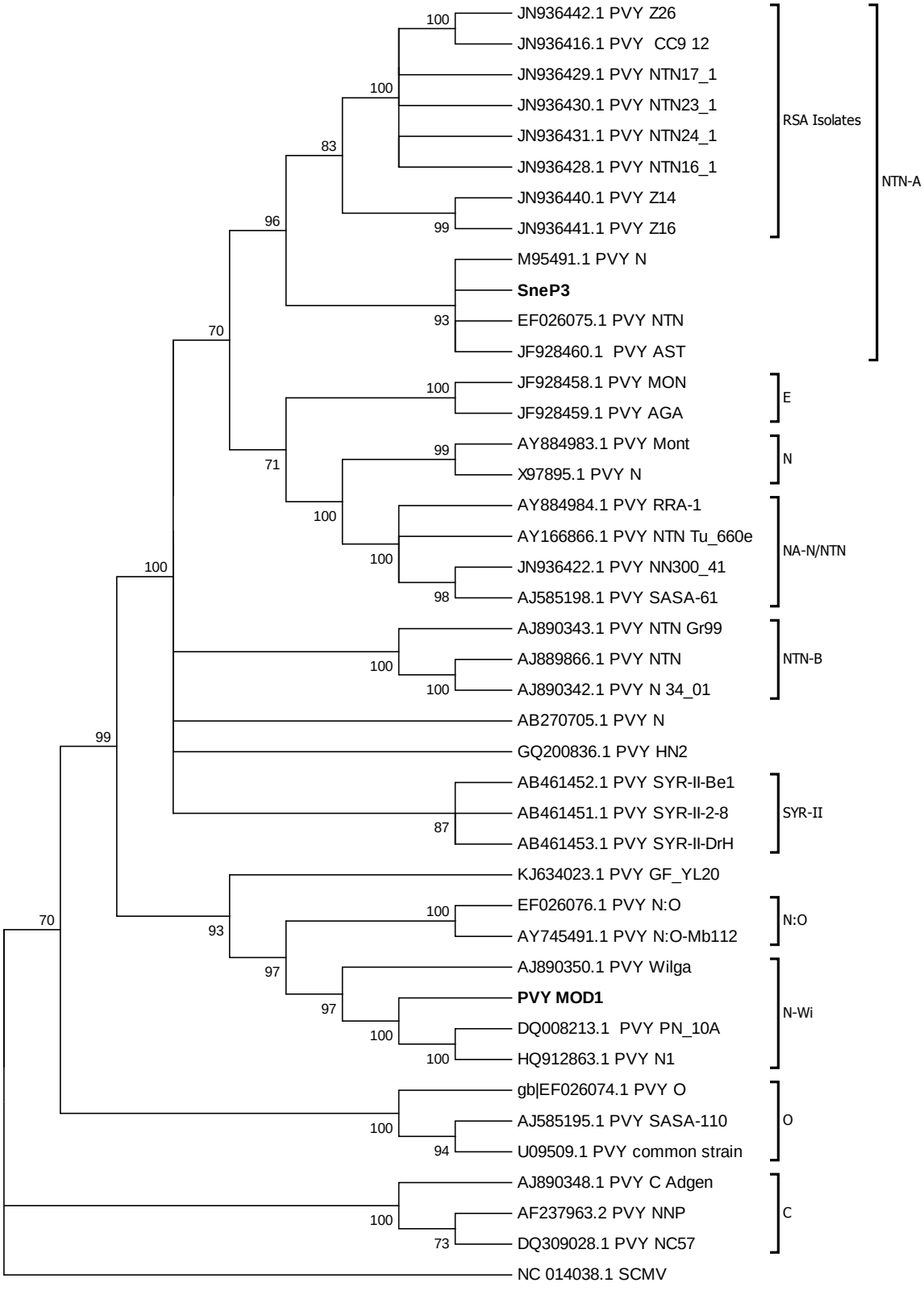

Figure 3.1. Maximum Likelihood Phylogenetic relationship of Msinga PVY isolates The bootstrap values are shown next to the branches.

3.3.3 Recombination analyses

According to the RDP4 program it was found that Isolate SneP3 had two recombination events (Figure 3.2). Event 1 occurs at 2437 nt to 5852 nt and consists of a major parent PVY^N 605 with 98.4% similarity to Isolate SneP3 and minor parent PVY Oz with a 98.6% similarity to Isolate SneP3. Event 2 occurs from 9203nt to 9710nt and consists of major parent PVY^N 605 with 93.7% similarity to Isolate SneP3 and a minor parent PVY^O SCRI with a 99.6% similarity to Isolate SneP3. Isolate PVYMOD1 had a single recombination event occurring from position 462 nt up to 2358nt and consists of major parent PVY Oz (98.7% similarity to Isolate PVYMOD1) and minor parent PVY Mont (99.2% similarity to Isolate PVYMOD1).

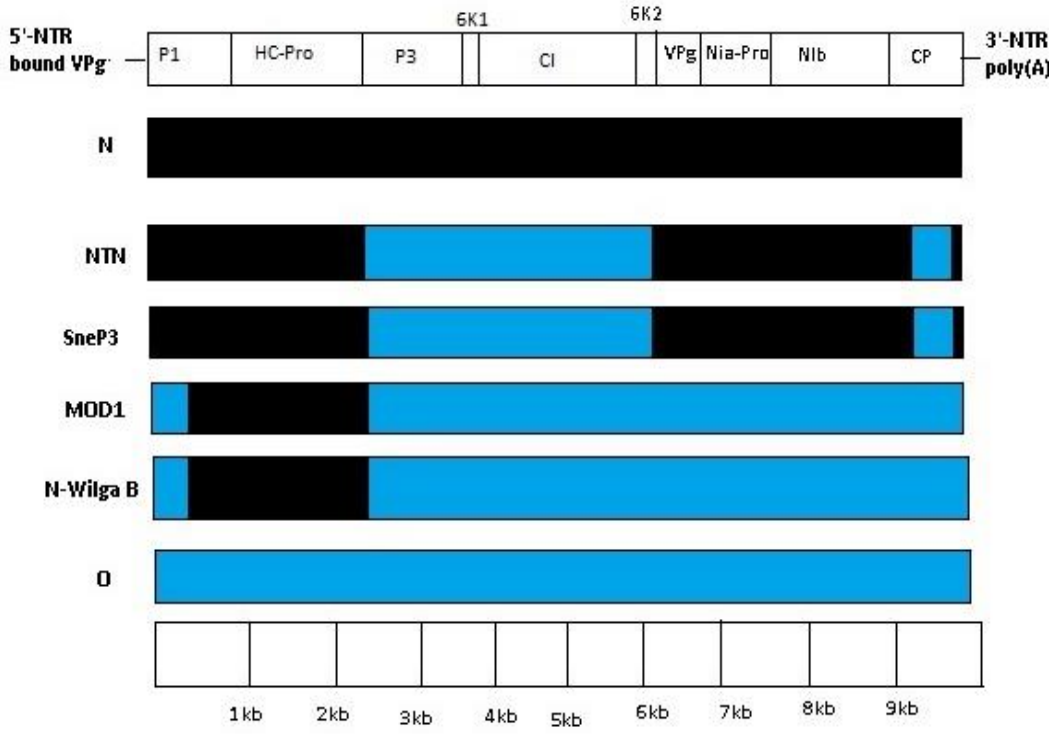

Figure 3.2: Recombination map of PVY genomes. Patterns of recombination between PVY parental strains PVY^N (black) and PVY^O (blue). Recombinant genomes are represented as combinations of these two colours, with vertical lines indicating known recombinant junctions. The top and bottom boxes indicate the

various genes of the PVY polyprotein and an indication of where exactly on the genome the different recombination events are occurring respectively.

3.4. Discussion

The accuracy of NGS is reportedly influenced by the quantity and quality of starting RNA. RNA is a thermodynamically stable molecule, which is, however, rapidly digested in the presence of RNase enzymes. Purity and integrity of RNA are critical elements for the overall success of RNA-based analyses. Starting with low quality RNA may strongly compromise the results of downstream applications which are often labour-intensive, time-consuming and highly expensive (Auer *et al*., 2003, Imbeaud *et al*., 2005, Raeymarkers, 1993).

It was noted in this study that the samples with the lowest RNA concentration also yielded the lowest percentage of PVY mapped reads when compared to those with high RNA concentration. Host genome data accounted for more than half of the data in some samples (Table 3.3) and was discarded. According to Kutnjak *et al* (2015) high background of host nucleic acids and the high diversity of viral populations complicate the reconstruction of a complete consensus viral genome sequence from NGS data. To overcome this problem, a step for the amplification of complete or partial viral genomes is included (McElroy *et al*., 2014). Such an approach enables achieving great sequencing depth, however, it can distort the variation occurring in primers annealing regions and affect detection of other variants, which are connected with those regions. Moreover, this approach requires specific knowledge about viral genome sequence, since the sequences of PCR primers used for the amplification of viral genome have to be known in advance. Another more generic solution is the enrichment of viral nucleic acids before sequencing library preparation, employing the characteristics of different viral sequence pools within the host.

Complete genome sequences are known for several PVY strains from the PVY^{O} and PVY^{N} groups, which are ≈8% different along the entire 9.7-kb genome. In addition to these main parental genomes, multiple recombinants have been discovered, with segments of PVY^{O} and PVY^{N} sequences spliced in their genomes

(Hu *et al*., 2009). A distinct genetic characteristic of positive RNA plant viruses is driven by three main factors namely mutation, recombination, and reassortment (Roossinck, 1997; Simon and Burjaski, 1994). PVY is a good example of an RNA virus using high replication rate and build-up of large populations to generate huge genetic diversity and, thus, survive and succeed in infecting multiple hosts and in various geographical areas (Hu *et al.,* 2009; Kerlan, 2006; Kerlan and Moury; 2008, and Wu *et al*., 2012). Strain differentiation can be achieved through the use of bioassays, serology or nucleic acid-based techniques (Chikh *et al*., 2010; Schubert *et al*., 2007). Variants and recombinants of PVY have been detected and NGS helps in identifying where recombination occurs as it has the ability to generate large data at a time in a very short space of time.

Full genome sequences of PVY were obtained and they consisted of two different strains belonging to PVY NTN-A and PVY^{N} Wilga strain groups. RDP results and phylogenetic analysis showed that Isolate PVY MOD1 belongs to PVY^{N} Wilga strain. PVY^{N} Wilga is a recombinant strain of PVY^{N} and PVY^{O} having serological properties of PVY^{O} but phenotypic properties of PVY^{N} (Ogawa *et al*., 2008; Schubert *et al*., 2007). This is in agreement with our findings where it was shown that Isolate PVYMOD1 had tested positive for PVY^{O} (Chapter 2). To our knowledge, this is the first report of a full genome sequence of PVY^{N} Wilga in South-Africa.

Based on RDP results and phylogenetic analysis, isolates SneP3, A4, and KD2 belong to PVYNTN-A. Phylogenetic analysis of the PVY^{NTN} strain group showed that this is not the first time it is reported in South Africa as other South African isolates belonging to the NTN-A strain group have been identified before (Visser *et al.*, 2012). Isolate SneP3 seems to differ from these previously identified South African isolates at molecular level because it is grouped differently to them. The nucleotide and amino acid sequence identities help us understand why Isolate SneP3 doesn't cluster with South African isolates that have been previously reported. It is also unclear how the nucleotide and amino acid sequence identities relate in terms of pathogenicity of the isolates found in Msinga.

The existence of PVY^{NTN} as the dominant strain in the Msinga region suggests that it may have been introduced earlier than PVY^{N} Wilga. Infected potato seed might have been given to Msinga farmers by one donor thus resulting in the wide spread distribution of PVY^{NTN}.

The findings of this study are significant in that they show the existence of PVY recombinants on a potato crop in a specific geographic area. These findings will have serious implications on how PVY diseases are managed in Msinga district. The importance of NGS in generating a lot of data that can be used to determine the diversity of viruses infecting a particular crop is clearly demonstrated. In conclusion the detection of PVY recombinant strains existing in the Msinga district is a huge concern for farmers as they rely heavily on potato farming for their livelihoods. PVY^{NTN} is capable of causing necrotic ringspots (potato tuber ringspot disease; PTNRD) on the surface of tubers. This renders the tubers unmarketable thus resulting in reduced income earnings, which negatively affects livelihoods.

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Chapter 4

General Overview

4.1 Major Findings

This study sought to determine the existence of *Potato Virus Y* strains infecting potatoes in Msinga. In Chapter 2, based on ELISA and RT-PCR analysis it was confirmed that PVY is prevalent in Msinga as it was detected in all farms where sampling took place. PVY^{N} was the most dominant strain with incidence levels of over 96%. PVY^{O} is also present but at very low levels. Host plant bioassays, SDS-PAGE analysis and electron microscopy studies confirmed the identity of the PVY in the samples analysed.

Results reported in Chapter 3 highlighted the advantage of using NGS over the traditional Sanger sequencing as a tool in Plant Virology. The total data generated was 11 gigabytes with the raw reads as many as 23 million. Recombinant analysis confirmed the presence of PVY^{NTN} and PVY^{N} Wilga recombinants. Isolate PVY MOD1, the isolate which had tested positive for PVY^{O} in Chapter 2 was found to belong to recombinant PVY^{N} Wilga while isolate SneP3 was found to belong to the PVY^{NTN} strain.

4.2 Implications of Findings

The existence of PVY in Msinga is likely to impact farming in a negative way. PVY^{NTN}, a recombinant found in this study is responsible for causing PTNRD, a disease that causes superficial necrotic rings on the surface of the tubers. This reduces the marketability of the potatoes thus resulting in fewer sales. Moreover, PVY^{NTN} causes degradation of tuber starch into simple carbohydrates thus resulting in reduced nutritional value of potatoes (Tomassoli *et al*., 1998).

4.3 Way Forward

Vector biology is important in that it can help farmers know when to plant their crops. Workshops on PVY control strategies can be run whereby farmers will be taught about PVY biology, control methods and factors in epidemiology.

Resistant cultivars can be developed and run question-answer panels with farmers on a regular basis. Extension officers can liaise directly with farmers and be readily available when farmers spot unusual symptoms on their crops which may be due to PVY.

More studies need to be done in the rural areas on small-scale farms because currently more focus is placed on studying the effects of PVY in commercial farms. This will allow for the identification of more PVY isolates whose sequences will be available on GenBank as presently there is fewer than 30 South African PVY isolates sequences that have been deposited.

Future research can focus sequencing all available samples in order to determine if there are no other recombinants. Additionally, NGS can be performed directly from potato plants instead of onto *N. tabacum* inoculated with field samples. It would be advisable to have a bigger sample size for analysis by collecting samples from all farming regions of Msinga to have a more complete picture of PVY in the district. All the collected samples can be sent for NGS provided there is enough funding.

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Appendix A

SDS-PAGE stock solutions

Solution A: Monomer [30% (m/v) acrylamide, 2.7% (m/v) Bis-acrylamide)

Acrylamide (14.0g)

Bis-acrylamide (0.4g)

Dissolved and made up to 50ml with distilled water

Stored in amber-coloured bottle at 4˚C

Filtered through Whatman No.1 filter paper before use

Solution B: 4x Running Gel buffer [1.5M Tris-Hcl,Ph 8.8]

Tris (9.070g)

Dissolved in 40ml distilled water

Adjust to ph 8.8 with HCl

Made up to 50ml

Filtered through Whatman No.1 filter paper before use

Solution C; 4x Stacking Gel Buffer [500Mm Tris-HCl, ph 6.8]

Tris (3g)

Dissolved in 20ml distilled water

Adjust to ph 8.8 with HCl

Made up to 25ml

Filtered through Whatman No.1 filter paper before use

Solution D: 10% (m/v) SDS

SDS (2.5g)

Dissolved in 25 ml distilled water with gentle heating

Solution E: Initiator [10% (m/v) ammonium persulfate]

Ammonium persulfate (0.1g)

Made up to 1ml just before use.

Solution F: Tank Buffer [250Mm Tris-HCl , 192Mm glycine,0.1% (m/v) SDS, PH 8.3]

Tris (6g)

Glycine (14.4g)

Made up to 1 litre

2.5ml of solution D was added prior to use

Solution G: Reducing Treatment Buffer [125 Mm Tris-HCl , 4% (m/v) SDS, 20% (v/v) glycerol,10% (v/v) 2-mercaptoethanol PH 6.8]

Solution C (0.25ml)

Solution D (0.4ml)

Glycerol (0.2ml)

2-mercaptoethanol (0.1ml)

Made up to 1ml in distilled water

Conferences to date

- Ximba, S.P.F. and Gubba, A. Detection, Differentiation and Genome analysis of *Potato virus Y* strains infecting potato (*Solanum tuberosum* L.) in the Msinga area in KwaZulu-Natal, Republic of South Africa. 13th International Plant Virus Epidemiology Symposium, Avignon France. 6-10 June 2016.