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Characterization of two plant rhabdoviruses not previously reported in South Africa

BY

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I declare that the thesis, which I hereby submit for the degree MSc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

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Abstract

Two previously uncharacterized plant rhabdoviruses, infecting Bermuda grass (*Cynodon dactylon* (L.) Pers) and soybean (*Glycine max*) respectively, have been found in South Africa. To determine the morphology and virion size of these viruses, embedded ultra-thin sections of infected plant samples were observed under a transmission electron microscope. The virion distribution within the cell, the bullet-shaped morphology and the virion sizes indicated that both these viruses might belong to the *Rhabdoviridae* family. Degenerate polymerase chain reaction (PCR) primers were designed by alignment of the polymerase gene sequences of several plant rhabdoviruses in order to identify conserved regions. Standard PCR and sequencing protocols were used to determine a partial polymerase gene sequence of the viruses that was then compared to the most closely related sequences available on Genbank. The analysis indicated that the *Cynodon* rhabdovirus was most closely related to known nucleorhabdoviruses; and the rhabdovirus-infecting soybean (Soybean blotching mosaic virus proposed name) was closely related to other known cytorhabdoviruses. These results indicate that both the viruses are new members to the *Nucleo-* and *Cytorhabdovirus* genera, respectively.

List of abbreviations:

aa	amino acid
acc. number	accession number
AMV	Avian Myoblastosis Virus
ARC	Agricultural Research Council
BLAST	Basic Local Alignment Search Tool
bp	base pair
CCSV	*Cynodon* chlorotic streak virus
CRV	*Cynodon* rhabdovirus
cDNA	complementary Deoxyribose nucleic acid
ddH_2O	double distilled H_2O
DNA	Deoxyribonucleic acid
dNTP	deoxynucleic triphosphate
et al.	and others
G	glycoprotein
h	hour
ICTV	International Committee for the Taxonomy of Viruses
IPTG	Isopropyl-B-D-1-thiogalactopyranoside
ISEM	Immunosorbent Electron Microsopy
L	polymerase (L) protein
LB	Luria-Bertani
LNYV	*Lettuce necrotic yellows virus*
M	matrix protein

M	Molar
mg	milligram
$MgCl_2$	Magnesium chloride
min	minutes
ml	millilitre
MMV	*Maize mosaic virus*
N	nucleoprotein
NCMV	*Northern cereal mosaic virus*
ng	nanogram
nm	nanometer
OFV	Orchid fleck virus
ORF	Open reading frame
P	phosphoprotein
PCR	Polymerase Chain Reaction
pmol	pico molar
RNA	Ribonucleic Acid
RSA	Republic of South Africa
RT-PCR	Reverse Transcriptase PCR
RYSV	*Rice yellow stunt virus*
SBMV	Soybean blotching mosaic virus
SCV	*Strawberry crinkle virus*
SYNV	*Sonchus yellows net virus*
Taq	Taq polymerase (*Thermus aquaticus*)
TaVCV	Taro vein chlorosis virus
TEM	Transmission Electron Microscope

TMV	*Tobacco mosaic virus*
U	Unit
UHQ water	Ultra high quality water
UV	Ultra-Violet
µg	microgram
µl	microlitre
µM	micro Molar
p-distance	pairwise distance

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Chapter 1: Literature Review

The plant rhabdoviruses

1.1 The family *Rhabdoviridae*

Rhabdoviruses, in the order *Mononegavirales*, family *Rhabdoviridae*, are viruses of medical and agricultural (veterinary health and plant production) importance. Other families in the order *Mononegavirales* include the *Bornaviridae*, *Filoviridae* and *Paramyxoviridae*. The four families are viruses that have monopartite, negative sense RNA genomes (ICTVdb 2006; Jackson et al., 2005; Pringle and Easton, 1997). The name *Rhabdoviridae* is derived from the Greek word *rhabdos* that refers to rod, or bullet, referring to the shape of the viral particles. Rhabdoviruses have a single-stranded, negative-sense RNA genome, 12 - 15kb long. The rhabdovirus particle sizes range from 45-100nm in width and 130-350nm in length. The genome encodes for at least five functional proteins (Fig. 1.1): the nucleocapsid protein (N), phosphoprotein (P), matrixprotein (M), glycoprotein (G) and the polymerase (L). Different rhabdoviruses vary considerably in size. Their virions consist of a tightly coiled nucleocapsid core that is enclosed by host-derived lipids with protruding glycoprotein spikes. The consensus functional genes (Table 1.1) are flanked by leader and trailer regions in the order 3'-leader-N-P-M-G-L-trailer-5' (ICTVdb 2006; Jackson et al., 2005).

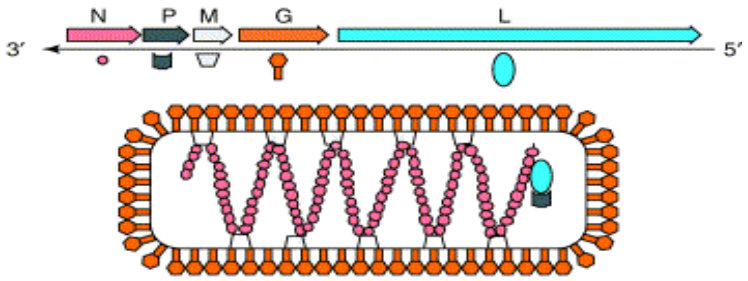

Fig 1.1: Representation of the minimal rhabdovirus genome (above) and virion (below). The minimal rhabdovirus genome (example vesiculoviruses) contains five structural protein genes: the nucleocapsid protein (N), the polymerase complex consisting of P and L, the matrix protein (M) and the glycoprotein (G). All of the fully sequenced plant rhabdoviruses contain one or more additional genes between P and M. Reprinted from Publication Trends in Microbiology, Volume 11 number 5, Author(s) Hogenhout et al, titled Plant and animal rhabdovirus host range: a bug's view, p. 265, Fig 1(c), Copyright (2003), with permission from Elsevier.

Table 1.1: Properties of the rhabdovirus-encoded proteins (Jackson et al., 2005; Poch et al., 1990; Posthuma et al., 2000):

Protein	Function
Nucleocapsid protein (N)	Assists in replication and encapsidates the genomic RNA.
Phosphoprotein (P)	It is a component of the viral nucleocapsid core. Forms complexes with the N and L proteins and therefore forms part of the moveable subunit of the polymerase.

Matrix protein (M)	Condenses the nucleocapsid and associates with the G protein during morphogenesis. Responsible for the assembly and budding of the virions.
Glycoprotein (G)	It forms the glycoprotein spikes of the virions. In vertebrate and arthropod rhabdoviruses, the G protein functions in host recognition by binding to host cell receptors. Glycosylation of the G protein is a prerequisite for viral budding.
Polymerase protein (L)	Contains the polymerase and RNA binding domains. Important for transcription and replication.

All the fully sequenced plant rhabdoviruses (to date) contain at least one extra gene, between the P and M genes (3'-leader-N-P-**X**-M-G-L-trailer-5') indicated by **X**. Therefore, all plant rhabdoviruses have at least six open reading frames (ORFs). The function of the extra gene(s) is still however open to speculation. It has been hypothesized is that the extra gene (or sixth protein) is required for cell-to-cell movement in plant rhabdoviruses as it resembled other plant virus movement proteins. Genes between P and M are absent in vertebrate-infecting rhabdoviruses (Jackson et al., 2005; Posthuma et al., 2000).

Rhabdoviruses have a very broad host range, infecting vertebrates, invertebrates and plants (Hogenhout et al., 2003; ICTVdb 2006; Jackson et al., 2005). The only other virus family that is similar in having a large host range are the bunyaviruses (*Bunyaviridae*), infecting a variety of arthropods, mammals and plants (ICTVdb 2006). There are a total of six accepted genera (Table 1.2) in *Rhabdoviridae* recognized by the ICTV, separated, amongst other citeria, according to their hosts. The mammalian genera include *Ephemerovirus*, *Lyssavirus* and *Vesiculovirus*. *Novirhabdovirus* are rhabdoviruses that infect fish, and the plant rhabovirus genera are the *Cyto-* and *Nucleorhabdoviruses*. Kondo et al. (2006) suggested a new genus, *Dicorhabdovirus*, to be included into the *Rhabdoviridae* family, with Orchid fleck virus (OFV) as the type species. This genus would include viruses that resemble rhabdoviruses except that the genome is divided (Kondo et al., 2006). However, not only is the genome of OFV bipartite, the viral capsid is also not enveloped (ICTVdb 2006). Therefore, despite the obvious sequence similiarities to plant rhabdoviruses, OFV will not be able to be classified as a rhabdovirus due to its bipartite genome.

Table 1.2: The genera within *Rhabdoviridae* (ICTVdb 2006; Hogenhout et al., 2003):

Genus	**Species**	**Hosts**	**Insect vectors**
Ephemerovirus	*Bovine ephemeral fever virus* (BEFV)	Cattle	Mosquitoes
Lyssavirus	*Rabies virus* (RaV)	Humans, mammals	None (to date)
Vesiculovirus	*Vesicular stomatis Indiana virus* (VSINV)	Cattle and swine	Blackflies, sandflies
Novirhabdovirus	*Viral hemorrhagic*	Fish	None

	septicemia virus (VHSV)		
Cytorhabdovirus	*Lettuce necrotic yellows virus* (LNYV)	Plants	Planthoppers, aphids, leafhoppers
Nucleorhabdovirus	*Sonchus yellow net virus* (SYNV)	Plants	Planthoppers, aphids, leafhoppers
*Dicorhabdovirus**	Orchid fleck virus (OFV)	Plants	Unknown

* New genus for *Rhabdoviridae*, suggested by Kondo et al. (2006).

Studies by Poch et al. (1990) revealed that the L protein of different species of rhabdoviruses show a very high degree of homology to each other. The polymerase gene is believed to be the most conserved among rhabdoviruses. The authors have compared the L protein between different rhabdoviruses and also paramyxoviruses, and found that there could be an evolutionary relationship between unsegmented negative strand RNA viruses (*Mononegavirales*). It was also shown that there are six blocks of high conservation in the L protein of the rhabdoviruses. The blocks of conservation were designated block I – VI, of which blocks II – V are considered to have high amino acid conservation and are located in the central region of the L gene. Block III of the polymerase gene was found to be conserved among all RNA-dependent RNA polymerases (Poch et al., 1990; Bouhry et al., 2005).

The phylogenetic similarity of the families in the order *Mononegavirales* is believed be due to a common ancestor. Because of the wide host range of rhabdoviruses, and the highly conserved polymerase gene, the L protein of rhabdoviruses is ideal to study the evolution of members of negative sense RNA viruses (Poch et al., 1990; Bourhy et al., 2005; Posthuma et al., 2000). There is also a sequence similarity of the L gene as

well as the N genes of viruses in the families *Orthomyxoviridae* and *Bunyaviridae* with the families of Mononegavirales, indicating a common ancestor for all negative, single stranded RNA viruses (ICTVdb 2006; Koonin et al., 2006; Pringle & Easton, 1997).

1.2 The plant rhabdoviruses: genera *Cytorhabdovirus* and *Nucleorhabdovirus*

Early classification of plant rhabdoviruses was simply based on electron microscopic observations and serological cross-reactivity, without any molecular information. As a result, about a 100 members have been characterized based on morphology alone (Jackson et al., 2005; Kuzmin et al., 2006). Microscopy has revealed that plant rhabdoviruses accumulate within membrane-bound vesicles and that there are two separate replication sites within the rhabdovirus infected plant cell; the virions either accumulate in the nuclear membranes or accumulated in the cytoplasm. Based on the replication sites of the viruses, plant rhabdoviruses are divided into two genera, *Nucleorhabdovirus* (from the latin word *nux* or *nucis* meaning "nut") and *Cytorhabdovirus* (from the greek word *kytos* meaning "cell"). It is not yet understood why the two genera have two different maturation sites, but this current classification is supported by the available genome sequence information and the cell biology (ICTVdb 2006; Jackson et al., 2005; Hogenhout et al., 2003; Posthuma et al., 2002).

There are differences in the replication cycle of the nucleo- and cytorhabdoviruses (Fig. 1.2). First, the rhabdoviruses gain entry into the plant host by means of an insect vector (see later). Uncoating takes place at the endoplasmic reticulum (ER)

membranes and then the nucleocapsid core is released into the cytoplasm. However, from here the two genera differ (Jackson et al., 2005):

Cytorhabdovirus:

- The newly released cores become transcriptionally active and associate with the ER and establish viroplasms.
- The viroplasms function in transcription of viral mRNA's and replication of genomic and antigenomic viral RNA's.
- Following translation, the viral proteins involved in replication accumulate in the viroplasm.
- Viral glycoproteins are targeted to the cytoplasmic membranes.
- Maturation of cytorhabdoviruses takes place at sites of G protein accumulation in the ER (or possibly to the outer nuclear envelope [ONE]).
- Budding occurs into proliferated ER associated with the viroplasms

Nucleorhabdovirus:

- The newly released cores are transported into the nucleus through nuclear pore complexes (NPC).
- Following transcription and export, the viral mRNA's are translated.
- The viral proteins are imported into the nucleus where they form large viroplasms.
- Morphogenesis occurs near the end of active transcription and replication and involves interactions with the M protein to coil the viral nucleocapsids and form associations with the membrane-associated G protein.

- Budding occurs through intact inner nuclear envelope (INE) resulting in an expansion of the outer nuclear envelope (ONE).
- Mature virions accumulate in the perinuclear spaces of infected cells where they may be reacquired by insect vectors during feeding.

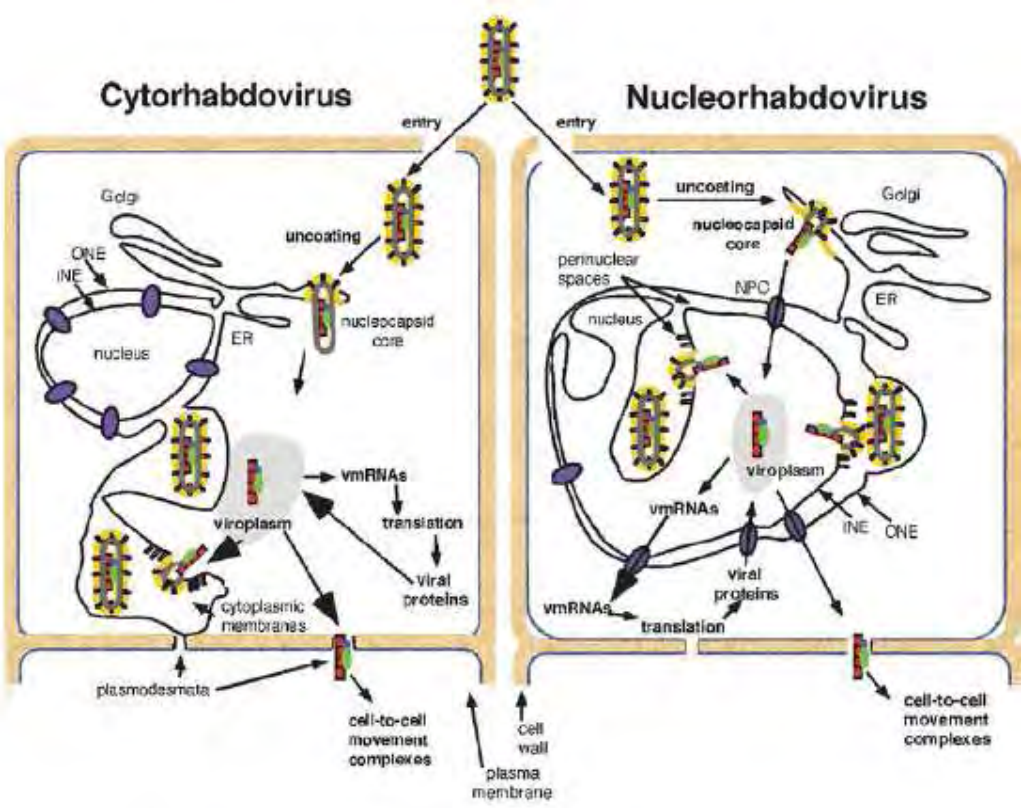

Fig. 1.2. Contrasts between the replication cycles of cyto- and nucleorhabdoviruses (Jackson et al., 2005) ER: endoplasmic reticulum; ONE: outer nuclear envelope; INE: inner nuclear envelope; NPC: nuclear pore complexes (permission for reproduction requested).

Additional biological, molecular and diagnostic examination needs to be performed in order to characterize and define the relationships of known and putative plant rhabdoviruses more clearly (Jackson et al., 2005).

It is believed that plant rhabdoviruses are globally dispersed, and there are still more than 75 unassigned plant rhabdoviruses to be described. There are 18 recognized plant rhabdoviruses (Jackson et al., 2005), but only 10 of these recognized plant rhabdoviruses have been fully sequenced. Orchid fleck virus (OFV), which is still an unclassified rhabdovirus, has also been sequenced (Table 1.3). The authors Kondo et al. (2005) proposed that a new genus, *Dicorhabdovirus*, should be included into the *Rhabdoviridae* family, based on OFV's bipartite genome. However, phylogenetic analysis based on L sequences, indicated that OFV clusters with other nucleorhabdoviruses (see Chapter 2, Fig. 2.7, p. 38; Kondo et al., 2006). The latest addition to the plant rhabdoviruses is the Lettuce yellow mottle virus, a new cytorhabdovirus (Heim et al. 2008).

Table 1.3: List of sequenced genomes of plant rhabdoviruses available on Genbank (www.ncbi.nlm.gov).

Unclassified *Rhabdoviridae*	Acc. Number
Orchid fleck virus (OFV)	NC009609
Lettuce yellow mottle virus (LYMoV)	EF687738
Taro vein chlorosis virus (TaVCV)	AY674964
Maize fine streak virus (MFSV)	AY618417
Nucleorhabdovirus	
Sonchus yellow net virus (SYNV)	L32603

Rice yellow stunt virus (RYSV)	AB001125
Maize mosaic virus (MMV)	AY618418
Cytorhabdovirus	
Lettuce necrotic yellows virus (LNYV)	NC007642
Northern cereal mosaic virus (NCMV)	NC002251
Strawberry crinkle virus (SCV)	AY250986

1.3 Plant rhabdoviruses are arthropod-borne.

Insects play an important role in the horizontal transfer of most rhabdoviruses. A high degree of vector specificity has been observed for the rhabdoviruses (Jackson et al., 2005) Generally one or two vector species of the same genus transmit a single rhabdovirus (Creamer & He, 1997). Five of the seven genera of *Rhabdoviridae* contain viruses that are insect-transmitted and/or have insect hosts. Some rhabdoviruses are vertically transmitted to insect progeny, but vertical transmission of rhabdoviruses does not occur in plants or vertebrates (Hogenhout et al., 2003). This suggests that an insect was the primary host of the rhabdovirus ancestor. The ancestor of all ssRNA(-) viruses probably emerged for the first time around 700 million years ago, when arthropods crawled out of the ocean to feed on plants. However, the evolution of *Rhabdoviridae* continues to be unclear (Hogenhout et al., 2003; Kuzmin et al., 2006).

The insect host transmits the rhabdoviruses in a circulative, propagative manner (Fig. 1.3). Upon feeding, the virus is acquired, internalised and actively transported across

several cell membranes. The virus ultimately associates with the vector's salivary glands to be inoculated into the host. Circulative, propagative viruses replicate in both the arthropod as well as in the plant or vertebrate host (Gray and Banerjee, 1999) and most rhabdovirus species have two natural hosts: either insects or plants, or insects and vertebrates. It is thought that the vertebrate or plant host is merely a temporary host and that it serves as a high titre source to permit the efficient infection of more arthropod hosts (Gray and Banerjee, 1999; Jackson et al., 2005).

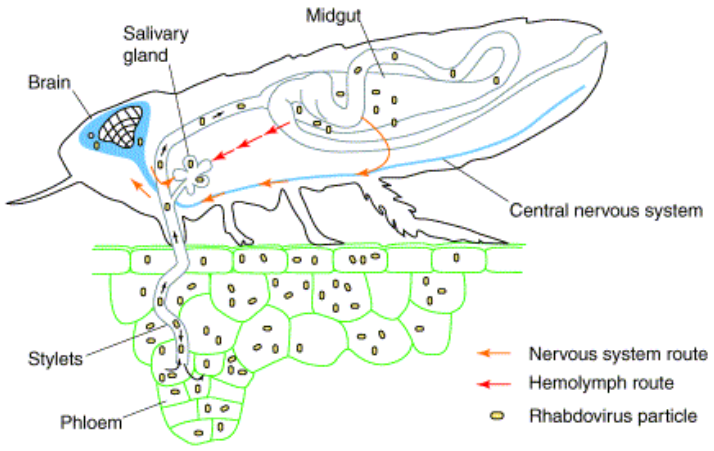

Fig. 1.3. Transmission of a plant rhabdovirus, by means of a leafhopper. The virus is acquired by the leafhopper from the plant cells through the stylet from the insect's mouthparts. From the stylet the viruses move from the midgut lumen through the epithelial cell layer into the hemolymph, and/or nerve cells and brain. It spreads throughout the insect and infect the salivary glands. From here they are re-introduced into a new plant host. Reprinted from publication Trends in Microbiology, Volume 11 number 5, Author(s) Hogenhout et al, titled Plant and animal rhabdovirus host range: a bug's view, p. 266, Fig 2(a), Copyright (2003), with permission from Elsevier.

Arthropod vectors play a crucial role in determining the host range of vertebrate and plant rhabdoviruses (Jackson et al., 2005). The natural host range of plant rhabdoviruses is determined by the vector specificity and feeding behaviours, therefore the vector dynamics have a major influence on the distribution, evolution and survival of the virus (Jackson et al., 2005; Gray and Banerjee, 1999). The worldwide distribution of plant rhabdoviruses is likely to be linked to the distribution of their arthropod vectors and their feeding behaviours (Jackson et al., 2005).

Studies have shown that plant rhabdovirus strains can be recovered after prolonged passage in plants with a loss in their capability to be transmitted by insects. This phenomenon could provide means for evolution of vector-less rhabdoviruses, in cases where infections become established in vegetatively propagated hosts (Jackson et al., 2005).

1.4 Plant rhabdoviruses in the Republic of South Africa (RSA)

Plant rhabdoviruses are globally dispersed and cause serious plant disease outbreaks all across the world (Creamer & He, 1997; Jackson et al., 2005). Amongst the recognized and unclassified plant rhabdoviruses, only SCV has been reported in South Africa (Posthuma et al., 2000). It is very likely that there are more known or unknown plant rhabdoviruses present in southern Africa. These viruses are not observed in this country because of a lack of surveillance or funds for research in plant rhabdoviruses. In the last two decades, reports of two probable plant rhabdoviruses infecting soybean plants (Pietersen et al., 1998) and the other on Bermuda grass (Pietersen, *unpublished*). Electron microscopy has revealed that both

these viruses have typical rhabdovirus morphology. The aim of this research project was to identify and characterize these two new putative rhabdoviruses, and to provide enough evidence to classify them within a *Rhabdoviridae* genus. If confirmed that both these viruses are indeed rhabdoviruses and if they are characterized at molecular level, it could motivate other virologists on the opportunities of describing new or unassigned plant rhabdoviruses, not just in South Africa, but also worldwide.

1.4.1 An unknown rhabdovirus found in *Cynodon dactylon*

Bermuda grass (*Cynodon dactylon* (L.) Pers) is a perennial species of the family *Poaceae* with a worldwide distribution (Hosseini et al., 2005). In South Africa Bermuda grass is mostly used for animal food and it also makes excellent coverage for lawns and sports fields. It plays an important role in conservation as it prevents soil erosion. In traditional medicine it is used for indigestion, heartburn and treatment of wounds. The Xhosa tribe developed a lotion from the grass for swellings, and the Sotho use the grass as a charm and to ward off sorcery (Mitich, 1989).

Rhabdovirus-like particles were found to be associated with chlorotic streak symptoms in *Cynodon dactylon* in the North West Province, South Africa in 1995. Further investigation through electron microscopy revealed that the rhabdovirus particles accumulated in the perinuclear spaces in very high numbers (Pietersen, *unpublished*). This is the first report of a virus in the *Nucleorhabdovirus* genus in South Africa.

1.4.2 An unknown rhabdovirus found in *Glycine max*

During a survey on soybean (*Glycine max*) viruses of South Africa, conducted in 1993, an unknown rhabdovirus in soybean plants was shown to be widespread in soybean fields in the Brits/Thabazimbi region, North West Province, South Africa, (Pietersen et al., 1998). This unknown virus showed blotchy mosaic symptoms on the leaves of the soybean plant. The soybean rhabdovirus has not been characterized, but electron microscopy has shown that the virions are bullet-shaped, typical of rhabdovirus morphology (Pietersen, *unpublished*).

1.5 Conclusion

The purpose of this research was to characterise the two viruses described in the above sections on such a level that they could be compared to other plant rhabdoviruses towards clarifying their taxonomic status and classification. Plant rhabdoviruses are infecting crops worldwide; several of these are serious diseases of major economic impact. The amount of sequence information that is available in the public domain is very limited and it is one of our objectives to add to this database of sequence information. If more sequence data should come available, it will permit a better understanding of the relationships among these unique viruses in the *Nucleo-* and *Cytorhabdovirus* genera. There are still many opportunities to extend our knowledge of plant rhabdoviruses, here in South Africa and the rest of the world.

Further characterization and proposed classification of the South African *Cynodon* rhabdovirus will follow in Chapter 2. Chapter 3 will deal with the characterization and proposed classification of the South African soybean rhabdovirus, with Chapter 4 as conclusion.

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Chapter 2:

Characterization of a *Nucleorhabdovirus* new to South Africa

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2.1 Abstract

A previously uncharacterized plant rhabdovirus, infecting Bermuda grass (*Cynodon dactylon* (L.) Pers) in the North West Province, South Africa, has been found. To determine the morphology and virion size of this virus, embedded ultra-thin sections of infected plant samples were observed under a transmission electron microscope. The virion distribution within the cell, its bullet-shaped morphology and its size (240 x 63 nanometres) indicated that this might be a rhabdovirus of the genus *Nucleorhabdovirus*. Degenerate polymerase chain reaction (PCR) primers were designed by alignment of the polymerase gene sequences of several plant rhabdoviruses in order to identify conserved regions. Standard PCR and sequencing protocols were used to determine a partial polymerase gene sequence of this virus sample which was then compared to the most closely related sequences available on Genbank. The analysis indicated that the virus was indeed most closely related to known nucleorhabdoviruses, with the highest homologies being to Taro vein chlorosis virus and *Maize mosaic virus.* Serological testing indicated that the South African *Cynodon* rhabdovirus had a close serological relationship with the nucleorhabdovirus *Cynodon chlorotic streak virus*.

2.2 **Introduction**

Bermuda grass (*Cynodon dactylon* (L.) Pers) is a perennial species of the family *Poaceae* with a worldwide distribution (Hosseini et al., 2005). Rhabdovirus-like particles were found to be associated with chlorotic streak symptoms in *Cynodon dactylon* in Morocco in 1974 and the virus, named *Cynodon* chlorotic streak virus (CCSV), was widely distributed in the Mediterranean area (Lockhart et al., 1985). Similar chlorotic streak symptoms were found in Bermuda grass in the North West Province of South Africa in 1995 and such plants also contained rhabdovirus-like particles. The aim of the study was to characterize this virus on the basis of antigenicity and genetic sequence comparisons. Utilizing the conserved regions of the L gene, degenerate RT-PCR primers were designed and subsequent analysis of the amplified sequence indicated that this virus was most closely related to the group of viruses within the genus *Nucleorhabdovirus*. Antisera specific to CCSV (Lockhart et al., 1985) reacted with the South African virus in ISEM analyses and confirmed a close relationship between the two viruses. This report is the first to describe the presence of a nucleorhabdovirus in South Africa.

2.3 Materials and Methods

2.3.1 **Collection of samples**.

Symptomatic Bermuda grass (Fig. 2.1), believed to be infected with an unidentified virus, was vegetatively propagated and maintained in a greenhouse at the University of Pretoria (Pretoria, South Africa). Fresh leaf samples were taken whenever needed.

Fig. 2.1. The symptoms associated with the unknown rhabdovirus infecting *Cynodon dactylon*, found in South Africa. The virus causes fine, chlorotic streaking along the younger and older blades of Bermuda grass.

2.3.2 **Electron microscopy**.

To determine the morphology and virion size, embedded ultra-thin sections of infected plant material were observed under a Philips EM301 TEM. Ultra-thin sections of infected Bermuda grass tissue were fixed under vacuum with 2.5% glutaraldehyde in a 0.075M phosphate buffer, pH 7.4, for 18 h at room temperature and then rinsed three times for 10 min each using wash buffer (0.075 phosphate buffer). The sections were then fixed in 0.5% (vv^{-1}) aqueous osmium tetraoxide (OsO_4) for 1 h and again washed with the wash buffer. Dehydration was performed in a successive increasing ethanol concentration series (50%, 70%, 90%, 3 x 100%, all

vv^{-1}) for 10 min each. Infiltration with 30% (vv^{-1}) Quetol 651 resin (SPI Suppliers, Canada) in ethanol for 1 h was followed by 60% Quetol 651 resin with ethanol for another 1 h, followed by infiltration with pure Quetol 651 resin for 4 h or longer. Polymerisation was done with pure Quetol 651 resin for 48 h (Van der Merwe et al., 1992). The material was then cut into ultra-thin sections (70 – 90 nm thickness with a light gold interference) using a diamond knife fitted to an ultramicrotome (Reichert Ultracut E, Leisa Mikrosysteme, Austria). Each section was collected on a 300 mesh copper grid. Grids were stained for 30 min 4% (vv^{-1}) aqueous uranyl citrate and, after rinsing with H_20, for 3 min with Reynolds' lead citrate, (pH 12, Reynolds, 1963), followed by a final wash step. Grids were immediately examined with a Philips EM301 transmission electron microscope at 60 kV. *Tobacco mosaic virus* (TMV) was used as an external standard to calibrate magnifications. TMV infected leaf pieces were negatively stained in 4% (vv^{-1}) uranyl citrate and observed with a Philips EM301 transmission electron microscope (Van der Merwe et al., 1992).

2.3.3 **Isolation of RNA**.

Viral RNA was extracted from infected plant material using SV Total RNA Isolation System (Promega, Wisconsin) according to the method described by the manufacturers.

2.3.4 **Oligonucleotide primers design**.

ClustalW in BioEdit version 7.0.0 (Hall, 1999) was used to perform a multiple alignment of the L gene of various *Nucleo-* and *Cytorhabdoviruses* available on Genbank (Table 2.1). Degenerate primers were designed to the most conserved

regions of the L genes and were expected to be broadly reactive to plant rhabdoviruses.

Table 2.1: L gene sequences (GenBank) of plant rhabdoviruses used for degenerate primer design. The abbreviation as well as the accession number of each of these viruses is also listed (www.ncbi.nlm.nih.org.)

Nucleorhabdovirus	Acc. Number
Sonchus yellow net virus (SYNV)	L32603
Taro vein chlorosis virus (TaVCV)	AY674964
Rice yellow stunt virus (RYSV)	AB001125
Maize mosaic virus (MMV)	AY618418
Cytorhabdovirus	
Lettuce necrotic yellows virus (LNYV)	NC007642
Northern cereal mosaic virus (NCMV)	NC002251
Strawberry crinkle virus (SCV)	AY250986

2.3.5 **PCR amplification**.

For cDNA synthesis, 10 µl *Cynodon* rhabdovirus RNA and 1µl RhabF (10 pmol) were denatured at 70 °C for 5 min and cooled on ice for 2 min. A mixture of AMV buffer, 40mM dNTP's, 50 U RNase Inhibitor and 40 U AMV-RT (Roche, Switzerland) was added to the reaction tube, and made up to 20 µl with ultra high quality DNAse and RNAse free water (UHQ water) and incubated at 25 °C for 10 min. The temperature was then increased to 42 °C and incubation at this temperature was for one hour. The AMV was then inactivated at 99°C for 5 min. PCR reactions of 50 µl reaction

volumes were prepared. The reaction mixture consisted of 5 µl of 10x reaction buffer (Bioline, UK), 50 mM $MgCl_2$, 40 mM dNTP's, 10 pmol of each primer, and 2.5U Taq polymerase (Bioline, UK). Finally 5 µl of the cDNA template was added, and the volume made up to 50 ul with UHQ water. The cycle conditions, on a ABI GeneAmp PCR System 2700 (PE Applied Biosystems, Connecticut), were as follows: one cycle at 94 °C for 2 min, and then 30 cycles of 94 °C for 30 s, 37 °C for 30 s, 72 °C for 90 s, followed by one final cycle at 72 °C for 7 min. Amplicons were analysed on a 1% agarose gel with a O'GeneRuler 100bp DNA Ladder Plus (Fermentas, Vilnius Lithuania) and viewed under a UV transilluminator (Vilber Lourmat, France). A *Strawberry crinkle virus* (SCV) clone was kindly supplied by M. Goodin (University of Kentucky, Lexington) and was used as a positive control in PCR reactions.

2.3.6 **Cloning of PCR products**.

PCR products were ligated into pGEM-T Easy Vector (Promega, Wisconsin) and the recombinant plasmids introduced into JM109 High Efficiency Competent Cells (Promega, Wisconsin). For ligation, 5 µl 2X Rapid Ligation buffer and T4 DNA Ligase, 1 µl pGEM-T Easy Vector (50 ng), 5 µl PCR product and 1 µl of T4 DNA Ligase (3 Weiss units^{-1}) were mixed together, and adjusted to a final volume of 10 µl with UHQ water. The reactions were incubated for 1 h at room temperature. For transformation, two LB/ampicillin/IPTG/X-gal plates were prepared for each ligation reaction according to the manufacturer's instructions (Promega, Wisconsin). The ligation reactions were centrifuged to collect the contents at the bottom of the tube. Ligation reactions (2 µl each) were transferred to a sterile 1.5 ml microcentrifuge tube on ice. Frozen competent cells were thawed for 5 min, and 100 µl added to each of the ligation reactions. Cells were gently mixed and placed on ice for 20 min. Cells were

heat-shocked at 42°C for 45 – 50 s, and placed on ice for 2 min. LB broth (900 µl) with ampicillin added to the transformants and incubated for 1.5 h at 37°C with shaking. Cell suspensions (100 µl) were plated onto duplicate LB/amp/IPTG/X-Gal plates and incubated overnight at 37°C (Promega, Wisconsin). Utilising blue/white selection, colonies were picked with sterile toothpicks and dropped into test tubes containing LB broth with 50 mg/ml ampicillin, followed by overnight incubation at 37°C, with shaking. Plasmid purification was with Wizard Plus SV Minipreps DNA Purification System according to the manufacturers' instructions (Promega. Wisconsin). To verify that insertion has taken place, the purified plasmids were digested with EcoRI (Promega, Wisconsin) and electrophoresed on a 1% agarose gel.

2.3.7 Sequencing of PCR products and clones.

Cloned inserts were sequenced in both directions using RhabF and RhabR primers (see below). The sequencing reactions were performed using ABI Prism BigDye Primer Cycle Sequencing Kits (PE Applied Biosystems, Connecticut), according to the manufacturers specifications. Sequencing was with an automated fluorescent sequencer (ABI 377 DNA Sequencer, PE Applied Biosystems, Connecticut) at the University of Pretoria's commercial sequencing facility.

2.3.8 Sequence analysis.

Consensus sequences from forward and reverse primers that were derived from a single clone, were created with the ContigExpress option from Vector NTI Advance 9. The sequences were analysed by using the basic local alignment search tool (BLAST) program available on the National Centre for Biotechnology Information website (http://ncbi.nlm.nih.gov/). The *Cynodon* rhabdovirus partial L gene sequence

was compared with all available polymerase gene sequences of cytorhabdoviruses and nucleorhabdoviruses by doing multiple alignments with ClustalX in BioEdit version 7.0.0 (Hall, 1999). Mega Version 3.1 (Kumar et al., 2004) was used to construct a neighbour-joining phylogenetic tree based on the cognate sequence regions.

2.3.9 **Serology**.

Antiserum, raised against CCSV in rabbits, was kindly supplied by Lockhart (Lockhart et al., 1985). Crude undiluted plant sap from infected Bermuda grass was used as antigen in an ISEM assay as follows: for trapping of the virus particles, different dilutions (1:100 or 1:1000 vv^{-1}) of the CCSV antisera, in phosphate buffer (0.02M, pH7), were placed onto dental wax. Freshly carbon coated Formvar clad grids were placed on the drops and left for an hour at room temperature. The grids were washed with phosphate buffer (0.02M, pH 7) and each of the grids were placed on a drop of crude plant sap extracts and left for one hour at 37°C. After another washing step, the grids were placed onto another set of antiserum dilutions (1:10 or 1:100 vv^{-1}) for particle decoration, and incubated for 1 h at 37°C. Staining of the grids with uranyl acetate for 5 min was followed with a final washing step with phosphate buffer as well as ddH20. Pre-immunisation serum prepared in rabbits was used as a negative control, in the same dilutions as mentioned above. The grids were viewed under an EM 002A ABT transmission electron microscope. Viral particles were counted to examine the enrichment of the particle trapping by the CCSV antiserum relative to the trapping by the pre-immune serum.

2.4 Results

Vegetatively propagated Bermuda grass samples, showing fine chlorotic streaking along the leaf veins, were sampled from the greenhouse of the University of Pretoria (Pretoria, South Africa) for electron microscopic analysis. Transmission electron micrographs (TEM) of ultrathin sections of these samples were used to determine the intercell distribution as well as the morphology of this virus. The virions were found to be present in the nucleus of infected cells and were present in high numbers (Fig. 2.2a). The viral particles were mostly bullet-shaped, and bacilliform particles were occasionally observed. It was apparent that size variation between individual particles was minimal, and 69 individual particles were measured, using TMV for external calibration of the electron micrographs. The sizes of the virions were found to be typical for members of the *Rhabdoviridae* family (Jackson et al., 2005) and were on average: 240nm (σ = 26) in length and 60nm (σ = 6.5) in width [Fig. 2.2b].

(a) (b)

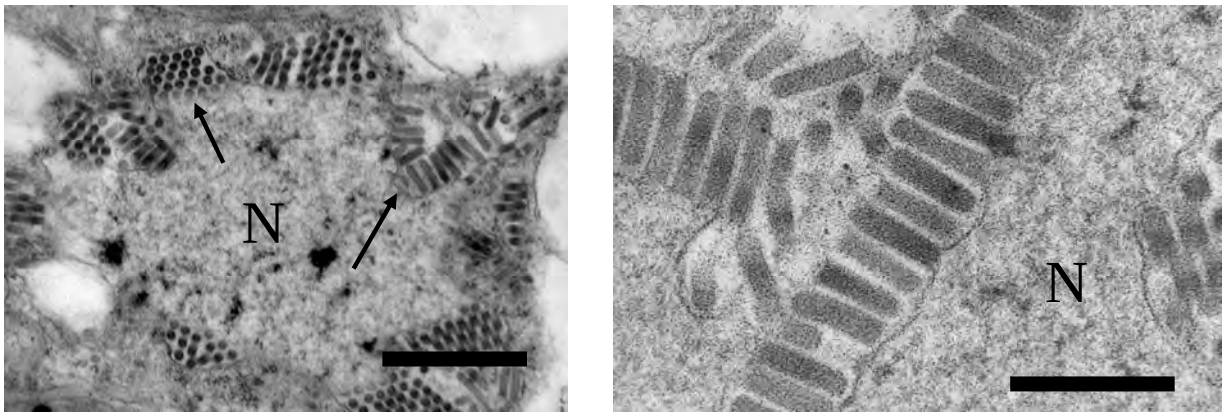

Fig. 2.2. TEM micrographs indicating the *Cynodon* rhabdovirus virions in the nucleus (N). (**a**).The arrows indicate viral particles that are in the process of budding through the nuclear envelope. Scale bar = 1µm. (**b**) The typical bullet-shaped virion particles of the *Cynodon* rhabdovirus. Nucleus (N) indicated. Scale bar = 300nm

2.4.1 **Oligonucleotide primers design**

For RT-PCR, conserved areas of known plant rhabdovirus L genes were targeted and degenerate primers were designed from blocks I and III (Fig. 2.3 a, b) of the aligned L genes. For two or more mismatches, the site was considered degenerate and the primers were: forward primer RhabF (5'-GGATMTGGGGBCATCC-3'), designed from block I; reverse primer RhabR (5'- GTCCABCCYTTTTGYC-3'), designed from block III. The expected amplicon from this primer set was approximately 900 bp depending on rhabdovirus species detected.

Fig. 2.3. (a)

Fig. 2.3. (b)

Fig. 2.3. Primer design: the L gene sequences used in this study of plant rhabdoviruses available on Genbank were aligned to detect relatively conserved sequences. (a) RhabF was designed from block I of the L gene from 6 plant rhabdoviruses and (b) RhabR was designed from block III of the L gene using the same plant rhabdoviruses.

2.4.2 **Amplification of *Cynodon* rhabdovirus and sequence analysis**.

RT-PCR was carried out with primers RhabF and RhabR on total RNA extracted from symptomatic Bermuda grass. The amplicons obtained with this primer set represented a ~900 bp segment from both the *Cynodon* sample and SCV (Fig. 2.4). The PCR product was purified from the gel and cloned into pGEM-T Easy Vector, and sequenced from both directions utilizing RhabF and RhabR. A contiguous sequence was produced and yielded a 791 bp fragment (Fig. 2.5). BLAST analysis confirmed that the cloned inserts were of viral RNA (polymerase gene) origin and that it was most closely related to sequences of various nucleorhabdovirus polymerase genes**.**

The accession number for this sequence is EU650683 from GenBank.

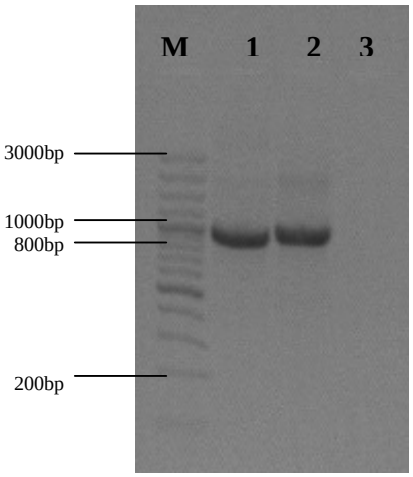

Fig. 2.4. Detection of the putative new rhabdovirus in Bermuda grass by RT-PCR. Samples were loaded onto a 1% agarose gel and electrophoresed for an hour. The O'GeneRuler 100bp DNA Ladder Plus from Fermentas (M), infected *Cynodon* rhabdovirus material (1), SCV (2) and the negative control (3) are indicated.

```
1   gcaattcaaggagatgttctttacatcataccataggattcacaagcattatcctctcca
61  caagatgttagacggcaggccggacagatcatatgttctggattcgttgaagaataacac
121 aactatcaatacaaaggtggttccctacatctttaaggattgggatttcgtaaagctcca
181 gaagaattttgaggtcccttattcatggaatctagtacacaatttgaaagataaggcaat
241 ctccccccacgaggaaggaaatcttcaacactttatcaagacggaataccatctttggggc
301 cgagaacaggcggggtattctcaaatctctcaaaatggagacggttcaattgagggagtt
361 tcttcaaaaggtgaatgatgagggaattgctgaagaagacaggattataggagtctatcc
421 caaggagagggagttgaagatcaaagcacgcctgttctcactaatgtctttcaaacttcg
481 tttgtatatcgtgtcgaccgaagcacttctaggggataagatcatcagatatttccctca
541 gataaccatgtctcttgacatgctatctatgatcaagaaaatgttccgggtatcatcaca
601 gacaaatcgtggagatgattccatcacggtgatcttcaatcttgattttgtaaaatggaa
661 cctgcaaatgcggaaagatatatgcagtccggtgttttcgcagttgggagagctctttgg
721 gatgcctttgatattcgaaaggacgcatgatatcttcaaaaacagcttgatctatctctg
781 ctcaggagagg
```

Fig. 2.5. The 791 bp sequenced fragment obtained from the *Cynodon* rhabdovirus (EU650683). The sequence was obtained from a PCR fragment that was cloned in a PGEM-T-Easy vector (Promega) and sequenced from both directions using RhabF and RhabR. BLAST analysis indicated that the *Cynodon* rhabdovirus sequence was most related to nucleorhabdoviruses.

The 791 bp sequenced fragment of the putative new *Cynodon* rhabdovirus was aligned with the L gene of known plant rhabdoviruses that were available on Genbank. For phylogenetic analysis, a 732 bp region of this alignment of the *Cynodon* rhabdovirus and selection of other plant rhabdoviruses was used. Pairwise distances were calculated and phylogenetic trees constructed as described in Materials and methods. The *Cynodon* rhabdovirus had the highest homology to the nucleorhabdoviruses *Maize mosaic virus* (MMV) and Taro vein chlorosis virus (TaVCV) [Table 2.2].

Table 2.2: Homology matrix of nucleotide sequences of the L genes of the South African *Cynodon* nucleorhabdovirus (indicated CRV) and other plant rhabdoviruses (TaVCV, LNYV, MFSV, MMV, NCMV, RYSV, SCV and the unclassified LYMoV and OFV). Relative homologies to the *Cynodon* rhabdovirus are indicated in bold print. The homology matrix was calculated using MEGA 3.1 (Kumar et al., 2004). The abbreviations as well as the accession numbers are listed in Table 1.3, p. 10

	TaVCV	LNYV	MFSV	MMV	NCMV	RYSV	SCV	SYNV	LYMoV	OFV
TaVCV										
LNYV	0.484									
MFSV	0.436	0.511								
MMV	0.299	0.511	0.44							
NCMV	0.496	0.459	0.5	0.526						
RYSV	0.467	0.497	0.5	0.473	0.51					
SCV	0.492	0.389	0.518	0.533	0.485	0.527				
SYNV	0.481	0.537	0.447	0.485	0.534	0.511	0.519			
LYMoV	0.496	0.302	0.514	0.531	0.459	0.496	0.37	0.541		
OFV	0.474	0.512	0.448	0.466	0.473	0.449	0.504	0.49	0.496	
CRV	**0.301**	**0.518**	**0.451**	**0.302**	**0.503**	**0.475**	**0.507**	**0.507**	**0.511**	**0.486**

Phylogenetically the panel of viruses could be divided into two distinct clades, correlating with the *Cyto*- and *Nucleorhabdovirus* genera (Fig. 2.6). The putative new *Cynodon* rhabdovirus grouped within the nucleorhabdovirus clade, where it was close to, but distinct from (MMV).

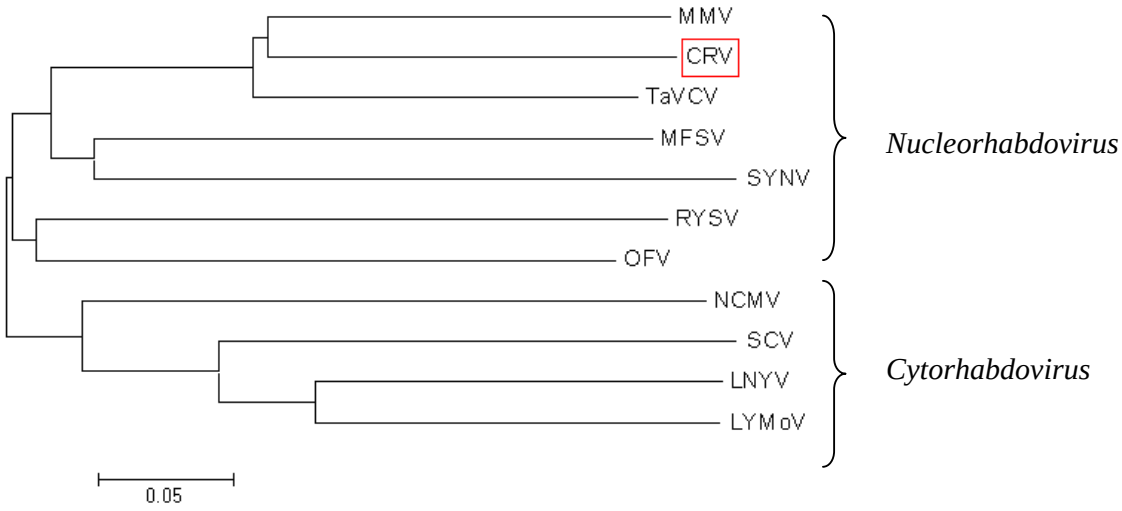

Fig. 2.6. Phylogenetic tree of partial L gene nucleic sequences of *Cynodon* rhabdovirus (CRV, blocked) and other plant rhabdoviruses. The tree was constructed using MEGA 3.1 using the neighbour-joining method (Kumar et al., 2004). The abbreviations as well as the accession numbers are listed in Table 1.3, p. 10.

2.4.3 **Serology**.

The antiserum that was raised against CCSV from Morocco reacted with the *Cynodon* rhabdovirus from South Africa, and decorated the *Cynodon* rhabdovirus particles in a dark halo, indicating a close serological relationship between CCSV and the South African *Cynodon* rhabdovirus. Counting of the trapped viral particles (at 1:100 serum dilutions vv^{-1}) indicated that the CCSV antisera resulted in 23.2 (±3.96) particles whereas the pre-immune serum only yielded particle counts of 6.6 (±2.88).

(a) (b)

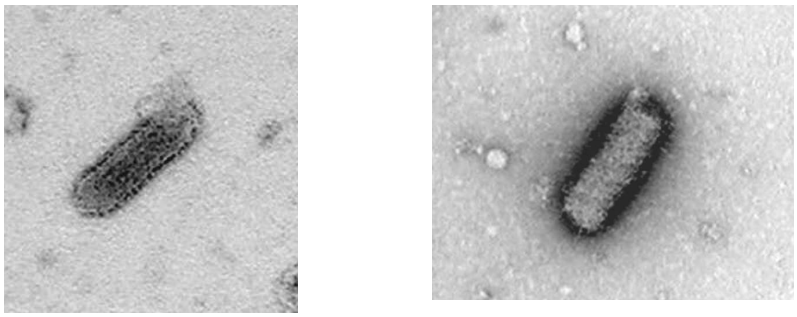

Fig. 2.7. TEM micrographs following serological testing. (a) Trapping and decorating with pre-immunisation serum produced no reaction, and viral particle counts were low. (b) Trapping and decorating with CCSV antiserum produced strong reactions indicating a strong serological relationship, and particle counts were high compared to trapping and decorating with pre-immunisation serum.

2.5 Discussion

We report the presence and identification of a bacilliform virus found in high numbers in the cell nuclei of infected *Cynodon dactylon* grass in South Africa. From our morphological, serological and sequence data, we concluded that this virus is a *Nucleorhabdovirus* from the family *Rhabdoviridae* and our report constitutes the first record of the presence of a member of this genus in South Africa.

Sequence analysis indicated that this *Cynodon* rhabdovirus was most closely related to the nucleorhabdoviruses MMV and TaVCV. From the literature, however, a nucleorhabdovirus of *Cynodon*, CCSV, was reported in 1985 and was found to be widely spread throughout the Mediterranean area (Lockhart et. al., 1985). These

authors also described the relatedness of CCSV and MMV. Unfortunately, CCSV has never been analysed on sequence level and no sequence data for this virus is available in the public domain. The source of the virus was however lost and therefore the sequences of the two viruses could not be compared at molecular level (B. E. L. Lockhart, *personal communication*). Although there appears to be slight differences in the particle sizes we have observed (240x60nm) and that reported for CCSV (280x80nm), our *Cynodon* rhabdovirus was found to be closely related to CCSV, based on serology. Based on our cumulative evidence of morphology, pathology, serology and phylogeny, it is likely that our *Cynodon* rhabdovirus is CCSV or a strain of CCSV. Since no sequence information is available for CCSV, this suggestion is speculative and indeed; sequence data is required to establish the true taxonomic status of the virus known as CCSV. Since sequence data of only 10 other plant rhabdoviruses are available in the public domain, our contribution of the first sequence data from a *Cynodon* nucleorhabdovirus is also the first of any nucleorhabdovirus from South Africa and adds to what should be a growing sequence database for these globally disseminated plant pathogens.

Planthoppers and leafhoppers are common insect vectors for nucleorhabdoviruses, and it is very likely that this is the case for the *Cynodon* rhabdovirus. MMV is transmitted by the leafhopper *Peregrinus maidis*. However, the vector for TaVCV is still unknown but is suspected that it is transmitted by the planthopper *Tarophagus proserpina* (Revill et al., 2005). The degenerate primers as well as the established RT-PCR protocol used in this study can be utilized to determine the vector species responsible for the horizontal transfer of the *Cynodon* rhabdovirus. The protocols used in this study could also be utilized as tools for detecting known, as well as new,

plant rhabdoviruses in field studies, broadening our understanding about the epidemiology of these viruses and addressing the problem of inconclusive classification and taxonomy. At present, it is not clear whether the South African *Cynodon* rhabdovirus holds any threat to the significant maize farming industry of southern Africa.

2.6 Acknowledgements

We thank B. E. L. Lockhart (Department of Plant Pathology, University of Minnesota, USA) for kindly supplying us with the CCSV antiserum and the Virology Unit at the ARC-Plant Protection Institute (Roodeplaat, South Africa) for supplying us with TMV. We also like to thank A. Hall and C. van der Merwe (Department of Microscopy and Microanalysis, University of Pretoria, Pretoria, South Africa) for their expertise in microscopic analysis

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Chapter 3:

Soybean blotchy mosaic virus, a proposed new *Cytorhabdovirus* found in South Africa

3.1 **Abstract**

A previously uncharacterised plant rhabdovirus, infecting soybean (*Glycine max*), was collected from a soybean field in Brits, North West Province, South Africa. To determine the morphology and virion size of this virus, embedded ultra-thin sections of infected plant samples were observed under a transmission electron microscope. The distribution of the virions within the cytoplasm, its bullet-shaped morphology and its size (336 x 48 nanometres) indicated that this might be a rhabdovirus of the genus *Cytorhabdovirus.* Degenerate polymerase chain reaction (PCR) primers were designed by alignment of the polymerase gene sequences of several plant rhabdoviruses in order to identify conserved regions. Standard PCR and sequencing protocols were used to determine a partial polymerase gene sequence of this virus sample that was then compared to related sequences available on Genbank. The analysis indicated that the virus was indeed most closely related to known cytorhabdoviruses, being the closest to Northern cereal mosaic virus but with only a 60.7% homology.

3.2 **Introduction**

Soybean (*Glycine max*) was introduced into South Africa in the early 1900s from China. Soybeans are now grown commercially on average 190 000 tonnes annually in areas the Amersfoort, Carolina, Koedoeskop, Thabazimbi, Beestekraal, Ermelo, Middelburg, Vrede, Bergville, Frankfort, Morgenson, Vryheid, Bethal, Hendrina, Newcastle, Warden, Brits, Hopetown, Paulpietersburg and Winterton. Of the 190 000 tonnes produced commercially, between 60 000 - 65 000 tonnes are processed for human consumption and the remainder used mainly for animal feed (www.sppcom.com/about_soy.htm).

A survey on soybean viruses of South Africa was conducted in 1993 to determine the relative abundance of soybean viruses. Among those detected were soybean mosaic potyvirus (SMV), peanut mottle potyvirus (PeMotV), cowpea aphid-borne mosaic virus (CAMV) and alfalfa mosaic alfamovirus (AMV). During the survey an unknown rhabdovirus in soybean plants was found to be widespread in soybean fields in the Brits/Thabazimbi region, North West Province, South Africa, (Pietersen et al., 1998). This unknown virus showed blotchy mosaic symptoms (Fig. 3.1) on the younger and older leaves of the soybean plants. The soybean rhabdovirus has not been characterized, but electron microscopy has shown that the virions are bullet-shaped – a morphological feature that is typical to rhabdoviruses (Pietersen, *unpublished*).

The main aim of this research was to classify this putative soybean rhabdovirus within a *Rhabdoviridae* genus on the basis of morphological and molecular data. Embedded ultra-thin sections of the virions were viewed under a transmission electron

microscope to examine the morphology of the virions. Degenerate RT-PCR primers were designed from conserved regions of the L gene of plant rhabdoviruses for amplification of the viral DNA. When analysed, it was found that the South African soybean rhabdovirus was closely related to the cytorhabdoviruses, in particular to Northern cereal mosaic virus (NCMV). We tentatively named this proposed new soybean virus the soybean blotchy mosaic virus (SBMV) and this name will be used throughout this thesis.

Fig. 3.1. The typical blotchy mosaic symptoms caused by the soybean blotchy mosaic virus (SBMV). This photo was taken in 2006 on the soybean farm in Brits (North West Province, South Africa) where all the SBMV samples were taken for this study.

3.3 Materials and Methods

3.3.1 Collection of samples:

Soybean leaves believed to be infected by the rhabdovirus, based on symptoms, were collected during the summer months in the beginning of 2007, at a soybean farm in Brits, North West Province, South Africa. The infected soybean material was stored at –70°C until required.

3.3.2 Electron microscopy:

To determine the morphology and virion size, embedded ultra-thin sections of infected soybean material were observed under a Philips EM301 TEM as described in section 2.3.2, p. 24. *Tobacco mosaic virus* (TMV) was used as an external standard to calibrate magnifications.

3.3.3 Isolation of RNA:

Viral RNA was extracted from infected plant material using SV Total RNA Isolation System (Promega, Wisconsin) according to the method described by the manufacturers.

3.3.4 Oligonucleotide primers design:

Refer to section 2.3.4 and Table 2.1 (p.25) for the design of the degenerate primers. These degenerate primers were expected to be broadly reactive to plant rhabdoviruses.

3.3.5 PCR amplification:

For cDNA synthesis, the reaction tube with 8.5 µl RNA and 2 µl RhabF (10 pmol) were heat treated at 65 °C for 10 min and cooled on ice for 2 min. Then 5x Expand Reverse Transcriptase Buffer, 100 mmol/L DTT, 10mM dNTP's, 40 U RNase Inhibitor and Expand Reverse Transcriptase (Roche, Switzerland) were added and incubated at 43°C for 1 h. The reaction was inactivated on ice for 2 min. *Lettuce necrotic yellows virus* (LNYV) RNA was kindly supplied by R. Dietzgen (University of Queensland, St. Lucia, Australia) to serve as a positive control in all protocols. cDNA synthesis of LNYV was also performed as mentioned above. LNYV was used as a cDNA and PCR control. PCR reactions of 50 µl reaction volumes were prepared. The reaction mixture consisted of 5 µl of 10x reaction buffer (Bioline, UK), 50 mM $MgCl_2$, 40 mM dNTP's, 10 pmol of each primer, and 2.5U Taq polymerase (Bioline, UK). Finally 5 µl of the cDNA template was added, and the volume made up to 50 ul with UHQ water. The cycle conditions, on a ABI GeneAmp PCR System 2700 (PE Applied Biosystems, Connecticut), were as follows: one cycle at 94 °C for 2 min, and then 35 cycles of 94 °C for 30 s, 37 °C for 30 s, 72 °C for 90 s, followed by one final cycle at 72 °C for 7 min. Amplicons were analysed on a 1% agarose gel with a O'GeneRuler 200bp DNA Ladder Plus (Fermentas, Vilnius Lithuania) and viewed under an UV transilluminator (Vilber Lourmat, France). A *Strawberry crinkle virus* (SCV) clone was kindly supplied by M. M. Goodin (University of Kentucky, Lexington) and was used as a DNA positive control in PCR.

3.3.6 Sequencing of PCR products:

PCR products of ~900bp were purified from the gels using Wizard SV gel and PCR clean-up system (Promega, Wisconsin). The sequencing reactions were performed

using ABI Prism BigDye Primer Cycle Sequencing Kits (PE Applied Biosystems, Connecticut) according to the manufacturers specifications. Sequencing was with an automated fluorescent sequencer (ABI 377 DNA Sequencer, PE Applied Biosystems, Connecticut) at the University of Pretoria's commercial sequencing facility.

3.3.7 Sequence analysis:

Samples were sequenced in both directions by the forward and reverse primers and contiguous sequences were created with the ContigExpress option from Vector NTI Advance 9 (Invitrogen, Carlsbad). The sequences were analysed by using the basic local alignment research tool (BLAST) program available on the National Centre for Biotechnology Information website (http://ncbi.nlm.nih.gov/). The SBMV partial L gene sequence was compared with the polymerase genes of all available cytorhabdoviruses and nucleorhabdoviruses by doing a multiple alignment with ClustalX in BioEdit version 7.0.0 (Hall T. A., 1999). The pairwise distance (p-distance) of cognate regions of SBMV to the plant rhabdoviruses were calculated. A neighbour-joining phylogenetic tree was constructed using the Mega Version 3.1 (Kumar S. et al., 2004).

3.4 Results

3.4.1 Electron microscopy:

Soybean leaves showing the blotchy mosaic symptoms that could be indicative of the rhabdovirus infection were collected. Transmission electron micrographs were taken of the SBMV to determine the distribution patterns as well as the morphology of this virus. TMV was used as an external standard to see whether the microscopes were calibrated correctly. The long bullet-shaped virions were found in the cytoplasmic areas of the collected plant material, albeit in low numbers (Fig 3.2 and Fig.3.3 a,b). The average sizes of the virions were found to fall within the *Rhabdoviridae* criteria (Jackson et al., 2005) with an average length of 346nm and an average width of 48nm (*n*=6).

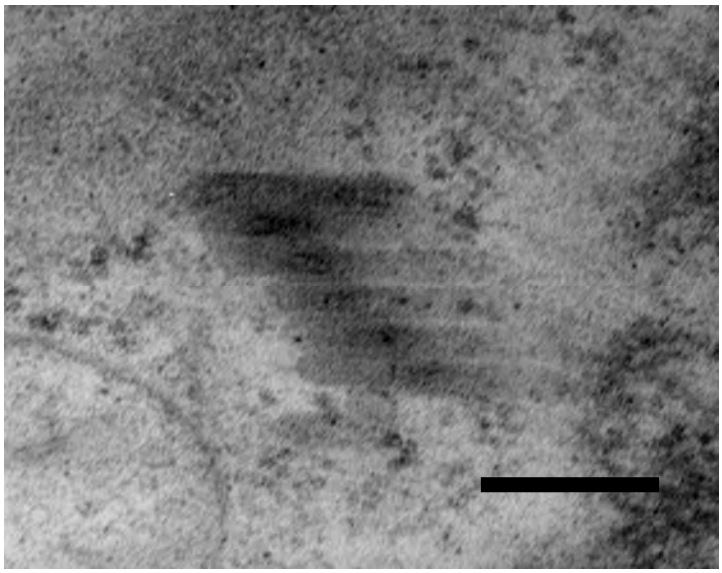

Fig. 3.2. TEM micrograph of SBMV in *Glycine max*. The bullet shaped virion particles were found in the cytoplasm in the cells. Scale bar = 250nm

(a)

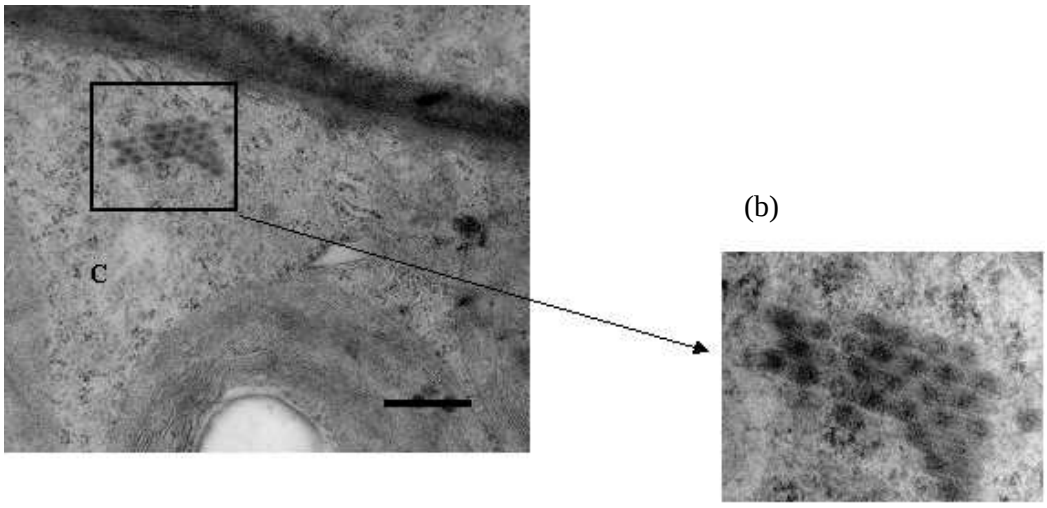

Fig. 3.3. Distribution of SBMV particles within the cytoplasm. The cytoplasmic area is indicated (C). Scale bar = 250nm. (a) TEM micrograph taken at a magnification of 9.8K, indicating aggregated SBMV particles. Scale bar = 1µm. (b) The same group of virion particles as in (a) but in a higher magnification at 75K.

3.4.2 PCR amplification and sequence analysis:

Degenerate primers RhabF and RhabR were designed and RT-PCR carried out on total RNA extracted from symptomatic soybean leaves. The amplicon obtained with this primer set was a ~900 bp segment from SCV, LNYV and SBMV (Fig. 3.4).

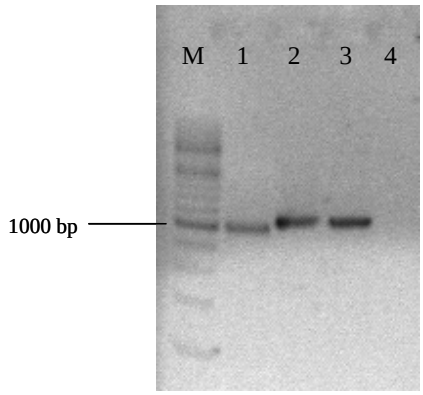

Fig. 3.4. Detection of the putative SBMV by RT-PCR. Samples were loaded onto a 1% agarose gel and electrophoresed for an hour. The O'GeneRuler 200bp DNA Ladder Plus (M), SCV (1), LNYV (2), SBMV (3) and water negative control (4) are indicated.

The PCR product was purified from the gel and sequenced from both directions utilizing RhabF and RhabR. The PCR product was not cloned as the previous chapter (section 2.3.6, p27), since a good clone could not be produced. A contiguous sequence was produced and yielded a 522 bp fragment. BLAST analysis confirmed that the PCR amplicon was of viral RNA (polymerase gene) origin and that it was most closely related to known *Cytorhabdovirus* polymerase genes. The SBMV sequence was submitted to GenBank (acc. number EU877231).

```
1   ATCATGATGACTTGATACAGTTTTCTGGTCTGTTACCGACCCAATTGAATTGATCGTCAA

61  ATTTAGGCATATAGCTCCCATCACTCAAATATAAAAGTGAATTTCTGAATATATCATATG

121 TTCTATTGTAGAGATGGGTCAGTCCAAACAGTCTTCCCAGGCTATCAAATACATGAAATG

181 TCACTTCCTTCCTAAAGTTCAGATTCCATTTTTCAAAGTCCATATTTATGCAGAAAGTCC

241 GGCCTCCCGGCTTTTGAGATTGTTGCCGTCTTGTGAATAGATTTAGTTTCTTGGTTAAAT

301 CTAACAGAGGCAAGGTCATAGAAATATTAGGGAAGTGCTTTAGTACATGCTTAGACAACA

361 TCTCTTCAGTGATAACAAAATAAGCCCTCATCATAAGTGTCATGAGAGAAAACATTCTGG

421 CCACCGGGTTGACTTCTCTTTCTTTCGGATAAAGCCCTATAATCAACCATATCAACGGGA

481 GTCCAGTTGGGCAAAGATTGCAATTATAATAGGAAGAAAACA
```

Fig. 3.5. The 522 bp sequenced fragment obtained from the SBMV. The sequence was obtained from a PCR fragment sequenced from both directions using RhabF and RhabR. BLAST analysis indicated that the SBMV sequence was most related to cytorhabdoviruses.

The 522 bp sequenced fragment of the putative SBMV was aligned with the L gene of known plant rhabdoviruses that were available on Genbank (see Appendix A, p.68), including the newly added *Cynodon* rhabdovirus, acc. number EU650683 (Lamprecht et al., 2008). From this alignment a 410 bp region of the SBMV was compared to the same 410 bp cognate region of other plant rhadoviruses for analysis. Pairwise distances of SBMV to other polymerase genes of different plant rhabdoviruses were calculated using the cognate region of 410 bp with MEGA 3.1 (Kumar et al., 2004). The SBMV had the highest homology to NCMV with 60.7 % followed by SCV, LYMoV and LNYV (58.5%, 56.8% and 59.7% respectively) and then followed by the nucleorhabdoviruses (Table 3.2).

Table 3.1: Homology matrix of nucleotide sequences of the L genes of the Soybean blotchy mosaic virus (SBMV) and all the other plant rhabdoviruses, including CRV. Relative homologies to SBMV are in bold print. The homology matrix was calculated using MEGA 3.1 (Kumar et al., 2004).

	LNYV	MMV	NCMV	RYSV	SCV	SYNV	OFV	TaVCV	MFSV	LYMoV	SBMV
LNYV											
MMV	0.478										
NCMV	0.410	0.500									
RYSV	0.493	0.437	0.463								
SCV	0.312	0.49	0.437	0.490							
SYNV	**0.534**	0.449	0.502	0.463	0.527						
OFV	0.512	0.444	0.459	0.454	0.507	0.410					
TaVCV	0.468	0.280	0.461	0.429	0.439	0.463	0.461				
MFSV	0.483	0.417	0.463	0.473	0.459	0.407	0.415	0.415			
LYMoV	**0.261**	0.495	0.420	0.461	0.300	0.524	0.495	0.485	0.478		
SBMV	**0.407**	**0.502**	**0.393**	**0.466**	**0.415**	**0.505**	**0.485**	**0.483**	**0.483**	**0.432**	
CRV	**0.493**	**0.302**	**0.485**	**0.437**	**0.461**	**0.456**	**0.483**	**0.290**	**0.427**	**0.488**	**0.512**

Bold print: relative homologies of plant rhabdoviruses to CRV and SBMV.

Bold and underlined: the highest relative homology for CRV and SBMV.

Blue: highest homology relationship, LNYV and LYMoV.

Pink: lowest homology relationship (most divergent sequence), LNYV and SYNV.

From Table 3.1 it can be seen, and as mentioned in previous chapters, that CRV shared the closest sequence relationship with TaVCV, and SBMV with NCMV. Also from Table 3.1 it can be deduced that LNYV and LYMoV (blue) shared the closest homology (73.9 % nucleotide identitiy), whereas SYNV and LNYV (pink) were the most divergent from all the plant rhabdoviruses, only sharing 46.6 % of their sequence. SBMV and CRV are also divergent, sharing a homology of 48.8% nucleotide identity in the 410 bp fragment. This indicates that these two viruses are

certainly in two different genera, and this observation is also backed by their different maturation sites within the plant cells shown in the TEM micrographs.

A phylogenetic tree (Fig. 3.6) was constructed with the same cognate region using Mega 3.1 (Kumar et al., 2004). The tree separated the selected plant rhabdoviruses into two distinct clades, correlating with the *Cyto*- and *Nucleorhabdovirus* genera. Within this analysis, the SBMV was most closely related to the cytorhabdoviruses, especially NCMV and then to SCV. It was interesting to note that Orchid fleck virus (OFV) clustered with the nucleorhabdoviruses, while it is still recorded as putative bipartite rhabdovirus according to the International Committee of Taxonomy of Viruses (ICTV) (ICTVdb, 2006). It is however highly unlikely that OFV will be classified as a plant rhabdovirus since it has a bipartite genome and the virions are enveloped (ICTVdb 2006).

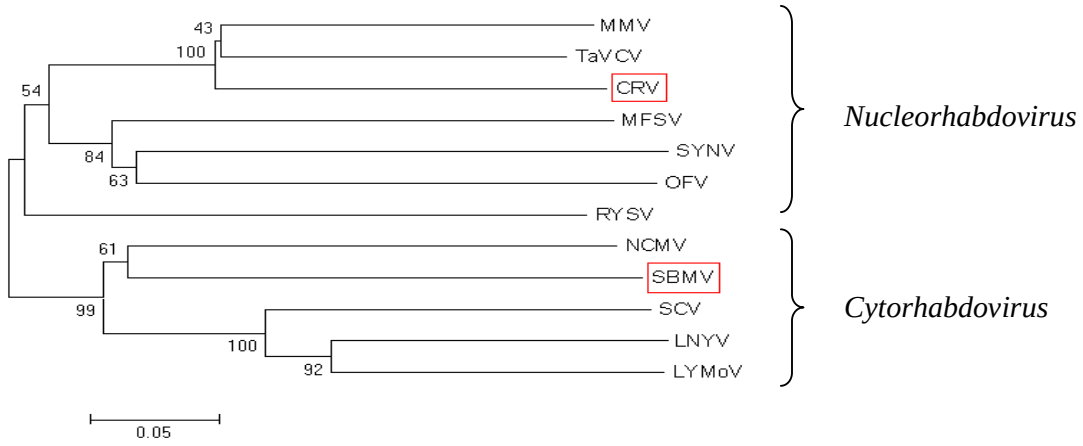

Fig. 3.6. A neighbour-joining phylogenetic tree based on the cognate region of 410 bp of several plant rhabdoviruses, including the newly characterized *Cynodon* rhabdovirus (CRV, acc. number EU600683) and Soybean blotchy mosaic virus (SBMV, acc. number EU877231) [Blocked]. The tree formed two distinct clades, for the two genera *Nucleo*- and *Cytorhabdovirus*. The two newly described plant rhabdoviruses each fell into the respective genera. The abbreviations and accession numbers are listed in Table 1.3, p. 10

3.5 Discussion

In this study, we reported the presence and identification of a proposed new cytorhabdovirus found in South Africa. The putative SBMV particles measured an approximate length of 350 nm and a width of 87 nm in ultra-thin sections. The virion particles were found in low numbers in the cytoplasm of infected *Glycine max* cells.

We also reported the first sequence information of SBMV. The sequenced fragment was obtained by using degenerate primers that would amplify conserved regions within the L gene of plant rhabdoviruses. The designed degenerate primers, RhabF and RhabR, were also able to amplify SCV and LNYV. Sequence analysis indicated that this SBMV is related to other cytorhabdoviruses, particularly to NCMV. Therefore, with morphological as well as sequence data, we concluded that the SBMV is a *Cytorhabdovirus* from the family *Rhabdoviridae.* Full or partial genome sequence data of only 11 (including CRV) other members of plant rhabdoviruses are available in the public domain. Of all the known plant rhabdoviruses, only SCV has been reported in South Africa. Now that another cytorhabdovirus has been reported in South Africa, plant rhabdoviruses should be receiving more surveillance and attention from virologists and it is likely that more plant rhabdoviruses may be identified in RSA and the African subcontinent at large.

Planthoppers, aphids, lacebugs and leafhoppers are common insect vectors for plant rhabdoviruses, and it is very likely that this is the case for SBMV. It is suspected that SBMV is transmitted by means of leafhoppers. It was investigated which leafhoppers

are common to the Brits area. It is suggested that the leafhoppers *Austroagallia cunneata*, *A. nigristerna* and *Peragallia carboverdensis* are the potential vectors for SBMV as they are commonly found in this area (Stiller M., *personal communication*). However, transmission studies were done with the leafhopper Igerna spp (Order Homoptera, Family Cicadellidae) and this leafhopper vector successfully transmitted SBMV (Pietersen, *unpublished*). The degenerate primers as well as the established RT-PCR protocol that were used in this study can be utilized to determine the vector species that are responsible for the horizontal transfer of SBMV. The protocol in this study could also be utilized for detecting known, as well as new, plant rhabdoviruses in field studies, broadening our understanding of the epidemiology of these viruses.

3.6 Acknowledgements

We would like to thank R. Dietzgen (University of Queensland) and M. M. Goodin (University of Kentucky) for supplying us with the LNYV RNA and SCV clone (respectively). We thank the Virology section of the ARC-Plant Protection Research Institute (ARC-PPRI) (Roodeplaat, South Africa) for supplying us with TMV. We also like to thank A. Hall and C. van der Merwe (Department of Microscopy and Microanalysis, University of Pretoria, Pretoria, South Africa) for their expertise in microscopic analysis.

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Chapter 4:

Concluding Remarks

Two unclassified plant rhabdoviruses were found in South Africa; a putative nucleorhabdovirus infecting *Cynodon dactylon*, better known as Bermuda grass, and a putative cytorhabdovirus infecting soybean, *Glycine max* (Jackson et al., 2005; VIDE database, http://biology.anu.edu.au/Groups/MES/vide/). The main aim of this MSc project was to identify and characterize these two rhabdoviruses on a morphological and molecular level, using standard electron microsopic (EM) and RT-PCR techniques.

Degenerate primers were designed for this study to be broadly reactive to most plant rhabdoviruses, as it was designed from the most conserved part (block III) of the highly conserved polymerase gene of plant rhabdoviruses (Poch et al., 1990). The relative binding sites of RhabF and RhabR on other plant rhabdoviruses are indicated in Fig. 4.1.

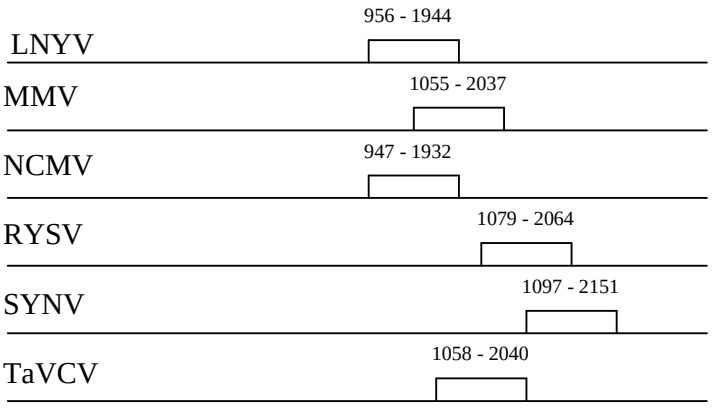


Fig. 4.1: The relative positions of where the degenerate primers RhabF and RhabR will bind on to plant rhabdovirus L gene sequences available on GenBank (Table 4.1)[www.ncbi.nlm.gov]. The numerals represent the base pair site where the primers would bind.

When tested, the primer set RhabF and RhabR was able to amplify four different plant rhabdoviruses, *Strawberry crinkle virus* (SCV), *Lettuce necrotic yellows virus* (LNYV), the South African *Cynodon* rhabdovirus (CRV) and Soybean blotchy mosaic rhabdovirus (SBMV). There is no doubt that the protocol with the degenerate RhabF and RhabR primers could be used as a tool to further discover or characterize new or known plant rhabdoviruses, and even used to determine their respective insect vectors. From this study a CRV clone was also produced and could be used as a positive control in the future study of potential new plant rhabdoviruses.

Early researchers have classified organisms based on the differences in their morphology and structural aspects by microscopic observations. When researchers first started to use molecular information, in particular DNA sequences, they thought that this system would be superior to morphology, as it had the potential for determining the phylogeny of all life. However, it was soon realised that there are advantages and disadvantages to using morphology and DNA data for systematics.

In virology, looking at organisms at a morphological level is still very important as it can depict the size of the virus, the cytopathology it causes and the distribution patterns within the cell, among others. EM techniques can still at present be more cost effective than the molecular methods used, and therefore EM could be used for the more classical epidemiological and surveillance studies, and for calculation of the incidence of the disease. In this study, EM observations have revealed that the two viruses are rhabdoviruses, based on their characteristic bullet-shaped particles, and

therefore it was known to which viral family degenerate primers had to be designed for.

Studying DNA sequences could reflect ancestry and phylogenetic relationships. Studying an organism at molecular level can give more specific and quantitative data, depicting the probable evolutionary relationships through phylogenetic trees, rates of evolution (molecular clock) and percentage divergence, among others. In other words, molecular data allows for direct and quantitative comparisons. Molecular information in this study has confirmed that the viruses were indeed related to rhabdoviruses, and could indicate the evolutionary relationship to other plant rhabdoviruses. If the full genomic sequences of CRV and SBMV should come available (and other new plant rhabdoviruses) they could be compared at a much accurate and meaningful level than comparing a partial sequence of 500-800bp.

At present it is recognized that the best classifications are based on different types of criteria, including morphological and molecular. The PCR designed in the course of this study has significantly increased our ability to detect and characterize plant rhabdoviruses, and will be extremely useful in further studies to establish the epidemiology and control strategies to these viruses. We have produced two new sequences for two different plant rhabdoviruses; a partial L gene sequence for the newly characterized CRV and SBMV. These viruses were described adequately to be classified into a *Rhabdoviridae* genus, that the *Cynodon* rhabdovirus is a new addition to the *Nucleorhabdovirus* genus, and SBMV is a new addition to the *Cytorhabdovirus* genus.

Communications

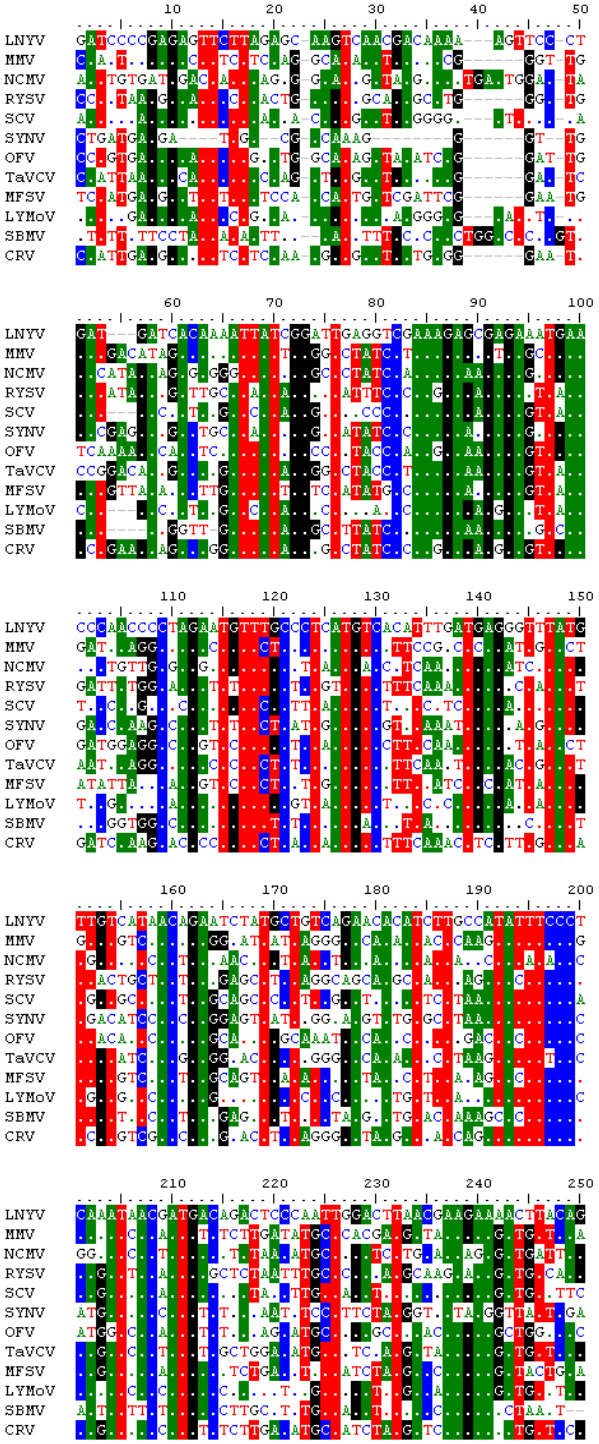
Lamprecht R., Nel L. H. and Pietersen G. 2007. Characterization of two previously unknown plant rhabdoviruses from South Africa. Molecular and Cell Biology Group Symposium. Pretoria, South Africa. 17 October 2007.

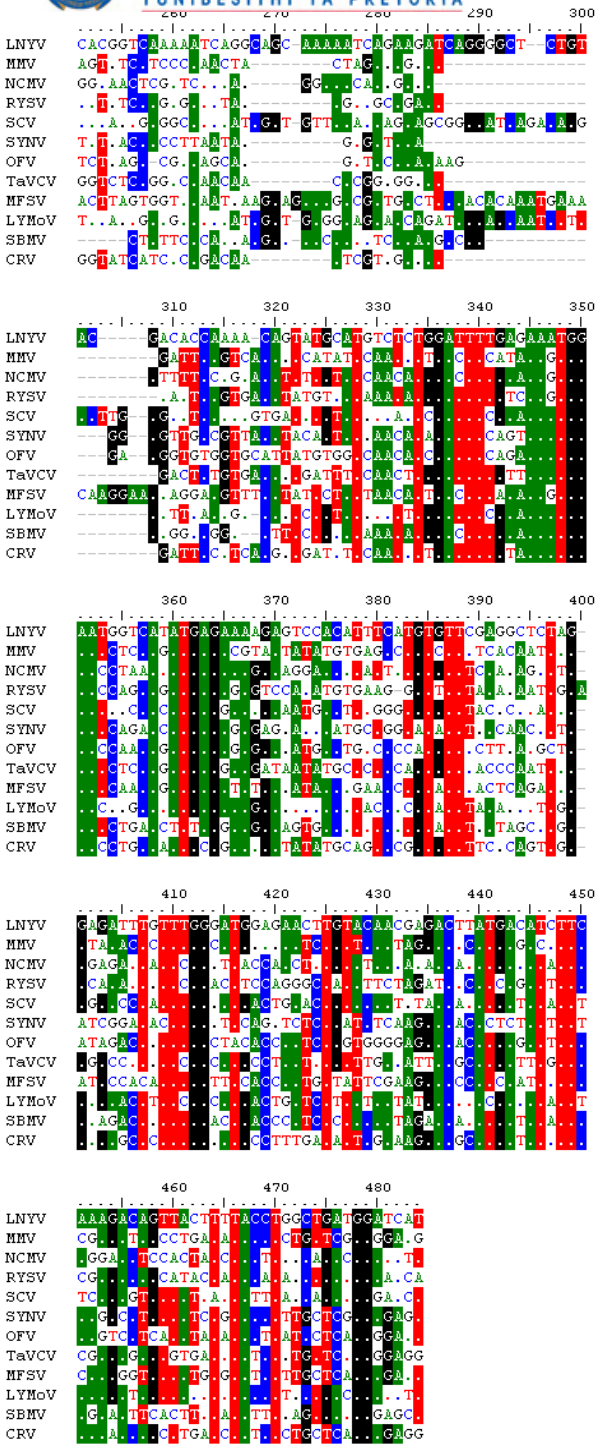
Publications

R. L. Lamprecht, G. Pietersen, G. G. F. Kasdorf, L. H. Nel. 2008. Characterisation of a proposed *Nucleorhabdovirus* new to South Africa. *European Journal of Plant Pathology* (2008). DOI 10.1007/s10658-008-9339-5.

Appendix A

Multiple alignment of several cyto- and nucleorhabdoviruses, including the newly characterized *Cynodon* rhabdovirus (CRV) and Soybean blotchy mosaic virus (SBMV). Homologous areas are highlighted. ClustalX in BioEdit version 7.0.0 (Hall, 1999).




LNYV
MMV
NCMV
RYSV
SCV
SYNV
OFV
TaVCV
MFSV
LYMoV
SBMV
CRV