Identification and Characterization of Viruses Infecting Cucurbits in the Province of KwaZulu-Natal, Republic of South Africa, with the Purpose of Developing Transgenic Virus-Resistant Cucurbits

By

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Thesis Summary

The continued presence of cucurbit-infecting viruses across the Province of KwaZulu-Natal (KZN), South Africa requires exploration of alternative methods of controlling these viruses. The aim of this research project was to identify and characterize the viruses infecting cucurbits in KZN with the intention of subsequently developing transgenic cucurbits with broad virus resistance. A systematic virus survey was therefore carried out in all cucurbit growing areas of KZN during the 2011, 2012 and 2013 growing seasons. Symptomatic leaves suspected to be of viral aetiology were sampled and tested using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR), in order to detect the common viruses causing mosaic and yellowing diseases. Samples were tested for the following viruses: *Cucumber mosaic virus* (CMV), *Squash mosaic virus* (SqMV), *Moroccan watermelon mosaic virus* (MWMV), *Watermelon mosaic virus* (WMV), *Zucchini yellow mosaic virus* (ZYMV), *Cucurbit yellow stunting disorder virus* (CABYV), *Beet pseudo-yellows virus* (BPYV), *Cucumber yellow stunting disorder virus* (CYSDV), *Lettuce infectious yellows virus* (LIYV), and members of the genera *Polerovirus* and *Carlavirus*. CMV, BPYV, ZYMV, and MWMV were detected, along with Pepo aphid-borne yellows virus (PABYV), a putative new species in the genus *Polerovirus* that has been reported in West Africa. MWMV was the most prevalent mosaic-inducing virus and PABYV was the most prevalent yellowing-inducing virus.

One common virus symptom consisted of shoe strings leaves and deformed fruits on baby marrows plants (*Cucurbita pepo* L.). However, the samples tested negative for all the viruses selected. These symptoms were further investigated using *Potyvirus* universal primers. This led to the detection of a tentative species in the *Papaya ringspot virus* (PRSV) cluster in the genus *Potyvirus*. The tentative potyvirus was named *Zucchini shoestring virus* (ZSSV). Next Generation Sequencing was later used in combination with Sanger Sequencing to elucidate the full genome sequences of MWMV, PABYV, and ZSSV. MWMV isolates from RSA were found to be more closely related to each other than to the isolate from Tunisia. Both PABYV and ZSSV were found to be distinct species in the genera *Polerovirus* and *Potyvirus,* respectively, on the basis of their genome organization and the species criteria for each genus.

Baby marrow was identified as the most susceptible cucurbit to viral infections in KZN. It was therefore decided to develop baby marrow plants with resistance to the potyviruses identified in the survey, using antisense post-transcriptional gene silencing. A portion of the 5' coding sequence of the coat protein genes of MWMV, ZYMV and ZSSV were amplified by RT-PCR and inserted into a plant expression vector pEPJ86-m/2N. The expression cassette on

pEPJ86-m/2N was subsequently sub-cloned into the plant transformation vector pGA482G before being introduced into *Rhizobium radiobacter*, formerly *Agrobacterium tumefaciens*, strain LBA4404 (pAL4404)(pBI121) by electroporation. *Rhizobium*-mediated transformation on baby marrow cotyledon explants was subsequently performed. The resultant putative transgenic regenerated baby marrows plants were subjected to different tests that included PCR to confirm transgene insertion, mechanical inoculation of each virus and DAS-ELISA to evaluate virus resistance. A total of 94 baby marrow plants were successfully regenerated from 250 explants. Out of the 94 plants, 84 were found to have the transgene based on the PCR results. Of these 84 lines, 76 showed resistance to the selected three viruses. Our preliminary results show the potential of using transgenic cucurbits with resistance to three potyviruses as an effective strategy to control virus diseases on cucurbits.

Declaration

I, **Jacques Davy Ibaba**, declare that:

i. The research reported in this thesis, except where otherwise indicated, is my original work.

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(Jacques Davy Ibaba, Candidate)

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(Prof Mark D. Laing, Co-Supervisor)

Signed: ……. Date: ……
(Dr Augustine Gubba, Supervisor)

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- The Pietermaritzburg Cornerstone Church for being a family to me;
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JD Ibaba

Dedication

I dedicate this thesis to the memory of my beloved father, Justin IBA-BA (1941 – 2010) for teaching the values of excellence, hard work and perseverance to his children, constantly encouraging us to further our education.

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Symbols, Abbreviations and Acronyms

%	:	percent
°C	:	Degree Celsius
µl	:	Microliters
µM	:	Micromolar
35S-enh	:	enhancer of CaMV 35S promoter
35S-Pro	:	CaMV 35S promoter
35S-ter	:	CaMV 35S terminator

A

A	:	Adenine
aa	:	Amino acid
AlMV	:	*Alfalfa mosaic virus*
ARC BTP	:	The Agricultural Research Council Biotechnology Platform
AWMV	:	*Algerian watermelon mosaic virus*

B

bp	:	Base pair
BCMV	:	Bean common mosaic virus
BPYV	:	*Beet pseudo yellows virus*

C

C	:	Cytosine
CABYV	:	*Cucurbit aphid-borne yellows virus*
CaMV	:	*Cauliflower mosaic virus*
cDNA	:	Complimentary DNA
CI	:	Cylindrical Inclusion protein
CMV	:	*Cucumber mosaic virus*
CP	:	Coat protein
CPm	:	Coat protein minor
CTV	:	*Citrus tristeza virus*
CuYV	:	*Cucumber yellows virus*
CYSDV	:	*Cucumber yellow stunting disorder virus*

D

DAFF	:	Department of Agriculture Forestry and Fisheries
DNA	:	Deoxyribonucleic acid
dNTP(s)	:	Deoxynucleotide(s)
dpi	:	Day post inoculation
dsRNA	:	double-stranded RNA

E

ELISA : Enzyme-linked immunosorbent assay

F

Fig. : Figure

G

G : Guanine

Gb : Gigabytes

H

h : Hour(s)

HC-Pro : Helper Component-Proteinase

Hel : Helicase

Hsp70h : Heat shock protein 70

I

ICTV : International Committee on Taxonomy of Viruses

J

K

Kb : Kilobase

KZN : KwaZulu-Natal

L

LABYV : Luffa aphid-borne yellows virus

LIYV : *Lettuce infectious yellows virus*

M

MABYV : *Melon aphid-borne virus*

MCL : Maximum composite likelihood

MCS : Multiple cloning site

miRNAs : MicroRNAs

min : Minutes

mM : Millimolar

MP : Movement protein

mRNA : Messenger RNA

MS : Murashige and Skoog

Mtr : Methyltransferase

MW : Molecular weight

MWMV : *Moroccan watermelon mosaic virus*

N

NCBI : National Center for Biotechnology Information

NCR : Non-coding region

NGS : Next generation sequencing

nm : Nanometres

nt : Nucleotides

O

OD : Optical density

ORF(s) : Open reading frame(s)

P

PABYV : Pepo aphid-borne yellows virus

PASC : Pairwise Sequence Comparison

PCR : Polymerase chain reaction

PDR : Pathogen-derived resistance

PLRV : *Potato leafroll virus*

P-Pro : Papain-like proteinase

PIPO : Pretty Interesting *Potyvirus* ORF

PRSV : *Papaya ringspot virus*

PSV : *Peanut stunt virus*

PTGS : Post-transcriptional gene silencing

Q

R

RDP : Recombination detection program

RdRp : RNA-dependent RNA polymerase

RISC : RNA-induced silencing complex

RNA : Ribonucleic acid

RNAi : RNA interference

RSA : Republic of South Africa

RTD : Readthrough domain

RT – PCR : Reverse transcription polymerase chain reaction

S

s : Seconds

SABYV : *Suakwa aphid-borne yellows virus*

siRNA : Small/short interfering RNA

SqMV : *Squash mosaic virus*

T

T: Thymine

TAE : Tris acetate EDTA

TGS : Transcriptional gene silencing

TSWV : *Tomato spotted wilt virus*

U

U : Uracil

USA : United States of America

UTR : Untranslated region

V

VPg : Viral Protein genome-linked

W

WMV : *Watermelon mosaic virus*

X

Y

Z

ZSSV : Zucchini shoestring virus

ZTMV : *Zucchini tigré mosaic virus*

ZYFV : *Zucchini yellow fleck virus*

ZYMV : *Zucchini yellow mosaic virus*

Thesis Introduction and Rationale for Study

Cucurbit is a term used to denote all species within the Family *Cucurbitaceae* (Robinson & Decker-Walters, 1997; Weng & Sun, 2012). Cucurbits are annual, herbaceous, tendril-bearing, frost-sensitive vines that are found in both temperate and tropical regions (Weng & Sun, 2012). Besides being recorded among the most ancient domesticated plants (Robinson & Decker-Walters, 1997), cucurbits are also among the most economically important vegetable crops worldwide (Weng & Sun, 2012). Cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), squash (*Cucurbita pepo* L.) and pumpkin (*Cucurbita maxima* Duch.) are the cucurbits commonly cultivated in the Republic of South Africa (RSA). These cucurbits play an important role in the daily diet of most humans. They are eaten in mature or mature in many ways that include fresh in salad or desserts, baked, fried, pickled, in jams, and candied. Various types of drinks, processed from cucurbit fruit juice, are available on the local market. The orange-flesh of some cucurbits are an excellent source of vitamin A (Weng & Sun, 2012). Cucurbits produced in RSA are sold locally and exported to neighbouring countries (DAFF, 2012).

Problem identification

Cucurbit production is affected by virus infections worldwide. Viruses are infectious, non-cellular pathogens that are too small to be seen under a light microscope (Bos, 1999). They are constructed of a core of nucleic acid, one or more molecules of either DNA or RNA, sheltered in a protective protein coat. Viruses are intracellular, obligate parasites that rely on their host's cellular machinery to replicate (Sastry, 2013). Plant virus infections result in a reduction in plant growth, lower yields, reduced flowering, fruit of inferior quality, increased susceptibility of the host plant to other pathogens, and economic losses to cucurbit growers (Sastry, 2013). Viral diseases in plants, unlike fungal diseases, have no treatment to control or even to slow down the pathogen. In extreme cases, virus diseases lead to fields being abandoned. In such cases, the economic losses suffered by the farmers cannot be overemphasized. Exacerbating the problem for growers is that the number of viruses infecting cucurbits has increased over the last two decades.

The current control of viral diseases of cucurbits in RSA relies on the use of cultivars with partial resistance, and on the control of the insect vectors that transmit the viruses. However, the current implementation of these strategies has not been effective. Viruses infecting cucurbit previously identified in the Province of KwaZulu-Natal (KZN) in RSA include *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Moroccan*

watermelon mosaic virus (MWMV) and *Cucumber mosaic virus* (CMV) (Cradock et al., 2001; Usher et al., 2012). There is now evidence that new viruses infecting cucurbits have emerged in other parts of the world. Based on symptoms observed in the field during recent surveys in KwaZulu-Natal, South Africa, it is possible that these emerging viruses are now present in RSA. This project sought to identify and characterize the common viruses infecting cucurbits in KZN, with the aim of subsequently developing transgenic virus-resistant cucurbits as a strategy to manage the diseases they cause.

RNA silencing has been demonstrated as an efficient way of conferring virus resistance to plants. Virus-resistant transgenic cucurbits have been developed in others parts of the world. Moreover, virus-resistant transgenic seeds, fruit or leaves are a commercial reality in the United States of America (Dias & Ortiz, 2013). The effectiveness of transgenic virus resistance in cucurbits has been demonstrated beyond doubt. RNA silencing has the added advantage of allowing the development of plants resistant to more than one virus. This approach will address the practical problem of multiple virus infections, which is common under field conditions.

Project objectives

The specific objectives of the proposed project were:

- To carry out a survey of viruses infecting cucurbits in KZN, RSA. This survey would provide an updated situation of the viruses infecting cucurbits in the face of the emergence of new viruses infecting these crops in other parts of the world.
- To characterize and determine the phylogeny of the viruses identified in the survey.
- To develop transgenic cucurbits with broad resistance to the commonly occurring viruses.

Organization of the thesis

This thesis is organized into six chapters. Chapter 1 is a review of current literature on the viruses infecting cucurbits with emphasis given to the viruses reported in KZN. Chapter 2 focuses on the survey of cucurbit-infecting viruses conducted in KZN. The Chapter 3 to 5 report on the genome sequence analysis and phylogeny of three viruses of cucurbits commonly occurring in KZN based on the survey. Chapter 6 includes the work performed towards developing transgenic baby marrow resistant to three viruses. The thesis ends with an overall discussion in the Thesis Overview.

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Chapter 1
Literature Review

1.1. The family *Cucurbitaceae*

The family *Cucurbitaceae*, one of eight in the order Cucurbitales, is made of 95 genera assembled in 15 tribes (Schaefer & Renner, 2011) of which several genera (Table 1.1) are of economic importance worldwide. Cucurbits are beneficial to humankind in diverse ways including, food, drink, household utensils, musical instruments, clothing, cosmetics, medicines, ornaments and decoration (Behera *et al*., 2012; Robinson & Decker-Walters, 1997; Weng & Sun, 2012).

Table 1.1. Important genera in the family *Cucurbitaceae*

Tribes	Genera
Benincaseae	*Citrullus* *Lagenaria* *Benincasa* *Praecitrullus* *Cucumis* *Coccinia*
Cucurbiteae	*Cucurbita* *Sicana*
Joliffieae	*Telfairia*
Momordiceae	*Momordica*
Sicyoeae	*Luffa* *Trichosanthes* *Sechium* *Cyclanthera*

1.2. Cucurbit importance in the Republic of South Africa

Cucurbits of economic importance, in the Republic of South Africa (RSA), belong to ten genera, namely: *Acanthosicyos, Citrullus, Cucumis, Cucurbita, Coccinia, Kedrostis, Lagenaria, Luffa, Momordica* and *Sechium*. The genera *Citrullus, Cucumis and Cucurbita* are the only ones that include commercial crop species (Table 1.2). (Trench *et al*., 1992; Van Wyk & Gericke, 2003; Weng & Sun, 2012).

Table 1.2. Cucurbits commercialized in RSA

Scientific names	Common names	Origins
Citrullus lanatus (Thunb.) Matsum. & Nakai	Watermelon	Southern Africa
Cucumis melo L.	Melon Spanspek Sweet melon Muskmelon	Asia
Cucumis sativus L.	Cucumber English cucumber	Asia
Cucurbita maxima Duch.	Pumpkin Hubbard	South America
Cucurbita moschata Duch.	Butternut Squash Caledon Ceylon pumpkin Amatanga esintu	South America
Cucurbita pepo L.	Marrow Baby marrow Zucchini Patty pan Gem squash Courgette	Southern Mexico

Cucurbit crops, in RSA, are cultivated countrywide by both subsistence and commercial farmers (Department of Agriculture Forestry and Fisheries (DAFF), 2014; DAFF, 2012a; Voster *et al*., 2007). Seeds from a comprehensive selection of hybrids and cultivars are available from local and international seed firms, depending on the market demand. Cucurbits produced in RSA are sold in fresh produce markets, wholesalers, supermarkets, retailers, informal markets, and exported in the neighbouring countries (DAFF, 2012a). The estimated South African production of selected cucurbits in 2012 is enumerated in Table 1.3. (DAFF, 2012b; DAFF, 2012c; DAFF, 2012d; DAFF, 2013).

Table 1.3. South African production of selected cucurbits in 2012

Crops	Production (Tons)
Butternut	97,718
Pumpkin	58,173
Hubbard	24,508
Gem squash	20,215
English cucumber	15,991

1.3. Viruses infecting cucurbits in RSA

Viruses have a negative impact on cucurbit production worldwide because of the yield reduction and/or compromised fruit quality they cause. (Lecoq, 2003). There are currently 59 well-characterized viruses from the different families and genera of plant viruses that are known to infect cucurbit crops (Lecoq & Desbiez, 2012). Typical viral symptoms in cucurbits are classified into three major groups, namely: mosaics, yellowing and necrosis. Symptoms in the mosaics group include mosaic on leaves often associated with distorted or reduced leaf and discoloured or deformed fruits. Yellowing symptoms start on the older and mature leaves but do not affect fruit quality. Necrosis appears as spots or wilt on either the leaves or the fruits (Lecoq & Desbiez, 2012; Lecoq, 2003). Mosaics and necrosis may cause significant economic loss. Cucurbit-infecting viruses identified in RSA belong to three virus family: *Potyviridae*, *Bromoviridae* and *Closteroviridae* (Cradock *et al*., 2001; de Vries, 2009; van der Meer, 1985; van der Meer & Garnett, 1987; van Regenmortel, 1960; von Wechmar, *et al.*, 1995). Losses due to virus infections on cucurbit crops in RSA depend on the type of cucurbit and the timing of the infection (Mather *et al.,* 2002).

1.3.1. Viruses of the family *Potyviridae* infecting cucurbit crops in RSA.

The family *Potyviridae* is made of eight genera (ICTV, 2012) and includes several important viruses infecting cucurbit worldwide. *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV) and *Moroccan watermelon mosaic virus* (MWMV) are the viruses of the family *Potyviridae* reported to infect cucurbit in RSA (Cradock *et al*., 2001; de Vries, 2009; van der Meer, 1985; van der Meer & Garnett, 1987; van Regenmortel, 1960; Usher *et al.*, 2012; von Wechmar, *et al.*, 1995). These three viruses belong to the genus *Potyvirus*, one of the two largest genera of plant viruses (Gibbs & Ohshima, 2010). Potyviruses are non-enveloped, flexuous filamentous viruses of about 680 – 900 nm long harbouring a monopartite genome consisting of a single positive sense RNA covalently linked to a viral protein genome (VPg) at its 5'end, and a polyadenyl tail at its 3'end. Eleven multifunctional proteins, described in Table 1.4. and Figure 1.1., are produced from the potyvirus genome through polyprotein expression and ribosome frameshifting strategies. Of all the potyvirus proteins, P1 is the least conserved in sequence and the most variable in size (Adams *et al.*, 2012).

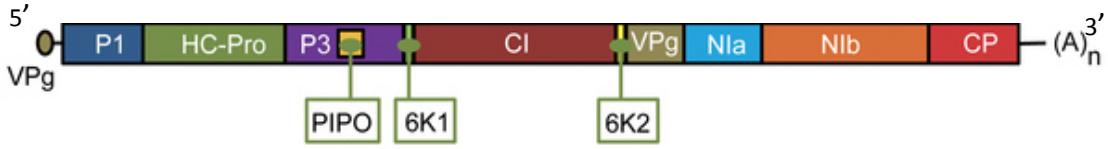

Figure 1.1. Genomic map of *Potyvirus* (Cuevas *et al*., 2012)

Table 1.4. *Potyvirus* gene products and their role in the virus life cycle

Gene products	Genome strategies	Role in virus life cycle	References
P1	Polyprotein expression	Virus replication; Symptomatology; C-terminal auto cleavage.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
Helper Component-Proteinase (HC-Pro)	Polyprotein expression	Polyprotein cleavage; Genome amplification; Local and systemic infectivity; Pathogenicity; Vector transmission; RNA silencing suppressor; RNA binding; Virus accumulation; Virus synergism.	Adams *et al.*, 2012; Hasiów-Jaroszewska *et al*., 2014; Urcuqui-Inchima *et al.*, 2001.
P3	Polyprotein expression	Virus replication; Pathogenicity.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
6K1	Polyprotein expression	Still unknown.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
Cylindrical Inclusion protein (CI)	Polyprotein expression	ATPase/RNA helicase; Virus intercellular movement.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
6K2	Polyprotein expression	Anchoring replication complex to the endoplasmic reticulum; Virus systemic infectivity.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
Viral Protein genome-linked (VPg)	Polyprotein expression	RNA silencing suppressor; Virus replication and translation; Virus systemic infectivity.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
NIa	Polyprotein expression	Polyprotein cleavage; Serine-like cysteine proteinase.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
NIb	Polyprotein expression	RNA-dependent RNA polymerase.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001.
Coat Protein (CP)	Polyprotein expression	Host specificity in systemic infectivity; Vector transmission; Virus assembly; Local and systemic movement.	Adams *et al.*, 2012; Urcuqui-Inchima *et al.*, 2001; Desbiez *et al.*, 2014.
Pretty Interesting Potyvirus ORF (PIPO)	+2 ribosome frameshifting	Virus intercellular movement.	Adams *et al.*, 2012; Vijayapalani *et al.*, 2012.

1.3.1.1. *Zucchini yellow mosaic virus* (ZYMV)

ZYMV is among the most economically important viruses infecting cucurbits. It was isolated for the first time in Northern Italy in 1973 (Desbiez & Lecoq, 1997; Romay *et al.*, 2014). Thereafter, researchers in numerous countries growing cucurbits across the globe reported the incidence of ZYMV with yield reductions reaching 94% (Romay *et al*., 2014; Simmons *et al*., 2011). ZYMV naturally infects cucurbits, mostly cultivated species and a few ornamentals and weeds, producing a variety of symptoms that include vein clearing, yellow mosaic, leaf deformation with blisters, enations or filiformism, stunting, misshaped fruit, internal marbling and hardening of the flesh and deformed seed (Lecoq & Desbiez, 2012). These symptoms are especially severe on zucchini infected plants.

Serological studies of ZYMV isolates revealed a minimum of sixteen different serotypes (Desbiez & Lecoq, 1997; Yakoubi *et al.,* 2008a). Phylogenetic relationships of the sequences of the coat protein of ZYMV isolates worldwide arranged them into three major groups, namely A, B and C, with group A being the largest (Figure 1.2.; Coutts *et al*., 2011a). ZYMV is spread by aphids (Lecoq & Desbiez, 2012), mechanically (Svoboda *et al*., 2014) and through seed transmission (Simmons *et al*., 2013). Aphid transmission of ZYMV occurs in a non-persistent manner. The aphid species *Aphis gossypii* Glover*, A. craccivora* Linnaeus, *Macrosiphum euphorbiae* Thomas and *Myzus persicae* Sulz have been identified as the most efficient vectors among 26 aphid species capable of transmitting ZYMV (Lecoq & Desbiez, 2012). Simmons *et al*. (2013) suggested that seed transmission might have significantly contributed to the spread of ZYMV through the cryptic infection of virulent ZYMV.

ZYMV in RSA was reported for the first in 1994 (Mather *et al.,* 2002). In 2001, ZYMV was the most prevalent virus infecting cucurbits in the KwaZulu-Natal (KZN) province (Cradock *et al*., 2001). ZYMV infects all cucurbit crops in RSA, with marrow being severely affected and butternut only showing symptoms on leaves. Sequence analysis of the spatial coding sequences of the coat protein of a KZN ZYMV isolate showed more than 90% similarity with the isolates from Central Europe, Indian, Singapore and Taiwan (Usher *et al*., 2012).

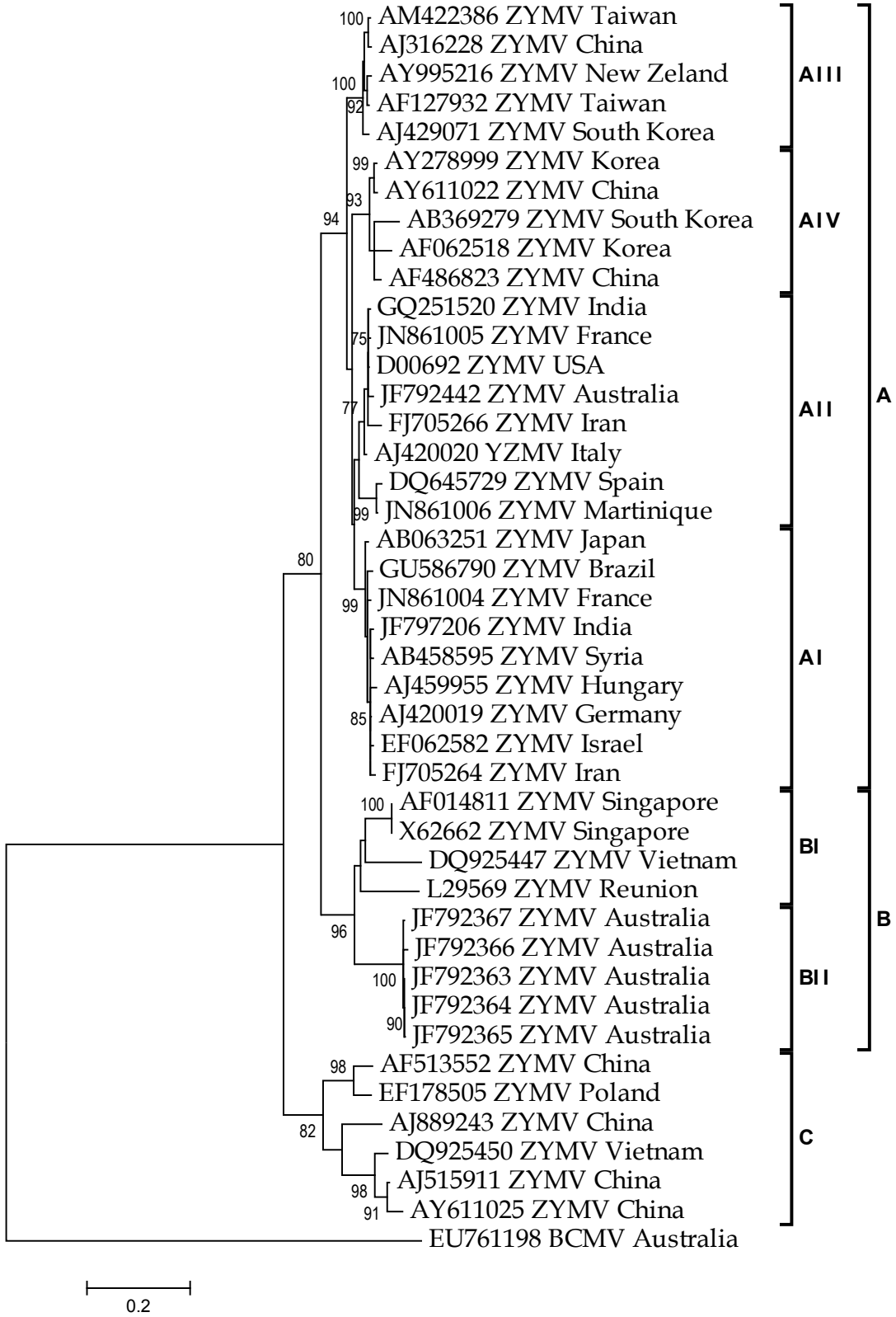

Figure 1.2. Neighbour-joining relationship phylogram of the coat protein of ZYMV isolates from GeneBank (Coutts *et al*., 2011a).

1.3.1.2. *Watermelon mosaic Virus* (WMV)

WMV, formerly WMV-2 is among the first described potyviruses infecting cucurbit crops (Desbiez *et al.*, 2007) and has become an economically virus of cucurbit crops in temperate and Mediterranean climates worldwide (Lecoq, 2003; Lecoq *et al*., 2011). WMV host range, beside cucurbit crops, also includes species from *Apiaceae* (Parry & Persley, 2005), *Chenopodiaceae*, *Fabaceae*, *Malvaceae*, *Orchidaceae* (Ali *et al.*, 2006; Laney *et al*., 2012) and several weeds (Desbiez *et al.*, 2007). WMV symptoms which vary depending on the isolates and the host (Lecoq & Desbiez, 2012) comprise mosaic, vein banding, necrosis, leaf deformation, fruit discolouration and distortion (Finetti – Sialer *et al.*, 2012; Lecoq *et al.*, 2011).

WMV isolates worldwide fall within four serotypes: 1, 2, 3, and 4, with serotype 1 being the most common. At a molecular level, they are distributed into three distinct phylogenetic groups denoted G1, G2 and G3 (Figure 1.3.) on the basis of the amino acid sequence of their coat proteins (Desbiez *et al.*, 2007). G1 and G3 were later referred as classic (CL) strains and emerging (EM) strains respectively. EM strains are frequently associated with severe symptoms (Finetti – Sialer *et al.*, 2012). Genetic drift and frequent recombination events are the driving factors of the genetic variation of populations of WMV (Desbiez & Lecoq, 2008; Glasa *et al*., 2011). *A gossypii* Glover, *A. craccivora* Linnaeus, *Myzus persicae* Sulz are the efficient vectors of WMV among 35 aphid species in 19 genera that transmit the virus in a non-persistent mode (Lecoq & Desbiez, 2012). Seed transmission has recently been reported with WMV and is thought to have been an important avenue for the dissemination of WMV (Laney *et al*., 2012).

Very little has been written on populations of WMV in RSA. The earliest report of WMV in RSA dates from the 1960s. WMV was subsequently identified in cucurbit crops cultivated in the Western Cape and KwaZulu-Natal provinces (Cradock *et al*., 2001; van der Meer, 1985; van Regenmortel, 1960).

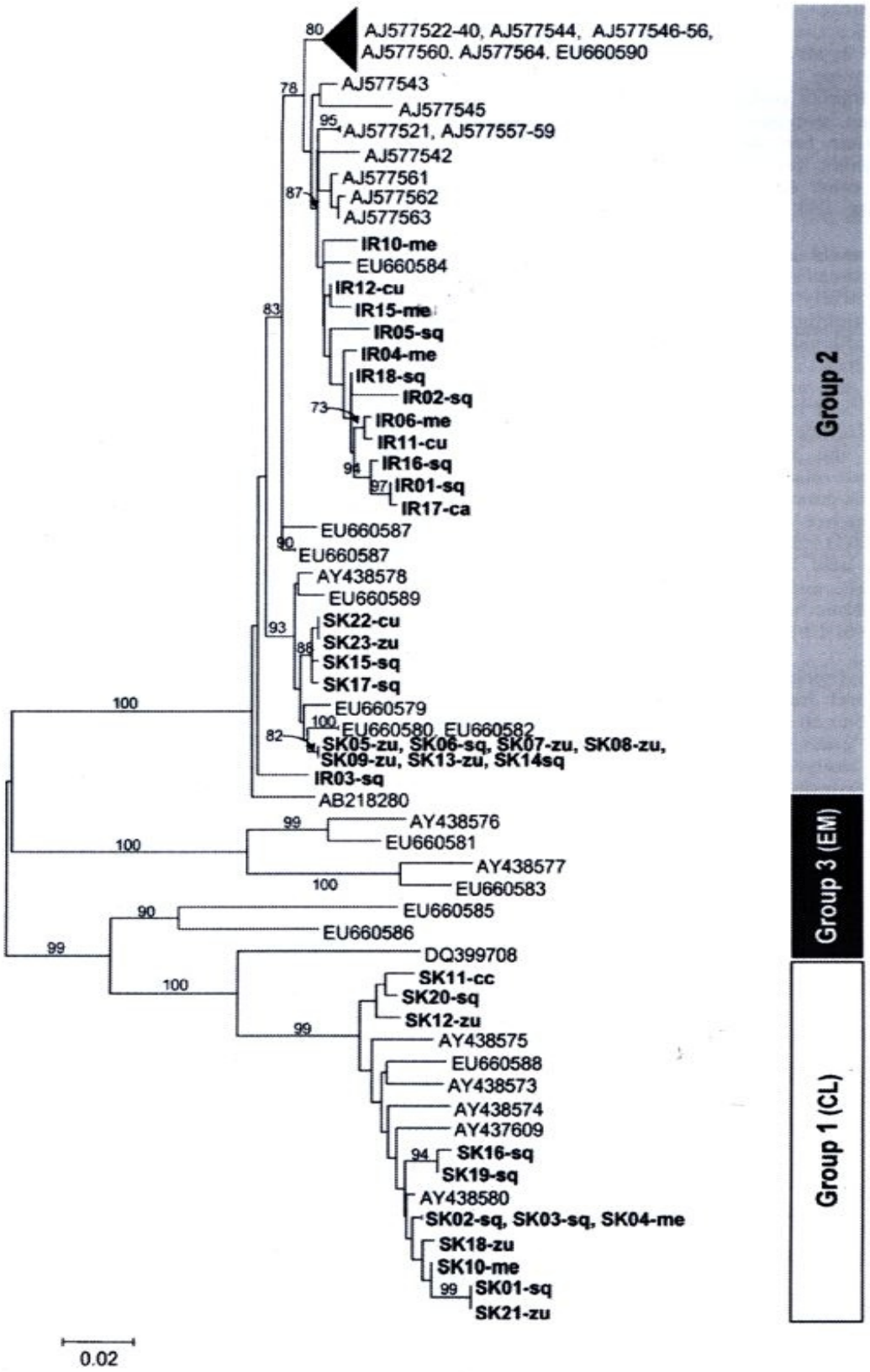

Figure 1.3. Phylogram of the coat protein of WMV isolates (Glasa *et al*., 2011).

1.3.1.3. *Moroccan watermelon mosaic virus* (MWMV)

MWMV was first reported in Morocco in 1972 as a strain of WMV (Arocha *et al*., 2008; Lecoq *et al*., 2001). A subsequent study of the nucleotide and amino acids sequences of its coat protein distinguished MWMV as a distinct potyvirus (Baum *et al.*, 1979; McKern *et al.*, 1993). MWMV's geographic distribution includes Cameroon, Canary Island (Lecoq *et al*., 2001), Democratic Republic of Congo (Arocha *et al*., 2008), France (Lecoq *et al*., 2008), Italy (Roggero *et al.*, 1998), Niger (Lecoq *et al*., 2001), Nigeria (Owolabi *et al.,* 2012), RSA (Cradock *et al*., 2001; van der Meer & Garnett, 1987), Spain (Quiot-Douine *et al.*, 1990), Sudan (Lecoq *et al*., 2001), Tanzania (Menzel *et al*., 2011), Tunisia (Yakoubi *et al.*, 2008b) and Zimbabwe (Lecoq *et al*., 2001). MWMV host range is restricted to cucurbits and papaya (*Carica papaya* L.). Symptoms associated with MWMV infection are severe and comprise interveinal chlorosis, leaf deformation and filiformism, necrosis, stunting, distorted fruit (Arocha *et al*., 2008; Lecoq, 2003; Lecoq *et al*., 2008; Lecoq *et al*., 2001).

MWMV is among the potyviruses that are serologically and biologically related to *Papaya ringspot virus* (PRSV) but molecularly different according to the species criteria (Lecoq & Desbiez, 2012; Yakoubi *et al.*, 2008c; Figure 1.4.). This relatedness and distinction between MWMV and PRSV are also reflected on the phylogenetic analyses of the full-length genome of a Tunisian MWMV isolate (Yakoubi *et al.*, 2008b). Phylogenetic analyses of the sequence of the coat protein of MWMV isolates from different geographical origins revealed three clusters: One cluster comprised isolates from the Mediterranean region, a second comprised isolates from Western and Central Africa, and a third one comprised isolate from Southern Africa. The Sudanese MWMV isolate, a highly divergent variant, is regarded as an evolutionary intermediate between MWMV and PRSV (Yakoubi *et al.*, 2008b). MWMV is efficiently transmitted on a non-persistent manner by *A. gossypii* Glover and *Myzus persicae* Sulz (Lecoq *et al*., 2001; Owolabi *et al.,* 2012; Yakoubi *et al.*, 2008b).

MWMV, which was first isolated in RSA in 1987, is among the most prevalent and damaging virus of cucurbits countrywide (Cradock *et al*., 2001; van der Meer & Garnett, 1987). South African MWMV isolates fall in the Southern Africa cluster, therefore suggesting a recent common origin of introduction (Yakoubi *et al.*, 2008a).

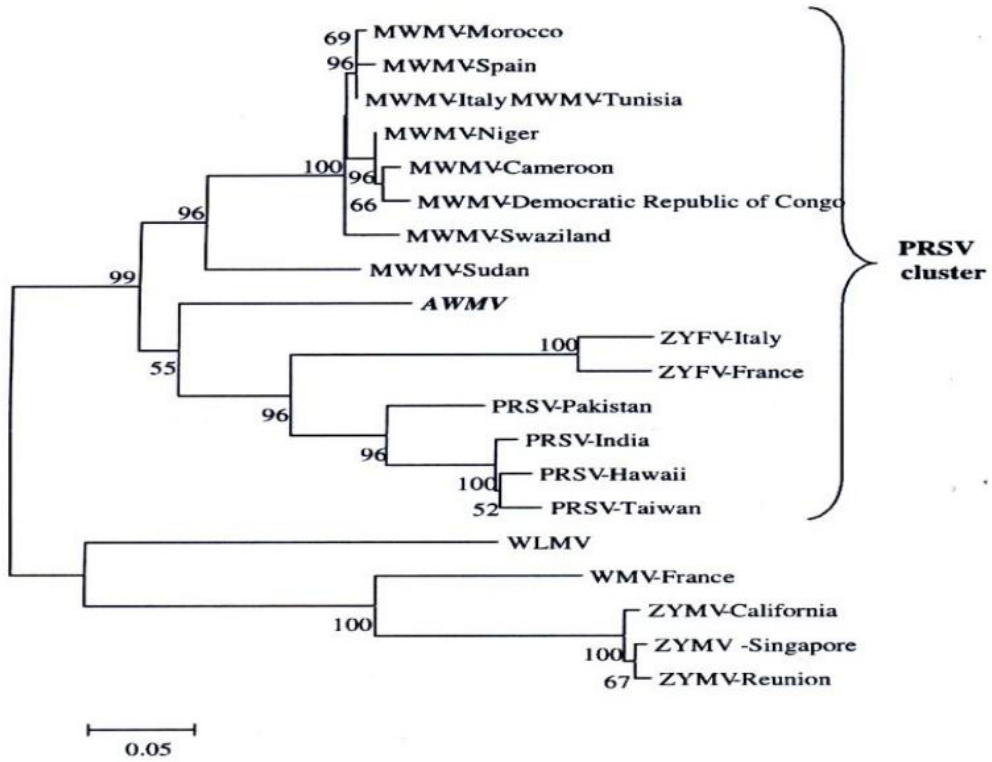

Figure 1.4. Neighbour-joining relationship phylogram of the coat protein of seven cucurbit-infecting potyviruses from GeneBank (Yakoubi *et al.*, 2008b).

1.3.2. Virus of the family *Bromoviridae* infecting cucurbit crops in RSA.

The family *Bromoviridae* is made up of six genera. *Cucumber mosaic virus* (CMV) is the only virus from this family that has been reported to infect cucurbits in RSA.

1.3.2.1. *Cucumber mosaic virus* (CMV)

CMV is the type member of the genus *Cucumovirus*. Virions are icosahedral particles of approximately 29 nm in diameter and are made of 180 subunits of a single capsid protein (Bujarski *et al.*, 2012; Jacquemond, 2012). The CMV genome, packaged in a separate particle (Bujarski *et al.*, 2012), consists of three linear positive sense single-stranded RNA molecules named 1 to 3 in order of decreasing size, with 5'- terminal cap structure and 3'- terminal tRNA-like structure (Bujarski *et al.*, 2012; Jacquemond, 2012). RNA 1 harbours a single ORF that encodes protein 1a. RNA 2 contains ORFs for proteins 2a and 2b. ORF 2b overlaps (+1 frameshift) the 3'terminal part of ORF 2a. Protein 2a is expressed from genomic RNA but protein 2b is expressed from subgenomic RNA 4a. RNA 3 encodes proteins 3a and 3b from genomic and subgenomic RNA 4 respectively (Bujarski *et al.*, 2012; Jacquemond, 2012; Mochizuki & Ohki, 2012; Figure 1.5.). The functions of CMV proteins are listed in Table 1.5.

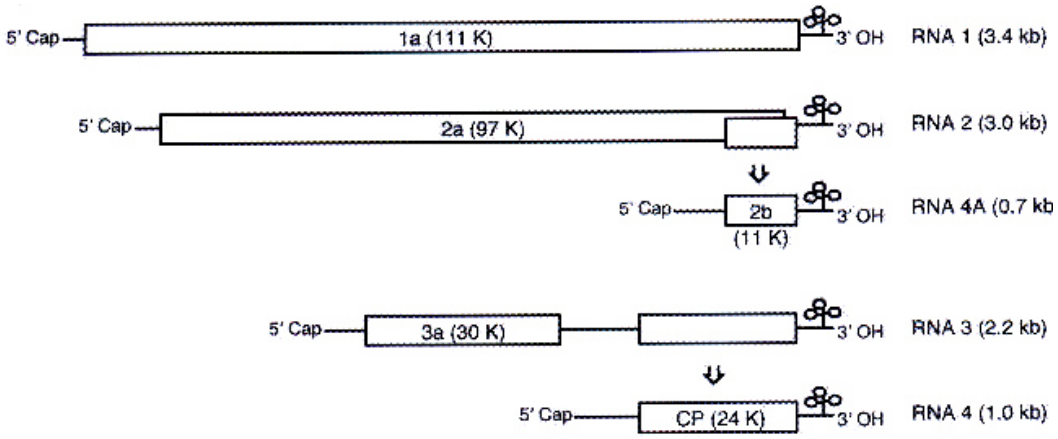

Figure 1.5. CMV genome organization (Mochizuki & Ohki, 2012)

Table 1.5. Role of CMV proteins in the viral life cycle

Protein	Role in virus life cycle	References
1a	Virus replication	Bujarski *et al.*, 2012.
2a	Virus replication; Host specific virulence	Bujarski *et al.*, 2012; Mochizuki & Ohki, 2012.
2b	PTGS suppressor; Long distance movement; Pathogenicity; Aphid transmission; Virus synergism	Kumari *et al.*, 2013; Jacquemond, 2012; Mochizuki & Ohki, 2012.
3a	intercellular movement; symptom determinant	Bujarski *et al.*, 2012; Mochizuki & Ohki, 2012.
3b (CP)	Virus assembly; Intercellular and long distance movement; Aphid transmission; Symptom expression.	Bricault & Perry, 2013; Kumari *et al.*, 2013; Jacquemond, 2012; Mochizuki & Ohki, 2012.

Some CMV isolates, in addition to viral genome RNAs, have two small linear single-stranded RNA molecules known as RNA 5 and satellite RNA (Bujarski *et al.*, 2012; Jacquemond, 2012; Mochizuki & Ohki, 2012). RNA 5, approximately 300 nucleotides long, is thought to have arisen by specific cleavages of RNA 2, 3 and 4 (Jacquemond, 2012). Satellite RNAs are about 332 to 405 nucleotides long. They have no sequence similarity to the viral genome and are

dependent on CMV for replication, encapsidation and vector transmission (Jacquemond, 2012; Mochizuki & Ohki, 2012; Nouri *et al.*, 2012). Satellite RNA may alter CMV pathogenicity by either attenuating or increasing the severity of the symptoms (Bujarski *et al.*, 2012; Nouri *et al.*, 2012).

CMV is the first reported cucurbit-infecting virus in 1916. Nowadays, CMV is among the devastating plant viruses in temperate and tropical areas worldwide (Kumari et al., 2013; Lecoq, 2003; Lecoq & Desbiez, 2012; Mochizuki & Ohki, 2012). CMV has probably the widest host range comprising over 1200 species within 101 plant families of monocots and dicots, which includes crop, vegetables, ornamentals and woody plants (Kumari et al., 2013; Jacquemond, 2012; Mochizuki & Ohki, 2012). CMV causes typical mosaic symptoms in most cucurbit crops: mosaic on leaves and/or fruit, yellow spot, leaf distortion, stunting, deformed fruit with pinpoint depression and wilting. These symptoms generally result in severe economic losses, especially in zucchini plants (Lecoq & Desbiez, 2012; Mochizuki & Ohki, 2012).

CMV isolates are classified into two subgroups namely I and II based on their biological, physical and serological properties. Molecular analyses confirmed the existing grouping and further divided the subgroup I into subgroup IA and IB (Jacquemond, 2012; Mochizuki & Ohki, 2012). The subgroups IA and IB share nucleotide sequence similarity between 92 and 94% whereas nucleotide sequence similarity between subgroup I and II ranges between 73 and 78% (Roossinck, 2001). Subgroup II is heat sensitive, they induce etch symptom on tobacco, and encapsidates the subgenomic RNA 4a (Bujarski *et al.*, 2012; Jacquemond, 2012). The subgroup IA and II have a worldwide distribution while subgroup IB has been identified in Asia mainly (Jacquemond, 2012; Mochizuki & Ohki, 2012).

Over 80 species of aphids in more than 30 genera transmit CMV in a non-persistent manner in the field (Bujarski *et al.*, 2012). Moreover, Mauck *et al*. (2010) observed that CMV-infected plants are more attractive to aphids, thus spreading the virus more rapidly. CMV is also spread by the dodder *Cuscuta* spp (Mochizuki & Ohki, 2012). Transmission of CMV through seed has recently been established in some cucurbit crops (Jacquemond, 2012).
CMV is among the threatening viruses of zucchini production in RSA. The incidence of CMV has been reported by von Wechmar, *et al.* (1995) and Cradock *et al*. (2001). There is no published literature on the serological and molecular identities of South African CMV isolates infecting cucurbits.

1.3.3. Virus of the family *Closteoviridae* infecting cucurbit crops in RSA.

The family *Closteoviridae*, composed of three genera, contains some of the emerging damaging viruses in agriculture (Martelli *et al.*, 2012; Tzanetakis *et al.*, 2011). Members of this family are characterized by a flexuous particle with cross banding, the five gene nodule and infection that are restricted to the vascular tissues (Martelli *et al.*, 2012; Medina *et al.*, 2003). *Beet pseudo yellow virus* (BPYV) is a virus of the family *Closteroviridae* that has been identified as infecting cucurbits in RSA.

1.3.3.1. *Beet pseudo-yellows virus* (BPYV)

BPYV is a member of the genus *Crinivirus* in the family *Closteroviridae* (Martelli *et al.*, 2012). BPYV particles are approximately 12 nm wide and 1500 to 1800 nm long (Liu & Duffus, 1990). Its genome consists of two linear, positive sense, single RNA strands, i.e., RNA 1 and RNA2 of about 7.6 to 8 kb, separately encapsidated (Martelli *et al.*, 2012). Both RNAs are required for infectivity (Lecoq & Desbiez, 2012). RNA 1 encodes for three ORFs: ORF1a, ORF1b and ORF2 (Figure 1.6). ORF1a yields a polyprotein that contains a papain-like proteinase, methyl transferase and helicase motifs. ORF 1b expressed from a +1 frameshift translation mechanism correspond to the RNA-dependent RNA polymerase. A putative 6 kDa is expressed from ORF2 through subgenomic RNA (Abrahamian & Abou-Jawdah, 2014; Lecoq & Desbiez, 2012; Martelli *et al.*, 2012). RNA 2 encodes 8 ORFs, i.e., the quintuple gene block hallmark of the family *Closteroviridae* (P9, P59, the heat shock protein 70 homolog, the coat protein and the minor coat protein) and three putative proteins: P6 P10 and P26 (Abrahamian & Abou-Jawdah, 2014; Lecoq & Desbiez, 2012; Martelli *et al.*, 2012). All ORFs on RNA2 are expressed through subgenomic RNAs (Martelli *et al.*, 2012). The role of BPYV proteins is described in Table 1.6.

BPYV was the first crinivirus identified in 1965 in California (Abrahamian & Abou-Jawdah, 2014; Tzanetakis *et al.*, 2013). Subsequently, BPYV has been responsible for significant economic losses in numerous countries in Asia, Europe, North America, Oceania and USA (Abrahamian & Abou-Jawdah, 2014; Wisler *et al.*, 1998).

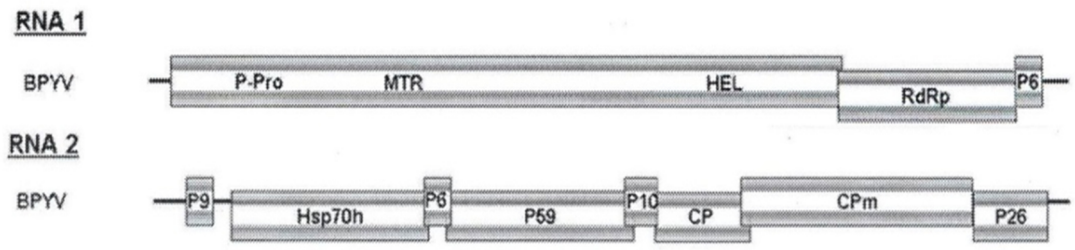

Figure 1.6. Genome organization of BPYV (Abrahamian & Abou-Jawdah, 2014)

Table 1.6. Role of BPYV proteins in the viral life cycle.

Proteins	Role in virus life cycle	References
Papain-like proteinase (P-Pro)	Proteinase; Replication enhancer; Systemic transport.	Martelli *et al.*, 2012.
Methyltransferase (Mtr)	Replication.	Abrahamian & Abou-Jawdah, 2014 ; Martelli *et al.*, 2012.
Helicase (Hel)	Replication.	Abrahamian & Abou-Jawdah, 2014 ; Martelli *et al.*, 2012.
RNA-dependent RNA polymerase (RdRp)	Replication.	Abrahamian & Abou-Jawdah, 2014 ; Martelli *et al.*, 2012.
Heat shock protein 70 homolog (Hsp70h)	Cell to cell movement; Virion assembly and disassembly.	Martelli *et al.*, 2012.
Coat Protein (CP)	Virus assembly; Gene silencing suppressor and protection from cellular degradation.	Abrahamian & Abou-Jawdah, 2014; Canizares *et al*., 2013.
Coat protein minor (CPm)	Assembly of the virion tail; Vector transmission.	Abrahamian & Abou-Jawdah, 2014.
P59	Virus assembly	Martelli *et al.*, 2012.
P6 P9 P10 P26	Unknown	Abrahamian & Abou-Jawdah, 2014.

BPYV has a broad host range that includes a number of cultivated crops (cucurbits, spinach, lettuce, endive, strawberry, blackberry, sugar beet and carrot), ornamentals (marigold and zinnia) and weeds (dandelion and cheese weed). Typical BPYV symptoms may be confused with physiological disorders, soil pH, nutritional disorders or deficiency, natural senescence and pesticide toxicity (Abrahamian & Abou-Jawdah, 2014; Wisler *et al.*, 1998). These symptoms, often restricted to the older leaves, start with interveinal chlorotic spots that eventually spread to the entire leaf. Fruit, not altered, are few in number with total weigh and sugar content significantly reduced (Tzanetakis *et al.*, 2013; Wisler *et al.*, 1998). Yield reduction of 30% to 40% has been associated with BPYV infection (Lecoq & Desbiez, 2012).

No biological variability has been reported for BPYV. Furthermore, insignificant genetic variability was found between the only two fully sequenced BPYV isolates (Lecoq & Desbiez,

2012). BPYV is efficiently transmitted by the green house whitefly *Trialeurodes vaporariorum* in a semi-persistent manner (Wisler et al., 1998).

BPYV has been positively identified in cucurbits crop in RSA (de Vries, 2009). However, very little is known on its physical, biological, serological and molecular characteristics.

1.4. Methods of detection of viruses infecting cucurbits

Techniques used to detect viruses infecting cucurbit are divided into serological and nucleic acid based methods (Albrechtsen, 2006). Serological based methods involve the use of antibodies raised against specific antigens. nucleic acid based methods, also referred to as molecular techniques, rely on the specific non-covalent hydrogen bonds form between the different bases (adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U)) that compose the nucleic acid molecules (Maroon-Lango, 2004; Webster *et al.*, 2004). Enzyme-linked immunosorbent assay (ELISA) is the main serological based methods. Nucleic acid based methods, on the other hand, encompass several techniques which can be used on both routine diagnosis and virus discovery (Boonham *et al*., 2014).

1.4.1. Serological-based method

1.4.1.1. ELISA

ELISA uses immunology to detect the presence of an antigen in a sample by means of specific monoclonal or polyclonal antibodies (Albrechtsen, 2006; Fegla & Kawanna, 2013). ELISA was performed for the first time in connection with plant viruses in 1977 by Clark and Adams (Boonham *et al*., 2014; Fegla & Kawanna, 2013). ELISA is performed on a solid phase and consist of sandwiching the tested virus between two antibodies specific to the targeted virus. Two types of ELISA, the direct and indirect, are differentiated depending on the approach used to detect the complex antibody-virus-antibody formed (Figure 1.7.). ELISA has become the most popular methods for virus routine testing due to several contributing factors such as high sensitivity and specificity, ease of use, speed, cost-effectiveness, robustness, the ability to scale it up to test a large number of samples and it is semi-quantitative for the pathogen (Albrechtsen, 2006; Boonham *et al*., 2014; Fegla & Kawanna, 2013). Regarding cucurbit-infecting viruses, a number of ELISA kits are commercially available from various suppliers (Lecoq & Desbiez, 2012).

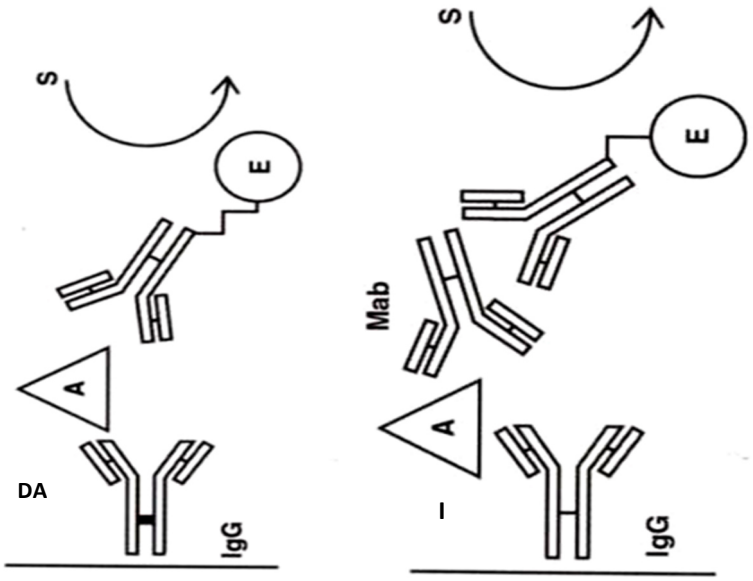

Figure 1.7. Type of ELISA. (D): Direct Elisa: the enzyme on the labelled-antibody reacts with the substrate to indicate a positive reaction. (I): Indirect ELISA: A positive reaction is detected upon addition of an enzyme-labelled anti-immunoglobulin antibody and the substrate-enzyme. (A) antigen; (E) enzyme; (IgG) antibody; (Mab) Monoclonal antibody; (S) Substrate (Albrechtsen, 2006).

1.4.2. Molecular-based methods

1.4.2.1. Polymerase chain reaction

Polymerase chain reaction (PCR) is an *in vitro* DNA synthesis whereby the copy number of a targeted nucleotide sequence is amplified through a series of repeated cycles using a DNA polymerase (Figure 1.8.) PCR is performed inside a thermocycler. PCR has become the basic technique used in the detection and diagnosis of plant viruses since its first use in the early 1990s (Boonham *et al*., 2014). PCR offers high levels of specificity and sensitivity allowing it to distinguish between viruses at the genus, species and strain level. Detection of RNA viruses using PCR requires the conversion of viral RNA into its complementary DNA (cDNA). This step is known as reverse transcription (RT) or first strand cDNA synthesis and is performed using an enzyme called reverse transcriptase. The cDNA synthesized serves as the template during PCR. Viruses infecting cucurbits are detected through PCR using Primers specific to any portion of the virus genome.

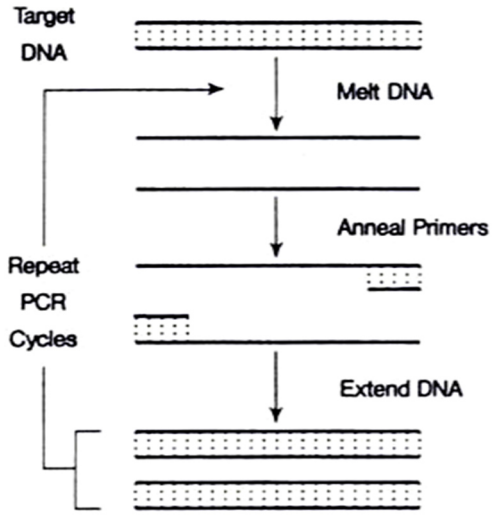

Figure 1.8. PCR's principle. Each cycle consists of a denaturation step that opens the DNA. Primers then anneal at their complementary sites and direct the synthesis of a new DNA that will act as a template in the next cycle (Henson & French, 1993).

1.4.2.2. Next generation sequencing (NGS)

NGS describes platforms, summarized in Table 1.7, that produce a large amount of DNA reads using various technologies (Barba *et al.*, 2014; Kobayashi *et al.*, 2012). It was introduced at the beginning of the 21st century (Barba *et al.*, 2014) and operates on the capillary electrophoresis principle (Prabha *et al.*, 2013). NGS is under continuous improvement to deliver fast and accurate genomic information at a very high level of resolution (Barba *et al.*, 2014). NGS has revolutionized virus identification and characterization (Barba *et al.*, 2014; Boonham *et al.*, 2014; Kobayashi *et al.*, 2012; Prabha *et al.*, 2013). The application of NGS combined with metagenomic analysis has led to the detection of several novel viruses infecting both agricultural and wild plants (Barba *et al.*, 2014; Prabha *et al.*, 2013). This feature of virus discovery is made easier because NGS may be performed without *a priori* knowledge of the causal agent of the disease (Prabha *et al.*, 2013). In this regards, NGS is the best alternative for investigating diseases of unknown viral aetiology (Barba *et al.*, 2014). NGS in RSA appears to be at an embryonic stage that is characterized by the presence of few platforms and facilities and a high operating cost per sample that is not affordable to the average plant pathology research unit.

Table 1.7. Current NGS platforms and technologies (Barba *et al.*, 2014).

Sequencing platform	Amplification method	Sequencing chemistry	Read length (bp)	Sequencing Speed/h	Maximum Output Per run	Accuracy (%)
454 (Roche)	Emulsion PCR	Pyrosequencing	400–700	13 Mbp	700 Mbp	99.9
Illumina (Illumina)	Bridge PCR	Reversible terminators	100–300	25 Mbp	600 Gbp	99.9
SOLiD (Life Technologies)	Emulsion PCR	Ligation	75–85	21–28 Mbp	80–360 Gbp	99.9
PacBio (Pacific Biosciences)	No amplification Single molecule real-time (or SMRT)	Fluorescently labeled nucleotides	4, 000–5,000	50–115 Mbp	200 Mb–1 Gbp	95
Helicos (Helicos Biosciences)	No amplification Single molecule	Reversible terminators	25–55	83 Mbp	35 Gbp	97
Ion Torrent (Life Technologies)	Emulsion PCR	Detection of released H	100–400	25 Mb–16 Gbp	100 Mb–64 Gbp	99
Nanopore (Oxford Technologies)	No amplification Single molecule		Very long reads up to 50 kbp	150 Mbp	Tens of Gbp	96

1.5. Management of viral diseases of cucurbits

Control strategies of cucurbit virus disease encompass prophylactic measures (Lecoq & Desbiez, 2012; Tzanetakis *et al.*, 2013), cross protection (Gal-On, 2007; Lecoq & Desbiez, 2012), the use of plant growth promoting fungi (Murphy *et al.*, 2008) and resistant cultivars (Gal-On, 2007; Lecoq & Desbiez, 2012). Some of these strategies are used to control diseases caused by several viruses from different families and genera while other are only used to control diseases caused by specific viruses.

1.5.1. Prophylactic measures

Prophylactic measures aim at keeping viruliferous vectors away from cultivated crops and are the primary approach to control diseases such as BPYV where no resistant cultivar has been developed by breeding nor genetic transformation (Lecoq & Desbiez, 2012). These measures include phytosanitation and cultural techniques such as the manipulation of planting date, planting upwind of infection sources, avoiding overlapping and side-by-side plantings, removal of potential virus and vector sources among weeds, deploying tall non-host barriers, use of plastic mulches that have a repelling action on vectors of the viruses, the use of insecticides and oil application (Coutts *et al.*, 2011b; Hansson *et al.*, 2013 Lecoq & Desbiez, 2012). Biological control is an alternative to chemical control. It has been achieved with the use of entomopathogenic fungi (Kim *et al.*, 2014; Rivas *et al.*, 2014), vector predators and parasitoids (Yang *et al.*, 2014). Several bioinsecticides targeting virus vectors have been developed and commercialized (Kim *et al.*, 2013).

1.5.2. Use of plant growth promoting fungi

The use of plant growth promoting fungi as a mean of controlling virus disease in cucurbits has only been documented with regards to CMV. The plant growth promoting fungi *Trichoderma asperellum* SKT-1, *Glomus mosseae*, *Fusarium equiseti* and *Penicillium simplicissimum* GP17-2 have all been reported to induce systemic resistance against CMV. The treated plants have developed reduced levels of disease and low virus titers compared to the control plants (Elsharkawy, 2013; 2012a; 2012b).

1.5.3. Cross protection

Cross protection consists in inoculating young seedling with a mild virus isolate in order to protect the plant from subsequent contamination by severe isolates of the same virus. In that regard, the natural mild strain (ZYMV-WK) and the artificial mild mutant [ZYMV-AG (AG) and ZYMV-2002] have been used at a commercial level to protect cucurbit crops against serologically related strains (Gal-On, 2007; Lecoq & Desbiez, 2012).

1.5.4. Use of resistant cultivars

The use of resistant cultivars remains the best control for virus diseases on cucurbit (Gal-On, 2007). Resistant cultivars are produced either through breeding or genetic modification, also referred to as pathogen-derived resistance (PDR).

1.5.4.1. Virus resistance breeding

Breeding for virus resistance requires good sources of resistance (Gal-On, 2007) which are generally found in wild and landrace cucurbits (Svoboda *et al.*, 2014). Virus resistance based on major genes had been identified, but some have been overcome already by virulent races of the viruses (Behera *et al*., 2012; Gal-On, 2007). Resistance genes identified in *C. moschata* cv Nigeria, *C. melo* WMR29 and *C. sativus* TMG have shown good durable resistance towards MWMV (Yakoubi *et al.*, 2008a). The recessive alleles zym^{TMG-1} and zym^{Dina-1} in the *zym* locus in cucumber seems to confer durable resistance against several ZYMV isolates worldwide (Amano *et al*., 2013; Gal-On, 2007; Svoboda *et al.*, 2014). Breeding for resistant WMV cultivars has had limited success due to the various limitations associated with it (Behera *et al*., 2012; Lecoq & Desbiez, 2012; Lecoq, 2003). Good resistance against CMV in cucurbit is scarce, partial, subgroup-specific and mostly polygenic and recessive (Essafi *et al.*, 2009; Jacquemond, 2012; Lecoq & Desbiez, 2012; Malik *et al.*, 2014), making it difficult to develop commercial cultivars (Malik *et al.*, 2014).

1.5.4.2. Pathogen-derived resistance (PDR)

The PDR concept, which is the use of genetic elements from a pathogen's own genome to confer resistance in an otherwise susceptible host via genetic engineering, was introduced by Sanford and Johnston in 1985 (Gottula & Fuchs, 2009). Its application in virus resistance was demonstrated for the first time in 1986 with the development of a transgenic tobacco plant resistant to *Tobacco mosaic virus* (TMV) by expressing the coat protein gene of TMV (Duan *et al.*, 2012; Gottula & Fuchs, 2009). Several virus-resistant transgenic plants have been developed subsequently, based on the PDR concept (Dietzgen & Mitter, 2006; Gottula & Fuchs, 2009; Prins *et al.*, 2008), making PDR the most powerful approach towards the control of plant virus infections (Saurabh *et al.*, 2014). PDR strategies are classified into two groups on the basis of their functional molecules: protein and RNA-mediated resistance (Duan *et al.*, 2012; Gottula & Fuchs, 2009; Prins *et al.*, 2008). While the mechanism of protein-mediated resistance remains unclear, RNA-mediated resistance has been intensively documented and was found to rely on an evolutionarily conserved defence mechanism naturally occurring in eukaryote *i.e.* RNA silencing (Duan *et al.*, 2012; Saurabh *et al.*, 2014; Pattanayak *et al.,* 2013).

RNA silencing is also referred as to gene-quelling and RNA interference (Duan *et al.*, 2012). Small non-coding RNAs classified as microRNA (miRNAs) and small/short interfering RNAs (siRNAs) function by either post-transcriptional gene silencing (PTGS), mostly by cleavage of target mRNA, or transcriptional gene silencing (TGS) through RNA-directed DNA methylation (Pattanayak *et al.,* 2013). miRNAs, 21 to 24 nt long, are involved in plant response to virus infection (Ramesh *et al*., 2014). miRNA biogenesis (Figure 1.9.) is initiated in the nucleus from long single-stranded RNA that contain imperfect stem-loop structures called pri-miRNA. Pri-miRNA, generated by the activity of RNA Pol II, is further processed into precursor miRNA (90 – 140 bp) and into smaller, double stranded, mature miRNA before being exported to the cytoplasm by HASTY 1. The conversion of pri-miRNA to mature miRNA involves many protein families including RNAseIII, Dicer-like-1, DAWDLE, HYL1, SERRATE and HEN. The mature miRNA in the cytoplasm is incorporated into the AGO 1 protein on the RNA-induced silencing complex (RISC), which unwinds the miRNA duplex to release one strand called the passenger, which is degraded. The effector miRNA on the RISC functions as a catch by binding on the target mRNA and causing its degradation (Masuta & Shimura, 2013; Ramesh *et al*., 2014).

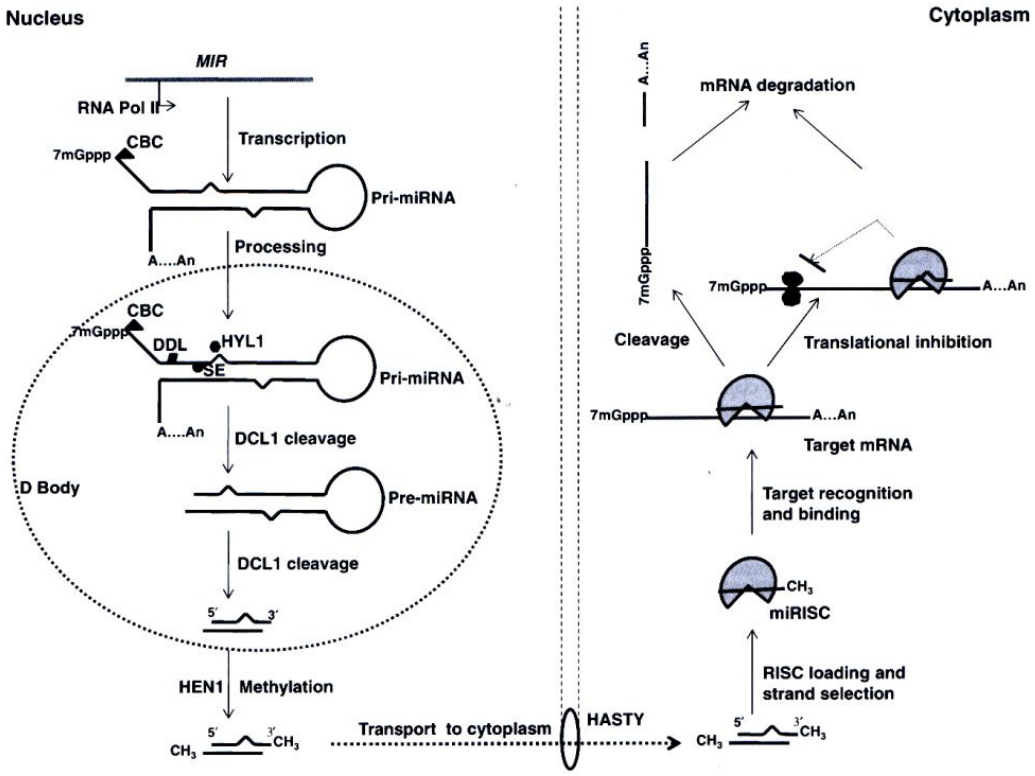

Figure 1.9. A simplified view of plant miRNA biogenesis. RNA polymerase II-mediated transcription of miRNA gene (*MIR*) generates primary precursor RNA, called Pri-miRNA. Processing of Pri-miRNA by Dicer-like (*DCL*) 1 takes place in D-body of the nucleus and a number of processing-associated proteins, cap-binding complex, serrate (*SE*), Dawdle and hyponastic leaves1 (*HYL1*), interact with DCL1 during processing. Two-step cleavage of Pri-miRNA by DCL1 generates duplex miRNA, which is methylated by Hua Enhancer1 (*HEN1*). Methylated duplex miRNA is transported into the cytoplasm, mediated by HASTY, and guide miRNA strand is loaded on miRNAinduced silencing complex (*miRISC*). miRNA guides miRISC for silencing of target mRNA by either cleavage or translational inhibition (Pattanayak *et al.,* 2013).

Virus resistance mediated by transgene-induced silencing in plants is highly specific, environmentally friendly and capable of targeting multiple virus pathogens (Zhou, 2012). The different approaches that have been used to express double-stranded RNA from viral genome for activation of RNA silencing include transgenes expressing viral sense, antisense, inverted repeat sequences and artificial miRNA precursors (Dietzgen & Mitter, 2006; Duan *et al.*, 2012; Gottula & Fuchs, 2009; Prins *et al.*, 2008). Coat protein-mediated transgenes have been a popular strategy to develop virus resistant cucurbits (Jacquemond, 2012; Lecoq & Desbiez, 2012; Morroni *et al*., 2008). Durable resistance in both the lab and in the fields has been recorded with a number of transgenic cucurbit crops against ZYMV (Dias & Ortiz, 2013; Fuchs *et al*., 1997; Leibman *et al*., 2011; Tricoll *et al*., 1995; Wu *et al.*, 2009; Yu *et al*., 2011), WMV (Dias & Ortiz, 2013; Fuchs *et al.*, 1997; Gaba *et al.*, 2004; Lin *et al.*, 2012) and CMV (Jacquemond, 2012; Lecoq & Desbiez, 2012; Morroni *et al*., 2008).

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Chapter 2

Incidence and Phylogeny of Viruses Infecting Cucurbit Crops in KwaZulu-Natal, Republic of South Africa[1]

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Abstract

Virus infections on cucurbits often result in substantial losses. Surveys were conducted throughout the province of KwaZulu-Natal (KZN) in the Republic of South Africa (RSA) during the 2011 - 2013 growing seasons to identify cucurbit-infecting viruses. Viruses were detected on sampled leaves displaying virus-like symptoms using double antibody sandwich enzyme-linked immunosorbent assay and reverse transcription polymerase chain reaction. The phylogenetic relationships of all detected viruses were also studied. *Cucumber mosaic virus* (CMV), *Beet pseudo-yellows virus* (BPYV), *Zucchini yellow mosaic virus* (ZYMV), *Moroccan watermelon mosaic virus* (MWMV) and a *Polerovirus* were detected at an incidence of 3.48 %, 10 %, 13.04%, 48.70 % and 41.67% respectively. Phylogenetic analyses identified CMV isolates as members of the Subgroup IA of the CMV lineage and ZYMV isolates as members of the subgroups AI and AII of the of ZYMV lineage. MWMV isolates formed a distinct clade within the Southern African group of the MWMV lineage. *Polerovirus* isolates were identified as *Pepo aphid-borne yellows virus* (PABYV) based on the sequence similarity and phylogenetic analyses. The information generated from this study will contribute towards the development of effective management strategies against viruses infecting cucurbits in KZN.

Keywords

ELISA

Mosaic

Phylogenetic analysis

RT-PCR

Yellowing

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2.1. Introduction

The term cucurbit generally designates any species belonging to the family *Cucurbitaceae* (Robinson and Decker-Walters, 1997; Weng and Sun, 2012), which includes indispensable crops that have been recorded as second in economic importance to solanaceous crops (Romay et al., 2014). Cucurbits are primarily found in the tropical and subtropical regions worldwide (Robinson and Decker-Walters, 1997; Weng and Sun, 2012) and play significant roles in human nutrition (Weng and Sun, 2012). Cucurbit species belonging to the genera *Citrullus, Cucumis* and *Cucurbita* are extensively cultivated by commercial and subsistence farmers throughout South Africa (Trench et al., 1992). Orange and yellow-fleshed cucurbits are important in the alleviation of vitamin A deficiency in rural communities (Voster et al., 2007).

Viruses are a major limiting factor of cucurbit production worldwide. The number of reported viruses infecting cucurbits worldwide has increased from approximately 35 in 1996 (Provvidenti, 1996) to more than 59 in 2012 (Lecoq and Desbiez, 2012). Typical viral symptoms in cucurbits are grouped into mosaic, yellowing and necrosis (Lecoq and Desbiez, 2012). Mosaic type symptoms are often associated with distorted or reduced leaf growth and discoloured or deformed fruits. Yellowing symptoms start on the older and mature leaves. Necrosis may appear as spots on leaves and fruits (Lecoq and Desbiez, 2012; Lecoq, 2003). Mosaic and necrosis diseases, that generally affect fruit quality, may result in more severe economic loses compared to yellowing diseases which only affect fruit production and composition (Lecoq and Desbiez, 2012; Tzanetakis et al., 2013; Wisler et al., 1998). Viruses from the genera *Carlavirus* (Nagata et al., 2010), *Polerovirus* (Knierim et al., 2010) and *Crinivirus* (Abrahamian and Abou-Jawdah, 2014; Tzanetakis et al., 2013; Wisler et al., 1998) have been associated with yellowing diseases on cucurbits.

Effective virus disease management can be initiated following the accurate identification of the causative virus (Rubio et al., 1999). *Beet pseudo-yellows virus* (BPYV), *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV) and *Moroccan watermelon mosaic virus* (MWMV) have been previously reported to infect cucurbit crops in RSA (Cradock et al., 2001; de Vries, 2009; Usher et al., 2012; van der Meer, 1985; van der Meer and Garnett, 1987; van Regenmortel, 1960; von Wechmar, et al., 1995). CMV, ZYMV, WMV and MWMV are transmitted in a non-persistent manner by various aphid species (Lecoq and Desbiez, 2012) while BPYV is transmitted in a semi-persistent manner by the greenhouse whitefly *Trialeurodes vaporariorum* (Wisler et al., 1998).

Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are the most successfully established methods in the routine diagnosis of plant viruses (Boonham et al., 2014). The last published study of viruses infecting cucurbits in KwaZulu-Natal (KZN) was conducted in 1998 (Cradock et al., 2001). Moreover, there is little information on the phylogeny of viruses infecting cucurbits in RSA. Against this background, the aim of this study was to evaluate the incidence and phylogeny of viruses infecting cucurbits in KZN.

2.2. Materials and Methods

2.2.1. Sample collection

Cucurbit leaves exhibiting symptoms of viral aetiology were collected from commercial and small holder farms, tunnels, plots and households throughout KZN (Fig. 1) during 2011 to 2013 growing seasons. Sampled crops included butternut (*Cucurbita moschata* Duch.), English cucumber (*Cucumis sativus* L.), baby marrows (*Cucurbita pepo* L.), pattypan (*Cucurbita pepo* L.) and various pumpkins (*Cucurbita maxima* Duch.). The samples were appropriately labelled and stored frozen at -80°C in sealable plastic bags until further use.

2.2.2. Virus detection

Samples displaying mosaic disease symptoms were individually tested for the presence of *Squash mosaic virus* (SqMV), CMV, MWMV, WMV and ZYMV using DAS - ELISA. Antibodies specific to the selected viruses were purchased from Neogen Corporation (USA) and used as per manufacturer's instructions. Tests that displayed optical density values (OD) three times the mean of the negative control OD were considered as positive.

Nucleic acid detection methods have been frequently used over serological-based methods in the diagnosis of viruses causing yellowing diseases (Abrahamian and Abou-Jawdah, 2014; Lotos et al., 2014). RT-PCR tests using gene-specific primers (Table 1) were performed on samples displaying yellowing symptoms. RNA was extracted using the SV total RNA isolation kit (Promega, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using gene-specific primers (Table 1) and the RevertAid Premium First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. PCR was performed using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., USA) as described in Table 1. PCR products were analysed on 1.5% agarose gel pre-stained with SYBR Safe.

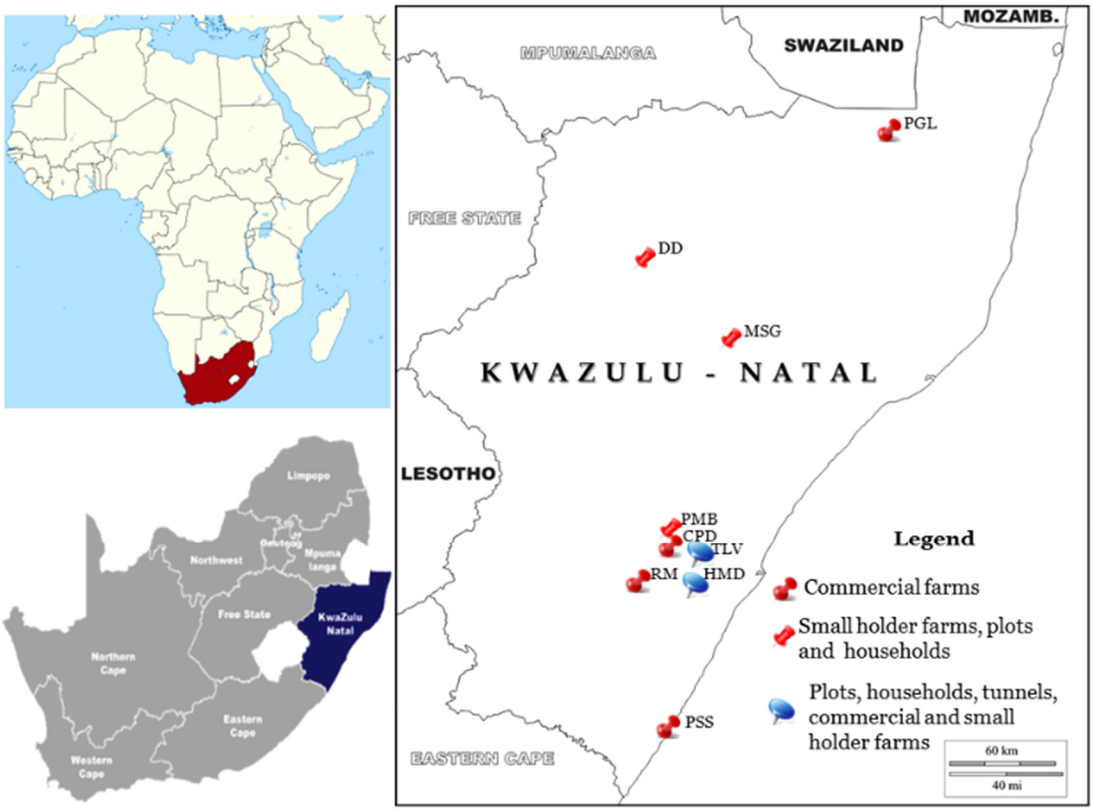

Fig. 1. Locations where samples were collected. CPD: Camperdown; DD: Dundee; HMD: Hammarsdale; MSG: Msinga; PGL: Pongola; PMB: Pietermaritzburg; PSS: Port Shepstone; RM: Richmond; TLV: Tala Valley.

2.2.3. Cloning, sequencing and phylogenetic analysis

The coat protein gene for each virus detected using DAS - ELISA was amplified using RT-PCR and gene-specific primers (Table 1) from randomly selected ELISA-positive samples. Each PCR product was cleaned using the DNA clean and concentrator-25 kit (Zymo Research, USA) prior to cloning into a pSMART HCKan vector (Lucigen, USA) according to the manufacturer's instructions. Four positive clones of each sample were sequenced at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA). Amplicons from RT-PCR tests performed to detect viruses causing yellowing diseases were randomly selected, cloned and sequenced.

Table 1. Primers used to detect viruses causing yellowing and to amplify the coat protein gene of the viruses causing mosaic that were detected with ELISA.

Disease	Name	5'- 3'sequence	Portion of the genome amplified	Amplicon size (kb)	PCR conditions (40 cycles)	References
Yellowing	CABYV up	GAACACTAGCCAAGCACACAC	Partial CP of CABYV	0.484	98°C for 5 s; 60°C for 5 s; 72°C for 15 s.	Boubourakas et al., 2006
	CABYV down	GGTAGGCCTTGAGTATTCCAG				
	BPYV I	TCGAAAGTCCAACAAGACGT	Partial Hsp70h of BPYV	0.251	98°C for 5 s; 55°C for 5 s; 72°C for 10 s.	Boubourakas et al., 2006
	BPYV II	CTGATGGTGCGCGAGT				
	410U	TTGGGCATGTGACAT	Partial Hsp70h of CYSDV	0.435	98°C for 5 s; 45°C for 5 s; 72°C for 15 s.	Celix et al., 1996
	410L	GAACACTAGCCAAGCACACAC				
	Pol-G-F	GAYTGCTCYGGYTTYGACTGGAG	RdRp, CP and MP of all Poleroviruses	1.1	98°C for 5 s; 60°C for 5 s; 72°C for 25 s.	Knierim et al., 2010
	Pol-G-R	GATYTTATAYTCATGGTAGGCCTTGAG				
	Cucurbit reverse	GTGTTHGAYAACCAAGTGTTTGG [(a)]	Partial RdRp of BPYV CYSDV LIYV	0.643 0.279 0.427	98°C for 5 s; 55°C for 5 s; 72°C for 15 s.	Wintermantel and Hladky, 2010
	BPYV	TGATGTCTGGTTTGATGACGGG				
	CYSDV	CTTAATGACCTTAGCCGACTTGAT				
	LIYV	GCACATACGACAGTTACAATGCTCC				
	Carl deg	TTTGCHGGBGATGACATGTG	Carlaviruses	3.1	98°C for 5 s; 50°C for 5 s; 72°C for 65 s.	Nagata et al., 2010
	M4T	GTTTTCCCAGTCACGACAATTAA(T)20				
Mosaic	ZY-2	GCTCCATACATAGCTGAGACAGC	CP and 3' UTR of ZYMV	1.186	98°C for 5 s; 60°C for 5 s; 72°C for 25 s.	Thomson et al., 1995
	ZY-3	TAGGCTTGCAAACGGAGTCTAATC				
	PfCMV-1163	ATGCTTCTCCRCGAGATT	CP OF CMV	0.870	98°C for 5 s; 55°C for 5 s; 72°C for 20 s.	Chang et al., 2011
	PrCMV-2034	GTAAGCTGGATGGACAAC				
	N1T	GACCACGCGTATCGATGTCGAC(T)17[(b)]	Nib C-terminal, CP and 3' UTR of MWMV	1.2	98°C for 5 s; 55°C for 5 s; 72°C for 25 s	Ha et al., 2008; Lecoq et al., 2008
	MWMV-5	AGCAAGCGCCATACTCTGA				
	N1	GACCACGCGTATCGATGTCGAC				

Cp: Coat Protein; Hsp70h: Heat shock protein 70 homolog; MP: Movement protein; RdRp: RNA-dependent RNA polymerase; UTR: untranslated region; (**a**): reverse primer used with BPYV, CYSDV and LIYV primers; (**b**): primer used in RT.

All generated sequences were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and compared with sequences selected from the GenBank database. Molecular Evolutionary Genetics Analysis (MEGA version 6) software (Tamura et al., 2013) and Clustal W were used to analyse and align the generated sequences. Nucleotide and protein sequence similarity were done on the SIAS server (http://imed.med.ucm.es/Tools/sias.html) using the default parameters. The phylogenetic trees were inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbour-Joining method and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site

2.3. Results

2.3.1. Virus detection

CMV, MWMV and ZYMV were positively identified using DAS-ELISA. In addition, BPYV and an unknown *Polerovirus* species were detected in some samples following RT-PCR (Table 2). Cucumber was the least infected crop which tested positive for BPYV only. Butternut was the most infected crop, testing positive for four viruses: MWMV, ZYMV, BPYV and a *Polerovirus*. Pattypan and pumpkin were both infected with the *Polerovirus* and MWMV while baby marrow was infected with the *Polerovirus*, CMV and MWMV. ZYMV was the most common virus infecting butternut; and MWMV was the most common in baby marrow, pattypan and pumpkin. MWMV had the highest incidence of 48.70 % among the five viruses detected in this survey, followed by the *Polerovirus* with 41.67 % incidence. ZYMV, BPYV and CMV had incidences below 20 % with CMV being the least common with an incidence of 3.48 %. Multiple infections accounted for 22.64 % of infected samples and consisted of double infections only. They were detected on butternut and baby marrow samples (Table 3).

Table 2. Cucurbit-infecting viruses detected in collected samples

Method of detection used	Virus identified (# sample tested)	Butternut	Baby marrow	Cucumber	Pattypan	Pumpkin	Total (%)
DAS- ELISA	CMV (115)	0	4	0	0	0	4 (3.48)
DAS-ELISA	SqMV (115)	0	0	0	0	0	0 (0)
DAS-ELISA	MWMV (115)	9	19	0	10	18	56 (48.70)
DAS-ELISA	WMV (115)	0	0	0	0	0	0 (0)
DAS-ELISA	ZYMV (115)	15	0	0	0	0	15 (13.04)
RT-PCR	BPYV (60)	1	0	5	0	0	6 (10)
RT-PCR	CABYV (60)	0	0	0	0	0	0 (0)
RT-PCR	CYSDV (60)	0	0	0	0	0	0 (0)
RT-PCR	LIYV (60)	0	0	0	0	0	0 (0)
RT-PCR	*Carlavirus* (60)	0	0	0	0	0	0 (0)
RT-PCR	*Polerovirus* (60)	10	6	0	5	4	25 (41.67)

Table 3. Multiple virus infections detected on cucurbit crops

Crops	{CMV + *polerovirus*}	{MWMV + *polerovirus*}	{ZYMV+*Polerovirus*}
Butternut	0	0	10
Baby marrow	1	1	0
Cucumber	0	0	0
Pattypan	0	0	0
Pumpkin	0	0	0

2.3.2. Phylogenetic analysis of detected viruses

The sequences of three BPYV, four CMV, six MWMV, five ZYMV and eight *Polerovirus* isolates were submitted to GenBank. Details of these sequences are summarized in Table 4.

Table 4. Description of the virus isolate sequences submitted to GenBank

Virus	Isolate name	Host	Accession number
BPYV RNAI	KZN112a	Cucumber	KJ789909
BPYV RNAI	KZN120a	Cucumber	KJ789912
BPYV RNAI	KZN125a	Butternut	KJ789914
BPYV RNAII	KZN112b	Cucumber	KJ789910
BPYV RNAII	KZN120b	Cucumber	KJ789913
BPYV RNAII	KZN125b	Butternut	KJ789915
CMV	KZN-BM1	Baby marrow	KJ789892
CMV	KZN-BM2	Baby marrow	KJ789893
CMV	KZN-BM3	Baby marrow	KJ789894
CMV	KZN-BM4	Baby marrow	KJ789895
MWMV	KZN24	Pattypan	KJ789896
MWMV	KZN31	Butternut	KJ789898
MWMV	KZN33	Butternut	KJ789899
MWMV	KZN54	Baby marrow	KJ789901
MWMV	KZN75	Baby marrow	KJ789905
MWMV	KZN105	Pumpkin	KJ789908
ZYMV	KZN-ND1	Butternut	KJ789916
ZYMV	KZN-ND2	Butternut	KJ789917
ZYMV	KZNN-TV1	Butternut	KJ789918
ZYMV	KZNN-TV2	Butternut	KJ789919
ZYMV	KZNN-TV3	Butternut	KJ789920
Polerovirus	KZN27	Pattypan	KJ789897
Polerovirus	KZN37	Butternut	KJ789900
Polerovirus	KZN60	Butternut	KJ789902
Polerovirus	KZN62	Pattypan	KJ789903
Polerovirus	KZN71	Baby marrow	KJ789904
Polerovirus	KZN81	Pumpkin	KJ789906
Polerovirus	KZN85	Pumpkin	KJ789907
Polerovirus	KZN115	Pattypan	KJ789911

The nucleotide sequences of the entire coat protein gene of the four CMV isolates detected in this study were aligned with the coat protein sequences of a South African CMV isolate and other CMV isolates from different geographical origins and phylogenetic subgroups; Subgroup IA, IB and II (Table 5).

Table 5. Virus sequences used in phylogenetic analyses of the CP sequences of CMV isolates

Virus	Accession number	Strain/ Isolate	Origin	Phylogenetic subgroup	references
CMV	AJ517802	Rs	Hungary	IA	Bashir et al., 2006.
CMV	AJ511990	Ns	Hungary	IA	Bashir et al., 2006.
CMV	AJ585517	237	Australia	IA	Thompson and Tepfer, 2009.
CMV	AM183119	Ri-8	Spain	IA	Thompson and Tepfer, 2009.
CMV	D00462	C	USA	IA	Bashir et al., 2006.
CMV	D10538	Fny	USA	IA	Thompson and Tepfer, 2009
CMV	U22821	Ny	Australia	IA	Bashir et al., 2006.
CMV	U32859	Banana	Colombia	IA	Bashir et al., 2006.
CMV	U66094	Sny	Israel	IA	Thompson and Tepfer, 2009
CMV	Y18137	I17F	France	IA	Thompson and Tepfer, 2009
CMV	AB008777	SD	China	IB	Bashir et al., 2006.
CMV	AF013291	As	Korea	IB	Bashir et al., 2006.
CMV	AY429437	Cs	China	IB	Bashir et al., 2006.
CMV	L36525	ABI	Korea	IB	Bashir et al., 2006.
CMV	AB006813	M2	Japan	II	Bashir et al., 2006.
CMV	AF063610	S	RSA	II	Madhubala et al., 2005
CMV	AF127976	LS	USA	II	Thompson and Tepfer, 2009
CMV	AF268598	Xb	China	II	Bashir et al., 2006.
CMV	U22822	Sn	Australia	II	Bashir et al., 2006.
CMV	U61285	Wemmershoek	RSA	II	Bashir et al., 2006.
CMV	Z12818	Kin	UK	II	Bashir et al., 2006.
PSV*	JF838186	K1	Korea	Outgroup	Bashir et al., 2006.
PSV*	U31366	W	USA	Outgroup	Bashir et al., 2006.

*: Peanut stunt virus

All four sequences of the CMV isolates did not display any genetic diversity among themselves and clustered within the Subgroup IA of the Group I lineage of CMV (Fig. 2).

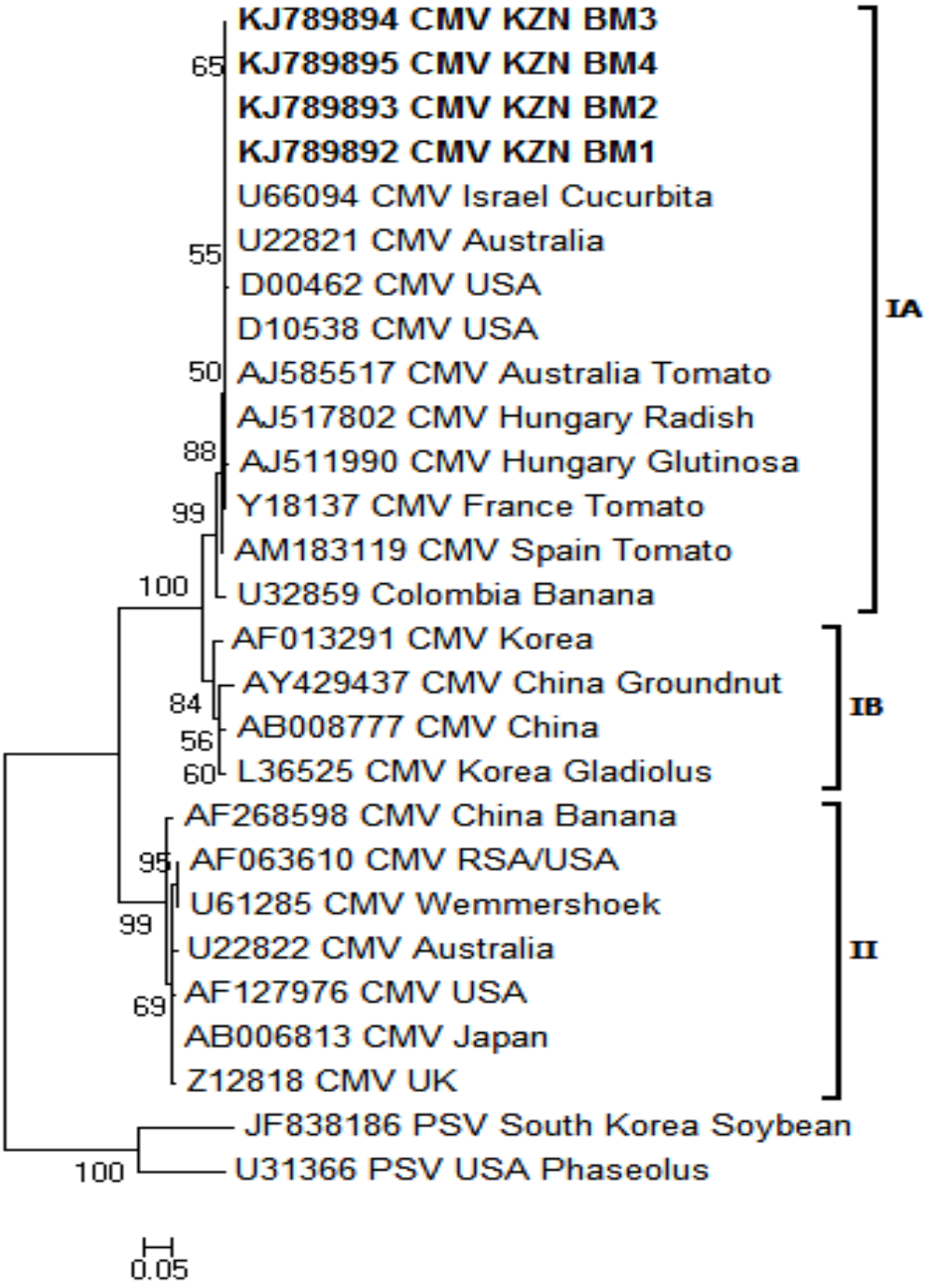

Fig. 2. Phylogenetic relationship of the coat protein sequences of CMV isolates from KwaZulu-Natal.

Likewise, the nucleotide sequence of the entire coat protein gene of the five KZN ZYMV isolates were aligned with the coat protein sequences of selected ZYMV isolates representing the phylogenetic groups that form the ZYMV lineage (Table 6).

Table 6. Virus sequences used in phylogenetic analyses of the CP sequences of ZYMV isolates

Virus	Accession number	Isolate name	Origin	Phylogenetic Subgroup	References
ZYMV	AB063251	M39	Japan	AI	Romay et al., 2014
ZYMV	AB458595	SYZY-1	Syria	AI	Romay et al., 2014
ZYMV	AJ420019	Berlin 1	Germany	AI	Romay et al., 2014
ZYMV	AJ459955	H272-5	Hungary	AI	Romay et al., 2014
ZYMV	EF062582	NAT	Israel	AI	Romay et al., 2014
ZYMV	FJ705264	Ker.Baf. S	Iran	AI	Romay et al., 2014
ZYMV	GU586790	ZTRICH	Brazil	AI	Romay et al., 2014
ZYMV	JF797206	APCU	India	AI	Romay et al., 2014
ZYMV	JN861004	E9	France	AI	Romay et al., 2014
ZYMV	AB188115	Z5-1	China	AII	Romay et al., 2014
ZYMV	AJ420020	Italy 1	Italy	AII	Romay et al., 2014
ZYMV	D00692	Conneticut	USA	AII	Romay et al., 2014
ZYMV	DQ645729	C-16	Spain	AII	Romay et al., 2014
ZYMV	EU561044	Zuy	Poland	AII	Romay et al., 2014
ZYMV	FJ705266	Ker.Ker.S	Iran	AII	Romay et al., 2014
ZYMV	GQ251520	Aligarh	India	AII	Romay et al., 2014
ZYMV	JF792442	Nt-3	Australia	AII	Romay et al., 2014
ZYMV	JN861005	E15	France	AII	Romay et al., 2014
ZYMV	JN861006	MT92-2	Martinique	AII	Romay et al., 2014
ZYMV	AB369279	RDA	Korea	AIII	Coutts et al., 2011
ZYMV	AF062518	cu	Korea	AIII	Coutts et al., 2011
ZYMV	AF486823	hainan	China	AIII	Coutts et al., 2011
ZYMV	AY278999	KR-PE	Korea	AIII	Coutts et al., 2011
ZYMV	AY611022	CH99/87	China	AIII	Coutts et al., 2011
ZYMV	AF127932	TW-TNML1	Taiwan	AIV	Coutts et al., 2011
ZYMV	AJ316228	SG	China	AIV	Coutts et al., 2011
ZYMV	AJ429071	A	South Korea	AIV	Coutts et al., 2011
ZYMV	AM422386	Begonia	Taiwan	AIV	Coutts et al., 2011
ZYMV	AY995216	New Zealand	New Zealand	AIV	Coutts et al., 2011
ZYMV	AF014811	Singapore	Singapore	BI	Coutts et al., 2011
ZYMV	DQ925447	VN/Cm3	Vietnam	BI	Coutts et al., 2011
ZYMV	L29569	Reunion	Reunion	BI	Coutts et al., 2011
ZYMV	X62662	S	Singapore	BI	Coutts et al., 2011
ZYMV	JF792363	Knx-1	Australia	BII	Coutts et al., 2011
ZYMV	JF792364	Knx-2	Australia	BII	Coutts et al., 2011
ZYMV	JF792365	Knx-3	Australia	BII	Coutts et al., 2011
ZYMV	JF792366	Knx-4	Australia	BII	Coutts et al., 2011
ZYMV	JF792367	Knx-5	Australia	BII	Coutts et al., 2011
ZYMV	AF513552	shandong	China	C	Coutts et al., 2011
ZYMV	AJ515911	WM	China	C	Coutts et al., 2011
ZYMV	AJ889243	LG1	China	C	Coutts et al., 2011
ZYMV	AY611025	BJ-03	China	C	Coutts et al., 2011
ZYMV	DQ925450	VN/Cm2	Vietnam	C	Coutts et al., 2011
ZYMV	EF178505	Zug	Poland	C	Coutts et al., 2011
BCMV[#]	EU761198	MS1	Australia	Outgroup	Coutts et al., 2011

#: Bean common mosaic virus

All sequences of the five KZN ZYMV isolates clustered within the Group A of ZYMV lineage with the sequences of isolates KZNND1 and KZNND2 clustering within the subgroup AI. The sequences of the isolates KZNNTV1, KZNNTV2 and KZNNTV3 clustered within the subgroup AII (Fig. 3).

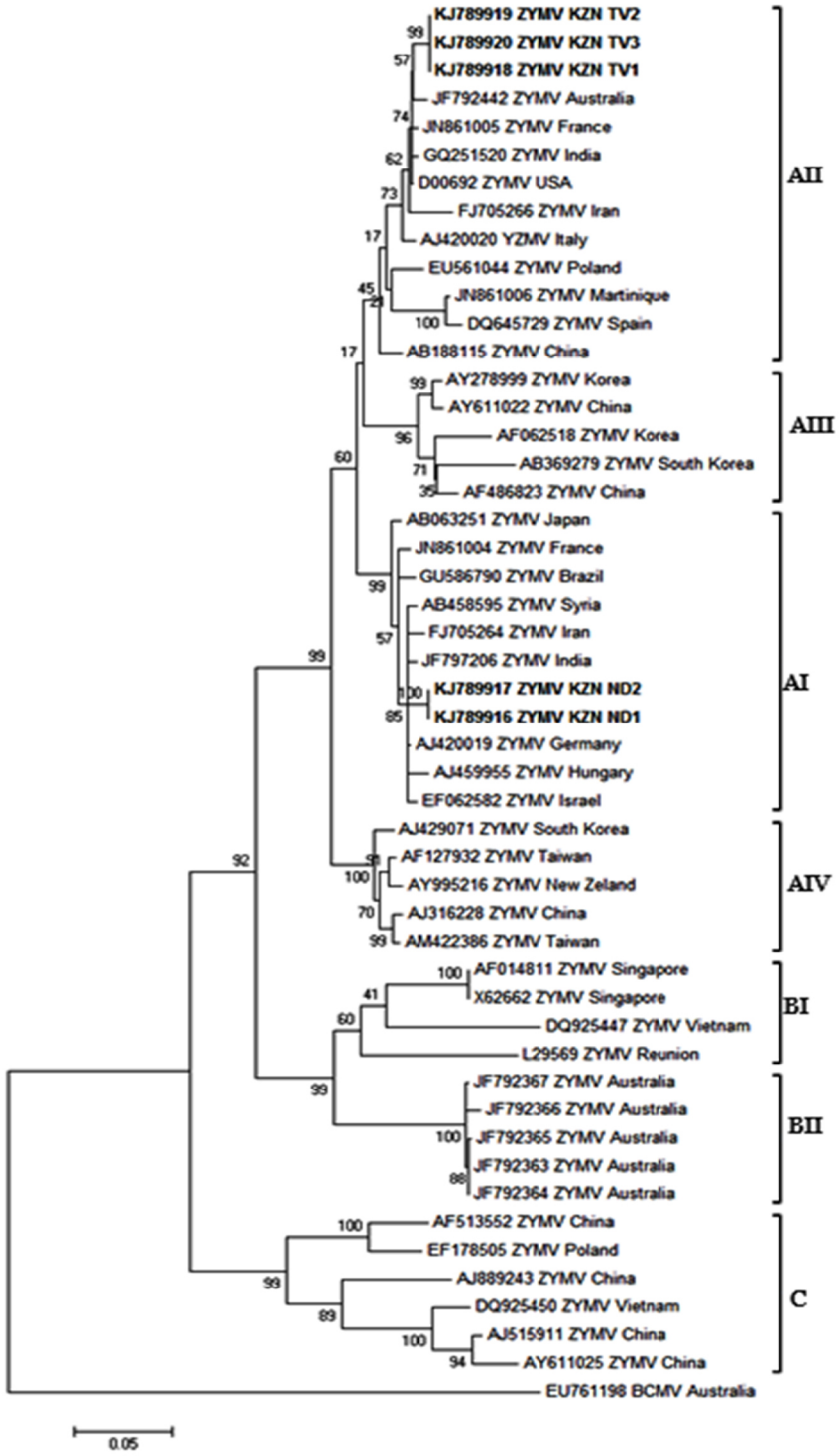

Fig. 3. Phylogenetic relationship of the entire coat sequences of ZYMV isolates from KZN.

The C-terminal portion of the polymerase coding region (Nib C-ter) and the N-terminal portion of the coat protein coding region, a variable potion of the genome frequently used in molecular studies of Potyviruses (Yakoubi et al., 2008), were selected in studying the phylogenetic relationships of the sequences of MWMV isolates due to the small number of MWMV coat protein gene sequences available in the GenBank database. The nucleotide sequences of the six MWMV isolates identified in this study were analysed in conjunction with nucleotide sequences of MWMV isolates from different geographic locations (Table 7).

Table 7. Virus sequences used in phylogenetic analyses of the sequences of MWMV isolates

Virus	Accession number	Isolate name	Origin	References
MWMV	AF307778	Sudan	Sudan	Yakoubi et al., 2008
MWMV	AF305545	Morocco	Morocco	Yakoubi et al., 2008
MWMV	EF579942	France	France	Yakoubi et al., 2008
MWMV	EF579943	Italy	Italy	Yakoubi et al., 2008
MWMV	EF579944	Spain	Spain	Yakoubi et al., 2008
MWMV	EF579945	Swaziland	Swaziland	Yakoubi et al., 2008
MWMV	EF579946	Cameroon	Cameroon	Yakoubi et al., 2008
MWMV	EF579947	Niger	Niger	Yakoubi et al., 2008
MWMV	EF579948	South Africa	South Africa	Yakoubi et al., 2008
MWMV	EF579949	TN05-73	Tunisia	Yakoubi et al., 2008
MWMV	EF579950	TN05-71	Tunisia	Yakoubi et al., 2008
MWMV	EF579955	TN05-76	Tunisia	Yakoubi et al., 2008
MWMV	EF211959	DRC	DRC	Yakoubi et al., 2008
MWMV	KF772944	Z-GR	Greece	Malandraki et al., 2014
PRSV^{+}	AY010722	strain W	Thailand	Yakoubi et al., 2008

+: Papaya ringspot virus used as an outgroup.

All MWMV isolates from KZN clustered within a unique clade in the Southern African group of the MWMV lineage (Fig. 4.).

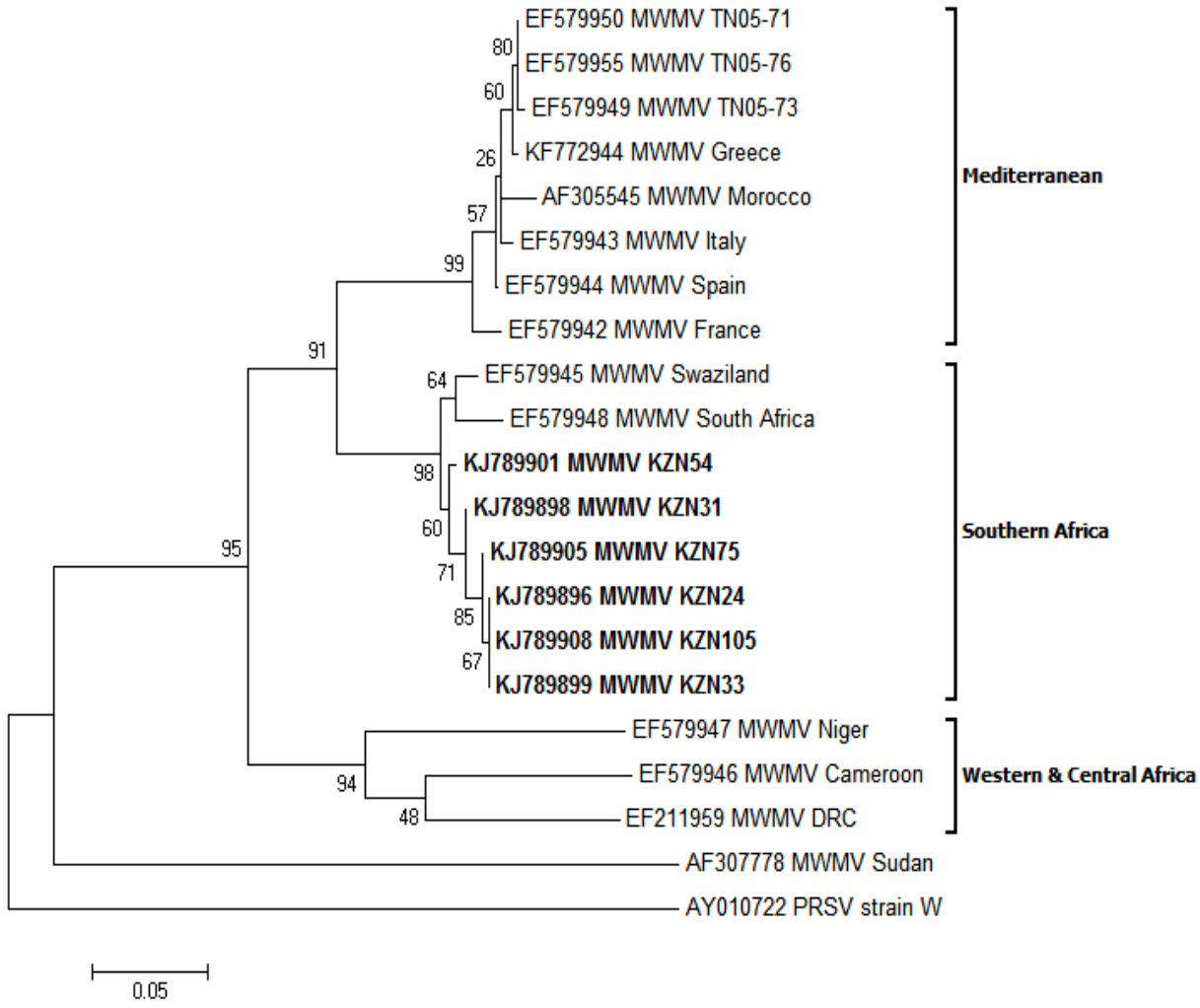

Fig. 4. Phylogenetic relationship of the Nib C-terminal and the coat protein N-terminal sequences of the MWMV isolates from KZN.

The portion of the genome sequenced from RNA1 (Heat shock protein 70 homolog; Hsp70h) and RNA2 (RNA-dependent RNA polymerase; RdRp) of the BPYV isolates were aligned with sequences of the only two available BPYV isolates for RNA1 and three BPYV isolates for RNA2 available on the GenBank database (Table 8). KZN isolates of BPYV clustered within the BPYV lineage (Fig. 5).

Table 8. Virus sequences used in phylogenetic analyses of the sequences of BPYV isolates

Virus	RNA	Accession number	Isolate	Origin	Reference
BPYV	1	AY330918	Strawberry	USA	Tzanetakis and Martin, 2004
CuYV	1	AB085613	CuYV	Japan	Hartono et al., 2003
CuYV	2	AB085612	CuYV	Japan	Hartono et al., 2003
BPYV	2	AY330919	Strawberry	USA	Tzanetakis and Martin, 2004
BPYV	2	Y15568	GreeK	Greece & USA	Livieratos et al., 1998
CTV	-	U16304	T36	USA	Karasev et al., 1994

CTV: Citrus tristeza virus used as an outgroup.

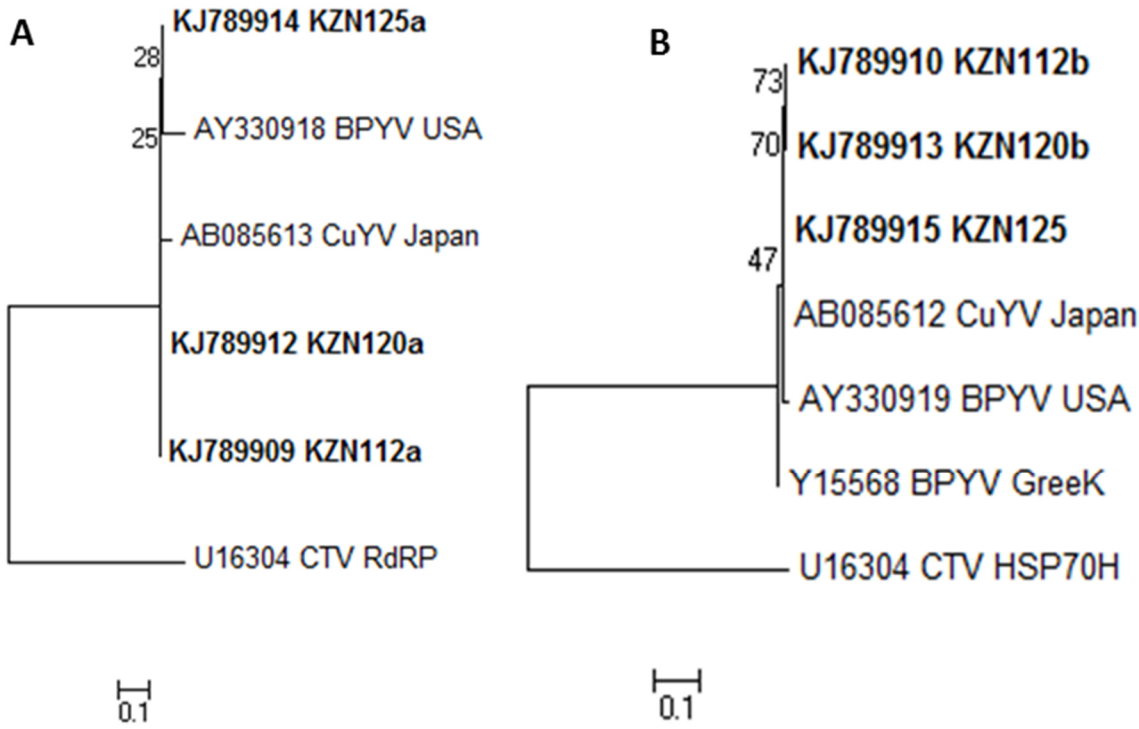

Fig. 5. Phylogenetic relationship of (A) RdRp sequences and (B) HSP70H sequences of the BPYV isolates from KZN.

The nucleotide sequences of the eight *Polerovirus* isolates identified in this study were aligned and compared with sequences from known *Polerovirus* infecting cucurbits (Table 9).

Table 9. Virus sequences used in phylogenetic analyses of the sequences of *Polerovirus* isolates

Virus	Accession number	Isolate name	Origin	References
PABYV	KF427698	ML13	Mali	Knierim et al., 2014
PABYV	KF427700	ML7	Mali	Knierim et al., 2014
PABYV	KF427699	ML3	Mali	Knierim et al., 2014
CABYV	KF791040	TH22	Thailand	Unpublished
CABYV	JQ700305	TW20	Taiwan	Knierim et al., 2013
CABYV	GQ221224	JAN	Japan	Unpublished
CABYV	EU636992	Xinjiang	China	Miras et al., 2014
CABYV	EU000535	CHN	China	Xiang et al., 2008
CABYV	X76931	N	France	Guilley et al., 1994
MABYV	JQ700307	TW1	Taiwan	Knierim et al., 2013
MABYV	KF827830	TH228	Thailand	Unpublished
MABYV	KF827829	TH242	Thailand	Unpublished
MABYV	FJ460217	HN	China	Shang et al., 2009
SABYV	JQ700308	TW19	Taiwan	Knierim et al., 2013
SABYV	HQ700825	PH11	Philippines	Knierim et al., 2014
SABYV	GU324109	TW58	Taiwan	Knierim et al., 2010
LABY	KF427701	TH24	Thailand	Knierim et al., 2014
LABYV	KF427702	TH6	Thailand	Knierim et al., 2014
LABYV	KF427703	TH8	Thailand	Knierim et al., 2014

All KZN *Polerovirus* isolates shared the highest nucleotide sequence similarity (varying between 94.05% and 94.75%) with PABYV isolates followed by SABYV isolates at 76.43 to 76.64%, MABYV isolates at 72.09 to 72.58%, CABYV isolates at 71.46 to 72.16% and lastly LABYV isolates with 58.53 to 59.09 %. Consequently, the highest amino acid sequence similarities of 90.09 to 91.5 %; 94.47 to 94.93 % and 97.46 to 98.47 % were recorded with PABYV isolates for the respective gene products MP, CP and RdRp. The amino acid sequence similarity of the respective gene products of the *Polerovirus* isolates in this study in relation to other isolates were below 80% except for the CP of SABYV, MABYV and CABYV that were 83.87 to 84.33 %, 81.1 % and 79.26 to 81.26 % respectively. All KZN *Polerovirus* isolates clustered in a separate clade within the PABYV group (Fig. 6).

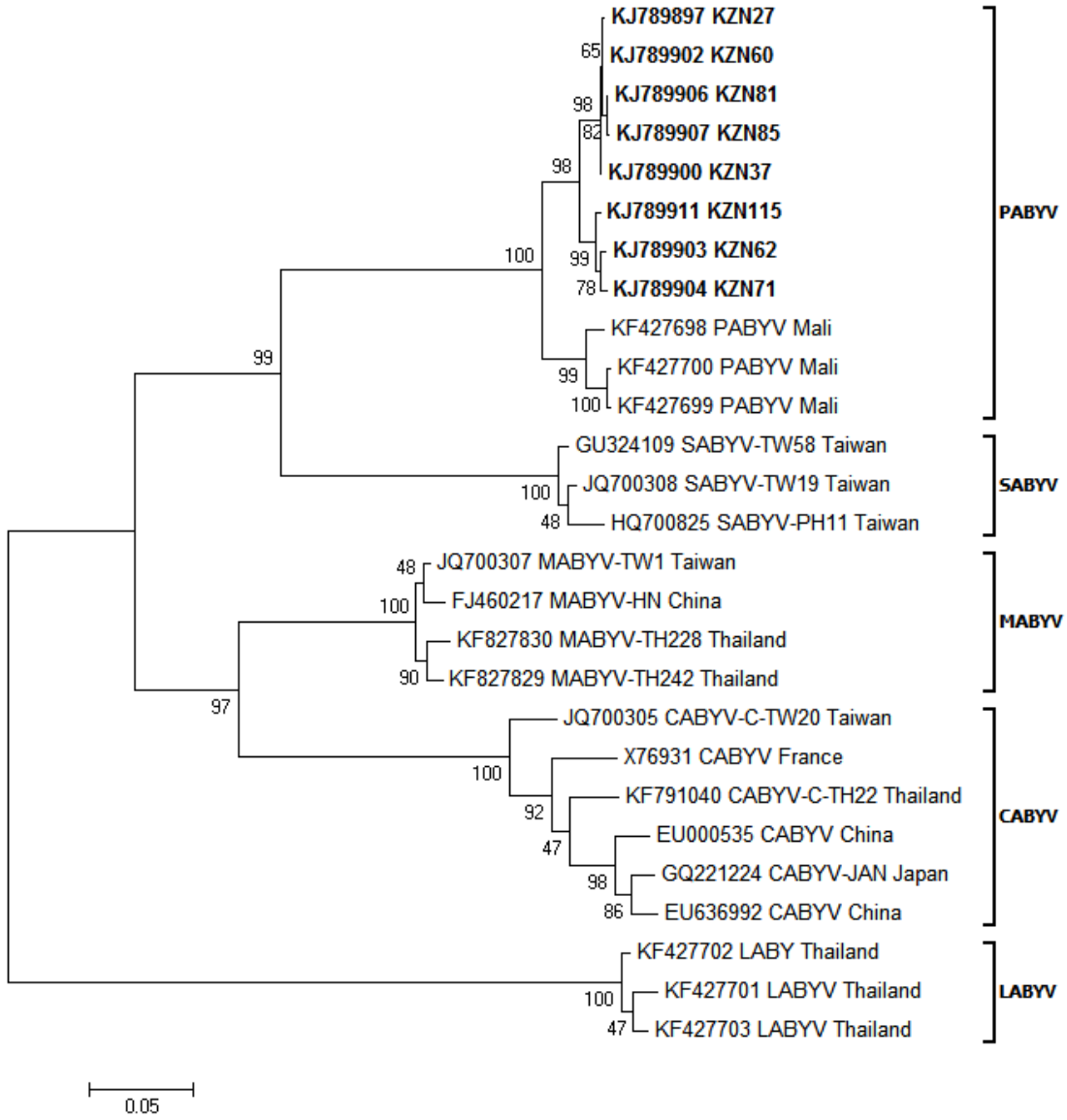

Fig. 6. Phylogenetic relationship of the sequence of the *Polerovirus* isolates from KZN.

2.4. Discussion

The survey undertaken in this study led to the identification of five viruses infecting cucurbits cultivated in KZN. These viruses which included CMV, MWMV, ZYMV, BPYV and PABYV were detected by DAS-ELISA and RT-PCR. These methods are widely used in routine testing and diagnosis of plant pathogens. Interestingly, four samples displaying mild mosaic symptoms reacted positively with antibodies specific to SqMV and WMV in ELISA tests. However all attempts to amplify the coat protein genes of these viruses with primers available in the literature (Chen et al., 2001; Desbiez et al., 2009; Ling et al., 2011; Meng et al., 2007) were unsuccessful. Subsequently, these samples were eliminated from the study on the basis of ELISA false positive results.

The increasing number of reported viruses infecting cucurbits in the world warranted an updated status of these viruses in KZN. Previous studies of this nature were reported by Cradock et al. (2001) who screened for viruses causing mosaic diseases by means of serological assays. In this study, serological and molecular based techniques were used to identify viruses causing mosaic and yellowing symptoms on cucurbits. Cradock et al. (2001) identified WMV in the previous survey, however, the virus was not identified during this study. Moreover, SqMV was not detected in both surveys. CMV, MWMV and ZYMV remained the mosaic viruses of cucurbits identified from both surveys. Results obtained during this survey showed that MWMV was the most prevalent virus infecting cucurbits in KZN contrary to previous reports by Cradock et al. (2001) in which ZYMV was the most prevalent. MWMV was detected in a variety of hosts including baby marrow, pattypan and pumpkin compared to ZYMV which was only detected in butternut.

Virus, vector, and host are the components that form the tripartite pathosystem of which each component interacts with the environment (Jones, 2014). All viruses identified in this study are vector transmitted. Unlike BPYV that is transmitted only by the greenhouse whitefly *T. vaporariorum* (Wisler et al., 1998), CMV; MWMV; ZYMV and PABYV are transmitted with varying efficiency by numerous species in various genera in the family *Aphididae* (Castle et al., 1992; Garzo et al., 2004; Lecoq and Desbiez, 2012). There is currently no information on the aphid species occurring in KZN and their efficiency in transmitting the viruses identified in this study. On the other hand, the increase in the number of cucurbit seed firms over the last decade has provided farmers with a comprehensive selection of hybrids and cultivars. Commercial growers actively pursue the use of tolerant varieties in order to mitigate virus damages on crops. Subsistence farmer selection of cultivars and varieties are primarily based on their affordability. The variation observed in the frequencies of virus incidence in KZN in regards to the factors involved in virus epidemics can only be speculative and could have been the result of variability in host and vector populations.

Phylogenetic analysis is of crucial importance in the molecular studies of plant viruses. The coat protein sequences of the CMV isolates detected in this study clustered within the Subgroup IA and are therefore different from the Wemmershoek isolate of CMV detected earlier in South Africa which belongs to the Subgroup II (Bashir et al., 2006). The coat protein sequence of the ZYMV isolates used in this study clustered within Group A of the three major groups that comprise the ZYMV lineage. The group A lineage of ZYMV, also referred to as the worldwide isolates, is further divided into four Subgroups; AI, AII, AIII and AIV (Coutts et al., 2011). Based on the coat protein sequences, the ZYMV isolates clustered within Subgroups AI and AII which consists of isolates from Asia, Europe, Oceania and North

America. Using Nib C-ter and the N-terminal portion of the coat protein coding region of the genome for analysis, the six MWMV isolates clustered within the Southern African lineage of MWMV which is consistent with previous studies conducted by Yakoubi et al. (2008) which supports the limited long distance dispersal hypothesis of MWMV.

Yellowing symptoms of cucurbits in KZN are the result of infection by the *Crinivirus* BPYV and a *Polerovirus* species. In this survey, BPYV was more frequently identified in samples from tunnels than in open fields. Phylogenetic analysis performed using all the available BPYV sequences in the GenBank database confirmed the identity of the sequences as BPYV.

Prior to the development of degenerate primers that target the species of the genus *Polerovirus*, CABYV was the main *Polerovirus* reported to infect cucurbits globally. The amplicon of the expected size obtained with the *Polerovirus* degenerate primers pointed to the presence of a *Polerovirus* different from CABYV as no amplification was obtained with the CABYV specific primers. The assumption was confirmed following the analysis of the respective sequence data. The sequences of these isolates shared 72 % similarity with CABYV sequences used in this study. The species criterion in the family *Luteoviridae* according to the International Committee on Taxonomy of Viruses (ICTV) is a difference in amino acid sequences of any gene product greater than 10 % (d'Arcy and Domier; 2012). PABYV was the only species among *Polerovirus* infecting cucurbits to share amino acid sequence similarity greater than 90 % for the different gene products analysed in this study. Therefore, the *Polerovirus* detected in this study should be considered as isolates of PABYV. This conclusion was supported by the phylogenetic results in which all KZN *Polerovirus* isolates clustered within the PABYV group. PABYV was the provisional name given to recent isolates of *Polerovirus* infecting cucurbit detected in Mali in West Africa (Knierim et al., 2014). Although no complete genome sequence of PABYV is currently available in the GenBank database, the high prevalence and broad host infectivity in cucurbits by PABYV recorded in this study may be an indication of its widespread distribution in the African continent.

The frequent identification of various cucurbit-infecting viruses across KZN is an indication of the lack of resistance in the locally cultivated varieties. This threat to cucurbit production requires the development of better virus control strategies. MWMV and ZYMV which were prevalent viruses of cucurbits detected in this study and are known to cause up to 100 % yield and up to 95 % fruit marketability losses when infections occur early in the season (Lecoq and Desbiez, 2012). These losses are mostly incurred by subsistence farmers who do not possess the knowledge or resources to effectively manage the diseases caused by these

viruses. The use of virus resistant cultivars can be effectively used in combination with existing methods to reduce the impact of these diseases (Gal-On, 2007). A study by Colvin et al. (2012), emphasizes the enormous socio-economic benefits that arise for resource-poor farmers from growing virus resistant varieties.

2.5. Conclusion

The survey undertaken in this study showed that the status of virus infecting cucurbits in KZN is different from what it was in 1998. The major changes were the absence of WMV and the detection of BPYV and PABYV. The use of resistant cultivars remains the best control for virus diseases on cucurbit (Gal-On, 2007). These resistant cultivars are produced either through breeding or genetic modification, also referred as pathogen-derived resistance. Virus resistance mediated by transgene-induced silencing in plants is highly specific, environmentally friendly and capable of targeting multiple virus pathogens (Zhou, 2012). Moreover, durable resistance has been recorded with transgenic cucurbits against several viruses in both *in vitro* and in the fields (Dias and Ortiz, 2013; Lecoq and Desbiez, 2012). The information provided in this study are intended to be used towards developing resistant cultivars using genetic engineering.

2.6. Acknowledgments

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Chapter 3

Genome Sequence Analysis of Two South African Isolates of *Moroccan Watermelon Mosaic Virus* Infecting Cucurbits[2]

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Abstract

Moroccan watermelon mosaic virus (MWMV) has been prevalent in cucurbits in the Republic of South Africa (RSA) since it was first reported in 1987. However, no genome studies of South African strains of the virus have been conducted previously. The full genomes of two isolates of MWMV infecting cucurbits (*Cucurbita pepo* L.) found in the province of KwaZulu-Natal, RSA, were analysed and compared with the genome of the Tunisian MWMV isolate available on the GenBank database. MWMV genomes of the isolates from RSA were elucidated using both next generation sequencing (NGS) and Sanger sequencing. NGS was run on the Illumina platform. Sanger sequencing was performed on amplicons generated by RT-PCR. Sequence analyses, performed on MWMV genomes included nucleotide and amino acids (aa) sequence homology, determination of genetic distances and selection pressure, detection of potential recombination, and phylogeny. The MWMV genome of the isolates from RSA is 9719 nucleotides long, excluding the poly(A) tail. Sequence homology, genetic distances and phylogenetic analyses indicated close relationships between the isolates from RSA. The information generated in this study may be useful in developing control strategies against further spread of MWMV.

Keywords: Potyvirus, Phylogenomics, Plant viruses, Next generation sequencing, Purifying selection

[2] Chapter 3 was submitted to Virus Gene and is currently under review.

3.1. Introduction

Some cucurbits are important food and cash crop in the Republic of South Africa (RSA). They are cultivated throughout the country by both subsistence and commercial farmers. However, virus diseases severely impede their cultivation [1, 2]. Viruses, especially from the genus *Potyvirus* in the family *Potyviridae*, can cause severe damage to cucurbits, with losses up to 100% when infection occurs early in the season [3].

Potyvirus virions are non-enveloped, flexuous filaments of a modal length varying between 650-900 nm, and a diameter of 11-15 nm with a helical pitch of about 3.4 nm [4]. Potyviruses have a single, positive RNA strand of about 10 kb. The 5' terminus of the potyvirus genome is covalently linked to a viral protein (VPg). A polyadenyl tail, made of 20 to 160 adenosines, is present at its 3' terminus. Two ORFs, a long and a very short one, have been identified on the potyvirus genome. The polyprotein, encoded from the longest ORF, is made up of ten multifunctional proteins: P1 proteinase; helper component proteinase (HC-Pro); P3; 6k1; cylindrical inclusions (CI); 6k2; VPg; Nia proteinase (NIa); nuclear inclusion b (NIb); and the coat protein (CP). These proteins are cleaved from the polyprotein by three viral-encoded proteinases [4]. The shorter ORF encodes PIPO, a protein expressed as a P3-PIPO fusion protein via ribosomal frame shifting [5].

Moroccan watermelon mosaic virus (MWMV), a potyvirus infecting cucurbits, was reported for the first time in Morocco in 1974, as a strain of *Watermelon mosaic virus* (WMV) on the basis of its host range [6]. It was later found to be a distinct potyvirus based on the relationship of its CP to related potyviruses [7]. At a molecular level, MWMV is part of the *Papaya ringspot virus* (PRSV) cluster [6]. MWMV was reported in cultivated cucurbits in RSA in 1987 [2]. Symptoms associated with MWMV on cucurbits include mosaic, stunting, dark-green blisters and vein banding, and leaf and fruit malformation [2, 6]. MWMV has been identified as the most prevalent potyvirus infecting cucurbits grown in RSA in various surveys [1, 2, 8]. Other countries where MWMV has been reported include Zimbabwe [6], Tanzania [9], DRC [10], Cameroon [6], Nigeria [11], Niger [6], Sudan [12], Canary Island [6], France [13], Greece [14], Italy [15], Portugal [6], and Spain [6].

Knowledge of the genetic variability and diversity of plant-infecting viruses is important for understanding their evolution [16], as well as developing efficient and sustainable control strategies [17]. Most molecular studies on the MWMV genome have been performed around the genome portion flanking the coding sequences of the C-terminal part of NIb to the N-terminal part of the CP [10-14]. The genome sequence of a Tunisian isolate of MWMV, accession number: EF579955, [6] is the only full genome sequence of MWMV available on the

GenBank database. The aim of this study was to compare the full genome sequence of two isolates of MWMV infecting cucurbits cultivated in the province of KwaZulu-Natal (KZN), RSA, with the isolate from Tunisia.

3.2. Materials and methods

3.2.1. Virus isolates

Baby marrow, (*Cucurbita pepo* L.), and patty pan (*C. pepo* L.) were identified as highly susceptible to MWMV during the virus surveys that were conducted in the cucurbit growing areas of KZN in the 2011, 2012 and 2013 growing seasons [1]. MWMV was detected in leaf samples by performing RT-PCR using primers specific to the CP coding sequence and the 3' non-coding sequence [1]. Consequently, two MWMV infected leaf samples, from a patty pan and a baby marrow, were randomly selected for next generation sequencing (NGS) to recover the full genome of MWMV.

3.2.2. Determination of the genome sequences of two South African isolates of MWMV

Total plant RNA was the template used for NGS. An extraction kit (Nucleo spin RNA plant kit, Macherey-Nagel, Germany) was used to extract total RNA from the selected samples. Sample preparation (treatment with Ribo-Zero Plant and libraries preparation) and NGS were performed at the Agricultural Research Council's Biotechnology Platform (ARC-BTP) in Pretoria (RSA). NGS was run on the Illumina Hiseq using paired-end chemistry 125x125bp reads.

NGS generated reads were assessed for quality using FastQC [18]. Trimmomatic (version 0.33) [19] was used to adjust the quality of the reads using the Pair end settings: {ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:9:1:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36}. *De novo* assembly was subsequently performed to assemble reads into contigs. This was achieved using SeqMan NGen (software version 12.3.1 build 48; DNASTAR Lasergene) with the default setting that includes the removal of contigs made of less than 100 reads. Contigs shorter than 1000 bp were also removed from the final results. Data from the host genome was excluded, during *de novo* assembly, by being treated as contaminants. Host genome data consisted of the complete genome of the *Cucurbita pepo* mitochondrion (NC_014050.1) and the Zucchini genome draft v3.2 (https://cucurbigene.upv.es/genome-v3.2/). Contigs, generated from *de novo* assembly, were blasted to the GenBank database using the Netsearch function on the SeqMan pro (software version 12.3.1 build 48 421; DNASTAR Lasergene). All contigs that matched MWMV were selected and aligned with the Tunisian MWMV isolate, using the Muscle program,

implemented on MEGA (software version 6 6.06 build 6140226), to generate the MWMV draft genome of the South African (SA) isolates.

Sanger sequencing was used to resolve any ambiguity observed on the MWMV draft genome of the SA isolates. Primers, flanking the portion of the genome showing ambiguity, were designed using Primer3 [20]. From the portion of genome targeted, amplicons were produced by RT-PCR, using total plant RNA as template for RT, and the generated cDNA as the PCR template. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and the respective reverse complement primers were used to perform RT. Phire Hot Start DNA polymerase (Thermo Scientific, USA) was used to do PCR. Both RevertAid First Strand cDNA Synthesis Kit and Phire Hot Start DNA Polymerase were used, as per the manufacturers' instructions. Direct Sanger sequencing of the amplicons was done at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA). The molecular weight of the proteins of the SA isolates of MWMV was estimated using the online tool Molecular Weight (http://www.bioinformatics.org/sms/prot_mw.html).

3.2.3. Sequence analyses and phylogeny of South African isolates of MWMV

The SA isolates of MWMV were compared to each other and to the Tunisian isolate. The online tool SIAS (http://imed.med.ucm.es/Tools/sias.html) was used to evaluate the nucleotide and amino acids (aa) homologies. Genetic variability was assessed by determining the genetic distance between MWMV isolates. This was done in MEGA6 [21] using the Maximum Composite Likelihood model [22]. The rate variation among sites was modelled with a gamma distribution, and all positions containing gaps and missing data were eliminated. The test statistic dN - dS was used on the coding sequences of the protein genes of all MWMV isolates for detecting codons that had undergone positive selection. This was also performed on MEGA, using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model [23] of codon substitution and General Time Reversible model [24] of nucleotide substitution. Maximum Likelihood computations of dN and dS were conducted using HyPhy software package [25]. Selected potyviruses that form the PRSV cluster were included in the test for determination of putative recombination breakpoints and phylogeny. The program RDP version 4.56 [26] was used to detect any putative recombination breakpoints on the MWMV genomes of all isolates, using the default settings except for the sequence type setting that was set to linear instead of circular. Multiple sequence alignment was performed using the program Muscle, also implemented on MEGA. The phylogeny of MWMV isolates was inferred by using the Maximum Likelihood method based on the General Time Reversible model [24] with a discrete Gamma distribution, and considering some sites to be evolutionarily invariable (+I). Bootstrap analysis was performed with 500 iterations.

3.3. Results

3.3.1. MWMV genome sequences of SA isolates

The longest MWMV identified contig in the patty pan sample was 9730 bp long. MWMV mapped reads from the patty pan sample were 3,453,027 (Median Coverage: 42054.34), and had an average length of 114 bp (Table 1). Regarding the MWMV mapped reads from the baby marrow sample, the longest contig identified was 9735 bp long, made up of 3,425,094 reads (Median Coverage: 37626.61) with a mean length of 113 bp . A gap from Position 6 to 11 on the genome of the Tunisian isolate was the only ambiguity spotted when aligning the MWMV isolates from RSA with the isolate from Tunisia. Direct sequencing of the RT-PCR amplicon obtained using the primers MWMVF1: 5'- AAA CAC TCA ACA CAA CAC AAC ATC -3', and MWMVR530: 5'- CCC TGT CTT GCT TCA GCT AAA TTC -3' produced the same nucleotide sequence as in the contigs. The 9.7 kb contigs from the baby marrow and patty pan samples were therefore considered as the consensus sequences of the full genome of the SA isolates of MWMV. In that regard, SA MWMV isolates have a genome made of 9719 nucleotides (nt), excluding the poly(A) tail, and consists of 150 non-coding nucleotides at its 5' terminus followed by 9375 nucleotides coding for the polyprotein, and 194 non-coding nucleotides at 3'terminus (Table 2). PIPO ORF starts at Genome Position 3018 and ends at Genome Position 3200. The two SA isolates had the same polyprotein cleaving sites as the Tunisian isolate. The complete genome sequences of the two SA isolates of MWMV were deposited in the GenBank database. They were given the accession number KU315175 and KU315176.

Table 1. NGS statistics

Sample	Pattypan	Baby marrow
Data size (compressed)	2.6 Gb	2.5 Gb
Number of raw reads	33,524,534	33,060,753
Average length	125 bp	125 bp
Number of reads after trimming and adapter removal	32,138,032	31,440,584
Average length	114 bp	113 bp
MWMV mapped reads	3,453,027	3,425,094
Median Coverage	42054.34	37626.61

Table 2. Genome organization of SA isolates of MWMV

Genome position (nt)	Genome feature	Protein MW (kDa)	
		Baby marrow isolate	Patty pan isolate
1 – 150	5' non-coding region	--	--
151 – 9525	Polyprotein ORF	354.06	354.07
9526 – 9719	3' non-coding region	--	--
151 – 1185	P1	38.69	38.68
1186 – 2556	HC-Pro	51.63	51.65
2557 – 3597	P3	39.71	39.75
3598 – 3753	6K1	6.05	6.05
3754 – 5658	CI	71.06	71.1
5659 – 5829	6K2	6.61	6.61
5830 – 6399	VPg	21.53	21.57
6400 – 7116	NIa	26.92	26.92
7117 – 8667	NIb	59.50	59.47
8668 – 9525	CP	32.52	32.42
3018 - 3200	PIPO	6.82	6.82

--: Not applicable.

3.3.2. Sequence analyses and phylogeny of SA isolates of MWMV

The nucleotide sequences of the genomes of the SA isolates of MWMV were almost identical to each other (Table 3). A 100% nucleotide sequence identity was observed between SA isolates for a number of genome features, including 5' and 3' non-coding regions (NCR), 6K1 and PIPO. The lowest nucleotide sequence identity, *i.e.* 98.93%, was recorded for P1. The nucleotide sequence identity of SA isolates of MWMV, compared with the homologous Tunisian isolate, varied between 80 and 95% (Table 3), with the highest nucleotide sequence identity recorded with PIPO, followed by the 3' NCR. The 5'non-coding region had the lowest nucleotide sequence identity for all MWMV isolates. In terms of aa sequence identity (Table 3), 100% sequence identity was observed between SA isolates of MWMV for 6K1, 6K2, Nia and PIPO. Vpg and P1 aa sequence identities were the lowest at around 98%. The sequence identity of aa between SA and the Tunisian isolates of MWMV varied between 82 and 99%.

Table 3. Percentage nucleotide and protein sequence identity between MWMV isolates

Genome features	Between SA isolates		Between baby marrow and Tunisian isolates		Between patty pan and Tunisian isolates	
Sequence type	nt	aa	nt	aa	nt	aa
5' NCR	100	--	80.66	--	80.66	--
P1	98.93	98.26	83.47	81. 73	83.28	82.02
HC-Pro	99.48	99.78	90.59	98.68	90.73	98.90
P3	99.61	99.42	91.54	96.25	91.35	96.25
6K1	100	100	87.82	98.07	87.82	98.07
CI	99.52	99.68	91.02	98.89	91.23	99.21
6K2	99.41	100	88.30	94.73	88.30	94.73
VPg	99.12	98.42	87.36	97.89	87.19	96.31
NIa	99.72	100	85.91	95.81	85.77	95.81
NIb	99.67	99.8	89.29	97.67	89.23	97.48
CP	99.64	99.64	90.05	94.73	90.40	95.08
PIPO	100	100	95.62	91.80	95.62	91.80
3'NCR	100	--	94.05	--	94.05	--

nt: Nucleotide, aa: amino acids, --: not applicable.

Similarly, the genetic distances between SA isolates were much lower than the distances between the SA and Tunisian isolates (Table 4). The longest distance between SA isolates was observed with P1. The genetic distance between SA isolates for PIPO and 6K1 was zero. Regarding distances between the SA and Tunisian isolates, they were as follows, in ascending order: PIPO, P3, CI, Nia, NIb, 6K1, VPg, HC-Pro, 6K2, P1, and CP.

Table 4. Nucleotide distances of the MWMV isolates

Genome features	Between SA isolates		Between baby marrow and Tunisian isolates		Between patty pan and Tunisian isolates	
	Distance	SE[#]	Distance	SE	Distance	SE
P1	0.0108	*0.0033*	0.2319	*0.1245*	0.2346	*0.1272*
HC-Pro	0.0053	*0.0021*	0.1886	*0.0399*	0.1842	*0.0405*
P3	0.0039	*0.0019*	0.0925	*0.0206*	0.0947	*0.0203*
6K1	0.0000	*0.0000*	0.1373	*0.0335*	0.1373	*0.0335*
CI	0.0048	*0.0018*	0.1000	*0.0214*	0.0973	*0.0206*
6K2	0.0059	*0.0055*	0.2135	*0.0680*	0.2079	*0.0652*
VPg	0.0089	*0.0043*	0.1455	*0.0356*	0.1480	*0.0371*
NIa	0.0016	*0.0020*	0.1144	*0.1260*	0.1157	*0.1257*
NIb	0.0032	*0.0015*	0.1213	*0.0278*	0.1221	*0.0279*
CP	0.0037	*0.0025*	0.6684	*1.2551*	0.6295	*1.2423*
PIPO	0.0000	*0.0000*	0.0456	*0.0164*	0.0456	*0.0164*

[#]: Standard error.

Results from the statistic dN – dS (Table S1) showed that most of the nucleotide substitutions on the genome of MWMV were synonymous, thereby rejecting the hypothesis of positive selection pressure. No recombination event was identified on the genome of all MWMV isolates. The phylogram of the MWMV isolates, from phylogenetic analyses, grouped the three MWMV isolates together but in different clusters (Figure 1).

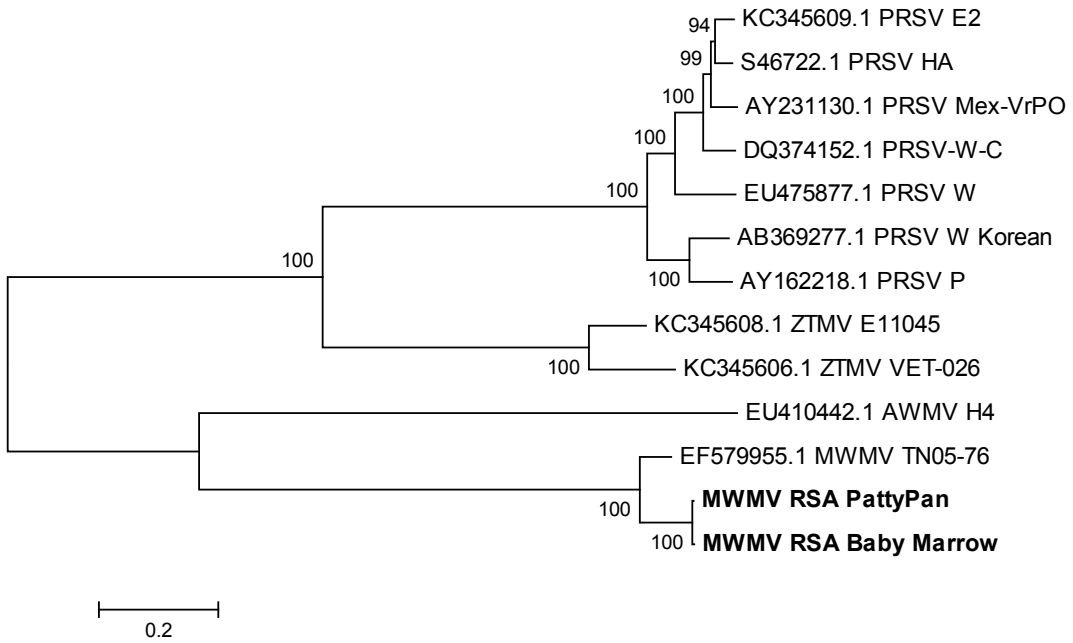

Figure 1. Phylogram of the MWMV isolates in the PRSV cluster. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. ZTMV: *Zucchini tigré mosaic virus* [26], AWMV: *Algerian watermelon mosaic virus* [27].

3.4. Discussion

The full genome sequences of two SA isolates of MWMV infecting cucurbits were ascertained in this study. The plant virus genome contains the genetic information of all the processes involved in the virus life cycle. In this regard, having knowledge of the full genome sequence of plant-infecting viruses may provide a deeper understanding of plant-virus and vector interactions. MWMV has been a prevalent and damaging virus of cucurbits in RSA. It was, therefore, important to have the full-length genome sequences of SA isolates of MWMV.

NGS was the primary tool used to sequence the genome of the MWMV genome of SA isolates. NGS is becoming a powerful tool in plant virology, which is now readily available to sequence whole plant virus genomes in a relatively short period of time [29]. The genomes of several plant viruses of several families have been sequenced using NGS [29-30]. The inconvenience of producing a high proportion of non-virus derived reads, associated with using total RNA as template [30-33], was not a serious issue in this study. MWMV derived reads accounted for 10.36 and 10.30% of the total reads generated from the baby marrow and patty pan samples, respectively, and were enough to generate the full genome sequence of each isolate.

De novo assembly of the reads was selected over the other option of mapping reads to a reference genome as a precautionary measure in case the MWMV genomes of SA isolates had undergone recombination, or had diverged considerably from the genome sequence of the Tunisian isolate. The 9.7 kb contigs generated from *de novo* assembly were subsequently considered as the accurate consensus genome sequences of the SA isolates of MWMV. Another factor that influenced that conclusion is that the contigs selected as the full genome sequence of the baby marrow isolate matched at 100 % the partial sequence (9.0 kb) of the MWMV genome of a baby marrow isolate that had been obtained earlier by Sanger sequencing (data not show).

The SA isolates are variants of MWMV, based on nucleotide and aa sequence identity. The percentage of nucleotides and aa sequence identity for each protein, *e.g.* HC-Pro, 6K1, CI and VPg (Table 3), between the SA and Tunisian isolates was an indication of synonymous substitutions. The statistic dN – dS performed on each codon of the polyprotein confirmed that hypothesis. It was therefore concluded that the SA isolates underwent purifying selection. RNA viruses are known to have high mutation rates, due to their error-prone replication, as a consequence of the absence of proofreading activity in RNA-dependent RNA polymerases [16]. However, purifying selection has been identified in virus population studies of a number of genera [16, 17, 34], and it has become apparent that the high mutation rates of RNA viruses are due to the need for rapid replication of their unstable RNA rather than being an evolution strategy [17, 35].

SA MWMV isolates were expected to be less diverse from each other than the Tunisian isolate. The genetic distances among MWMV isolates confirmed this hypothesis (Table 3). The highlight of the test of the genetic diversity was the high level of divergence of the CP between the SA and Tunisian isolates. This diversity had already been reported in the studies conducted by Yakoubi et al. [6] and Ibaba et al. [1]. Genetic diversity between SA and the Tunisian isolates were also reflected on the phylogram (Figure 1), with strong bootstrap support, because the three MWMV isolates were grouped together but in different clusters.

3.5. Conclusion

MWMV was first reported in RSA in 1987. Its host range appears to be limited to cucurbits. A number of CP coding sequences of MWMV from RSA have been studied and are available on the GenBank database. This study reports for the first time the full genome sequences of two isolates of MWMV from RSA. Although the analyses performed in this study do not explain the origin of MWMV in RSA, the information generated may be useful in developing control strategies against further spreading of MWMV.

3.6. Supplementary materials

Table S1: Test of the positive selection pressure on the codon of the MWMV polyprotein is attached in the CD at the back of this thesis.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 4

Pepo aphid-borne yellows virus: A Distinct Polerovirus, Based on Virus Morphology and Genome Organization[3]

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Abstract

Pepo aphid-borne yellows virus (PABYV) has been proposed as a putative member of the genus *Polerovirus* in the family *Luteoviridae*. The aim of this study was to characterize PABYV based on its virion morphology and genome organization. Virus purification, performed on selected PABYV infected cucurbit leaf samples, yielded polyhedral particles of about 25 nm upon visualisation using transmission electron microscopy. Total RNA, extracted from PABYV infected baby marrow and patty pan leaf samples, was subjected to next generation sequencing (NGS) on the Illumina platform. Sanger sequencing was subsequently used to authenticate the integrity of PABYV's genome generated from *de novo* assembly of NGS data. PABYV's genome was shown to consist of 5813 nucleotides and to display a genome organization typical of poleroviruses. A comparison of sequence homology strongly supported the classification of PABYV as a distinct species in the genus *Polerovirus*. Our results showed that PABYV's genome may have evolved from a recombination event between *Cucurbit aphid-borne yellows virus* as the likely minor parent and an unidentified major parent. This hypothetical recombination event, detected using the program RDP, was reflected on the phylograms from the phylogenetic analyses performed. The confirmation of PABYV as a distinct species in the genus *Polerovirus* has increased the number of polerovirus infecting cucurbits to five.

Keywords: Cucurbit-infecting virus, South Africa, RNA viruses, Emerging viruses, Phylogenomics, Next generation sequencing

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4.1. Introduction

The genus *Polerovirus*, one of the three that compose the family *Luteoviridae* (d'Arcy & Domier, 2012), is an important genus of emerging plant-infecting viruses (Distéfano *et al*., 2010). Polerovirus virions are non-enveloped particles of about 25 nm in diameter and display an icosahedral symmetry. The polerovirus genome consists of a single strand of positive RNA that is covalently linked to a viral protein genome (Vpg) at its 5'end, but it has neither a poly(A) tail, nor does it display a tRNA-like structure at its 3'end. Six major open reading frames (ORFs 0 - 5) have been identified on the polerovirus genome. The ORFs 1, 2 and 3 are all located at the 5'proximal of the genome and are translated from the genomic RNA (d'Arcy & Domier, 2012). Expression of ORF1 and ORF4 occurs through a leaky scanning mechanism. ORF2 overlaps ORF1 and is translated via a -1 ribosomal frameshift (Smirnova *et al*., 2015). A slippery site, usually a heptanucleotide, and a downstream pseudoknot, are required for stimulation of efficient ribosomal frameshifting, and have been identified on the genome of poleroviruses (Giedroc & Cornish, 2009). A subgenomic mRNA, produced in infected cells, allow for the translation of the ORFs 3, 4 and 5. ORF4 is embedded within ORF3 but in a different reading frame. ORF5 is translated by in-frame readthrough of the ORF3's stop codon (d'Arcy & Domier, 2012). Smirnova *et al*. (2015) recently reported the existence of an additional short ORF (ORF3a) near the 5' end of the subgenomic mRNA. ORF3a is highly conserved among poleroviruses and its expression is initiated at a non-AUG codons (Smirnova *et al*., 2015). Each ORF encodes for a single protein product, these being P0 from ORF0, P1 or Vpg from ORF1, P1-P2 fusion protein or RNA-dependant RNA polymerase (RdRp) from ORF2, P3 or the coat protein (CP) from ORF3, P3a from ORF3a, P4 or the movement protein (MP) from ORF4, and P3-P5 fusion protein or the readthrough domain (RTD) from ORF5 (d'Arcy & Domier, 2012). Poleroviruses are known to cause yellowing disease on economically important cucurbits (Knierim *et al.,* 2010; Xiang *et al*., 2008). Transmission of poleroviruses from plant to plant occurs only through specific aphid vectors in a circulative, non-propagative manner (d'Arcy & Domier, 2012).

Next generation sequencing (NGS), also referred as high throughput or deep sequencing, describes more efficient and faster DNA sequencing platforms that perform beyond the reach of standard and traditional Sanger sequencing technologies that were developed in the late 1970s. NGS data output usually consists of millions to billions of DNA reads of variable lengths, depending on the platform used (Barba *et al*., 2014.). The use of NGS technologies combined with bioinformatics tools has been beneficial to many areas of plant virology. Furthermore, NGS has the added advantage of not requiring *a priori* knowledge of the virus, thereby providing a powerful tool for the identification and characterization of both known and unknown viruses (Barba *et al*., 2014; Massart *et al*., 2014). The approaches that have been used

in studying viruses using NGS technologies include total host nucleic acids (DNA or RNA), virion-associated nucleic acids purified from virus-like particles, double-stranded RNAs and virus-derived small interfering RNAs (Barba *et al*., 2014; Massart *et al*., 2014; Prabha *et al*., 2013; Roossinck *et al*., 2015).

Degenerate primers that hybridize to the conserved region of the polerovirus genome have been used in routine diagnosis of plant-infecting viruses, and have confirmed the diversity of species in the genus *Polerovirus* that infect cucurbits. The number of species of polerovirus infecting cucurbits recognised by the International Committee on Taxonomy of Viruses (ICTV) has consequently increased from one, that being the *Cucurbit aphid-borne yellows virus* (CABYV), to three, with the addition of the *Melon aphid-borne yellows virus* (MABYV) and *Suakwa aphid-borne yellows virus* (SABYV) (ICTV, 2015a; Knierim *et al*., 2014). Two more species, also infecting cucurbits, provisionally named Luffa aphid-borne yellows virus (LABYV) and Pepo aphid-borne yellows virus (PABYV), have been proposed as putative poleroviruses (Knierim *et al*., 2014). PABYV was reported for the first time in Mali in West Africa (Knierim *et al*., 2014). It was then detected in the Republic of South Africa (RSA) (Ibaba *et al*., 2015) and lately in Cote d'Ivoire (Kone *et al*., 2015). Little is known about PABYV beside the portion of its genome, spanning from the RdRp 3' end to the CP, which is available on GenBank (Ibaba *et al*., 2015; Knierim *et al*., 2014; Kone *et al*., 2015). Therefore, this study aimed at establishing the taxonomic status of PABYV on the basis of its particle morphology, genome organization and phylogeny.

4.2. Materials and methods

4.2.1. Sources of PABYV isolates

PABYV infected leaves were collected from both commercial and small-scale farms in the province of KwaZulu-Natal (KZN), RSA in the 2011, 2012 and 2013 growing seasons. PABYV was detected in the sampled leaves using a combination of *Polerovirus* degenerate primers and phylogenetic analyses (Ibaba *et al*., 2015).Virus isolates were extracted from PABYV infected cucurbit leaves that were initially kept in labelled plastic bags at -80°C.

4.2.2. Virus Purification and visualisation under the transmission electron microscope

The protocol described by Hammond *et al*. (1983) was adopted for the purification of PABYV virions from 100 g cucurbit-infected leaf samples with the following adaptations: liquid nitrogen was used to freeze the samples prior to grinding; and cellulase and pectinase were used at concentrations of 2% and 4% (w/w), respectively. Low and high-speed centrifugation were performed using an Avanti J-26XPI and an Optima L-100XP centrifuge, respectively. Aliquots from sucrose density gradient centrifugation that produced a peak of UV

absorbance at 260 nm were selected for TEM examination. Preparations of purified virions were negatively stained using phosphotungstic acid before visualization, using a transmission electron microscope (TEM; JEOL JEM-1400) at the UKZN Microscopy and Microanalysis Unit (Pietermaritzburg, RSA).

4.2.3. RNA isolation and NGS sequencing

Isolates from two PABYV-infected leaf samples from patty pan (*Cucurbita pepo* L.) and baby marrow (*C. pepo* L.) were selected for NGS. Total RNA was extracted from the selected samples using a Nucleo spin RNA plant kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The RNA concentration of each sample was estimated using a Nanodrop ND2000 (Thermo Scientific, USA). Thereafter, the RNA samples were shipped on dry ice to the Agricultural Research Council's Biotechnology Platform (ARC-BTP) in Pretoria (RSA) for NGS library preparation and sequencing. The samples were run on an Illumina Hiseq with paired-end chemistry 125x125bp reads.

4.2.4. NGS data analysis

The quality of NGS data generated from each sample was assessed using FastQC (Andrews, 2010). Trimmomatic (version 0.33) (Bolger *et al*., 2014.) was subsequently used on data that needed trimming, filtering and removal of adapters using the pair end settings {ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:9:1:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36}. The pair-end sequence data henceforth was used as single reads in the removal of host sequences and *de novo* assembly. SeqMan NGen (software version 12.3.1 build 48; DNASTAR Lasergene) was run in "Transcriptome RNASeq assembly project" to remove the host data from the reads and perform *de novo* assembly. The complete genome of *Cucurbita pepo* mitochondrion (NC_014050.1) and the Zucchini genome draft v3.2 (https://cucurbigene.upv.es/genome-v3.2/) were used for the removal of the host sequences. *De novo* assembly was performed using the default settings, with the exception that the minimum contigs length was set to 500 bp. All generated contigs were identified by performing a non-redundant nucleotide BLAST on NCBI using Netsearch implemented in the SeqMan program (software version 12.3.1 build 48 421; DNASTAR Lasergene). Contigs that matched poleroviruses were selected and analysed on MEGA (software version 6.06 build 6140226) to generate the consensus genome sequence of PABYV.

4.2.5. Sanger sequencing

Sanger sequencing of cloned overlapping amplicons was performed to assess the integrity of the PABYV genome generated from NGS analysis. Six primer pairs (Table 1) were used to generate overlapping amplicons flanking the entire PABYV genome. Amplicons were

produced through a two-step RT-PCR. Total RNA was isolated from PABYV infected leaf samples using a Nucleo spin RNA plant kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. cDNA synthesis was carried out using the respective sequence-specific complementary primers and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. PCR was performed in 20 µl reactions containing 1x reaction buffer, 200 µM dNTPs, 0.3 µM of each primer, 0.4 µl Phire Hot Start DNA Polymerase and 1 µl cDNA. PCR was run according to the cycling parameters listed in Table 1. Amplicons of the expected size, determined upon visualization of 1.5% agarose gel in TAE buffer, were cloned into the pJET1.2/blunt vector (Thermo Scientific, USA). Amplicons were cleaned using DNA Clean & Concentrator™-5 (Zymo Research, USA) prior to ligation to pJET1.2/blunt vector. 5 µl of the ligation mixture was used to transform competent *Escherichia coli* strain Zymo 5α (Zymo Research, USA). Transformed *E. coli* cells were incubated on LB agar plates containing 100 $\mu g.mL^{-1}$ ampicillin. Six overnight colonies were transferred into LB broth supplemented with the same selecting antibiotic. Plasmid extraction, using the QIAprep Spin Miniprep kit (QIAGEN, Germany), was performed 12 hrs post incubation on broth. True recombinant clones were checked by PCR using the pJET1.2 sequencing primers. Two clones of each amplicon were sequenced in both directions, using the pJET1.2 primers, at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA).

4.2.6. Determination of PABYV genome organization

The ORFs on the PABYV genome sequence were elucidated using ORF Finder (Rombel *et al*., 2002; Wheeler *et al*., 2003). KnotInFrame (Janssen & Giegerich, 2015) was used to identify potential slippery nucleotide sequences and the downstream pseudoknots that are required to trigger -1 ribosomal frameshifting. Slippery nucleotide sequences and pseudoknots were searched for on the PABYV genome using the "Andronescu model, 2007". Nucleotide and protein sequence homology of the detected ORFs were assessed using the online tool SIAS (http://imed.med.ucm.es/Tools/sias.html). A protein molecular weight online tool (http://www.bioinformatics.org/sms/prot_mw.html) was used to estimate the molecular weight (MW) of the ORFs derived protein products.

4.2.7. Sequence analysis and phylogeny of PABYV

The program Muscle, implemented in MEGA (software version 6.06 build 6140226) was used to perform all multiple sequence alignments. Potential recombination on PABYV's genome was checked using the program RDP version 4.56 (Martin *et al*., 2015). Sequences checked for recombination were treated as linear in the RDP parameters. RDP default settings for the detection of recombination events were used. Only recombinant events detected by more than two methods were considered to be real. MEGA (software version 6.06 build 6140226)

was used to infer phylogenetic trees using the Maximum Likelihood method based on the best substitution model, with 500 bootstrap replicate (Tamura *et al*., 2013). The best substitution model for the sequence data used in this study was also identified using MEGA.

Table 1. Primers used to generate overlapping amplicons flanking PABYV's genome

Primer names	Sequence (5'-3')	Portion of the genome amplified	PCR cycling conditions[χ]	Amplicon sizes (bp)
Pol G F*	GAYTGCTCYGGYTTYGACTGGAG	2759-3879+	98°C - 5s 60°C - 5s 72°C - 16s	1121
Pol G R*	GATYTTATAYTCATGGTAGGCCTTGAG			
1F	GGAACTGATAGAGAGTYGAGTATTTAC	1-1396§	98°C - 5s 60°C - 5s 72°C - 20s	1396
R 1396	GGATCCGGACCCCTGAGC			
Pol637 F	TGTGGAGCTTCATTCTCTGGG	604-3145§	98°C - 5s 60°C - 5s 72°C - 30s	2542
Pol R#	TGAAATGATGCGAACAAAACTC			
F3168	CGGTCAATGTCGGAAAGATGTTA	3168-4371+	98°C - 5s 60°C - 5s 72°C - 17s	1204
R 4371	ATTCGCCTGGAGATTGATAGTGTC			
F4320	TATGTACAAGTGGGAAAATGAAAAGTGG	4320-5782+	98°C - 5s 60°C - 5s 72°C - 20s	1463
R 5782	GTCATCGGATATTGTACGCTGGTCTGTG			
F5412	GAAACGTGACGAGCGAATCTT	5412-5813+	98°C - 5s 60°C - 5s 72°C - 10s	402
3end1#	ACACGCGAGTGCACAGATCAACTCT			

χ: PCR consisted of 35 cycles; *: Primers designed by Knierim et al. (5), Primer select software (DNASTAR, Lasergene) was used to designed the rest of the primers; #: primer used for cDNA synthesis; +: cDNA generated using 3end1primer was used as template in PCR; §: cDNA generated using Pol R primer was used as template in PCR.

4.3. Results

4.3.1. Virus Purification and visualization under the transmission electronic microscope

Polyhedral particles measuring about 25 nm (Fig. 1) were observed upon examination of the purified virions under TEM.

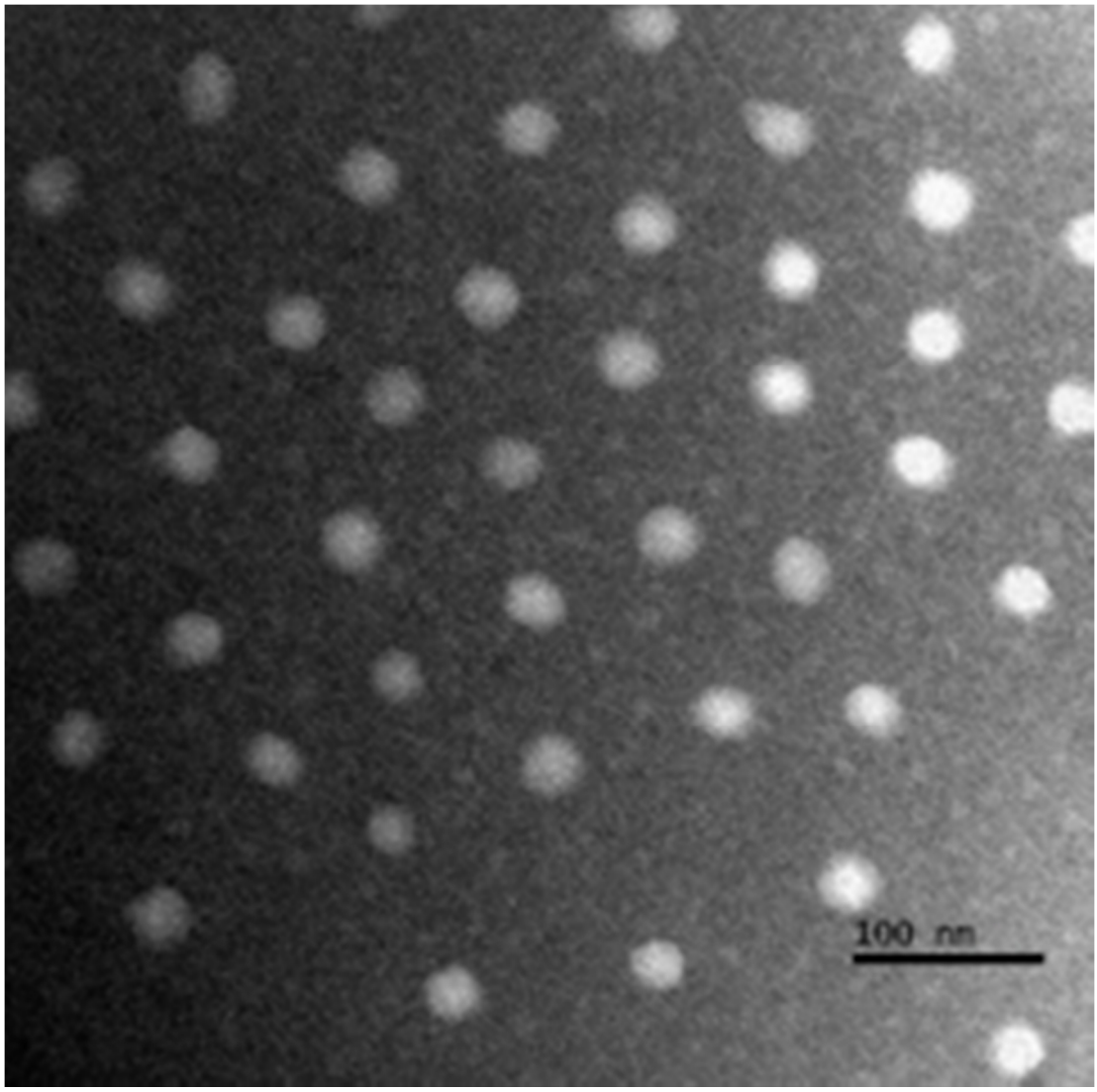

Fig 1. Electron micrograph of purified virions from PABYV infected cucurbit leaf samples

4.3.2. NGS data analysis

A total of 33.05 and 14.48 million raw reads were generated from NGS of the baby marrow and patty pan samples, respectively (Table 2). *De novo* assembly of the trimmed reads from the baby marrow sample yielded 541 contigs, of which 5 were identified as polerovirus. Four contigs out of the 260 contigs produced from *de novo* assembly of the trimmed reads from the patty pan sample matched poleroviruses. A contig of 5813 nucleotides (nt) among the identified polerovirus contigs was observed in both samples. The rest of the identified polerovirus contigs had sizes ranging between 1000 and 1500 nt. A deeper analysis of these

short polerovirus contigs on MEGA revealed that they were all complementary to the 5813 nt contig sequence. The 5813 nt contig sequence, which matched at a 99% sequence identity with the portion of the genome sequence of PABYV deposited on the GenBank (accession number: KJ789903, KJ789904 and KJ789911) with an E-value of zero, was therefore considered as the complete genome sequence of PABYV.

Table 2. PABYV read-mapping from *de novo* assembly

Samples	Raw reads	Reads after filtration	PABYV reads-mapped
Baby marrow	33,055,678	31,440,584	716,455
Patty pan	14,482,628	13,786,082	826,155

4.3.3. Sanger Sequencing

Primers were designed to produce overlapping amplicons flanking the entire 5813 nt sequence of the PABYV's genome. All primer combinations produced amplicons of the expected sizes from PCR. Sanger sequencing results of all cloned amplicons matched the nucleotide sequence of PABYV that was generated from *de novo* assembly, with no major ambiguities.

4.3.4. PABYV genome organization

ORFs identified on PABYV's genome using ORF Finder matched *Polerovirus* ORFs and protein products. KnotInFrame predicted the heptanucleotide GGGAAAC at PABYV Genome Position 1649 as the slippery sequence. The corresponding pseudoknot structure is situated between Nucleotides 1656 and 1696. With that background information, PABYV's genome consists of 55 non-coding nucleotides at its 5'end followed by the 5' proximal ORFs block (Fig. 2(a)). Theses ORFs include ORF0 [genome position: 56-847], ORF1 [210-2081] and ORF2 [210-1655, 1655-3343]. P0, with a predicted MW of 22.46 kDa, is encoded from ORF0 and harbours the conserved domain of the Luteo P0 superfamily (accession number: pfam04662) that has been found to be involved in suppression of post-transcriptional gene silencing (Csorba *et al*., 2010; Pfeffer *et al*., 2002). ORF1 encodes P1 protein (68.92 kDa) that possesses the Peptidase S39 superfamily conserved domain (accession number: pfam02122). ORF2 is translated via a -1 ribosomal frameshift, thus encoding a 115.75 kDa p1-p2 fusion protein that contains the RNA-dependent RNA polymerase (RdRp) superfamily domain (accession number: pfam02123). The ORFs at the 3'proximal of PABYV's genome include ORF3 [3568-4167], ORF4 [3596-4171] and ORF5 [3568-5619]. PABYV 3'end non-coding region (NCR) consists of 194 nucleotides (Fig 2(a)). ORF3 encodes PABYV CP with a predicted MW of 22.01 kDa. P4, encoded from ORF4, is a 21 kDa putative MP (accession

number: pfam01659). The protein product from the translation of PABYV ORF5 matched the RTD of *Polerovirus* (accession number: pfam01690). The non-UAG initiated ORF3a, discovered recently in poleroviruses (Smirnova *et al*., 2015), was also identified on PABYV's genome (Fig. 2(a)). ORF3a starts at an ACG codon located at genome position 3450 and ends at the next in-frame stop codon situated at genome position 3587. PABYV protein P3a, expressed from the translation of ORF3a, has a predicted MW of 4.88 kDa. The discovery of ORF3a has resulted in the length of the intergenic region of the polerovirus genome being reduced to about100 nt. PABYV genome organisation was the same for the baby marrow and patty pan isolates. The consensus sequences of the genomes of the two PABYV isolates were submitted to GenBank. They were assigned the following accession numbers: KU315177 for PABYV infecting patty pan, and KU315178 for PABYV infecting baby marrow.

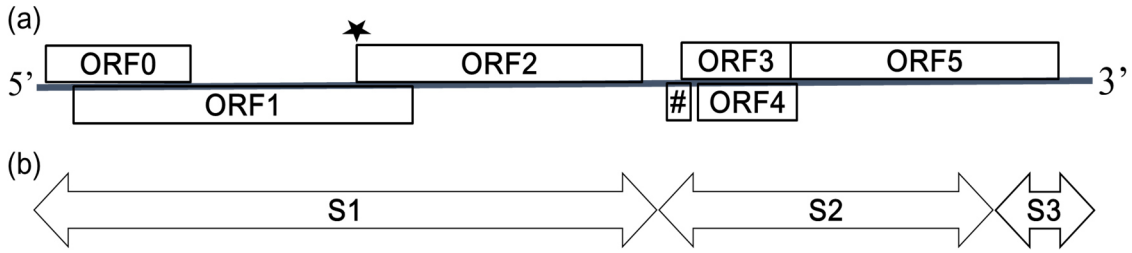

Fig. 2. Schematic representation of PABYV'S genome. (a) Genome organization. (b) Recombination profile of PABYV's genome. A star indicates the position of the slippery heptanucleotide; # indicates the non-UAG-initiated ORF3a.

Blast results of all PABYV's ORFs and NCR matched homologous regions of polerovirus infecting cucurbits. Consequently, the reference sequences (RefSeq) of every polerovirus infecting cucurbits, available from GenBank, were selected for nucleotide and amino acids (aa) sequence homology studies. Regarding nucleotide sequence homologies, PABYV 5'NCR, ORF0, and ORF1 shares sequence identity below 50% with poleroviruses infecting cucurbits (Table 3). ORF4 and ORF3 share the greatest sequence identity of 86.11% and 85.66%, respectively, with CABYV. ORF3a has between 73 and 75% nucleotide sequence identity with homologous regions of all poleroviruses selected. ORF5 shares 60 to 64% sequence identity with CABYV, SABYV and MABYV. 3'NCR shares 53.6 and 52.06% with corresponding sequences from LABYV and SABYV, respectively.

Table 3. Nucleotide sequence identities between the genomes of PABYV isolates and poleroviruses infecting cucurbits

Genome features	Polerovirus infecting cucurbits	PABYV baby marrow	PABYV patty pan
5'NCR	CABYV NC003688.1	30.00%	30.00%
	LABYV NC027703.1	40.00%	41.81%
	MABYV NC010809.1	30.00%	30.00%
	SABYV NC 018571.2	42.55%	42.55%
ORF0	CABYV NC003688.1	39.33%	40.72%
	LABYV NC027703.1	35.84%	35.97%
	MABYV NC010809.1	40.35%	40.63%
	SABYV NC 018571.2	35.60%	35.77%
OFR1	CABYV NC003688.1	42.94%	42.84%
	LABYV NC027703.1	42.41%	42.41%
	MABYV NC010809.1	42.68%	42.04%
	SABYV NC 018571.2	49.89%	49.83%
ORF2	CABYV NC003688.1	48.68%	48.87%
	LABYV NC027703.1	49.84%	49.87%
	MABYV NC010809.1	49.76%	50.17%
	SABYV NC 018571.2	56.43%	56.52%
ORF3a	CABYV NC003688.1	74.63%	74.63%
	LABYV NC027703.1	75.36%	75.36%
	MABYV NC010809.1	74.63%	74.63%
	SABYV NC 018571.2	73.91%	73.91%
ORF3	CABYV NC003688.1	85.66%	85.66%
	LABYV NC027703.1	51.50%	51.83%
	MABYV NC010809.1	82.83%	83.16%
	SABYV NC 018571.2	82.50%	82.50%
ORF4	CABYV NC003688.1	86.11%	86.11%
	LABYV NC027703.1	26.04%	25.69%
	MABYV NC010809.1	74.47%	74.82%
	SABYV NC 018571.2	27.6%	27.77%
ORF5	CABYV NC003688.1	64.22%	64.12%
	LABYV NC027703.1	38.40%	38.49%
	MABYV NC010809.1	59.53%	59.63%
	SABYV NC 018571.2	60.41%	60.41%
3'NCR	CABYV NC003688.1	42.23%	42.23%
	LABYV NC027703.1	53.60%	53.60%
	MABYV NC010809.1	41.31%	41.31%
	SABYV NC 018571.2	52.06%	52.06%

TABLE 4 Amino acid sequence identities between the proteins of PABYV isolates and poleroviruses infecting cucurbits

Proteins	Polerovirus infecting cucurbits	PABYV baby marrow	PABYV patty pan
P0	CABYV NC003688.1	21.75%	21.33%
	LABYV NC027703.1	17.06%	17.85%
	MABYV NC010809.1	22.31%	22.72%
	SABYV NC 018571.2	20.07%	20.45%
P1	CABYV NC003688.1	28.52%	28.52%
	LABYV NC027703.1	26.44%	26.44%
	MABYV NC010809.1	28.52%	28.52%
	SABYV NC 018571.2	37.82%	37.98%
P1-P2	CABYV NC003688.1	41.7%	41.51%
	LABYV NC027703.1	41.09%	41.28%
	MABYV NC010809.1	43.61%	43.61%
	SABYV NC 018571.2	51.85%	51.66%
P3a	CABYV NC003688.1	78.26%	78.26%
	LABYV NC027703.1	78.26%	78.26%
	MABYV NC010809.1	78.26%	78.26%
	SABYV NC 018571.2	76.08%	76.08%
P3	CABYV NC003688.1	82.5%	82.5%
	LABYV NC027703.1	43.50%	43.50%
	MABYV NC010809.1	82.5%	82.5%
	SABYV NC 018571.2	84.00%	84.00%
P4	CABYV NC003688.1	76.56%	76.56%
	LABYV NC027703.1	27.60%	28.64%
	MABYV NC010809.1	70.83%	71.87%
	SABYV NC 018571.2	72.39%	72.39%
P3-P5	CABYV NC003688.1	64.52%	64.52%
	LABYV NC027703.1	25.73%	25.73%
	MABYV NC010809.1	57.68%	58.68%
	SABYV NC 018571.2	58.85%	58.85%

Similar results were observed from aa sequence homology studies (Table 4). PABYV CP aa sequence identity ranges between 82 and 84% to SABYV, CABYYV and MABYV CP. PABYV P3a shares 76 to 78% aa sequence identity with all polerovirus selected in this study.

PABYV RTD has the highest aa sequence identity of 64.52% with CABYV homologous region. Aa sequence identity for the P1-P2 fusion protein of PABYV in comparison to the known polerovirus infecting cucurbits was around 52%. The lowest aa sequence identity, below 33%, was recorded for P0 and P1 proteins.

4.3.5. Sequence analysis and phylogeny of PABYV

Recombination analysis suggested that PABYV's genome may have evolved through a recombination event between CABYV, probably as the minor parent, and an unknown major parent. The hypothetical recombination event was detected, in both PABYV isolates, with BootScan (average p-Val: 8.38×10^{-5}) (Martin *et al*., 2005), MaxChi (average p-Val: 1.177×10^{-12}) (Smith, 1992), Chimaera (average p-Val: 2.306×10^{-12}) (Posada & Crandall, 2001) and SiScan (average p-Val: 1.57×10^{-96}) (Gibbs *et al*., 2000). PABYV's genome was subsequently split into three segments as proposed by the RDP results (Fig. 2(b)). The first segment (S1) consisted of the first 3379 nucleotides. The second segment (S2) included nucleotides from Genome Position 3380 to 5219. The last segment (S3) consisted of the last 594 nucleotides of the PABYV's genome.

Phylogenetic analyses were performed based on the profile generated from the recombination analyses. *Potato leafroll virus*, the type member of the genus *Polerovirus,* was used as an outgroup in phylogenetic analyses. The General Time Reversible, with a discrete Gamma distribution and an evolutionarily invariable sites model, was identified by MEGA as the model describing the substitution pattern the best. Consequently, it was used for phylogenetic analyses. Trees generated from phylogenetic analyses of the different PABYV genome segment displayed different phylogenic topologies (Fig. 3). Both the isolates of PABYV from baby marrow and patty pan clustered together in all phylogenetic trees. Phylogenetic analyses of the segment S1 indicated a distant relationship between the PABYV isolates and SABYV (Fig. 3(a)). The phylogenetic tree from analyses of the segment S2 put the CABYV and PABYV isolates together but in different clusters (Fig. 3(b)). From the phylogenetic analyses of the S3 segment, PABYV isolates form a unique cluster (Fig. 3(c)). All relationships displayed on the phylogenetic trees were supported by very high bootstrap values.

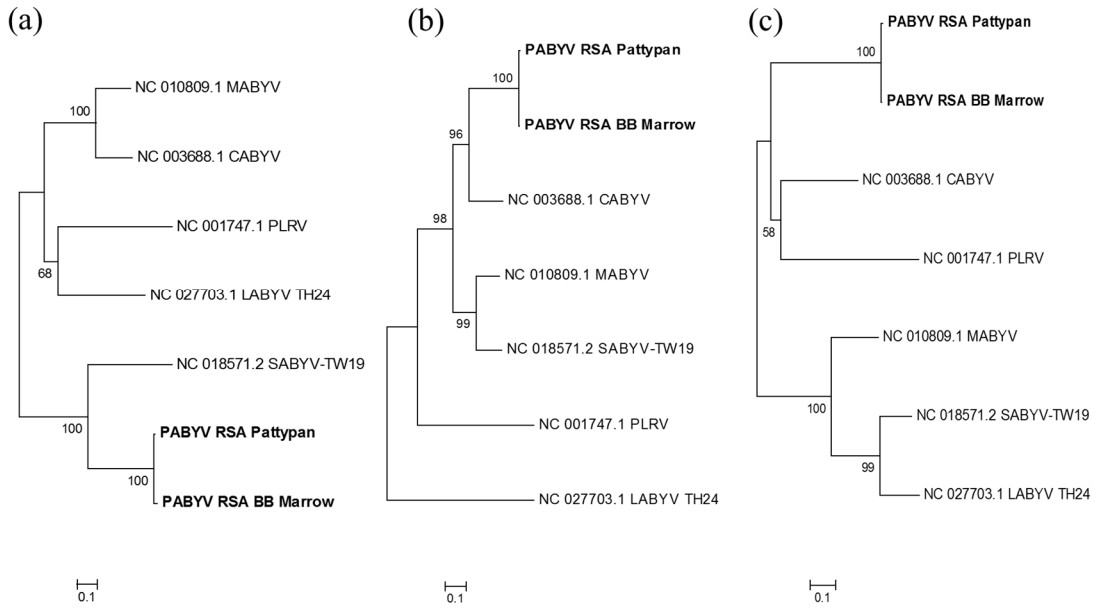

Fig. 3. Phylogram of PABYV's genome. (a) S1 fragment, (b) S2 fragment, (c) S3 fragment. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

4.4. Discussion

Virus diseases remain a significant threat in agriculture (Thottappilly, 1992). Damages on agricultural production, due to virus diseases, can have a negative socio-economic impact on communities (Vurro *et al*., 2010). One of the aims of plant pathology is to manage plant viruses and the diseases they induce in plants of economic importance. This objective is achieved through the development of various methods and strategies that prevent or limit infection and disease spread (Wilson, 2014). Accurate knowledge of each virus causing disease is usually required as a starting point for the effective control of the disease.

Disease surveys, conducted in cucurbit growing regions of KZN during the 2011, 2012 and 2013 growing seasons, led to the detection of a putative polerovirus that had previously been identified in Mali as PABYV (Ibaba *et al*., 2015). This study aimed to confirm the taxonomic status of PABYV by looking at its morphology and genome organization. The analyses of PABYV's virion was achieved using TEM. Purification of the virion was essential prior to visualization under the TEM. Polyhedral particles of about 25 nm (Fig. 1) were observed under TEM. The morphology of PABYV's virion is in line with the morphological description of virions from the genus *Polerovirus* (d'Arcy & Domier, 2012).

The advent of nucleotide sequence determination has revolutionized biology and largely rationalized taxonomy, including that of viruses (ICTV, 2015b). NGS was performed

on total RNA extracted from PABYV infected baby marrow and patty pan plants in order to recover the PABYV genome. RNA from purified virions would have been a better alternative for this step. This option was not possible because a two-year gap between when virus purification was performed, and when it was decided to perform NGS. PABYV's genome consists of 5813 nt based on both *de novo* assembly and Sanger sequencing. The genome length was the same in both PABYV isolates analysed in this study. Genera in the family *Luteoviridae* are distinguished on the basis of the arrangement of the ORFs (d'Arcy & Domier, 2012). The presence of both ORF0 and ORF4 on the genome distinguishes the genus *Polerovirus* from the other genera (d'Arcy & Domier, 2012). Therefore, PABYV is a member of the genus *Polerovirus* based on the presence of these two ORFs on its genome (Fig. 2(a)). This conclusion is furthermore supported by the identical genome organization that was observed in both PABYV isolates. The species demarcation criterion in the family *Luteoviridae,* according to the Ninth Report of the ICTV, is a 10% threshold difference in aa identity in any of the viral protein (d'Arcy & Domier, 2012). All PABYV protein products have a difference in aa identity greater than 10% to known poleroviruses (Table 4). This confirms the identification of PABYV as a distinct species in the genus *Polerovirus*.

ORF0, ORF1 and ORF2 are located at the 5'proximal portion of PABYV genome, and had lower nucleotide and aa sequence identity than the 3' proximal ORF block (Table 3 and 4). The 5'proximal portion of the genome of polerovirus has been recognized as the most variable region for a number of polerovirus species, even within species infecting the same host (Knierim *et al*., 2013). Nonetheless, the viral proteins of the corresponding ORFs contained the conserved domain common to members of the genus *Polerovirus*. These conserved domains are involved in various stages of the virus life cycle.

Recombination events have become a recognized feature of the poleroviruses (Smirnova *et al*., 2015). They are responsible for the viral genomic regions with a very similar sequence observed in several poleroviruses (Dombrovsky *et al*., 2013). These recombination events occur within the ORFs block located at the 3' proximal of the polerovirus genome (Dombrovsky *et al*., 2013; Knierim *et al*., 2013). A hypothetical recombinant event involving CABYV as the likely minor parent and an unknown parent was detected on the PABYV's genome of both isolates analysed in this study (Fig. 2(b)). Up to date, no report of CABYV has been recorded in Southern Africa. The hypothetical recombination event, detected on the genome of PABYV, may have taken place at an earlier time in the evolution of poleroviruses, probably in a mixed infection of a common host plant. Recombination should not be the sole factor responsible for PABYV's genome evolution. Other factors that have played a role in PABYV's genome evolution include both positive and purifying selection constraints, as

proposed by Dombrovsky *et al*. (2013). Exclusion of recombinant sequences in phylogenetic analyses improves both the relationship between taxa and bootstrap support (Ogawa *et al*., 2008). For that reason, the profile of PABYV's genome resulting from the recombination analyses was considered for phylogenetic analyses instead of each ORFs and NCR. The phylograms generated from phylogenetic analyses display different phylogenetic topologies (Fig. 3), which also support the recombination theory.

The report of the complete genome sequence of PABYV in this study, and LABYV (Knierim *et al*., 2015), confirmed that they are distinct species in the genus *Polerovirus.* This has increased the number of poleroviruses infecting cucurbits to five. All five poleroviruses infecting cucurbits have been associated with yellowing symptoms (Ibaba *et al*., 2015; Knierim *et al*., 2014). CABYV is the most widespread and has been reported in several countries from all continents (Omar & Bagdady 2012). LABYV, MABYV and SABYV have only been reported in Asia (Knierim *et al*., 2014). PABYV seemed to have been confined in Africa until it was identified on watermelon grown in Greece, in Europe (Lotos *et al*., 2016). The detection of PABYV in countries geographically distant from each other could be an indication of a much wider distribution of this virus. A comprehensive study of PABYV isolates from all geographical regions where it occurs may provide more understanding of its evolution.

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Chapter 5

Molecular evidence that Zucchini shoestring virus is a distinct potyvirus in the *Papaya ringspot virus* cluster[4]

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Abstract

Zucchini shoestring virus (ZSSV) was proposed as a putative potyvirus in the *Papaya ringspot virus* (PRSV) cluster, on the basis of the sequence identity of its coat protein to related potyviruses. ZSSV has been associated with the outbreak of a damaging disease on baby marrow (*Cucurbita pepo* L.) that had been observed throughout the province of KwaZulu-Natal (KZN), in the Republic of South Africa (RSA). The aim of this study was to decipher the complete genome sequence of ZSSV and to study its relationship with other potyviruses in the PRSV cluster. ZSSV genome sequence was elucidated from *de novo* analysis of data generated from NGS of total RNA, extracted from an infected baby marrow. ZSSV genome sequence is made of 10295 nucleotides excluding the poly(A) tail, and displays a genome organization typical of potyviruses. Nucleotide and amino acids sequence identity of each protein support the differentiation of ZSSV as a distinct species in the genus *Potyvirus*. This taxonomic position was also confirmed using the online PASC tool on the NCBI website. Phylogenetic analyses of the CP and the polyprotein coding sequence of ZSSV consistently grouped ZSSV together with *Algerian watermelon mosaic virus* and *Moroccan watermelon mosaic virus* but in different clusters. ZSSV is the second cucurbit-infecting virus in the PRSV cluster present in RSA.

[4] Chapter 5 was submitted to Archives of Virology and is currently under review.

5.1. Introduction

Potyviruses are species of the genus *Potyvirus*, one of eight genera of the family *Potyviridae* (Adams et al., 2012). Besides being one of the two largest genera of plant-infecting viruses (Gibbs and Ohshima, 2010; Hasiów-Jaroszewska et al., 2014), the genus also includes important viruses of agricultural crops (Gibbs and Ohshima, 2010; Hasiów-Jaroszewska et al., 2014; Ivanov et al., 2014). Potyviruses are non-enveloped, flexuous filament of a modal length varying between 650 and 900 nm with a diameter of 11 to 15 nm. Each virion comprises a single copy of the monopartite genome that is a single-stranded positive-sense RNA molecule of about 10, 000 nucleotides (nt). The potyvirus genome is covalently linked, at its 5'terminus, to a viral protein (VPg), and is polyadenylated at its 3'terminus (Adams et al., 2012). Eleven multifunctional proteins, all required in the infection cycle, are produced from the potyvirus genome through translation frameshifting and a polyprotein strategy (Adams et al., 2012). The polyprotein, encoded by the potyvirus genome, is proteolytically processed, at specific sites, by three encoded viral proteinases (P1, HC-Pro, and Nia) to yield 10 mature proteins (Adams et al., 2005; Urcuqui-Inchima et al., 2001). P3N-PIPO is the potyvirus protein produced by +2 ribosomal frameshifting at the 5'terminal part of the P3 encoding gene (Chung et al., 2008). Potyviruses are transmitted in a non-persistent, non-circulative manner by several aphid species (Adams et al., 2012; Ng and Perry, 2004). 76% nucleotide sequence identity in the coat protein (CP) or over the whole genome is the threshold for the species demarcation in the genus *Potyvirus*, according to the International Committee on Taxonomy of Viruses (Adams et al., 2012).

The number of cucurbit-infecting viruses worldwide has increased over the last two decades (Ibaba et al., 2015a). The advent of next-generation sequencing (NGS) and its application in areas of plant virology have accelerated the discovery of novel viruses (Barba et al., 2014; Prabha et al., 2013; Roossinck et al., 2015). Zucchini shoestring virus (ZSSV) has been proposed as a putative potyvirus infecting cucurbits, on the basis of the nucleotide sequence identity of its CP to related cucurbit-infecting potyviruses (Ibaba et al., 2015b). ZSSV was detected in the province of KwaZulu-Natal (KZN), in the Republic of South Africa, during virus surveys conducted in the cucurbit growing areas between 2011 and 2013. Symptoms associated with ZSSV include severe leaf filiformy and fruit deformation on baby marrow (*Cucurbita pepo* L.) (Ibaba et al., 2015b). These symptoms were observed throughout the survey and in all growing areas. Losses up to 100% were recorded in cases when the infection occurred before fruit formation. The aim of this study was to elucidate and analyse the genome sequence of ZSSV.

5.2. Material and methods

5.2.1. Virus source

The source of the ZSSV isolate used in this study was a baby marrow leaf sample displaying filliformy (shoestring) symptoms, randomly selected, from the samples collected during the virus surveys conducted between 2011 and 2013. All leaf samples collected during the survey were kept at -80°C in sealed and labelled plastic bags.

5.2.2. Determination of ZSSV genome sequence and organization

5.2.2.1. NGS

NGS was used to recover the ZSSV genome using total RNA as the template. Nucleo spin RNA plant kit (Macherey-Nagel, Germany) was used to extract total RNA from the selected leaf sample. RT-PCR was performed on the extracted RNA, using the forward 5′-CAC CCG TGC GAC ACA AGA C-3′ and reverse 5′-ACA GGT TTC AAG GGA TAC TCA A-3′ primers (Ibaba et al., 2015b) to confirm the presence of ZSSV. Upon confirmation of the presence of ZSSV, the RNA sample was shipped to the Agricultural Research Council's Biotechnology Platform (ARC-BTP) in Pretoria (RSA) for NGS library preparation and sequencing on the Illumina Hiseq platform, using paired-end chemistry 125x125bp reads.

5.2.2.2. NGS data analysis

FastQC (Andrews, 2010) was used to access the quality of the NGS data generated. Removal of adapters from the reads, trimming and quality filtering were performed using Trimmomatic version 0.33 (Bolger et al., 2014) according to the following settings: {ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:9:1:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36}. The pair-end sequences were subsequently used as single reads in *de novo* assembly that was performed on SeqMan NGen (software version 12.3.1 build 48; DNASTAR Lasergene) according to the following default parameters: Match Size: 21; Match Spacing: 50; Minimum Match Percentage: 95; Match Score: 10; Mismatch Penalty: 20; Gap Penalty: 30; Max Gap: 6 and removal of contigs shorter than 1000 nt, and made of less than 100 reads. The contigs generated from *de novo* assembly were blasted on the NCBI database using the Netsearch function on the program SeqMan pro (software version 12.3.1 build 48 421; DNASTAR Lasergene). Potyvirus identified contigs were selected to determine the consensus genome sequence of ZSSV.

5.2.2.3. Sanger sequencing

Direct sequencing of overlapping amplicon flanking the consensus genome of ZSSV was performed to validate the integrity of ZSSV genome. Primers were designed using Primer select software version 12.3.1 build 48 421 (DNASTAR Lasergene). Amplicons were produced

by RT-PCR. A RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA), the respective reverse complement primers and total RNA, extracted from ZSSV infected leaf samples, were used to synthesize first strand cDNA. PCR was performed using the synthesized cDNA as the template, the respective primer pair and Phire Hot Start DNA Polymerase. Both cDNA synthesis and PCR were done according to the manufacturer's instructions. Amplicons were checked on 1.5% agarose gel in TAE buffer, pre-stained with GelRed. Amplicons of the expected sizes were gel purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) according to the manufacturer's instructions. Direct sequencing of these amplicons was performed at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA).

5.2.2.4. Determination of ZSSV genome organization

The Open Reading Frame (ORF) Finder (Rombel et al., 2002; Wheeler et al., 2003) was used to predict the ORFs on the ZSSV genome. The cleavage sites, on the ZSSV polyprotein, were identified, using the data provided by Adams et al. (2005) and Romay et al. (2014). Compute pI/Mw (http://web.expasy.org/compute_pi/) was used for the computation of the molecular weight (Mw) of the polyprotein and the mature individual proteins.

5.2.3. Sequence analysis and phylogeny of ZSSV

The molecular taxonomic position of ZSSV was determined by comparing its genome sequence with the full-length genome sequences of all potyviruses using PAirwise Sequence Comparison (PASC) (Bao et al., 2014). Multiple sequence alignments were performed using the program Muscle, implemented in MEGA 6.06 build 6140226 (Tamura et al., 2013). Nucleotides and amino acids (aa) sequence similarities between ZSSV and the viruses of the *Papaya ringspot virus* (PRSV) cluster were computed using the online tool SIAS (http://imed.med.ucm.es/Tools/sias.html). Any putative recombination junctions were also checked on ZSSV genome with RDP (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), MaxChi (Smith, 1992), BootScan (Martin et al., 2005), and SIScan (Gibbs et al., 2000) included in the RDP v4.56 package (Martin et al., 2015). The phylogeny of ZSSV was conducted in MEGA6 and was inferred by using the Maximum Likelihood method based on the General Time Reversible model with a discrete gamma distribution and invariable sites with 500 bootstrap replicates.

5.3. Results

5.3.1. ZSSV genome sequence and organization

A 10,308 kb contigs matched the ZSSV CP coding sequence, accession number: KP723639.1, with an E-value of 0.00, and was therefore considered as the ZSSV draft genome. The ZSSV draft genome was constructed of 15332 reads (Median Coverage: 162.61) out of

32,138,032 filtered reads from 33,532,786 raw reads. Overlapping amplicons flanking the ZSSV draft genome were obtained using primers, and according to the conditions listed in Table 1. Direct sequencing of these amplicons yielded highly similar sequences with minor ambiguities. All nucleotides ambiguities were solved by considering the most abundant nucleotide at that position from the de novo assembly. ORF Finder identified the polyprotein encoding ORF and all the potyvirus conserved motifs on the ZSSV draft genome. Information from Chung et al. (2008), and Wen and Hajimorad (2010) were helpful in identifying PIPO. It was therefore concluded that ZSSV genome consists of 10295 nucleotides excluding the poly(A) tail, and is organized as follows: 5' non-coding region (NCR): nucleotide 1 - 66; polyprotein ORF: nucleotide 67 – 10104; 3'NCR: nucleotide 10105 – 10295; PIPO: nucleotide 3609 – 3791 (Fig. 1). The ZSSV genome sequence is available on the GenBank database under the accession number: KU355553.

Table 1.

Primers used to generate overlapping amplicons flanking ZSSV genome and PCR conditions

Primer names	5'- 3'sequences	Genome Portion (Amplicon size (bp))	Cycling conditions*
F1	CAA CAA ATC AAA GCA AAC GAA	1 – 846 (846)	98°C-5s 53°C-5s 72°C-15s
R846[c]	ATC CTT CAT GAC CTT ATC C		
F268	TTT CTG TCC TTG CCT AAT GG	268 – 2036 (1,769)	98°C-5s 55°C-5s 72°C-25s
R2036[c]	AGC ACA CTC CTC AAC CGA CTC		
F1526	CGC AGC GTG ATT TAG ATA C	1526 - 2257 (732)	98°C-5s 53°C-5s 72°C-15s
R2257[c]	GCT TTT TCG TTG CTT CAC CT		
F2035	CTC ATC AGC TAC GGG GGT TCG	2035 - 3976 (1,942)	98°C-5s 64°C-5s 72°C-25s
R3976[c]	ATT GTG CGC TCC TTC CTA TCT GC		
F3776	TTGTGAAAGACGTTAAAGAGGTTGTT	3776 - 4396 (621)	98°C-5s 60°C-5s 72°C-15s
R4396[c]	AATCGATTGTTAGTTGTTTGTCTTCA		
F4016	CTA AAG CAA AGC AAG ATA AGG A	4016 - 5993 (1,978)	98°C-5s 53°C-5s 72°C-25s
R5993[c]	TGG TCA ATA GTG GCA AGA G		
F5623	ATGATTGGTTTAGTGGCTCCTGATG	5623 - 7588 (1966)	98°C-5s 58°C-5s 72°C-25s
R7588[c]	GGC TAC TCC AAC TTA AAC TCT CC		
F7366	TCT AGC CAT ATT TCC GAC AG	7366 - 9320 (1955)	98°C-5s 53°C-5s 72°C-25s
R9320[c]	TTCTCTTTTTCTTTCTTTTCTTTGCTCT		
S[a]	GGN AAY AAY AGY GGN CAR CC	8620 - 10308 (1,689)	98°C-5s 55°C-5s 72°C-25s
N1T[b,c]	GACCACGCGTATCGATGTCGAC$(T)_{17}$		

*: 40 cycles PCR were run; [a]: Chen et al., 2001; [b]: Ha et al., 2008; [c]: primers used for RT.

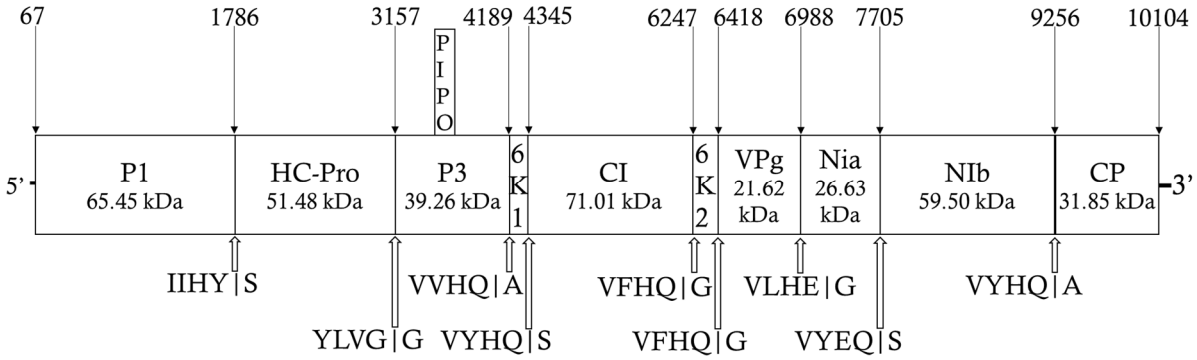

Fig.1. Schematic representation of the ZSSV genome organization. The number on the diagram indicate the starting nucleotide position predicted for the gene. The cleavage sites in the polyprotein are indicated below. The cleavage sites are indicated by the symbol ''|'' between the fourth and the fifth aa.

5.3.2. Sequence analysis and phylogeny

PASC classified ZSSV as a distinct potyvirus in the PRSV cluster. The PRSV cluster includes *Algerian watermelon mosaic virus* (AWMV) (Yakoubi et al., 2008a), *Moroccan watermelon mosaic virus* (MWMV) (Yakoubi et al., 2008b), PRSV, *Zucchini tigré mosaic virus* (ZTMV) (Romay et al., 2014) and *Zucchini yellow fleck virus* (ZYFV) (Desbiez et al., 2007). PRSV isolates are referred to as either W or P strains for isolates infecting cucurbits, or Papaya (*Carica papaya* L.), respectively (Romay et al., 2014). Sequence homology analyses showed that the ZSSV genome shares the highest nucleotide sequence identity of 65.68% with AWMV (Table 2). The lowest nucleotide sequence identity was recorded with PRSV. Regarding the respective protein-coding sequences, ZSSV shares the highest nucleotide sequence identity with AWMV for 8 out of the 11 potyvirus proteins (Table 2). The highest nucleotide sequence identity for the others three potyvirus protein was shared with MWMV isolates (Table 2).

Table 2.

Nucleotide sequence identity (%) between ZSSV and potyviruses of the PRSV cluster

Viruses	FLG	5'NCR	3'NCR	P1	HC-Pro	P3	6K1	CI	6K2	VPg	Nia	NIb	CP	PIPO
EU410442.1 AWMV H4	**65.68**	42.42	**62.31**	41.64	**70.02**	**60.91**	70.51	**68.98**	**57.73**	67.19	**67.78**	**73.69**	**69.49**	**72.13**
KU315175 MWMV RSA	63.75	**59.09**	56.54	**47.82**	63.96	57.55	**75.64**	68.45	51.46	68.07	64.99	72.14	68.9	62.84
KU315176 MWMV RSA	63.70	**59.09**	56.54	47.63	63.74	57.65	**75.64**	68.40	50.87	68.07	65.13	71.95	69.02	62.84
EF579955.1 MWMV TN05-76	63.28	56.06	56.93	46.76	64.98	57.36	74.35	67.29	50.87	**68.42**	66.10	70.53	68.78	62.84
AY010722.1 PRSV strain W	59.23	45.45	49.51	39.60	62.36	53.19	62.17	65.03	52.63	66.84	61.62	68.21	66.31	57.92
AY027810.2 PRSV W CI	59.54	37.87	48.54	39.91	62.87	52.9	62.17	65.03	52.63	66.31	61.90	67.76	67.37	59.01
NC 001785.1 PRSV-P	59.10	45.45	49.51	39.54	61.85	52.61	64.74	63.51	50.29	65.25	62.88	68.47	67.96	59.56
KC345606.1ZTMV VET-026	59.04	48.48	59.16	39.66	62.72	49.32	69.87	64.35	53.8	62.25	60.36	68.85	64.78	55.19
NC_023175.1 ZTMV Re01-25	59.15	54.54	57.73	39.34	63.09	4951	69.23	64.93	54.38	63.66	58.68	68.47	66.19	55.19
GQ165514.1 ZYFV 23-Sicily	--	--	--	--	--	--	--	--	--	--	--	--	64.98	--
DQ641511.1 ZYFV Fr	--	--	--	--	--	--	--	--	--	--	--	--	64.6	--

FLG: Full-length genome, --: data not available, highest nucleotide sequence identities are indicated in bold

Table 3.

Amino acid sequence identity (%) between ZSSV and potyviruses of the PRSV cluster

Viruses	Pol	P1	HC-Pro	P3	6K1	CI	6K2	VPg	Nia	NIb	CP	PIPO
EU410442.1 AWMV H4	**69.22**	27.16	**78.33**	**55.55**	**80.76**	**77.44**	**60.71**	74.21	**75.56**	**84.13**	71.73	**59.01**
KU315175 MWMV RSA	67.87	**38.26**	70.45	50.58	75.00	75.70	50.87	**76.84**	71.96	80.07	76.67	37.70
KU315176 MWMV RSA	67.80	37.68	70.45	50.58	75.00	75.70	50.87	**76.84**	71.96	79.88	**77.03**	37.70
EF579955.1 MWMV TN05-76	67.58	36.23	70.45	50.87	75.00	75.23	50.87	76.31	71.92	80.27	75.97	39.34
AY010722.1 PRSV strain W	58.34	25.95	67.39	43.60	63.46	69.55	49.12	67.19	62.60	74.08	67.49	39.43
AY027810.2 PRSV W CI	58.36	25.41	68.05	42.44	63.46	69.71	54.38	67.19	63.02	74.08	68.90	40.98
NC 001785.1 PRSV-P	57.93	24.13	66.08	42.73	63.46	69.40	49.12	66.13	64.70	74.46	69.25	42.62
KC345606.1ZTMV VET-026	58.72	24.64	67.83	40.11	67.30	71.29	47.36	68.25	62.18	75.24	66.78	42.62
NC_023175.1 ZTMV Re01-25	59.14	26.70	68.05	41.27	65.38	71.13	43.85	66.13	62.18	75.24	70.67	42.62
GQ165514.1 ZYFV 23-Sicily	--	--	--	--	--	--	--	--	--	--	66.54	--
DQ641511.1 ZYFV Fr	--	--	--	--	--	--	--	--	--	--	66.90	--

Pol: polyprotein, --: data not available, highest aa sequence identity are indicated in bold

No recombination junctions were detected on the ZSSV genome. Regarding aa sequence identity, the highest sequence identity (69.22%) was recorded with AWMV. It was again noticed that ZSSV shares the highest aa sequence identity with AWMV for 8 potyvirus encoded proteins (Table 3). A number of conserved motifs among potyviruses have been described. The conserved motifs identified on the ZSSV genome include the following: 3069D-A-G3071 on the N-terminal part of the CP (Atreya et al., 1990 and 1991), which is known to be involved in aphid transmission; 753F-R-N-K756 box and 820L-A-I-G-N824, in the domain II of the HC-Pro, which are involved in the suppression of RNA silencing (Shiboleth et al., 2007) and the amplification process (Cronin et al., 1995; Kasschau et al., 1997) . The motifs 624R-I-T-C627 and 882P-T-R884 were found in the central part of the HC-Pro instead of the highly conserved K-I-T-C and P-T-K motifs. ZSSV, like MWMV and AWMV, does not have the extra aa between the Nib and CP, as identified on PRSV. Phylograms of the CP and the polyprotein coding sequence of ZSSV consistently grouped ZSSV together with AWMV and MWMV but in different clusters (Fig. 2).

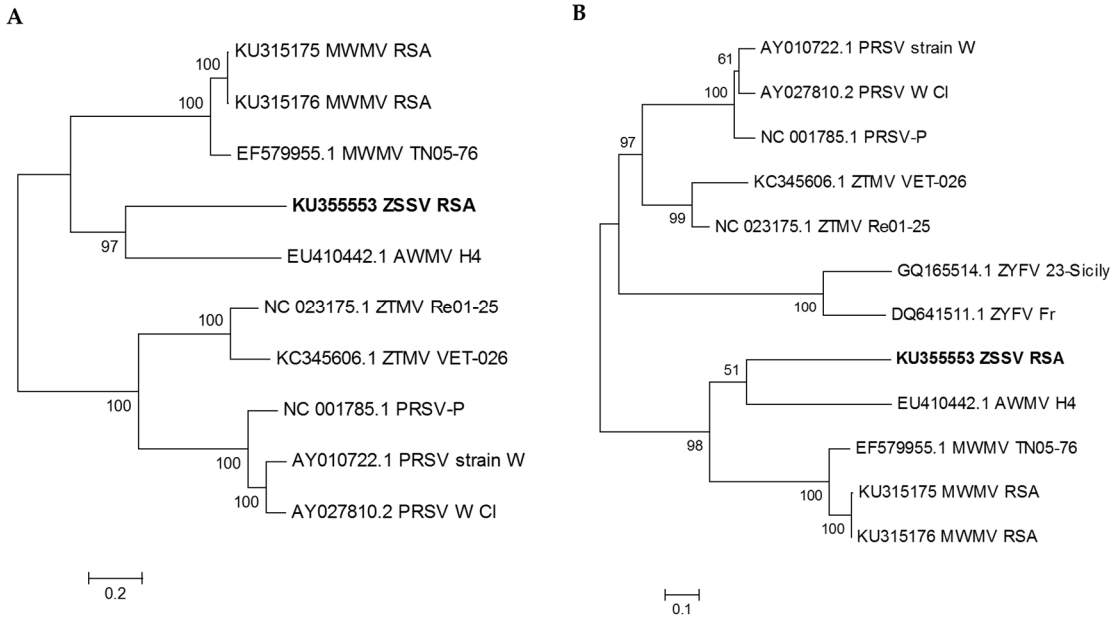

Fig. 2. Phylograms of the ZSSV polyprotein coding sequences (A) and CP coding sequence (B). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

5.4. Discussion

Accurate identification of the pathogenic viruses responsible for disease outbreaks contributes towards establishing effective strategies to control further spread of these pathogens. The genome organization of ZSSV was elucidated in this study. NGS made the

recovery of the entire ZSSV genome from total RNA extracted from infected leaf sample relatively easy, cheap and quick because no specific knowledge of the pathogenic virus was required prior to running NGS. Considering that ZSSV-mapped reads only accounted for 0.046% of the raw reads, it can be speculated that ZSSV may be a low titer virus. Another plausible explanation for the low mapped-read percentage could be that ZSSV was in low concentration in the leaf sample selected for RNA extraction. After Sanger sequencing of the overlapping amplicons, the ZSSV draft genome generated from *de novo* assembly was considered to be the accurate, complete genome sequence of ZSSV. The shoestring symptom cannot be solely attributed to ZSSV at this stage because a tobamovirus and another potyvirus were also detected in the sample analysed in this study (data not shown).

ZSSV has a genome organization typical of a potyvirus, containing the only two known ORFs that encode for the polyprotein and PIPO (Fig. 1). ZSSV polyprotein comprises 3345 aa. The cleavage sites detected on the ZSSV polyprotein point to the production of the 10 mature potyvirus proteins (Fig. 1). The CI|6K2 and Nia|NIb cleavage sites of ZSSV were unique when compared to the cleavage sites of all the virus species that compose the PRSV cluster. P1|HC-Pro, P3|6K1, 6K2|VPg, and VPg|Nia cleavage sites were identical to those of AWMV, MWMV, PRSV and ZTMV, respectively. The HC-Pro|P3 cleavage site of ZSSV was identical to that of AWMV and ZTMV. The NIb|CP cleavage site of ZSSV was the same as that of AWMV and MWMV. Furthermore, the MW of ZSSV mature proteins falls within the range of potyvirus proteins (Adams et al., 2012). The motifs ^{624}R-I-T-C^{627} and ^{882}P-T-R^{884}, on the central part of the HC-Pro of ZSSV, instead of the highly conserved K-I-T-C and P-T-K motifs, had also been identified in AWMV and MWMV. However, it was found that the presence of these substitute motifs in AWM and MWMV did not abolish aphids' ability to transmit these viruses (Yakoubi et al., 2008a, 2008b)

The ZSSV genome should be considered to be unique because no recombination junctions were detected in its genome. The threshold for the demarcation criterion in the genus *Potyvirus*, with regard to the whole genome, is set to 76% nucleotide sequence (Adams et al., 2012). PASC showed that AWMV has the closest related genome sequence to ZSSV. Nucleotide sequence identity across the genomes of ZSSV and AWMV was 65.68% (Table 2.), thereby qualifying ZSSV as a distinct species in the genus *Potyvirus*. ZSSV shares the highest nucleotide and amino acid sequences of its encoded proteins with AWMV and MWMV. Some phylogenetic relationships were therefore expected between these isolates and this is confirmed by our findings.

5.5. Conclusion

ZSSV is the second cucurbit-infecting virus of the PRSV cluster to occur in RSA, and the third one reported in Africa after MWMV in 1974 (Yakoubi et al., 2008a), and AWMV in 2008 (Yakoubi et al., 2008b). Yakoubi et al. (2008b), in addressing the diversity of members of the PRSV cluster in Africa, noted that tropical and subtropical Africa is known to be the centre of origin of several cucurbit crops, and therefore, it could also be the centre of origin of the members of the PRSV cluster. Further studies of ZSSV should include the determination of its biological and serological properties as well as its geographic distribution.

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Chapter 6

Use of a Chimeric Transgene Construct to Confer Broad Resistance in Zucchini (*Cucurbita pepo* L.) Plants Against Cucurbit-infecting Potyviruses Occurring in KwaZulu-Natal, Republic of South Africa

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Abstract

The frequent identification of cucurbit-infecting viruses across the Province of KwaZulu-Natal (KZN) in the Republic of South Africa (RSA) necessitates developing effective methods of controlling these diseases. Putative transgenic baby marrow *(Cucurbita pepo* L.) plants resistant to *Zucchini yellow mosaic virus* (ZYMV), *Moroccan watermelon mosaic virus* (MWMV), and Zucchini shoestring virus (ZSSV), three potyviruses occurring in KZN, were developed in this study using the antisense post-transcriptional gene silencing. A portion of the 5' coding sequence of the coat protein of ZYMV, MWMV, and ZSSV was amplified by RT-PCR and inserted into the plant expression vector pEPJ86-m/2N. The segment containing the expression cassette was sub-cloned into the binary vector pGA482G before being introduced into *Rhizobium radiobacter*, formerly *Agrobacterium tumefaciens*, strain LBA4404 (pAL4404)(pBI121). Baby marrow cotyledon explants, transformed using *Rhizobium*-mediated transformation, were regenerated *in vitro* using various media supplemented with 100 $mg.l^{-1}$ kanamycin and 500 $mg.l^{-1}$ cefatoxin. The regenerated putative transgenic baby marrows were subjected to polymerase chain reaction, mechanical inoculation and double antibody sandwich enzyme-linked immunosorbent assay to confirm transgene insertion and evaluate virus resistance. A total of 94 putative transgenic marrows lines were successfully regenerated from 250 explants. PCR results showed that 84 out of the 94 lines had the transgene. Of these 84 lines, 76 showed resistance after mechanical inoculation with the viruses under study. Our preliminary results confirm the findings of previous studies that have done similar work, and show the potential of using transgenic cucurbits with resistance to three potyviruses as an effective strategy to control potyvirus diseases on cucurbits.

Keywords: pGA482G; pEPJ86-m/2N; *Moroccan watermelon mosaic virus*; *Zucchini yellow mosaic virus*; Zucchini shoestring virus

6.1. Introduction

The use of resistant cultivars is often the best option to control plant virus diseases (Gal-On, 2007). Breeding and genetic engineering resistance are two approaches that have been used to develop virus-resistant plants. Genetic engineering, on one hand, has the advantage of addressing some of the constraints faced by growers, which may not be easily addressed through conventional plant breeding alone (Silva Dias and Ortiz, 2014). The discovery that RNA silencing, also known as RNA interference (RNAi) or small interfering RNA (siRNA), plays a role in the antiviral defense mechanism in plants has opened new avenues in engineering virus resultant cultivars (Duan et al., 2012). The RNA silencing mechanism is highly conserved in eukaryotic organisms, and has been exploited by others to develop transgenic virus-resistant plants (Duan et al., 2012; Galvez et al., 2014). Gene silencing in plants is triggered by double-stranded RNA (dsRNA) that are cleaved by Dicer-like proteins into 21 – 24 nucleotide siRNA. The siRNA antisense strand is then loaded into the RNA-induced silencing complex (RISC), which subsequently induces degradation of the homologous mRNA, thus, preventing its translation (Duan et al., 2012; Galvez et al., 2014). This pathway is known as Post-transcriptional gene silencing (PTGS) (Galvez et al., 2014).

Virus-resistant cucurbits are produced by means of plant tissue culture and genetic transformation. A *de novo* regeneration system from *in vitro* culture is required in order to develop transgenic virus-resistant cucurbits (Nuñez-Palenius et al., 2008). Regeneration of economically important cucurbit plants, *in vitro*, is a delicate exercise. Regeneration techniques of cucurbits have been found to vary among researchers with regards to the type of explant used and the concentrations of the different growth regulators applied (Abrie and van Staden, 2001). Consequently, several protocols have been described for the *in vitro* regeneration of cucurbits using either somatic embryogenesis, organogenesis or both regeneration pathways (Ananthakrishnan et al., 2003; Kathiravan et al., 2006; Nuñez-Palenius et al., 2008; Zhang et al., 2008). Although the level of *in vitro* regeneration is influenced by various biological and physical factors, regeneration of cucurbits has been reported from cotyledons, adventitious buds, somatic embryos, shoot primordia, protoplasts, and axillary buds (Ananthakrishnan et al., 2003; Nuñez-Palenius et al., 2008; Zhang et al., 2008).

RNA silencing in plants has been achieved using dsRNA-expressing vectors for the selected virus. *Rhizobium*-mediated transformation or particle bombardment are the two most commonly used methods of introducing potent inducers of PTGS (Senthil-Kumar and Mysore 2011). RNA silencing has been found to be highly specific, environmentally friendly and capable of targeting multiple viruses (Zhou, 2012). A number of virus resistant transgenic cucurbits have been produced in other parts of the world. These include cucurbits with

resistance to *Squash mosaic virus* (Jan et al., 2000; Pan et al., 2000), *Papaya ringspot virus* (Krubphachaya et al., 2007), and ZYMV (Wu et al., 2009). Multiple virus resistance has been reported in two transgenic watermelons (Lin et al., 2012a; Yu et al., 2011) and a melon (Wu et al., 2010). Chimeric transgene harbouring a portion of the genes of the targeted viruses were used in developing these transgenic cucurbits with multiple virus resistance. Virus-resistant transgenic squash has allowed growers to achieve yield similar to those obtained in virus free environment with a net profit of US $22 million in 2005 (Silva Dias and Ortiz, 2014).

Plant-infecting viruses are a serious problem affecting cultivation of various cucurbit crops in the Republic of South Africa (RSA), regardless of the type of farming practice used, from small-scale subsistence farms to large commercial farms. In virus surveys conducted during 2011, 2012 and 2013 growing seasons in the cucurbit-growing areas in the Province of KwaZulu-Natal (KZN), RSA, widespread impact of virus infections was observed. Symptoms of mosaic and yellowing, typical of viruses were detected in cucurbit-growing areas throughout KZN (Ibaba et al., 2015a; 2015b). Three potyviruses, *Zucchini yellow mosaic virus* (ZYMV), *Moroccan watermelon mosaic virus* (MWMV), and Zucchini shoestring virus (ZSSV), were the most common mosaic-inducing viruses detected during these surveys (Ibaba et al., 2015a; 2015b). It was also observed from these surveys that baby marrows (*Cucurbita pepo* L.), also known as zucchini in several parts of the world, was the most susceptible cucurbit. Diseases from infection by these potyviruses, in single or mixed infections, resulted in 100% losses in some cases. Against that background, the goal of this study was to develop baby marrow lines resistant to the potyviruses infecting cucurbits in KZN, using a single untranslatable chimeric construct made of the partial sequences of the coat proteins of the selected potyviruses.

6.2. Material and methods

6.2.1. Generation of the untranslatable chimeric transgene construct

A portion of the the 5' coding sequence of the coat protein (CP) of MWMV (216 bp), ZYMV (212) and ZSSV (198 bp) was amplified from their respective CP clones, using specific primers (Table 1) and Phire™ Hot Start II DNA Polymerase (Thermo Scientific, USA) according to the manufacturer's instructions. The CP clones of the selected viruses had been prepared previously (Chapter 2 and 5) and were kept at -20°C in labelled microfuge tubes. The Amplicon sizes, from all PCR performed towards the construction of the untranslatable chimeric transgene construct, were checked on 1.5% agarose gel in TAE buffer, pre-stained with SYBR safe (Life Technologies, USA), before downstream applications were performed. PCR reactions were cleaned-up, using DNA Clean & Concentrator™-25 (Zymo Research, USA), before performing any enzyme restriction digestion or ligation. The amplified portion of the CP of MWMV and ZYMV were first ligated to each other for 3 h at 23°C using T4 ligase

(Thermo Scientific, USA) shortly after *Xho*I digestion in individual reactions. One µl of the ligation mixture was used in 30 µl PCR to confirm successful ligation, using the forward primers I1F (Table 1) and the reverse primers I2R (Table 1). The resultant amplicon was subjected to *Sal*I digestion, separately with ZSSV's CP amplicon before being ligated using T4 ligase (Thermo-Scientific). Successful ligation of the chimeric construct (Figure 1) was established by running a PCR using 1µl of the latter ligation reaction with the forward primer I1F (Table 1) and the reverse primer I3R (Table 1). A stop codon was introduced in the primers I1F to make the chimeric construct untranslatable.

Table1. Primers and PCR settings used to get the CP portion of MWMV, ZYMV, and ZSSV.

Name	Sequence 5' – 3'	PCR conditions
*Xba*I-MWMV F (I1F)	AGAAG**TCTAGA***TAA*ACGGAGCGTTACATGCCTCG	98°C - 1s 60°C - 5s 72°C - 10s
*Xho*I-MWMV-R (I1R)	TATG**CTCGAG**CTCTCCGTGTTCTCTTCTCC	
*Xho*I-ZYMV-F (I2F)	TACA**CTCGAG**GCCGAGGTATGGTTTGCTTC	98°C - 1s 60°C - 5s 72°C - 10s
*Sal*I-ZYMV-R (I2R)	TAAA**GTCGAC**CAGTGTGCCGTTCAGTGTCT	
*Sal*I-ZSSV-F (I3F)	ATAT**GTCGAC**CACCCGTGCGACACAAGAC	98°C - 1s 60°C - 5s 72°C - 10s
*Sma*I-ZSSV-R (I3R)	ATA**CCCGGG**ACAGGTTTCAAGGGATACTCAA	

Restriction enzyme sites are in bold and termination codon is italicized.

The chimeric construct was inserted into the multiple cloning sites (MCS) of the plant expression vector pEPJ86-m/2N. pEPJ86-m/2N was designed by Jan (1998, Chapter six) and consists of an expression cassette that was inserted in the *lacZ* coding region of pUC18 (Figure 1). The expression cassette on pEPJ86-m/2N comprises a double enhancer of *Cauliflower mosaic virus* (CaMV) 35S promoter (520 bp), the 35S promoter of CaMV (94 bp), the 5'untranslated region (UTR) of *Alfalfa mosaic virus* (AlMV) CP (34 bp), the middle half N gene of *Tomato spotted wilt virus* (TSWV) which acts a silencer DNA (232 bp), an MCS, and the CaMV 35S terminator (215 bp; Figure 1).

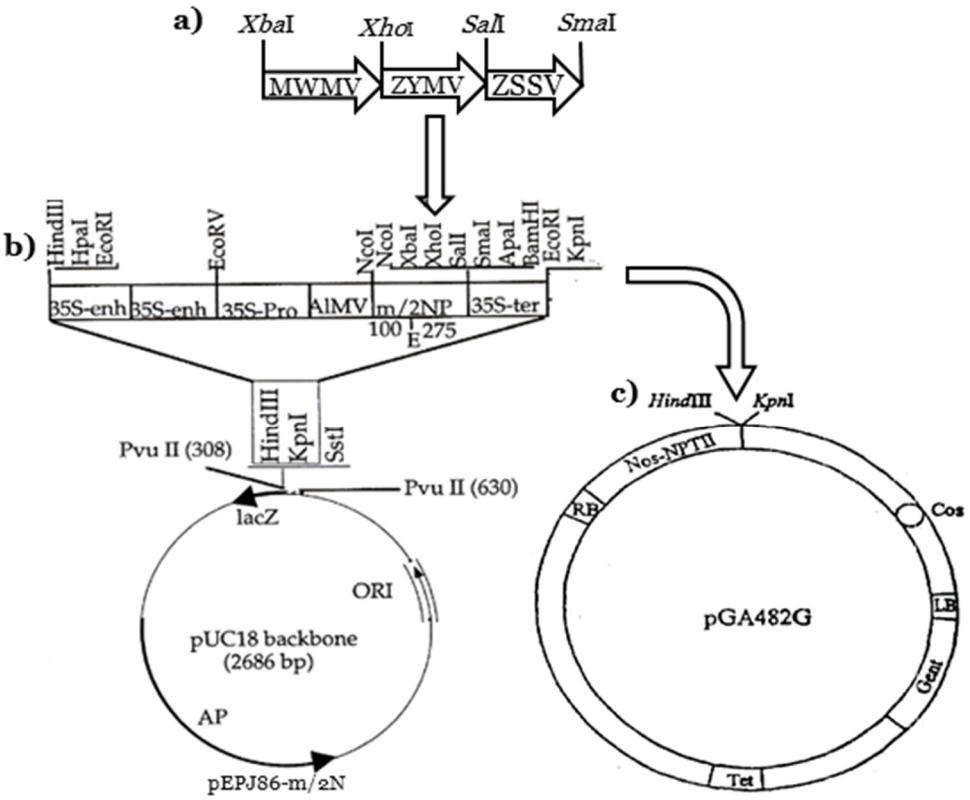

Figure 1. a) Schematic representation of the untranslatable chimeric construct containing fragments of MWMV, ZYMV, and ZSSV CP coding sequences. **b)** pEPJ86-m/2N vector map showing the expression cassette located between HindIII and KpnI restriction sites. 35S-enh: enhancer of CaMV 35S promoter, 35S-Pro: CaMV 35S promoter, AlMV: AlMV's 5'UTR, 35S-ter: CaMV 35S terminator, m/2N: middle half N gene of TSWV, AP: bacterial ampicillin resistance gene. **c)** pGA482G plant transformation binary vector. RB: right border, LB: left border, Gent: bacterial gentamicin resistance gene, Tet: bacterial tetracycline resistance gene, Nos-NPTII: Plant expressible neomycin phosphotransferase gene, cos: Enterobacteria phage λcos site.

pEPJ86-m/2N and the chimeric construct were separately subjected to double digestion with *Xba*I and *Sma*I restriction enzymes before being ligated in 10 µl reactions using T4 ligase (Thermo Scientific, USA). 1 µl of the ligation mixture was used to transform *E. cloni*® 10GF' electro-competent Cells using the MicroPulser™ Electroporator (BIO-RAD, RSA). Six colonies were subsequently picked for plasmid extraction using QIAprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer's instructions, after an overnight incubation on YT plates containing 200 mg/L ampicillin. PCR was performed on the extracted plasmid using the forward and reverse primer M13. The insertion of the chimeric construct was confirmed by performing a nested PCR, using the M13 amplicon as the template, the forward primer 97-1 (5'-TGC GCA AGC TTG TTA ACG AAT TCA ATT GAG ACT TTT CAA C-3') , and the reverse primer 97-2 (5'-ACC AGG TAC CGA ATT CTA GTA CTG GAT TTT

GGT TTT A-3'). The primers 97-1 and 97-2 amplify the whole expression cassette (Jan, 1998, Chapter Six). The cassette containing the chimeric construct was released from pEPJ86-m/2N by *Hind*III - *Kpn*I digestions and ligated with the plant transformation vector pGA482G (Jan, 1998, Chapter Six; Figure 1) that had been digested with the same restriction enzymes to generate pGA482G-POTYKZN. After incubation, the ligation mixture was cleaned up, using DNA Clean & Concentrator™-5 kit (Zymo Research, USA), before transforming *Rhizobium radiobacter* strain LBA4404 (pAL4404)(pBI121) by electroporation. Transformed *R. radiobacter* LBA4404 (pAL4404)(pBI121) that grew on YEP agar plates containing 50mg.l^{-1} gentamycin was subjected to colony PCR, using the primers 97-1 and 97-2, to identify true recombinants that were needed to transform baby marrow explants.

6.2.2. Generation of transgenic baby marrow lines

The transformation protocol described by Lin et al. (2012a) was used with minor modifications. Baby marrow seeds (8023, Starke Ayres) were manually peeled to remove their seed coats before sterilization for 5 min in 1% JIK solution followed by three washes in sterile water. The sterile seeds were placed onto MS/2 medium [MS basal medium M5519-50l (Sigma; RSA), 3% sucrose, 0.8% agar, pH 5.7] for germination under controlled environmental conditions (25 °C, 18 h light, 6 h dark cycles). The explants consisted of the junction of the hypocotyl and proximal half of the cotyledon that contain the organogenic competent cell. These explants were cut from cotyledons of 3 to 5 days old seedlings as described by Ananthakrishnan et al. (2003). The baby marrow explants were dipped in 20 ml MS medium containing 200 µl recombinant *R. radiobacter* LBA4404 (pAL4404)(pBI121) and 200 µM acetosyringone for 15 min. The explants were then transferred onto the co-culture medium [MS medium containing 1 mg.l^{-1} 6-Benzylamino-Purine (Sigma, RSA)] until they started swelling. The explants were then transferred onto the selection medium [co-culture medium containing 100 mg.l^{-1} kanamycin and 500 mg.l^{-1} cefatoxin] until adventitious shoots started appearing. Regenerated explants were transferred onto the elongation medium [MS medium containing 0.05 mg.l^{-1} 6-Benzylamino-Purine (Sigma, RSA), 1 mg.l^{-1} gibberellic Acid (Sigma, RSA), 100 mg.l^{-1} kanamycin and 500 mg.l^{-1} cefatoxin] for 4 weeks. Elongated shoots were individually transferred onto the rooting medium [MS medium containing, 1 mg.l^{-1} Indole-3-butyric acid, and 500 mg.l^{-1} cefatoxin]. Plantlets growing in rooting medium were considered to be putative transgenic marrow and were transferred onto sterile soil in 18.7 cm diameter pot and kept in a growth room, at the UKZN CEF facilities, for further assessments.

6.2.3. Test performed on the putative transgenic baby marrow lines

6.2.3.1. Screening of the putative transgenic baby marrow lines for transgene insertion

The confirmation of the insertion of the antisense transgene in putative transgenic marrow lines was carried out by PCR using Phire Plant Direct PCR kit (Thermo Scientific, USA) and the primers I1F and I3R to amplify the chimeric construct according to the manufacturer's instructions. The Thermo Scientific™ Phire™ Plant Direct PCR Kit is designed to perform PCR directly from plant leaves without prior DNA purification.

6.2.3.2. Evaluation of the virus resistance in the putative transgenic baby marrow lines

In order to evaluate the level of resistance, ZYMV, MWMV, and ZSSV were mechanically inoculated separately into all the putative transgenic baby marrow lines and non-transformed 8023 baby marrows. Each virus inoculum consisted of cucurbit leaves infected with the viruses of interest that had been collected during the virus surveys undertaken during 2011 to 2013 growing seasons. Virus infections in the collected leaf samples were confirmed using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and RT-PCR (Ibaba et al., 2015a; 2015b). The inoculum of the respective viruses was ground in 50 mM potassium phosphate buffer (pH 7.4) containing 2% sodium sulphite before being rubbed onto two different leaves that had been dusted with carborundum. Symptoms were monitored over a 7 week period. DAS-ELISA was performed on all inoculated plants to test for ZYMV and MWMV 30 days post inoculation using antibodies and positive controls of the respective viruses. ZSSV infection could not be assessed using DAS-ELISA because antibodies for this virus have not been developed yet.

6.3. Results

6.3.1. Generation of the untranslatable chimeric construct

All the PCR tests performed to evaluate the untranslatable chimeric construct were successful. Bands that matched the expected size of the portion of the CP of the viruses under study were observed on the agarose gels (Figure 2A). PCR of the ligation mixture of the portion of the CP coding sequences of MWMV and ZYMV yielded the expected amplicon of 428 bp (Figure 2B). The successful creation of the untranslatable chimeric construct was confirmed by the production of a 626 bp amplicon, expected, using the primers I1F and I3R (Figure 2C). Three out of six pEPJ86-m/2N clones had the chimeric construct since they produced amplicons with correct sizes from both PCR tests performed, using the M13F/R and 97-1/ 97-2 primers (Figure 3A and 3B). Colony PCR of 9 transformed colonies of *R. radiobacter* LBA4404 (pAL4404)(pBI121) displayed the expected amplicon (Figure 3C), confirming their successful transformation.

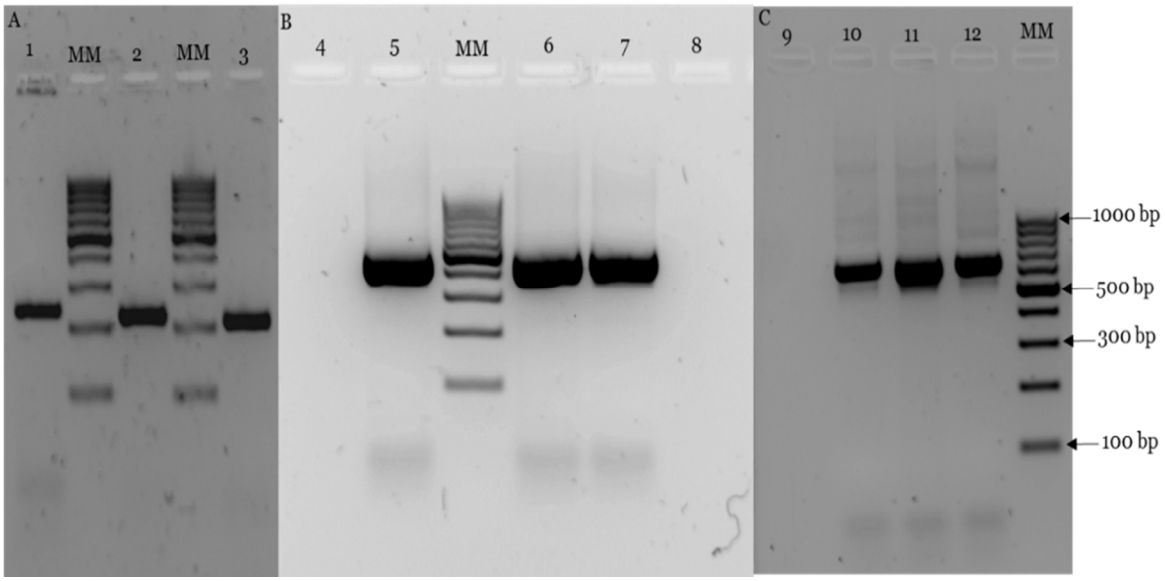

Figure 2. Generation of the untranslatable chimeric construct. **A.** amplification of the fragment CP of MWMV (lane 1), ZYMV (lane 2), and ZSSV (lane 3). **B** amplification of the ligation of (MWMV+ZYMV) CP amplicons (lanes 5, 6, 7). **C** amplification of the whole chimeric construct of the portions of the coding sequences of MWMV, ZYMV, and ZSSV CP (lanes 10 - 12). MM: 100 bp DNA ladder (Thermo Scientific, USA). Lane 9: no template control, lane 4 and 8: empty.

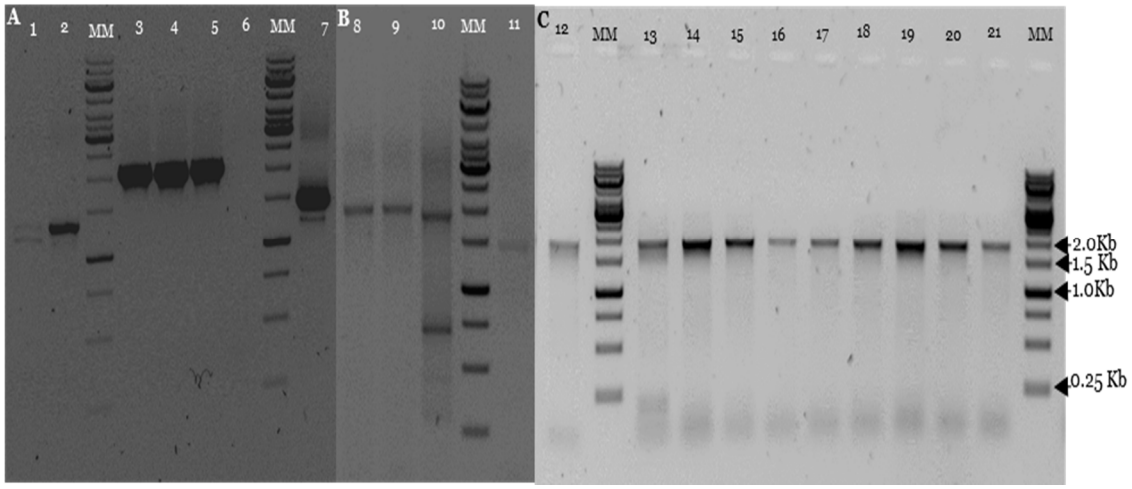

Figure 3. Detection of the untranslatable chimeric construct in the expression cassette of recombinant pEPJ86-m/2N clones using **A** M13 primers (lanes 1, 2, 3 – 6: plasmid DNA). **B** using 97-1 and 97-2 primers in nested PCR. Lanes 8-10: amplicon from lanes 3-5 as templates; Lanes 7 and 11: non-transformed and undigested pEPJ86-m/2N. **C** Colony PCR results of transformed *R. radiobacter* LBA4404 (pAL4404)(pBI121) (Lanes 13-21; lane 12: positive control from a recombinant pEPJ86-m/2N clone). MM: 1 Kb DNA ladder (Thermo Scientific).

6.3.2. Generation of the transgenic baby marrow lines

Transformed cotyledon explants of baby marrow (Figure 4A) started swelling 3 days post-incubation onto the co-cultivation medium (Figure 4B). Adventitious shoots were visible, at the junction of the hypocotyl and cotyledon (Figure 4C), at about 2 weeks post-incubation onto the selection medium. These shoots were subsequently extended on the elongation

medium (Figure 4D). Roots started developing at 2 weeks post-incubation onto the rooting medium (Figure 4E). Out of 250 transformed explants, 94 putative transgenic baby marrow lines grew well under greenhouse conditions after being transferred to soil (Figure 4F and G).

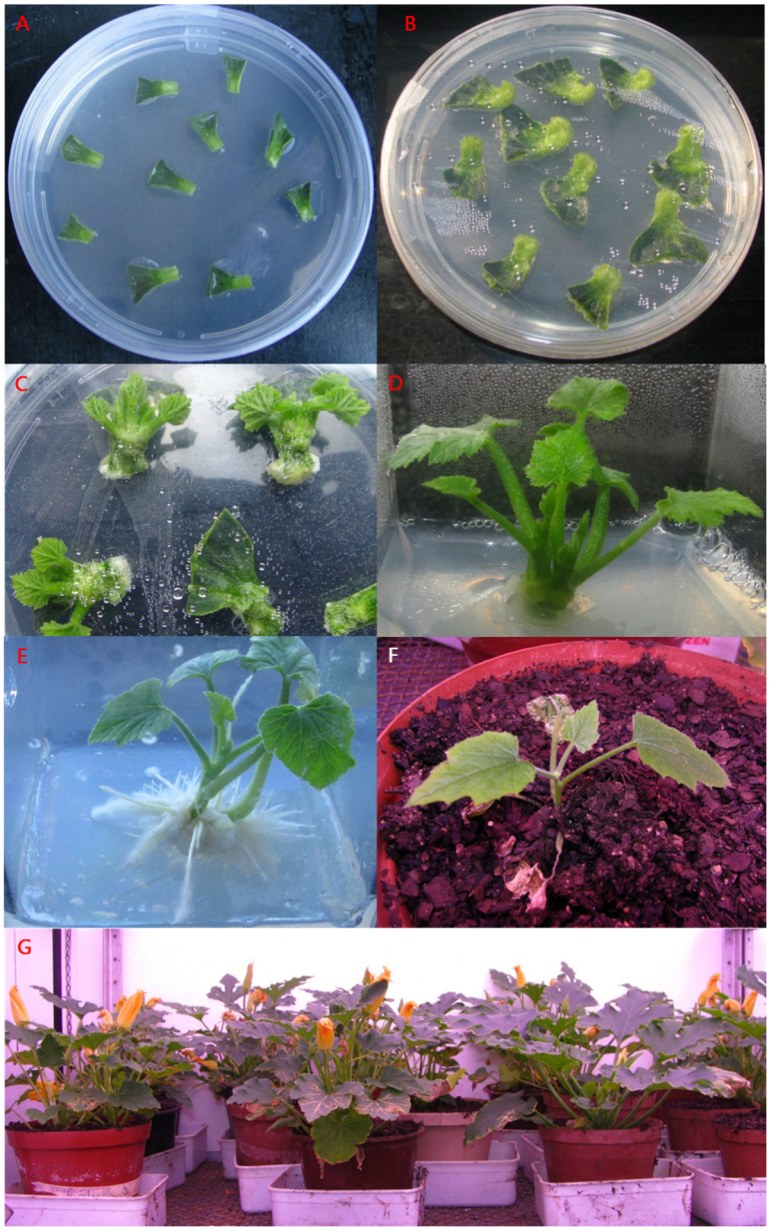

Figure 4. *In vitro* organogenesis of transgenic baby marrow lines. **A**. Baby marrow explants on the co-cultivation medium, **B**. Swollen explants on selection medium, **C**. Explants producing adventitious shoots. **D**. Shoots extending on the elongation medium and then producing roots on the rooting medium (**E**). Putative transgenic marrow lines transferred on soil (**F**), and actively growing in growth room (**G**).

6.3.3. Tests performed on the putative transgenic baby marrow lines

The 94 regenerated baby marrow lines were all tested for transgene insertion and evaluated for virus resistance. PCR results indicated that 84 of the 94 lines had the transgene because they produced the expected amplicons of 626 bp. Of these 84 lines, 76 did not display

any visual symptoms, and reacted negatively with ELISA, after being mechanically inoculated with the viruses under study (Figures 5 and 6).

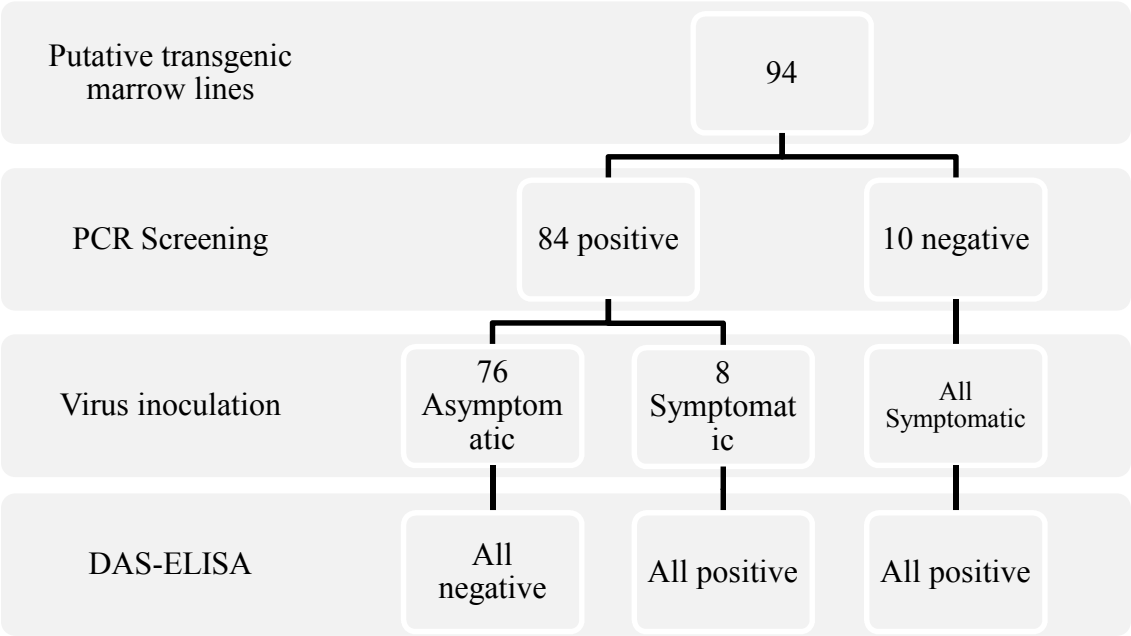

Figure 5. Results of PCR, virus inoculation, and DAS-ELISA performed on putative transgenic marrow lines.

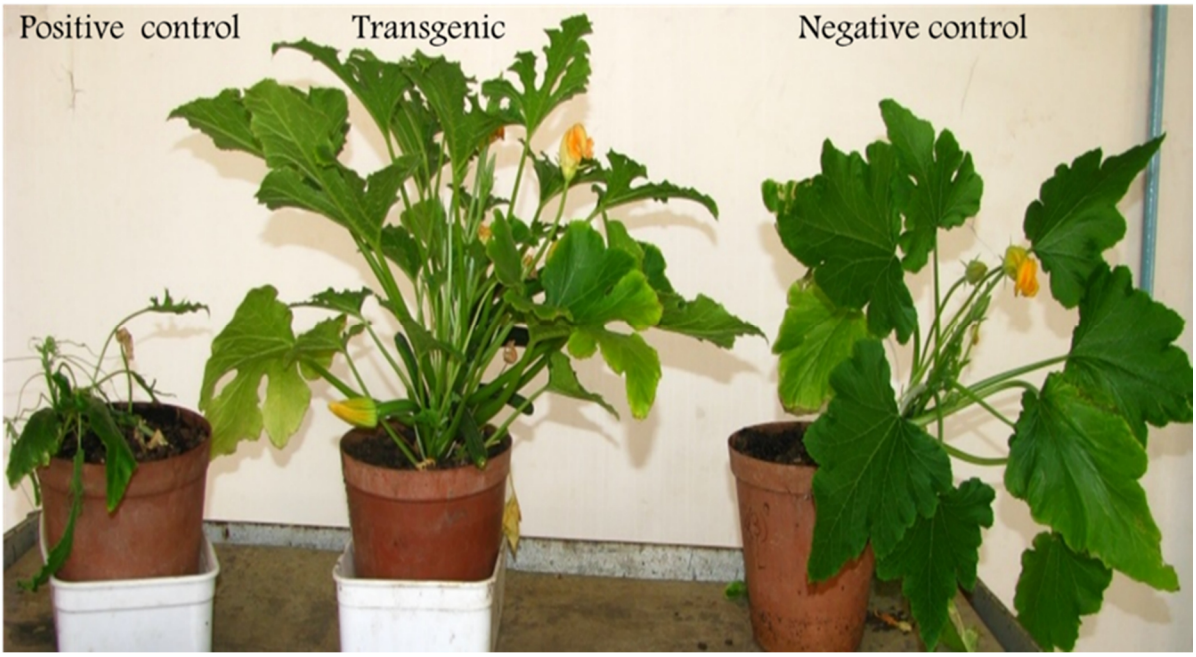

Figure 6. Virus inoculation results. The transgenic baby marrows line in the middle of the picture is looking as healthy as the non-transgenic marrow plant (Negative Control) compared to the Positive Control, which shows severe stunting and leaf filiformy. The Positive Control consists of a non-transformed marrow plant inoculated with MWMV, ZYMV, and ZSSV.

6.4. Discussion

The continued presence of cucurbit-infecting viruses across KZN is an indication of the lack of durable and strong resistance in the locally cultivated cucurbit varieties (Ibaba et al., 2015a). There is, therefore, a need to develop better strategies to manage the diseases caused by viruses on cucurbits. pGA482G-POTYKZN, a pGA482G vector containing a chimeric untranslatable construct made of a portion of the CP of the three potyvirus-infecting cucurbits in KZN, was successfully created in this study. Both pEPJ86-m/2N and pGA482G had successfully been used in several previous studies (Lin et al., 2012a; 2012b; 2011; Sivparsad and Gubba, 2014). Virus constructs for antisense post-transcriptional gene silencing studies either use a portion (Lin et al., 2012a; Yu et al., 2011) or the full coding sequence of the CP (Wu et al., 2009). The portions of the CP of three potyviruses used in this study were selected randomly.

Cotyledons have been used as the source of explants in several transgenic studies (Ananthakrishnan et al., 2003; Lin et al., 2012; Nuñez-Palenius et al., 2008; Zhang et al., 2008). The removal of the complete apical bud region from the explants was critical in ensuring that the regeneration observed was *de novo* (Ananthakrishnan et al., 2003). The 6-Benzylamino-Purine is the most popular cytokinin class of plant growth regulator used for *in vitro* regeneration of cucurbits from cotyledon explants. A concentration of 1 $mg.l^{-1}$ was enough in our experiment to trigger the production of adventitious shoots that were visible from seven days post incubation on the regeneration medium. The swelling of the explants before producing adventitious shoots is a common phenomenon in organogenesis of cucurbits using cotyledon explants (Ananthakrishnan et al., 2003; Nuñez-Palenius et al., 2008; Zhang et al., 2008). The organogenic competent cell of the baby marrow regenerated in this study was restricted to the hypocotyl and cotyledon junction because shoot production was observed only in that region of the explant. This observation has also been reported in previous studies of this kind. A total of 94 putative transgenic baby marrow lines grew on soil from an initial number of 250 explants. The putative transgenic baby marrow lines were hand-pollinated and produced seeded fruits.

The putative transgenic baby marrow plants generated in this study did not show any visual symptoms upon mechanical inoculation with the three potyviruses. Furthermore, the resistance observed on the putative transgenic baby marrow lines was confirmed by the results from the DAS-ELISA that did not detect any of the two viruses tested for. The resistance observed in virus-resistant transgenic plant developed through antisense post-transcriptional gene silencing has been attributed to the production of transgene siRNAs. siRNAs are generally detected, in virus-resistant transgenic plants, by Northern blots analysis of small RNAs. Since

a Northern blot test was not performed in this study, we can only speculate on the mechanism triggering the resistance observed in the transgenic marrow lines. However, considering that the production of transgene siRNAs as the mechanism of resistance was proven in previous studies involving pEPJ86-m/2N pGA482G (Lin et al., 2012a; 2012b; 2011), it is likely that the resistance observed in the transgenic marrow lines against MWMV, ZYMV and ZSSV was triggered by siRNAs through PTGS.

6.5. Conclusion

Our findings should be considered as a first attempt towards developing transgenic virus-resistant cucurbits. Our preliminary results confirmed the findings of previous studies that showed the potential of using transgenic cucurbits with resistance to three potyviruses and that this is an effective strategy to control potyvirus diseases on cucurbits. There is a need to carry out further tests on the putative transgenic plants developed in this study. These tests that include Northern and Southern blots will provide more evidence to the transgenic status of the baby marrow plants developed in this study.

6.6. Acknowledgements

The plant expression vector pEPJ86-m/2N and the plant transformation vector pGA482G were kindly provided by Emeritus Professor Dennis Gonsalves (Cornell University). Antibodies and positive controls used for DAS-ELISA were provided by Hervé Lecoq (INRA France).

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Thesis Overview

The experiments conducted in this thesis were initiated with regards to the increasing number of reported viruses infecting cucurbits worldwide. The first four research chapters, *i.e.* Chapters 2-5, depict a comprehensive status of the viruses infecting cucurbits in the Province of KwaZulu-Natal (KZN) in the Republic of South Africa (RSA). The information generated from theses chapters will contribute to the body of knowledge of viruses infecting cucurbits locally and internationally. Two articles, Ibaba et al., 2015a and 2015b, have already been published out of the data generated from these chapters. Chapter 2 to 5 have been written as discrete research papers and have since been submitted to different peer-reviewed journals.

Viruses infecting cucurbits in KZN

The information on the viruses infecting cucurbits in this thesis underlined how serious the virus disease problem is for farmers growing cucurbits in KZN. The seriousness of this situation was demonstrated not only by the continuing presence of known viruses infecting cucurbits but also by the detection of new viruses. Two viruses, Pepo aphid-borne yellows virus (PABYV) and Zucchini shoestring virus (ZSSV), were reported in this thesis as novel viruses in their respective genus of viruses. The discovery of these viruses put the number of the known viruses infecting cucurbits in KZN to six. The study relied on new technologies available in molecular biology to characterize the novel virus species, especially Next Generation Sequencing (NGS), for the sequencing of the nucleic acids of the viruses. In this thesis, NGS was used to confirm the taxonomic rank of the two novel cucurbit-infecting viruses that had been detected, and to establish the full genome sequence of two isolates of a potyvirus that is highly prevalent in the KZN.

Development of transgenic baby marrow lines with broad resistance to potyviruses occurring in KZN

Virus diseases of cucurbits, especially those caused by potyviruses, are a serious concern for all farmers in KZN. This situation necessitates exploring alternatives methods of controlling these viral diseases. Antisense post-transcriptional gene silencing has been shown to be an efficient way of conferring virus resistance in plants. Moreover, antisense post-transcriptional gene silencing has the added advantage of allowing the development of plants resistant to more than one virus. It was with that regards that transgenic baby marrow lines resistant to the three potyviruses prevalent in KZN were developed, using the principles of antisense post-transcriptional gene silencing (Chapter 6). To our knowledge, this is the first time that such project has been undertaken in RSA with regards to cucurbits-infecting viruses.

The way forward for the transgenic baby marrow plants and the viruses infecting cucurbits

Three important tests, the Southern and Northern blots, are still to be done. The Southern blots will give an indication on the integration of the transgene. Reverse-transcription polymerase chain reaction polymerase to check the expression of the antisense transgens. The Northern blot, performed on small RNA, will establish PTGS as the mechanism conferring resistance in the putative transgenic baby marrow lines. The same tests should also be performed on their progeny to confirm that the transgenes are stable.

Cucurbits are cultivated throughout RSA, including subsistence crops such as pumpkins, and commercial crops such as butternut gem squash and baby marrow. It would be interesting to conduct a country-wide survey to determine the status of the viruses infecting cucurbits of the other eight provinces. The information generated from such a study would be invaluable to creating a complete picture of presence and distribution of cucurbit viruses across RSA.

Zucchini shoestring virus (ZSSV) is a novel potyvirus that was identified from the analyses performed in this thesis. Although ZSSV has been associated with severe leaf filiformy in baby marrow, it was not possible to attribute this symptom to ZSSV alone. Establishing the aetiology of ZSSV is our next objective towards understanding ZSSV.

Management of viral diseases is also achieved by controlling virus vectors. However, there is very limited information on the transmission of several viruses reported in RSA including the viruses infecting cucurbits. Developing knowledge of the vectors transmitting viruses infecting cucurbits in RSA may contribute in designing integrated control strategies against viral diseases of cucurbits.

In conclusion, the information provided in this thesis has enriched the body of knowledge on viruses infecting cucurbits in KZN and, at the same time, laid a platform for developing sustainable and effective control strategies for the diseases caused by these viruses.

References

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