# THE DEVELOPMENT OF TRANSGENIC SWEET POTATO (*Ipomoea batatas* L.) WITH BROAD VIRUS RESISTANCE IN SOUTH AFRICA

by

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# FRONTISPIECE


Effect of Sweet potato virus disease (SPVD) on storage roots of sweet potato (*Ipomoea batatas* Lam.) plants grown on small-scale farms in KwaZulu-Natal (KZN).


Comparison of virus-challenged non-transgenic and transgenic sweet potato (*Ipomoea batatas* Lam.) plants.

# ABSTRACT

Sweet potato (*Ipomoea batatas* Lam.) is ranked as the seventh most important food crop in the world and its large biomass and nutrient production give it a unique role in famine relief. However, multiple virus infection is the main disease limiting factor in sweet potato production worldwide. The main objective of this research project was to develop a transgenic sweet potato cultivar with broad virus resistance in South Africa (SA).

A review of current literature assembled background information pertaining to the origin, distribution and importance of the sweet potato crop; viruses and complexes infecting sweet potato; and the strategies used in sweet potato virus detection and control.

A survey to determine the occurrence and distribution of viruses infecting sweet potato (*Ipomoea batatas* Lam.) was conducted in major sweet potato-growing areas in KwaZulu-Natal (KZN). A total of 84 symptomatic vine samples were collected and graft inoculated onto universal indicator plants, *Ipomoea setosa* Ker. and *Ipomoea nil* Lam. Six weeks post inoculation, typical sweet potato virus-like symptoms of chlorotic flecking, severe leaf deformation, stunting, chlorotic mosaic, and distinct interveinal chlorotic patterns were observed on indicator plants. Under the transmission electron microscope (TEM), negatively stained preparations of crude leaf sap and ultra-thin sections from symptomatic grafted *I.setosa* plants revealed the presence of elongated flexuous particles and pinwheel type inclusions bodies' that are characteristic to the cytopathology of Potyviruses. Symptomatic leaf samples from graft-inoculated *I. setosa* and *I. nil* were assayed for Sweet potato feathery mottle virus (SPFMV), Sweet potato mild mottle virus (SPMMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato chlorotic fleck virus (SPCFV), Sweet potato virus G (SPVG), Sweet potato mild speckling virus (SPMSV), Sweet potato caulimo-like virus (SPCaLV), Sweet potato latent virus (SPLV), Cucumber mosaic virus (CMV), and Sweet potato C-6 virus (C-6) using the nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA). The majority of leaf samples (52%) tested positive for virus disease and showed the

occurrence of SPFMV, SPMMV, SPCSV, SPCFV, SPVG, SPMSV, and SPCaLV. Of these 7 viruses, the most frequently detected were SPFMV (39%), SPVG (30%), followed by SPCSV (13%) and SPMMV (12%). SPCaLV and SPCFV at 10% and SPMSV at 7% were found exclusively in samples collected from one area. SPFMV, SPVG, SPCSV, and SPMMV were identified as the most prevalent viruses infecting sweet potato in KZN.

The genetic variability of the three major viruses infecting sweet potato (*Ipomoea batatas* Lam.) in KZN was determined in this study. A total of 16 virus isolates originating from three different locations (Umbumbulu, Umfume and Umphambanyomi River) in KZN were analyzed. These comprised of 10 isolates of Sweet potato feathery mottle virus (SPFMV), five isolates of Sweet potato virus G (SPVG) and one isolate of Sweet potato chlorotic stunt virus (SPCSV). The phylogenetic relationships of the SPFMV, SPVG and SPCSV isolates from KZN relative to isolates occurring in SA and different parts of the world were assessed. The division of SPFMV into four genetic groups (strains) according to the phylogenetic analysis of coat protein encoding sequences revealed mixed infections of the O (ordinary) and C (common) strains in sweet potato crops from KZN. All SPFMV isolates showed close lineage with isolates from South America, East Asia and Africa. The SPVG isolates showed high relatedness to each other and close lineage with other isolates, especially those from China and Egypt. Analysis of the partial sequence of the Heat shock protein 70 homologue (*Hsp70*h) gene indicated that the SPCSV isolate from KZN belongs to the West African (WA) strain group of SPCSV and showed close relatedness to an isolate from Argentina. The knowledge of specific viral diversity is essential in developing effective control measures against sweet potato viruses in KZN.

Multiple virus infections of Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato virus G (SPVG) and Sweet potato mild mottle virus (SPMMV) cause a devastating synergistic disease complex of sweet potato (*Ipomoea batatas* Lam.) in KZN. In order to address the problem of the multiplicity and synergism of sweet potato viruses in KZN, this study aimed to develop transgenic sweet

potato cv. Blesbok with broad virus resistance. An efficient and reproducible plant regeneration protocol for sweet potato (*Ipomoea batatas* Lam.) cultivar Blesbok was also developed in this study. The effect of different hormone combinations and type of explants on shoot regeneration was evaluated in order to optimize the regeneration protocol. Coat protein (CP) gene segments of SPFMV, SPCSV, SPVG and SPMMV were fused to a silencer DNA, the middle half of the nucleocapsid (N) gene of Tomato spotted wilt virus (TSWV) and used as a chimeric transgene in a sense orientation to induce gene silencing in the transgenic sweet potato. Transformation of apical tips of sweet potato cv. Blesbok was achieved by using *Agrobacterium tumefaciens* strain LBA4404 harboring a modified binary vector pGA482G carrying the plant expressible neomycin phosphotransferase II gene (*npt*II), the bacterial gentamycin-(3)-N-acetyl-transferase gene and the expression cassette. A total of 24 putative transgenic plants were produced from the transformed apical tips via *de novo* organogenesis and regeneration into plants under 50mg/L kanamycin and 200 mg/L carbenicillin selection. Polymerase chain reaction (PCR) and Southern blot analyses showed that six of the 24 putative transgenic plants were transgenic with two insertion loci and that all plants were derived from the same transgenic event. The six transgenic sweet potato plants were challenged by graft inoculation with SPFMV, SPCSV, SPVG and SPMMV- infected *Ipomoea setosa* Ker. Although virus presence was detected using NCM-ELISA, all transgenic plants displayed delayed and milder symptoms, of chlorosis and mottle of lower leaves when compared to the untransformed control plants. These results warrant further investigation under field conditions.

# DECLARATION

I, **Benice Sivparsad**, declare that

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

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(Supervisor)

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## DEDICATION

To my parents for all the love and encouragement throughout my studies.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AIMV | Alfalfa mosaic virus |
| ARC | Agricultural Research Council |
| BAP | 6-benzylaminopurine |
| BM | Basal media |
| bp | Base pairs |
| C | Common |
| CaMV | Cauliflower mosaic virus |
| cDNA | Complementary DNA |
| CERU | Controlled environmental research unit |
| CIP | International Potato Center |
| cos | Bacteriophage lamda cos site |
| CP | Coat protein |
| CMV | Cucumber mosaic virus |
| cv. | Cultivar |
| C-3 | Sweet potato C-3 virus |
| C-6 | Sweet potato C-6 virus |
| DAS | Double antibody sandwich |
| DCL | Dicer-like enzyme |
| DIBA | Dot blot immunobinding assay |
| DIG | Digoxigenin |
| DNA | Deoxyribonucleic acid |
| DR | Direct repeat |
| dsRNA | Double stranded RNA |
| EA | East African |
| EDTA | Ethyledediaminetetra-acetic acid disodium salt |
| ELISA | Enzyme-linked immunosorbent assay |
| EM | Electron microscope |
| G | Guanine |
| gent | Bacterial gentamycin-(3)-N-acetyl-transferase gene |

| | |
|---|---|
| GMO | Genetically modified organism |
| h | Hour |
| hpRNA | Hairpin RNA |
| hsp70h | Heat shock protein 70 homologue |
| ICLCV | Ipomoea crinkle leaf curl virus |
| ICTV | International Committee for the Taxonomy of Viruses |
| IR | Inverted repeat |
| ISEM | Immunosorbent electron microscopy |
| IYVV | Ipomoea yellow vein virus |
| KARI | Kenya Agricultural Research Institute |
| kb | Kilobases |
| kBp | Kilo base pairs |
| kDa | Kilodalton |
| KZN | KwaZulu-Natal |
| LB | Left border |
| M | Molar |
| MIBA | Membrane immunobinding assay |
| miRNA | MicroRNA |
| mRNA | messenger ribonucleic acid |
| MS | Murashige and Skoog |
| MWM | Molecular weight marker |
| m/2NP | Middle half of the nucleocapsid protein |
| N | Nucleocapsid |
| NAA | Naphthalene acetic acid |
| NCBI | National Center for Biotechnology Information |
| NCM | Nitrocellulose membrane |
| nm | nanometers |
| nptII | Neomycin phosphotransferase ll gene |
| nt | nucleotide |
| NTR | Non translatable region |
| O | Ordinary |

| | |
|---|---|
| OFSP | Orange fleshed sweet potatoes |
| PCR | Polymerase chain reaction |
| PDR | Pathogen derived resistance |
| PGR | Plant growth regulator |
| PMB | Pietermaritzburg |
| PTGS | Post transcriptional gene silencing |
| RB | Right border |
| RC | Russet crack |
| RdRp | RNA dependent RNA polymerase |
| RFLP | Restriction fragment length polymorphism |
| RH | Relative humidity |
| RIM | Root induction medium |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| rpm | revolutions per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SHP | Small hydrophobic protein |
| SIM | Shoot induction medium |
| siRNA | Small interfering RNA |
| SPCaLV | Sweet potato caulimo-like virus |
| SPCD | Sweet potato chlorotic dwarf disease |
| SPCFV | Sweet potato chlorotic fleck virus |
| SPCSV | Sweet potato chlorotic stunt virus |
| SPFMV | Sweet potato feathery mottle virus |
| SPLV | Sweet potato latent virus |
| SPLCV | Sweet potato leaf curl virus |
| SPLCGV | Sweet potato leaf curl Georgia virus |
| SPLCSCV | Sweet potato leaf curl South Carolina virus |
| SPLSV | Sweet potato leaf speckling virus |
| SPMMV | Sweet potato mild mottle virus |

| | |
|---|---|
| SPMSV | Sweet potato mild speckling virus |
| SPRSV | Sweet potato ringspot virus |
| SPSMD | Sweet potato severe mosaic disease |
| SPVCV | Sweet potato vein clearing virus |
| SPVD | Sweet potato virus disease |
| SPVG | Sweet potato virus G |
| SPV2 | Sweet potato virus 2 |
| SPVMV | Sweet potato vein mosaic virus |
| SPYDV | Sweet potato yellow dwarf virus |
| T | Thymine |
| TEM | Transmission electron microscope |
| Ter | Terminator |
| Tet | Bacterial tetracycline resistance gene |
| TGS | Transcriptional gene silencing |
| TMV | Tobacco mosaic virus |
| ToLTWCV | Tomato leaf curl Taiwan virus |
| TSV | Tobacco streak virus |
| TSWV | Tomato spotted wilt virus |
| TuMV | Turnip mosaic virus |
| µg | Microgram |
| UKZN | University of KwaZulu-Natal |
| µl | Microliter |
| µm | Micrometer |
| UV | Ultraviolet |
| V | Volts |
| WA | West African |
| X-Gal | 5-bromo-4-chloro-3-indoyl-ß-D-galactoside |
| YT | Yeast Tryptone |

# INTRODUCTION

## Global perspective on plant viruses

Pests and pathogens affect virtually all crops, causing substantial and often devastating losses. In terms of economic importance, viral crop diseases rank second only to those caused by fungi. Actual losses due to plant viruses are difficult to assess, but estimates indicate total economic damage as high as several billion U.S. dollars per year (Waterworth & Hadidi, 1998). However, due to the insidious nature of these pathogens, it is likely that they are responsible for far greater losses than generally recognized. Losses due to viruses are of particular importance in developing countries that are highly dependent on agricultural production for food security, employment and export earnings (Thresh, 2003). At present, over a thousand plant viruses have been recognized worldwide. For the past decades, plant virologists have provided information on the various strategies to control these viruses. Some viruses may be controlled using virus-free plant material, excluding or minimizing virus inoculum from a geographical area, or by incorporating virus-resistant genes into plants. However, these strategies have been met with limited success due to the rapid spread of viruses into new areas, lack of effective certification and/or quarantine programs, lack of sources of resistance in many commercial cultivars, and frequent breakdowns of resistance by the emergence of virulent strains of the virus (Waterworth & Hadidi, 1998). The recent advent of genetically engineered resistance has shown great promise as one of the major strategies for controlling plant viruses. For this reason, a growing number of plant virologists are directing their efforts towards developing and producing genetically engineered virus-resistant plants.

## Problem identification

Africa is a continent rich in human and natural resources. Yet, it is also a hungry continent where because of famine, disease and rapidly growing populations, almost 200 million people are undernourished and 33 million children go to sleep malnourished

and hungry every night (Thomson, 2008; Eicher *et al*., 2009). In the constant quest for food and the struggle for human survival, the sweet potato has historically played a critical role as a food staple for many people in Africa (Loebenstein *et al*., 2009).

In South Africa (SA), sweet potato is predominately grown by small-scale farmers (Du Plooy *et al*., 1996). Because of its good performance under adverse farming conditions and high carbohydrate and vitamin content, the sweet potato has been identified as an important source of nutrition and food security among poor and rural households in SA (Naylor *et al*., 2004). The incidence of viral disease in major sweet potato growing provinces has hindered successful cultivation and production of this important crop. Due to vegetative propagation of sweet potato, pathogens can accumulate in the planting material resulting in a decline in yield and/or quality of the crop over time (Clark *et al*., 2002). This phenomenon, commonly referred to as 'cultivar decline', has been experienced by many sweet potato farmers across SA. Soon after the release of sweet potato cultivars by the Agricultural Research Council (ARC)-Roodeplaat in 1960, a progressive degeneration in yield and quality was encountered. The decline was attributed to virus infection and was of such degree that commercial production became uneconomical and popular cultivars were wiped out of the market (Laurie *et al*., 2002). Moreover, the problem is exacerbated by the frequent occurrence of multiple viruses infecting sweet potato in SA (Domola *et al*., 2008). Such synergistic viral complexes result in a substantial reduction in yield of up to 90% (Clark *et al*., 2002).

Despite significant efforts in conventional breeding and production of clean planting materials, viral diseases still remain a threat to sweet potato production in SA. Biotechnology-based strategies have the potential to serve as valuable intervention in sweet potato virus control. Current genetic engineering efforts to develop sweet potatoes with virus resistance have used the coat protein gene of SPFMV and/or SPCSV (Loebenstein *et al*., 2002). However, given the multiplicity of viruses occurring under field conditions, this approach has had limited success. Therefore, future efforts should focus on developing a transgenic sweet potato cultivar with resistance to multiple virus infection.

## Project objectives

Genetic engineering for virus resistance has rarely been applied to sweet potato. The few attempted transgenic approaches have not resulted in sweet potato cultivars with sustainable resistance to multiple viruses (Loebenstein *et al*., 2002). Against this backdrop, the general objective of this project was to develop a transgenic sweet potato cultivar with broad virus resistance in KwaZulu-Natal (KZN), SA. In order to achieve this, certain specific objectives had to have been met. These objectives included:

1) Identification and characterization of viruses infecting sweet potato in KZN
2) Phylogenetic analysis of the most prevalent viruses infecting sweet potato in KZN
3) Development of an efficient tissue culture protocol for the regeneration of sweet potato
4) Designing a segmented gene construct using portions of the coat protein genes of the most prevalent viruses infecting sweet potato in KZN
5) Ligation of these constructs into expression and transformation vectors
6) *Agrobacterium*-mediated transformation of the construct into sweet potato
7) Greenhouse evaluation of transformed cultivars for virus resistance

## Organization of the dissertation

This dissertation is organized into five chapters. Chapter 1 presents a review of current literature pertaining to the origin, distribution and importance of the sweet potato crop; viruses and virus complexes infecting sweet potato; and the strategies used in sweet potato virus detection and control. Chapters 2 and 3 address specific objectives 1 and 2 respectively; Chapter 4 covers objectives 3-7. Chapter 5 provides an overview of the major findings of the research, identifies information gaps, and suggests future research to fill these gaps.

## References


**Clark, C. A, Valverde, R. A., Fuentes, S., Salazar, L. F. & Moyer, J. W. (2002).** Research for improved management of sweet potato pests and diseases: cultivar decline. *Acta Horticulturae* **583,** 103-113.

**Domola, M. J., Thompson, G. J., Aveling, T. A. S., Laurie, S. M., Strydom, H. & Van den Berg, A. A. (2008).** Sweet potato viruses in South Africa and the effect of viral infection on storage root yield. *African Plant Protection* **14,** 15-23.

**Du Plooy, C. P., Wagner, J. J. & Bosch, S. E. (1996).** Cultivation of sweet potato. In *Vegetable Production in South Africa,* pp. 1-7. Pretoria: ARC Roodeplaat.

**Eicher, C. K., Maredia, K. & Sithole-Niang, I. (2009).** Crop biotechnology and the African farmer. *Food Policy* **31,** 504-527.

**Laurie, L. M. (2002).** Overview of breeding and evaluation of orange-fleshed sweet potato in South Africa. http://www.cipotato.org/Vitaa?Proceedings/VITAA-paper-SLAURIE-FINAL-11Feb2002.pdf.

**Loebenstein, G., Thottappilly, G., Fuentes, S. & Cohen, J. (2009).** Viruses and phytoplasma diseases. In *The Sweet Potato*, pp. 114. Edited by Loebenstein, G. & Thottappilly, G. The Netherlands: Springer publications.

**Naylor, R. L., Falcon, W. P., Goodman, R. M. Jahn, M. M., Sengooba, T., Telera, H. & Nelson, R. J. (2004).** Biotechnology in the developing world: a case for increased investments in orphan crops. *Food Policy* **29,** 15-44.

**Thomson, J. A. (2008).** The role of biotechnology for agricultural sustainability in Africa. *Philosophical Transactions of the Royal Society B* **363,** 905-913.

**Thresh, J. M. (2003).** The impact of plant virus diseases in developing countries. In *Viruses and Virus-like Diseases of Major Crops in Developing Countries*, pp. 223-226. Edited by Loebenstein G. & Thottappilly G. USA: Kluwer Academic Publishers.

**Waterworth, H. E. & Hadidi, A. (1998).** Economic losses due to plant viruses. In *Plant Virus Disease Control*, pp. 1-13. Edited by Hadidi, A., Khetarpal, R. K. & Koganezawa, H. USA: APS Press.

# CHAPTER ONE
# LITERATURE REVIEW

## 1.1 GENERAL INTRODUCTION

Root and tuber crops are basic to the diets of millions of people in developing countries. Among these crops, sweet potato (*Ipomoea batatas* Lam.) is one of the world's highest yielding crops with total food production per unit area exceeding that of rice (Woolfe, 1992). Its high nutritional value coupled to its good quality performance under marginal and low input conditions; give it exciting potential in combating food shortage and malnutrition in Africa (Wambugu, 2003). In South Africa (SA), the crop is mainly grown at subsistence level and provides household security to many resource-poor farmers.

Infection by viruses is a key constraint to sweet potato production in SA. Sweet potatoes are vegetatively propagated and farmers from SA and other countries often propagate vines from their own field year after year. Thus, virus diseases are inevitably transmitted with the propagation material to the newly planted field, resulting in a decreased yield. Often, these fields are infected by multiple viruses interacting in a synergistic complex, thereby compounding the effect on yields (Loebenstein *et al*., 2009).

Detection and control of sweet potato viruses is complicated by the frequent occurrence of mixed infections, synergistic complexes and the constant emergence of new viral species and strains (Tairo, 2006; Gutièrrez & Valverde, 2007). Progress made in the control of sweet potato viruses through cultural means and conventional breeding has been relatively slow. Genetic engineering of sweet potato plants with virus resistance can accelerate crop improvement and ensure future food security in SA.

The chapter presents a review of current literature pertaining to the origin, distribution and importance of the sweet potato crop; viruses and complexes infecting sweet potato; and the strategies used in sweet potato virus detection and control.

## 1.2 THE SWEET POTATO CROP

Sweet potato (*Ipomoea batatas* Lam.) is a dicotyledonous, perennial plant that produces edible tuberous roots and leaves. It belongs to the *Convolvulaceae* or Morning glory family in the genus *Ipomoea* (Austin, 1987). Of the 55 genera and more than 1000 species of the *Convolvulaceae*, the sweet potato is the only food crop of economic importance (Woolfe, 1992).

### 1.2.1 Physiology and Cultivation

The sweet potato plant is a herbaceous vine bearing alternate leaves and trumpet-shaped flowers. The edible tuberous roots can vary in shape and colour according to cultivar and environmental conditions. These roots are normally long and tapered with a smooth skin whose colour ranges between cream, yellow, orange, brown, pink, red and purple. The flesh colour can be white, cream, yellow or orange depending on the cultivar and the amounts of carotenoid (orange) and anthocyanin (purple) pigments present (Laurie & Niederwieser, 2004).

Sweet potato is a versatile and hardy crop that grows best in warm tropical climates at average temperatures of 24$^{o}$C (Van den Berg & Laurie, 2004). It performs well in relatively poor soils with few inputs and has a short growing season (Jana, 1982). Due to its resilient and adaptable nature, it can be grown in several agroecological zones hence providing farmers with an 'insurance crop' to fall back on when other crops fail (Jana, 1982; Laurie, 2004; Tairo, 2006).

### 1.2.2 Origin and Distribution

Sweet potato is believed to have originated from central or northern South America at least 5000 years ago, (O' Brien, 1972; Srisuwan *et al*., 2006; Rännäli *et al*., 2008) and may be one of the earliest domesticated plants (Austin, 1987). By the time of European contact, sweet potato was cultivated throughout the American tropics and had spread to

the Easter Islands, Hawaii and New Zealand (Austin, 1987; Srisuwan *et al*., 2006). It was rapidly introduced to Europe after the first voyage of Columbus in 1492 (O'Brien, 1972; Srisuwan *et al*., 2006). In the 16$^{th}$ century, Portuguese explorers transferred sweet potato to Africa, India, South East Asia and the East Indies (Austin, 1987; Srisuwan *et al*., 2006). Today, thousands of cultivars of sweet potato are grown in all tropical and subtropical climatic regions of the world (Van den Berg & Laurie, 2004).

### 1.2.3 Economic Importance

Sweet potato, with an annual production of 150 million tons (Mt) globally in 2010, is ranked as the seventh most important food crop in the world (Woolfe, 1992; FAOSTAT, 2011). It is the third most important root crop grown globally after potato (*Solanum tuberosum* L.) and cassava (*Manihot esculenta* Crantz) (FAO, 2004). Among the major starch staples, sweet potato has the largest rates of biomass and nutrient production per unit area per unit time (Woolfe, 1992), making it one of the crops with a unique role in famine relief (Karyeija *et al*., 1998; Loebenstein *et al*., 2009).

Although sweet potatoes are grown in over 100 countries worldwide, developing countries account for over 95% of the total production (FAO, 2004). The annual world production was 140 million tons in 2009 with 91% produced in Asia (128.8Mt), 7% in Africa (9.1Mt), 1% in Central/ North America (1.1Mt), 1% in South America (1.2Mt), 0.5% in Oceania (0.59Mt) and only 46,000t in Europe (Laurie, 2004; FAOSTAT, 2011). China is the biggest sweet potato producer country in the world, accounting for 90% of the world production (Wang *et al*., 2010). In addition to human consumption, sweet potato is also used as livestock feed, raw material for alcohol production and processed industrial products (Srisuwan *et al*., 2006; Tairo, 2006). Uganda is the biggest producer of sweet potato in Africa, where is predominately grown as a household subsistence crop (FAO, 2004; Aritua *et al*., 2006).

Sweet potatoes are a good source of carbohydrates and vitamins, and both the storage root and foliage are nutritious foods. Orange-fleshed sweet potatoes (OFSP) are

particularly nutritious, ranking highest in nutrient content of all vegetables for vitamins A and C, folate, iron, copper, calcium and fiber (Woolfe, 1992; Mwanga *et al*., 2002). However, the major nutritional element of OFSP is the caroteniod, ß-carotene, a vitamin A precursor (Woolfe, 1992; Allemann *et al*., 2004). Vitamin A (retinol) is essential for the proper functioning of the immune system, the retina and mucous membranes. A deficiency in vitamin A is the principle cause of childhood blindness in developing countries (Ziegler, 2001). OFSP has been recognized as a potential means for saving thousands of children in Africa from vitamin A deficiency-related diseases such as night blindness (Van Jaarsveld *et al*., 2005; Kapinga *et al*., 2009). A study in South Africa has shown that daily consumption of OFSP provided about 2.5 times the vitamin A requirement for 4-8 year old children, and improved their liver vitamin A stores (Van Jaarsveld *et al*., 2005).

The low agricultural input requirement coupled to high productivity and good nutritional value make sweet potato an ideal starch staple in subsistence economies (Mukasa *et al*., 2003; Wambugu, 2003).

### 1.2.4 Sweet Potato in South Africa

Sweet potato was introduced to South Africa after Jan Van Riebeeck colonized the Cape in 1652 (Du Plooy, 1986; Laurie & Niederwieser, 2004). Today, it is grown by both small-scale and commercial farmers in all provinces of SA with the major production areas being Limpopo, Mpumalanga, Western and Eastern Cape, KwaZulu-Natal and Free State provinces (Du Plooy *et al*., 1996; Laurie, 2004).

In total, 22 commercial SA cultivars have been released by a sweet potato breeding programme created by the ARC-Roodeplaat Vegetable and Ornamental Plant Institute. The popular leading cultivars Blesbok, Bosbok, Ribbok, Koedoe and Mafuta have all been bred at ARC-Roodeplaat (Laurie, 2004). The cultivar Blesbok, released in 1989 is a top selling cultivar, accounting for 80% of national production with yields averaging 45 t/ha when grown commercially (Laurie, 2002).

South Africa has an annual production of 62,688 tonnes (FAOSTAT, 2011). However, large quantities of sweet potato are produced and sold by the informal sector, which is not reflected in the official figures (Alleman *et al*., 2004; Laurie, 2004). Sweet potato plays an important role as a food security crop in resource-poor farming in SA. Therefore, the total production can be estimated at 120 000 tonnes per annum (Alleman *et al*., 2004). The sweet potato industry in SA is, however, small compared to that of other developed countries and there is still vast potential for expansion (Du Plooy *et al*., 1996).

## 1.3 VIRUSES INFECTING SWEET POTATO

Virus infection is the main disease limiting factor in sweet potato production worldwide (Allemann *et al*., 2004). Moreover, viral diseases rank second after sweet potato weevils as biotic factors and can cause considerable yield reduction of up to 98% in sweet potato cultivars (Carroll *et al*., 2004; Aritua *et al*., 2006). Worldwide, at least 20 different viruses have been reported to infect sweet potato (Table 1.1) (Kreuze & Fuentes, 2008; Loebenstein *et al*., 2009). However, this number will increase with additional surveys and indexing germplasm collections (Karyeija *et al*., 1998; Kreuze & Fuentes, 2008). In SA, resource-poor farmers usually obtain low yields due to virus infection. This is a result of vegetative propagation of the crop causing a build-up of virus infection (Allemann *et al*., 2004; Laurie *et al*., 2001). Samples collected during a recent field survey in SA, reported that 81% of cuttings were found to be positive for virus infection and nine viruses have been identified in sweet potato in SA (Domola *et al*., 2008). Sweet potato feathery mottle virus (SPFMV), Sweet potato mild mottle virus (SPMMV), Sweet potato virus G (SPVG), and Sweet potato latent virus (SPLV) have been recorded as the most common viruses infecting sweet potato in SA (Thompson & Mynhardt, 1986; Jericho & Thompson, 2000; Domola *et al*., 2008).

### 1.3.1 Sweet potato feathery mottle virus (SPFMV)

Sweet potato feathery mottle virus (SPFMV, genus *Potyvirus*, family *Potyviridae*) is the most common sweet potato virus, found nearly everywhere sweet potatoes are grown (Moyer & Salazar, 1989; Karyeija *et al*., 1998; Kreuze & Fuentes, 2008). Some of the synonyms used for SPFMV include russet crack virus, sweet potato virus A, sweet potato ringspot virus, sweet potato leaf cork virus and internal cork virus (Clark & Moyer, 1988; Moyer & Salazar, 1989).

SPFMV has flexuous filamentous particles between 830-850nm in length. They contain a single-stranded, positive-sense RNA genome of about 10.6kb and a coat protein (CP) of 38kDa, both of which are larger than the genome and CP of the average potyvirus (Loebenstein *et al*., 2003; Kreuze & Fuentes, 2008). Transmission of SPFMV occurs by several species of aphids in a non-persistent manner (Clark & Moyer, 1988; Karyeija *et al*., 1998). The virus is transmitted by grafting but not by seed, pollen or by contact between plants (Loebenstein *et al*., 2003). SPFMV has a narrower host range than most potyviruses and is mostly limited to the family *Convolvulaceae*, and especially to the genus *Ipomoea*, although some strains have been reported to infect *Nicotiana benthamiana* Gray and *Chenopodium* spp (Kreuze & Fuentes, 2008). Symptoms, serology and host range have been used to differentiate SPFMV isolates into two strains: the common (C) strain and the more severe russet crack (RC) strain (Karyeija *et al*., 1998). However, phylogenetic analyses of the CP sequences have distinguished SPFMV into four phylogenetic lineages: RC, O (ordinary), EA (East Africa), and C (Kreuze *et al*., 2000; Mukasa *et al*., 2003).

Leaf symptoms in sweet potato are generally mild and may consist of the classic irregular chlorotic patterns (feathery mottle; Fig. 1.1A), chlorosis of older leaves and vein clearing (Clark & Moyer, 1988; Moyer & Salazar, 1989; Karyeija *et al*., 1998). Some infected plants may even be symptomless (Moyer and Salazar, 1989; Gibson *et al*., 1997). In other *Ipomoea* species, including the indicator plants *I. setosa* and *I. nil*, symptoms of vein clearing (Fig. 1.1B), mosaic and distortion are more pronounced

(Karyeija *et al*., 1998). Some strains of SPFMV cause necrotic lesions on the root exterior (russet crack disease, Fig. 1.1C), while another strain produces symptoms on the root interior (internal cork disease) (Fig. 1.1D; Clark & Moyer, 1988; Moyer & Salazar, 1989). The main economic loss due to SPFMV is when it acts with Sweet potato chlorotic stunt virus (SPCSV) in a synergistic virus complex known as sweet potato virus disease (SPVD) (Karyeija *et al*., 1998; Kreuze & Fuentes, 2008).

### 1.3.2 Sweet potato chlorotic stunt virus (SPCSV)

Sweet potato chlorotic stunt virus (SPCSV, genus *Crinivirus*, family *Closteroviridae*) is one of the most devastating viruses infecting sweet potato worldwide (Winter *et al*., 1992; Mukasa *et al*., 2006; Untiveros *et al*., 2007). In single infections SPCSV can reduce yields by 50%, causing mild stunting combined with slight yellowing or purpling of older leaves (Kreuze & Fuentes, 2008). However, what makes SPCSV one of the most damaging viruses of sweet potato is its ability to break down the natural resistance of sweet potato to other viruses and mediate severe synergistic viral diseases with other sweet potato viruses (Karyeija *et al*., 2000b; Kreuze *et al*., 2008). The most common and severe of these diseases is SPVD and is caused by co-infection with SPFMV (Gibson *et al*., 1998; Karyeija *et al*., 1998; Kreuze *et al*., 2008).

SPCSV, previously known as Sweet potato sunken vein virus, is phloem-limited and is transmitted in semi-persistent manner by whiteflies (Cohen *et al*., 1992; Loebenstein *et al*., 2003; Valverde *et al*., 2004a; Gamarra *et al*., 2010). Transmission can occur through grafting but not by mechanical inoculation or by contact between plants. Similar to most sweet potato-infecting viruses, the host range of SPCSV is limited mainly to the family *Convolvulaceae*, and the genus *Ipomoea*, although *Nicotiana* spp. and *Amaranthus palmeri* are susceptible (Brunt *et al*., 1996; Loebenstein *et al*., 2003; Kreuze & Fuentes, 2008).

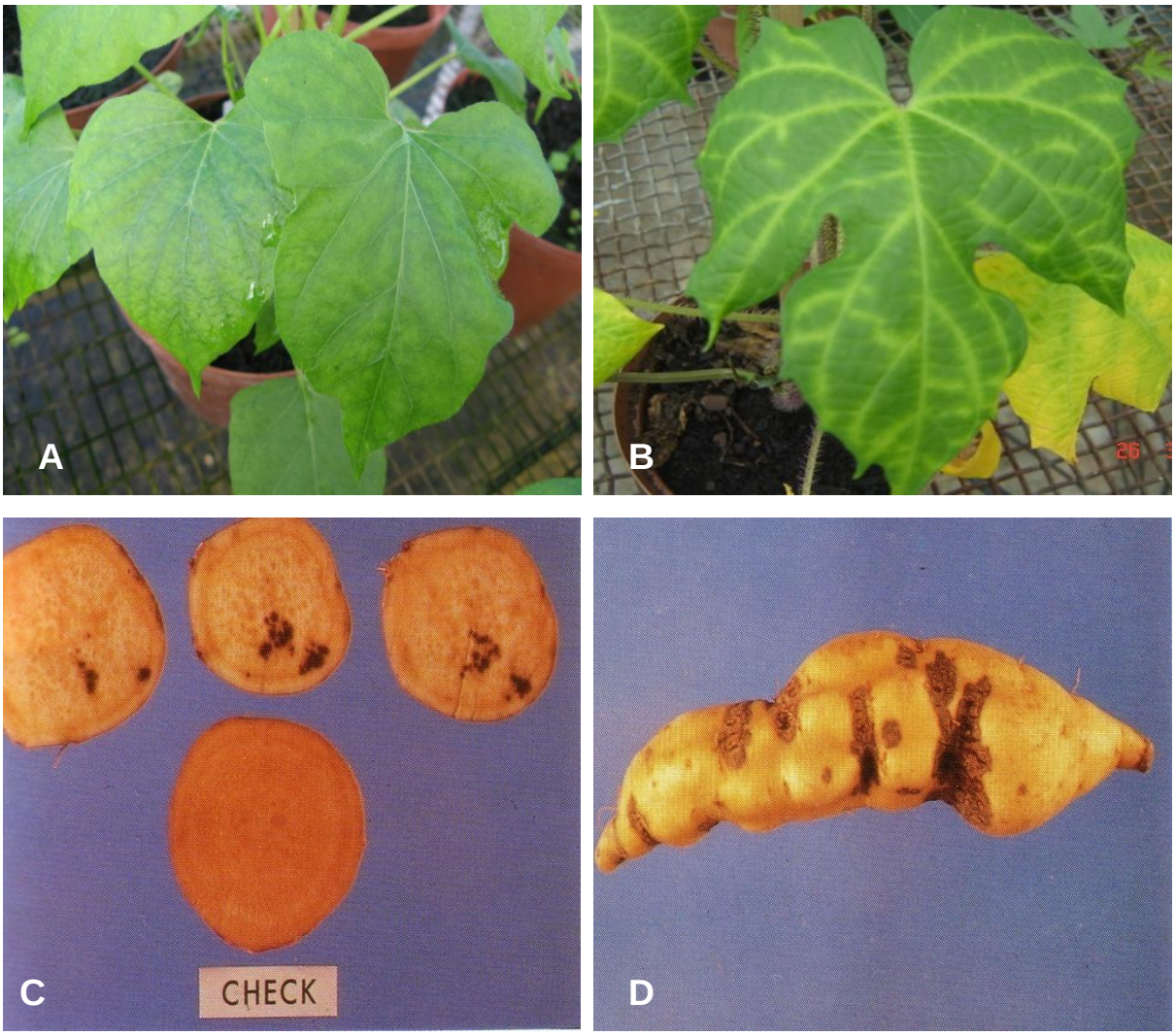



**Figure 1.1** Foliar (A-B) and root (C-D) symptoms of Sweet potato feathery mottle virus (SPFMV) on sweet potato (*Ipomoea batatas* Lam.). (A) Chlorotic patterns (feathering) on sweet potato leaves. (B) Vein clearing on symptomatic leaves of indicator plant, *Ipomoea setosa* (Sivparsad, 2011). (C) External root necrosis induced by the russet crack strain of SPFMV. (D) Internal root necrosis typical of internal cork disease (Clark & Moyer, 1988).

SPCSV has flexuous particles of 850-950nm in length and 12nm in diameter. The bipartite genome of SPCSV consisting of RNA1 (9,407nt) and RNA2 (8,223nt) is encapsidated by a 33kDa major CP (Cohen *et al*., 1992; Brunt *et al*., 1996; Kreuze *et al*., 2002). Based on molecular and serological analyses, SPCSV can be differentiated into East African (EA), and West African (WA) serotypes (Alicia *et al*., 1999; Ishak *et al*., 2003; Tairo *et al*., 2005).

### 1.3.3 Sweet potato mild mottle virus (SPMMV)

Sweet potato mild mottle virus (SPMMV, family *Potyviridae*, genus *Ipomovirus*), previously referred to as Sweet potato virus T or Sweet potato virus B, was first detected in East Africa from sweet potato showing leaf mottling, veinal chlorosis, dwarfing and poor growth (Sheffield, 1957; Hollings *et al*., 1976; Brunt *et al*., 1996). The virus has since been reported to occur in West Africa, South Africa, Indonesia, China, Philippines, Papua New Guinea, India, New Zealand and Egypt (Hollings *et al*., 1976; Carey *et al*., 1997; Fletcher *et al*., 2000; Domola *et al*., 2008). Virions of SPMMV are flexuous rod shaped particles, 800-950nm in length. It contains a positive single-stranded RNA genome of 10.8kb and a coat protein of 37.7kDa (Loebenstein *et al*., 2009). Although SPMMV was originally described as being transmitted by whiteflies in a persistent manner (Hollings *et al*., 1976), later studies have failed to confirm its whitefly transmissibility (Tugume *et al*., 2010). The virus can also be transmitted by grafting and mechanical inoculation but not by seed, pollen or by contact between plants (Brunt *et al*., 1996; Loebenstein *et al*., 2003). In comparison to other sweet potato-infecting viruses, SPMMV has an exceptionally wide host range, including species in 14 different families (Brunt *et al*., 1996). *Chenopodium quino*, *I. setosa*, *N. tabacum*, *N. benthamiana*, *N. glutinosa* and *N. clevelandii* are good experimental hosts for SPMMV. Symptoms of SPMMV- infected *I. setosa* are similar to those induced by SPFMV, which include vein clearing (Fig. 1.1B) and distortion (Hollings *et al*., 1976; Clark & Moyer, 1988).

### 1.3.4 Sweet potato virus G (SPVG)

Sweet potato virus G (SPVG, family *Potyviridae*, genus *Potyvirus*) is a relatively new sweet potato infecting virus that has gained little attention thus far (Colinet *et al*., 1994; Rännäli *et al*., 2008). It was originally described in China (Colinet *et al*., 1994). SPVG has since been reported to occur in Egypt (Ishak *et al*., 2003), the USA (Souto *et al*., 2003, Kokkinos and Clark, 2006b), Peru (Untiveros *et al*., 2007), Spain (Trenado *et al*., 2007), Tanzania (Ndunguru & Kapinga, 2007), Japan, Ethiopia, Nigeria, Barbados (Loebenstein *et al*., 2009), South Africa (Domola *et al*., 2008), and islands of the Pacific Ocean (Rännäli *et al*., 2008). The virus is transmitted mechanically and by aphids in a non-persistent manner. SPVG causes mottling in *I. nil* and chlorotic spotting in *I. setosa* (Souto *et al*., 2003). A partial sequence of SPVG showed around 70% and 80% identity with the amino acid sequence of the conserved core of the CP of SPFMV (Colinet *et al*., 1994). Biological properties (including host range and symptomatology) and viral characteristics have not been reported yet (Loebenstein *et al*., 2009).

### 1.3.5 Sweet potato virus 2 (SPV2)

Sweet potato virus 2 (SPV2) is a tentative new member of the genus *Potyvirus*, family *Potyviridae* (Ateka *et al*., 2004). The virus, previously referred to as Sweet potato virus Y and Ipomoea vein mosaic virus, was first isolated from sweet potato plants in Taiwan and Nigeria (Rossel & Thottappilly, 1988). Isolates of SPV2 have since been found in China, Portugal, South Africa, China, USA (Souto *et al*., 2003), Spain (Trenado *et al*., 2007), Australia (Tairo *et al*., 2006), Peru (Untiveros *et al*., 2007) and New Zealand (Perez-Egusquiza *et al*., 2009). SPV2 has filamentous particles of 850nm in length. The virus was non-persistently transmitted by aphids and mechanically to several species of genera *Chenopodium*, *Datura*, *Nicotiana*, and *Ipomoea*. SPV2 causes vein clearing and leaf distortion on *N. benthamiana*, chlorotic local lesions on *Chenopodium* species, and vein mosaic in *I. nil* and *I. setosa* (Souto *et al*., 2003; Ateka *et al*., 2007). The significance of SPV2 in sweet potato production remains unclear as similar symptoms may be induced by other viruses, and sweet potato cultivars inoculated with

SPV2 under greenhouse conditions failed to exhibit prominent symptoms (Souto *et al*., 2003; Ateka *et al*., 2004). However, SPV2 has been found to increase symptom severity when it interacts synergistically with SPCSV (Kokkinos & Clark, 2006b, Tairo *et al*., 2006), suggesting that SPV2 might be economically important in areas where SPCSV occurs (Loebenstein *et al*., 2009).

### 1.3.6 Sweet potato caulimo-like virus (SPCaLV)

Sweet potato caulimo-like virus (SPCaLV) is a possible member of the genus *Caulimovirus* as it shares certain properties with the genus such as an isometric shape with a diameter of 50-52nm, a double-stranded DNA genome and a coat protein of 42-44kDa (Brunt *et al*., 1996). The virus has been found in widely scattered geographical locations: Madeira, New Zealand, Papua New Guinea, Solomon Islands (Atkey & Brunt, 1987), China (Gao *et al*., 2000), Uganda (Aritua *et al*., 2006), Kenya, Nigeria, Egypt, and Puerto Rico (Kreuze & Fuentes, 2008; Loebenstein *et al*., 2009). No diagnostic symptoms on sweet potato have been shown to be associated with SPCaLV. Early symptoms on *I. setosa* include chlorotic flecks along the minor veins and interveinal chlorotic spots which may develop into general chlorosis, wilting and premature death of leaves (Clark & Moyer, 1988). SPCaLV is known only to be transmitted by grafting. The virus is not transmitted by aphids, mechanical inoculation, contact between plants or seed (Clark & Moyer, 1988; Brunt *et al*., 1996). Future characterization studies will allow for the elucidation of additional viral characteristics and subsequent definitive classification (Clark & Moyer, 1988).

### 1.3.7 Sweet potato mild speckling virus (SPMSV)

Sweet potato mild speckling virus (SPMSV, family *Potyviridae*, genus *Potyvirus*) was isolated from sweet potato plants in Argentina showing chlorosis, dwarfing, vein clearing, and leaf distortion (Alvarez *et al*., 1997). These symptoms are typical of Sweet potato chlorotic dwarf disease, a viral disease complex caused by the synergistic interaction of SPFMV, SPCSV and SPMSV. The severity of the disease depends on the presence of

SPMSV in the complex (Di Feo *et al*., 2000). SPMSV has been found in Peru, Argentina, Philippines, China, Indonesia, Egypt, South Africa, Nigeria and New Zealand (Alvarez *et al*., 1997; Nome *et al*., 2006; Loebenstein *et al*., 2009). Virion particles of SPMSV are flexuous and 790-800nm in length (Nome *et al*., 2006; Loebenstein *et al*., 2009). SPMSV is transmitted mechanically and by aphids in a non-persistent manner. The host range of SPMSV is restricted to *Convolvulaceae*, *Chenopodiaceae*, and *Solanaceae*. *I. setosa* and *I. nil* infected with SPMSV exhibit vein clearing, blistering, leaf deformation and mosaic (Alvarez *et al*., 1997; Loebenstein *et al*., 2009).

### 1.3.8 Sweet potato chlorotic fleck virus (SPCFV)

Sweet potato chlorotic fleck virus (SPCFV, tentative member of genus *Carlavirus*, family *Flexiviridae*) has been detected in samples from Peru, Japan, China, Korea, Taiwan, Cuba, Panama, Bolivia, Colombia, Brazil, Uganda, Philippines, India, Australia, New Zealand and South Africa (Usugi *et al*., 1991; Fuentes & Salazar, 1992; Gibson *et al*., 1997; Fletcher *et al*., 2000; Aritua *et al*., 2007; Jones & Dwyer, 2007; Domola *et al*., 2008). Virions of SPCFV are filamentous rods measuring 750-800 by 12nm in size consisting of a 9,104nt long genome encapsidated by a CP of 33.5kDa (Fuentes & Salazar, 1992; Aritua *et al*., 2007). The virus has a narrow host range in the families *Convolvulaceae* and *Chenopodium* and is transmitted mechanically but not by seed or aphids (Loebenstein *et al*., 2009). SPCFV can also be referred to as Sweet potato symptomless virus due to the lack of symptoms induced by infection on its natural host (Aritua *et al*., 2009). Infected *I. nil* display fine chlorotic spots (flecks) and vein clearing on the first and second true leaves (Loebenstein *et al*., 2009).

### 1.3.9 Sweet potato latent virus (SPLV)

Sweet potato latent virus (SPLV, family *Potyviridae*, genus *Potyvirus*), formerly known as Sweet potato N is widespread in China and has also been reported to occur in Taiwan, Korea, Indonesia, Japan, Philippines, Uganda, Kenya, South Africa, India, Egypt and New Zealand (Clark & Moyer, 1988; Loebenstein *et al*., 2009). As the name suggests,

infection of sweet potato by SPLV may not result in prominent foliar symptoms but the plants remain infected (Moyer & Salazar, 1989). SPLV induces systemic mosaic and stunting on *N. benthamiana* and systemic mottle on *I. setosa* (Wang *et al*., 2007; Loebenstein *et al*., 2009). Viral particles are flexuous rods, 750-790nm in length with a CP of 36kDa (Clark & Moyer, 1988). SPLV isolates from Japan and China were transmitted by aphids (Usugi *et al*., 1991). The virus can also be transmitted by mechanical inoculation and grafting but not by seed (Clark & Moyer, Loebenstein *et al*., 2009).

### 1.3.10 Viral synergism and disease complexes

Infection of plants by multiple viruses is a common phenomenon (Hull, 2002). Co-infection of plants with two or more unrelated viruses often results in a situation where one virus affects the co-infecting virus, allowing an increase in its accumulation in the plant by facilitating its replication or movement to tissues that it could otherwise not invade (Karyeija *et al*., 2000a). This phenomenon is known as viral synergism and often results in a more severe disease than the sum effect of infection with each virus alone (Mukasa *et al*., 2006; Untiveros *et al*., 2007). In sweet potato, most single virus infections induce mild or no symptoms and consequently, no obvious yield losses are observed. As a result, single virus infections are often unrecognizable by farmers who unknowingly spread the viruses through propagation of infected vines. This occurrence has led to the reports of numerous viral co-infections in almost every sweet potato growing area. However, despite these reports of great yield losses, only a few synergistic viral interactions in sweet potato have been studied (Untiveros *et al*., 2007).

**Table 1.1** Summary of viruses known to infect sweet potato (*Ipomoea batatas* Lam.)

| Virus | Genus | Vector | Distribution | References |
|---|---|---|---|---|
| Sweet potato feathery mottle virus (SPFMV) | Potyvirus | Aphids | Worldwide | Brunt *et al*., 1996; Kreuze & Fuentes, 2008 |
| Sweet potato chlorotic stunt virus (SPCSV) | Crinivirus | Whiteflies | Worldwide | Cohen *et al*., 1992; Brunt *et al*., 1996; Gibson *et al*., 1998 |
| Sweet potato mild mottle virus (SPMMV) | Ipomovirus | Whiteflies? | Africa, Indonesia, China, Philippines, Papua New Guinea, India, New Zealand, Egypt | Sheffield, 1957; Hollings *et al*., 1976; Brunt *et a*l., 1996 |
| Sweet potato virus G (SPVG) | Potyvirus | Aphids | China, Egypt, USA, Peru, Spain, Tanzania, Japan, Ethiopia, Nigeria, Barbados, South Africa, areas of the Pacific Ocean | Colinet *et al*., 1994, Rännäli *et al*., 2008; Loebenstein *et al*., 2009 |
| Sweet potato virus 2 (SPV2) | Potyvirus | Aphids | Taiwan, Nigeria, China, Portugal, South Africa USA, Spain, Australia, Peru, New Zealand | Ateka *et al*., 2004, 2008; Souto *et al*., 2003; Tairo *et al*., 2006 |
| Sweet potato caulimo-like virus (SPCaLV) | Caulimovirus? | Unknown | Madeira, New Zealand, Papua New Guinea, Solomon Islands, China, Uganda, Kenya, Nigeria, Egypt, Puerto Rico | Atkey & Brunt, 1987; Clark & Moyer, 1988; Brunt *et al*., 2006 |
| Sweet potato mild specking virus (SPMSV) | Potyvirus | Aphids | Peru, Argentina, Philippines, China, Indonesia, Egypt, South Africa, Nigeria, New Zealand | Alvarez *et al*., 1997; Di Feo *et al*., 2000 Nome *et al*., 2006 |
| Sweet potato chlorotic fleck virus (SPCFV) | Carlavirus | Unknown | Peru, Japan, China, Korea, Taiwan, South Africa Panama, Bolivia, Colombia, Brazil, Uganda, Philippines, India, Australia, New Zealand, Cuba | Fuentes & Salazar, 1992; Fletcher *et al.,* 2000; Aritua *et al*., 2009 |
| Sweet potato latent virus (SPLV) | Potyvirus | Aphids | Taiwan, Korea, Indonesia, Japan, Philippines, New Zealand, Uganda, Kenya, South Africa, India, Egypt | Clark & Moyer, 1988; Wang *et al*., 2007 Loebenstein *et al*., 2009 |
| Sweet potato leaf curl virus (SPLCV) | Begomovirus | Whitefies | Japan, Taiwan, USA, Far East, Brazil, Mexico, Korea, Puerto Rico, India, Kenya, Peru, China | Lotrakul *et al*., 2002; Luan *et al*., 2007 |

*Table 1.1 continued . . .*

| | | | | |
|---|---|---|---|---|
| Sweet potato leaf curl Georgia virus (SPLCGV) | Begomovirus | Whiteflies | USA, Puerto Rico, India | Lotrakul *et al*., 2003; Loebenstein *et al*., 2009 |
| Ipomoea yellow vein virus (IYVV) | Begomovirus | Unknown | Spain, Italy | Banks *et al*., 1999; Valverde *et al*., 2004a |
| Ipomoea crinkle leaf curl virus (ICLCV) | Geminivirus | Whiteflies | Israel, North America | Cohen *et al*., 1997; Loebenstein *et al*., 2009 |
| Sweet potato yellow dwarf virus (SPYDV) | Ipomovirus | Whiteflies | Taiwan, Far East, Brazil | Liao *et al*., 1979; Clark & Moyer, 1989 |
| Sweet potato vein mosaic virus (SPVMV) | Potyvirus | Aphids | Argentina | Nome, 1973; Brunt *et al*., 1996 |
| Sweet potato leaf speckling virus (SPLSV) | Luteovirus? | Aphids | Peru, Cuba | Fuentes *et al*., 1996; Kreuze & Fuentes, 2009 |
| Sweet potato ringspot virus (SPRSV) | Nepovirus | Unknown | Papua New Guinea, Kenya? | Brown *et al*., 1988; Brunt *et al*., 1996 |
| Sweet potato C-6 virus (C-6) | Carlavirus? | Unknown | Dominican Republic, Peru, Uganda, Cuba, USA, Philippines, Indonesia, Egypt, Kenya, South Africa, New Zealand, Puerto Rico | Fuentes, 1994; Loebenstein *et al*., 2009 |
| Sweet potato C-3 virus (C-3) | Unknown | Unknown | Brazil | Fuentes & Salazar, 1989 |
| Cucumber mosaic virus (CMV) | Cucumovirus | Aphids | Israel, Egytp, Kenya, Uganda?, Japan, South Africa, New Zealand | Loebenstein *et al*., 2009 |
| Tobacco mosaic virus (TMV) | Tobamovirus | None | USA | Clark & Moyer, 1988 |
| Tobacco streak virus (TSV) | Ilarvirus | Thrips | Guatemala | Clark & Moyer, 1988 |

*Sweet potato virus disease (SPVD)*

The most devastating viral disease affecting sweet potatoes worldwide is sweet potato virus disease (SPVD) (Kokkinos *et al*., 2006; Miano *et al*., 2008). This disease is caused by the synergistic interaction between SPFMV and SPCSV. It is the most economically important viral disease of sweet potato because infected plants display chlorosis, small deformed leaves, severe stunting (Fig. 1.2A-B) and an almost 99% reduction in tuber yield (Fig. 1.3A-B) (Gibson *et al*., 1998; Karyeija *et al*., 1998; Tairo *et al*., 2005). SPVD was first reported by Schaefers and Terry (1976) in Nigeria and is currently the most important viral disease complex in East Africa where sweet potato is often the main food staple (Karyeija *et al*., 1998; Loebenstein *et al*., 2009). The disease has since been reported in Israel (Loebenstein & Harpaz, 1960), Peru (Gutiérrez *et al*., 2003), USA (Abad *et al*., 2007), Spain (Trenado *et al*., 2007), Italy (Parella *et al*., 2006), and Egypt (Ishak *et al*., 2003).

*Sweet potato chlorotic dwarf disease (SPCD)*

Another recognized viral disease complex known as chlorotic dwarf (CD) was described as an important disease occurring in Argentina (Di Feo *et al*., 2000). This synergistic complex is caused by the infection of SPCSV and SPFMV and/ or SPMSV (Di Feo *et al*., 2000; Kreuze & Fuentes, 2009). SPCD is characterized by symptoms of chlorotic mosaic, leaf area deduction and deformation and plant dwarfism (Di Feo *et al*., 2000; Nome *et al*., 2007). The combined action of all three viruses produces more severe disease symptoms and yield reductions may reach up to 80% (Di Feo *et al*., 2000).


**Figure 1.2** Sweet potato (*Ipomoea batatas* Lam.) plants affected by viruses in Peru. (a) Sweet potato field with a large number of plants affected by Sweet potato virus disease (SPVD) and (b) a close up of an infected plant showing stunting, mosaic, and leaf deformation (Kreuze & Fuentes, 2009).


**Figure 1.3** Effect of Sweet potato virus disease (SPVD) on (A) sweet potato (*Ipomoea batatas* Lam.) plants and (B) yield of storage roots (Kreuze & Fuentes, 2009).

*Sweet potato severe mosaic disease (SPSMD)*

The dual infection of SPCSV and SPMMV results in the synergistic viral complex known as Sweet potato severe mosaic disease (SPSMD). The disease was initially reported in East Africa and induces severe symptoms of chlorosis, rugosity, leaf strapping and dark green islands. SPSMD-infected plants showed an 80% reduction in storage root yield when compared to healthy plants under greenhouse conditions in Uganda (Mukasa *et al*., 2006).

*Other viral disease complexes*

Other viral disease complexes have also been described, which consistently seem to involve SPCSV (Kreuze & Fuentes, 2008). More commonly, in all recognized viral synergistic diseases of sweet potato, infection with a single virus causes mild or no disease symptoms while severe disease is induced in the presence of SPCSV. The role of SPCSV in catalyzing severe disease in a viral complex has been documented and evaluated (Mukasa *et al*., 2006; Untiveros *et al*., 2007). SPCSV was shown to break host resistance in sweet potato in favour of other co-infecting viruses (Mukasa *et al*., 2006). As a result, in addition to SPFMV and SPMMV, SPCSV has been shown to cause synergistic diseases in sweet potato with CMV, SPCFV and C-6 virus (Untiveros *et al*., 2007). In the Philippines, SPCSV together with several other viruses causes a disease known locally as Camote kulot (Kreuze & Fuentes, 2008). In Israel and Egypt, SPCSV and usually SPFMV is found infecting sweet potato together with CMV, producing symptoms similar to SPVD and causing up to 80% reduction in yield (Milgram *et al*., 1996).

## 1.4 DETECTION OF SWEET POTATO VIRUSES

Viral disease poses a serious threat to sweet potato production worldwide (Loebenstein *et al*., 2009). Early detection of the appearance of viral disease followed by rapid and accurate identification of the causal viral agent is vital if correct control measures are to be employed. This is particularly true for newly identified sweet potato viruses where novel control strategies may have to be developed alongside characterization of the new

virus (Kreuze *et al*., 2009). The detection and identification of sweet potato viruses is complicated by the frequent occurrence of mixed infections, synergistic complexes and the constant emergence of new viral species and strains (Tairo, 2006; Gutièrrez & Valverde, 2007). Traditional methods such as biological indexing, enzyme-linked immunosorbent assay (ELISA), molecular hybridization and polymerase chain reaction (PCR) are mostly used. However, due to their limited reliability and sensitivity, a combination of methods is required to certainly identify the viral etiology present in a diseased sweet potato plant (Tairo, 2006).

### 1.4.1 Biological indexing

Biological indexing is one of the oldest methods that employ reactions of plants when infected by viruses. One way is by visual inspection of the material to be tested for characteristic symptoms. The other approach is by indexing for infective virus on indicator hosts and their examination for symptoms (Bos, 1999). In sweet potato, graft inoculation onto *Ipomoea* species is widely used to assay for many sweet potato viruses (Tairo, 2006). *Ipomoea setosa* Ker is a convenient and nearly universal host for sweet potato viruses. In grafting, the sweet potato plant suspected of viral disease is marked and a vine segment is taken. The base of the segment is cut and shaped into a wedge (scion) and then inserted into the lateral slit of the stem of the indicator plant (rootstock). Characteristic symptoms of mosaic, curled or wrinkled leaves and vein clearing on graft-inoculated *I. setosa* is an indication of viral infection (Feng *et al*., 2000).

Some sweet potato viruses may also be detected by mechanical inoculation of the indicator plants *I. setosa* and *I. nil*. In addition, mechanical inoculation of *Nicotiana* spp. such as *N. tabacum* and *N. benthamiana* can also be used in a sweet potato virus assay. *N. benthamiana* is known to be a good propagative host for SPFMV, *N. benthamiana* and *N. tabacum* are useful propagative hosts for SPMMV and the host range of SPLV includes some *Nicotiana* species including *N. benthamiana* (Clark & Moyer, 1988). However, some sweet potato viruses such as the phloem-confined

SPCSV cannot be transmitted by mechanical inoculation (Loebenstein *et al*., 2009). Transmission to indicator hosts by grafting is an alternative for such viruses.

Biological indexing has routinely been used to detect virus-infected sweet potato plants in China (Feng *et al*., 2000). Although indexing is simple and suitable to detect virus-infected sweet potato samples which do not show any obvious symptoms, it requires time and the production of similar symptoms makes it difficult to distinguish different sweet potato viruses. However, when indexing is combined with serological methods, detection efficiency can be improved (Feng *et al*., 2010; Tairo, 2006).

### 1.4.2 Serological tests

Serological methods, based on the specific interaction of an antibody and antigen, are widely used in the diagnosis of plant viral diseases (Bos, 1999). The advantages of such methods are that they are highly specific, rapid and sensitive to small amounts of viral antigen in the plant material (Yao & Hortense, 2005). The microplate method of the enzyme-linked immune-sorbent assay (ELISA) is a popular method for the detection and assay of plant viruses (Esbenshade & Moyer, 1982; Flint *et al*., 2000). In a microplate ELISA, detection of the viral antigen or antibody can be accomplished by solid phase methods in which the viral specific antibody or protein is absorbed to a plastic surface. To detect the viral antigen, a 'capture' or secondary antibody, directed against the virus, is linked to a solid support, a plastic dish or bead. The specimen is added to the plastic support, and if viral antigens are present, they will be captured by the bound antibody. The bound viral antigen is detected by using a second antibody linked to an enzyme. A substrate molecule is then added and is converted by the enzyme to an easily detectable coloured product (Voller *et al*., 1976; Wang & Gonsalves, 1990; Matthews, 1992). However, interferences between undiluted plant saps and the final colouration hinder the accuracy of this method in the detection of sweet potato viruses. Therefore, in order to bypass that difficulty, serological techniques on a nitrocellose membrane have been developed with a few variations (MIBA, Membrane Immune-binding Assay; DIBA, Dot Blot Immuno-binding Assay). These methods are based on the specific

antibody-antigen reactions and positive reactions are characterized by the appearance of dark bands on the membrane. The absence of colouration allows the use of undiluted plant saps with no interference problems (Gibb & Padovan, 1993, Yao & Hortense, 2005).

A membrane immuno-binding assay known as nitrocellulose membrane enzyme-linked immune-sorbent assay (NCM-ELISA) has been used with success for the detection of sweet potato viruses (Gutièrrez & Valverde, 2007). This method has been adapted for practical virus detection by the International Potato Center (CIP, Peru). To date, a kit containing antiserum for 10 sweet potato viruses is available from the CIP together with a standardized NCM-ELISA protocol. However, the accuracy of the serological detection of sweet potato viruses in hampered by the presence of interfering phenolic substances and inhibitors (Gibb & Padovan, 1993), low concentration and erratic distribution of viruses in infected sweet potato plants (Esbenshade & Moyer, 1982) and the frequency of multiple infections of viruses. Thus, subsequent testing is needed to dissolve discrepancies between assays and confirm positive results (Tairo, 1996).

### 1.4.3 Electron microscopy

Electron microscopy is useful in revealing virus structure, and in showing the cytoplasmic effect of viruses in infected tissue (Kado & Agrawal, 1972). Such characteristics are often specific to viral families and are valuable in virus identification. For instance, the long flexuous particles of sweet potato infecting potyviruses can be directly identified by transmission electron microscopy (TEM) of leaf-dip preparations or in thin sections of infected *I. setosa* plants. Alternatively, these viruses can be identified by the production of characteristic pinwheel cytoplasmic inclusions induced by Potyvirus infection (Nome *et al*., 2006). Immunosorbent electron microscopy (ISEM) has combined the ease of TEM with the specificity of serology. This technique which focuses on the specific trapping of viral particles on the grid that have been pretreated with antiserum has increased the sensitivity and specificity of TEM up to 10,000 fold (Bos, 1999). Both TEM and ISEM have been used to detect SPFMV, SPLV, TMV (Yang

*et al*., 1995). However, these methods are not specific in identifying individual viruses and also require expensive equipment. Therefore, TEM and ISEM is often used only to classify and study sweet potato viruses and not for routine diagnosis (Feng *et al*., 2000).

### 1.4.4 Molecular techniques

The most recent approaches used for the detection of sweet potato viruses are those based on molecular biology techniques. Techniques such as polymerase chain reaction (PCR), quantitative real-time PCR (qPCR), rolling-circle amplification (RCA) and deep-sequencing of small RNAs (siRNA) have emerged as powerful methods in the identification and characterization of viruses infecting sweet potato (Colinet *et al*., 1998; Valverde *et al*., 2004b; Clark *et al*., 2012). These techniques show great promise and may circumvent the problems associated with serological and biological indexing (Colinet *et al*., 1998).

PCR is an effective technique for detecting sweet potato viruses such as SPFMV, which are usually irregularly distributed and present at a low titre in the infected sweet potato plants (Souto *et al*., 2003). This procedure involves the enzymatic amplification of cDNA from templates of viral nucleic acid, with oligonucleotide primers complementary to the viral nucleic acid. Following the amplification, the specific DNA products can be detected by agarose gel electrophoresis (Sankaran *et al*., 2010). Depending on the choice of primers, PCR assists in the detection of a single species or many members of a group or family of related viruses (Colinet *et al*., 1998). Sequence information and the knowledge of conserved viral sequences have simplified the design of oligonucleotide primers that enable specific and rapid identification of sweet potato viruses (Colinet *et al*., 1998; Tairo *et al*., 2006). The value of PCR for rapid identification and characterization of sweet potato viruses has been demonstrated for potyviruses, criniviruses and geminiviruses (Colinet & Kummert, 1993; Colinet *et al*., 1998; Li *et al*., 2004; Kokkinos and Clark, 2006a; Tairo *et al*., 2006; Opiyo *et al*., 2010). Different types of PCR based techniques have been developed. The restriction fragment length polymorphism (RFLP) technique is a sensitive and simple diagnostic procedure that

involves the restriction analysis of PCR generated amplicons. RFLP analysis has been used in the rapid identification and differentiation of potyvirus complexes in sweet potato (Colinet *et al*., 1998; Tairo *et al*., 2006).

Quantitative real-time PCR (qPCR) is a sensitive method for the detection of viruses in the plant tissues whereby the amplification of mRNA or coding sequence of a host gene is included as an internal control in the detection of RNA and DNA viruses, respectively (Clark *et al*., 2012). This method has been used in the detection and quantification of SPCSV, SPFMV, SPMMV, SPVG, SPV2 and SPLCV directly from infected sweet potato plants (Kokkinos & Clark, 2006a; Mukasa *et al*., 2006; McGregor *et al*., 2009; Perez-Egusquiza *et al*., 2009). qPCR has been shown to be more efficient and sensitive in detecting sweet potato viruses than conventional PCR (Kokkinos & Clark, 2006a). The method has also been improved and modified to enable the detection of three potyviruses (SPFMV, SPVG and SPV2) in a multiplex assay by mixing primers and using probes labeled with distinct fluorophores and also developed generic primers and TaqMan probes to detect all sweet potato viruses whose sequences were available on GenBank (Lin *et al*., 2010). qPCR offers a high degree of detection sensitivity and can reduce time, money and labor expenditure. However, its use in routine virus detection may be restricted by the sequence specificity of the TaqMan probes and primers, as well as the expensive reagents and instruments (Clark *et al*., 2012).

The rapid evolution of viruses has caused significant problems in the design of PCR primers to detect all viral strains (Zhang & Ling., 2011). To this end, rolling-circle amplification (RCA), sometimes combined with RFLP, is emerging as a powerful tool for the detection of sweet potato viruses (Paprotka *et al*., 2009; Clark *et al*., 2012). RCA/RFLP combined with sequencing has enabled the identification of novel variants, strains and species of sweet potato viruses (Haible *et al*., 2006; Lozano *et al*., 2009; Paprotka *et al*., 2009; Albuquerque *et al*., 2011).

Novel DNA sequencing techniques, referred to as “next-generation” sequencing (NGS), have become available in the last few years and these involve an unbiased approach to

plant viral disease diagnosis which requires no prior knowledge of the host or pathogen (Adams *et al*., 2009). They have been widely used in many projects, e.g., whole genome sequencing, metagenomics, small RNA discovery and RNA sequencing. Their common feature is to provide high speed and throughput that can produce an enormous volume of sequences with many possible applications in research and viral diagnostics (Barzon *et al*., 2011). An interesting strategy to discover viruses exploits the property of invertebrates and plants to respond to infection by processing viral RNA genomes into small RNAs (siRNAs) of discrete sizes. A recent study on small RNA libraries sequenced by NGS platforms (Wu *et al*., 2010) showed that viral small silencing RNAs produced by invertebrate animals are overlapping in sequence and can assemble into long contiguous fragments of the invading viral genome. Based on this result, an approach of virus discovery in plants by deep sequencing and assembly of total small RNAs was developed and utilized in the analysis of contigs (i.e., a contiguous length of genomic sequences in which the order of bases is known) assembled from available small RNA libraries (Barzon *et al*., 2011). The use of deep-sequencing of siRNA has been described by Kreuze *et al*. (2009) as a novel means to detect sweet potato viruses. In the study, assembly of the overlapping 21- or 22nt long virus-derived siRNA sequences to contigs was used to compile the complete genome of a new strain of SPFMV. In addition, two previously unknown badnaviruses and a mastrevirus were also detected by comparing the contigs made from the siRNA sequences against the sequences available in common gene bank databases. PCR amplification and Sanger sequencing of the products confirmed the accuracy of the assembled viral sequences (Kreuze *et al*., 2009; Clark *et al*., 2012). The technique has since been used to determine complete genome sequences of SPVG, SPV2, SPLV, and SPFMV-RC, and also to detect new sweet potato viruses, badnaviruses, a cavemovirus, and a solendovirus (CIP, unpublished), and new strains of SPCSV from South America (Cuellar *et al*., 2011; Clark *et al*., 2012).

## 1.5 CONTROL OF SWEET POTATO VIRUSES

Control of viral disease in sweet potato is complicated by the frequent occurrence of mixed synergistic viral complexes. In addition, vegetative propagation of infected roots or vines provides a perfect means of perpetuating viruses within the production cycle. Therefore, effective and durable disease control methods for sweet potato are based on prevention (Clark & Moyer, 1988). These methods include: (a) decreasing the amount of virus (inoculum) by cultural methods, (b) protecting plants from infection using cross protecting viral strains, (c) distribution of virus-indexed propagation material and (d) the use of resistant cultivars. However, no single management tool is available that provides adequate control against the natural viral complexes that infect sweet potato.

### 1.5.1 Cultural methods

A series of cultural methods such as weed control, intercropping and roguing (destroying) of infected plants have been proven effective in minimizing losses due to viral disease in sweet potato (Karyeija *et al*., 1998; Ndunguru & Alyoce, 2000; Gibson *et al*., 2004). Weeds play an important role in the incidence and spread of sweet potato viruses as they might serve as alternate hosts of insect vectors and virus. Removal of reservoir weed hosts in a wide area around a crop may relieve the infection pressure (Peters, 2003). The incidence of SPFMV and SPCSV was shown to decrease when weeds, especially wild *Ipomoea* species, were removed in and around sweet potato fields (Karyeija *et al*., 1998). The use of intercropping to reduce the number of infectious insect vectors attacking the sweet potato crop may help to reduce viral incidence by delaying vector onset and build-up. A sweet potato/maize cropping pattern was shown to have lowered SPVD incidence in traditional sweet potato farming systems (Ndunguru & Alyoce, 2000). A recent on-farm site trial in Uganda showed that roguing of diseased cuttings within a month of planting and isolating new crops (15-20m apart) from diseased crops, can considerably decrease the spread of SPVD to susceptible cultivars (Gibson *et al*., 2004). However, despite these initial successes, neither cultural control

method has been shown to be durable and feasible against the multitude of viral complexes that infect sweet potato.

### 1.5.2 Cross protection

The term cross protection refers to the general phenomenon of protection provided to a plant by infection with a mild or attenuated strain (protecting strain) of a virus from subsequent infection by a more severe strain (challenging strain) of the same virus (Lecoq, 1998; Agrios, 2005). Its application in controlling viral disease has been shown with some success in cross protecting many different crops, including those propagated by vegetative reproduction (Agrios, 2005; Yamasaki *et al*., 2009). In sweet potato, an isolate of SPFMV from *I. batatas* roots that induced mild or undetectable disease was characterized and identified as SPFMV-O. This isolate was shown to be effective at protecting plants from subsequent infection with a virulent SPFMV isolate (SPFMV-S) (Yamasaki *et al*., 2009). However, cross protection has not gained widespread use for controlling viral disease in sweet potato. This is because mild strains of sweet potato viruses have not been identified and may not be effective viruses and exist in a synergistic complex. In addition, there is danger of mutations towards new and more virulent strains that might spread to other crops (Agrios, 2005).

### 1.5.3 Distribution of virus-indexed material

At present the best way to control viral diseases in sweet potato is to supply growers with virus-indexed propagation material (Cohen *et al*., 2008; Loebenstein *et al*., 2009). Such material can be produced by the meristem tip or shoot tip culture techniques, which are based on the uneven or low concentration of viruses in the youngest tissues of the shoot apex (Wang & Valkonen, 2008). Recently, the combination of meristem tip culture with cryo- or thermotherapy was shown to drastically enhance the efficiency of virus elimination in sweet potato (Wang & Valkonen, 2008; Mashilo, 2009; Feng *et al*., 2011). Worldwide, there are many programs that produce and distribute meristem-derived, virus-free propagation material for sweet potato cultivation (Loebenstein *et al*.,

2009). In SA, a sweet potato improvement scheme was implemented by the ARC-Roodeplaat in 1971 with the task of establishing virus-free market stock which is supplied to registered sweet potato growers and producers (Joubert *et al*., 1996). However, in SA and in the rest of Africa, such programs are operating on a limited scale, because sweet potatoes are grown mainly as a subsistence crop, and not commercially (Loebenstein *et al*., 2009).

### 1.5.4 Resistance

Cultural practices, cross protection and the distribution of virus-indexed propagation material have only been marginally effective in the management of the multitude of viruses that infect sweet potato. The development of resistant sweet potato varieties is the most promising means of controlling viral disease in the long term (Loebenstein *et al*., 2009).

#### *1.5.4.1 Natural resistance*

Little success has been reported in the development of sweet potato cultivars with broad virus resistance. All that has been reported, primarily concern SPFMV and SPVD (Gibson *et al*., 1998; Gibson *et al*., 2004; Karuri *et al*., 2009). Breeding for virus resistance in sweet potato involves the introduction of resistant genes into cultivated varieties without changing any of its desirable characteristics. Wild relatives of sweet potato (e.g., *I. trifida*) may serve as a source of resistant genes (Agrios, 2005). Breeding programs in Uganda have worked at combining SPVD resistance with desirable agronomical traits such as yield, earliness and acceptable culinary quality in sweet potato cultivars (Karyeija *et al*., 2000; Mwanga *et al*., 2002; Loebenstein *et al*., 2009). Although progress has been made, it remains to be seen if these cultivars will retain their resistance when challenged by differing strains of the SPVD viral components, which may occur in different geographical locations (Loebenstein *et al*., 2009). In addition to virus variation, conventional breeding is hindered by the amount of time and expense required (Lomonosoff, 1995). Moreover, genetic sources of resistance are

scarce and the incorporation of such resistance from the wild diploid species into polyploid sweet potato is a complicated task (Kreuze, 2002).

#### *1.5.4.2 Engineered resistance*

As natural resistance to viruses in sweet potato seems to be of limited use, alternate strategies for obtaining virus resistance through biotechnological means have been attempted (Kreuze, 2002). Most of these strategies are based on the concept of 'pathogen-derived resistance' (PDR), which proposes that pathogen resistance genes may be developed from the pathogen's own genetic material (Sanford & Johnston, 1985). Selected genes from a virus, when inserted into the host plant genome, may render that host transgenic and resistant to the virus (Lin *et al*., 2007). PDR for plant viruses can roughly be divided into resistance mediated by the expression of transgenic proteins (protein-mediated PDR) and resistance mediated by the expression of RNA (RNA-mediated PDR) (Shepherd *et al*., 2009; Collinge *et al*., 2010).

*Protein-mediated PDR*

In protein-mediated PDR, an accumulation of the transgene protein product is required for the resistance phenotype (Kreuze, 2002). The underlying principle of protein-mediated resistance is that certain pathogen-derived proteins [e.g., coat protein (CP), replicase and movement protein] play a crucial role in viral pathogenicity. Functional or non-functional (mutant) forms of such proteins could act in an overriding negative manner to interfere with virus replication, assembly, disassembly or movement. Therefore, expression of transgene derived proteins in host cells could confer viral resistance in the plant (Baulcombe *et al*., 1996; Lin *et al*., 2007). In general, protein-mediated resistance has been shown to confer relatively broad resistance to related viral strains (Fitchen & Beachy, 1993).

*RNA-mediated PDR*

As a result of initially unexplained effects observed in protein-mediated PDR approaches, the role of RNA transcripts derived from viral transgenes exposed an

entirely new dynamic field in biology involving sequence-specific RNA degradation (Prins *et al*., 2008). This post-transcriptional gene silencing (PTGS) process, also known as RNA interference (RNAi) or RNA silencing, is the mechanism of RNA-mediated PDR (Tenllado *et al*., 2004; Lindbo & Dougherty, 2005; Fuchs & Gonsalves, 2007).

PTGS is a specific RNA degradation mechanism of any organism that breaks down abberant, excess or foreign RNA intracellulary in a homology-dependent manner (Dasgupta *et al*., 2003). Induction of PTGS using a viral transgene enables a specific degradation of the genome of the invading virus and those that have high sequence homology with the virus transgene, resulting in a resistance phenotype (Fuchs & Gonsalves, 2007). The PTGS pathway (Fig. 1.4) is initiated by the generation of double-stranded RNAs (dsRNAs) by RNA-dependent RNA polymerases (RdRps) that are then digested into 21-25nt, known as small interfering RNAs (siRNAs). These siRNAs interact with various host proteins to form RNA-induced silencing complexes (RISC) that guide in the specific binding and subsequent degradation of target RNAs (Lin *et al*., 2007; Shepherd *et al*., 2009; Frizzie & Huang, 2010; Nui *et al*., 2010).

In transgenic plants, PTGS is triggered by the expression of dsRNAs homologous to viral sequences (Shepherd *et al*., 2009; Gaba *et al*., 2010). The generation of dsRNAs can be achieved by transgenes in the sense or antisense orientation. Antisense RNA was initially thought to inhibit endogenous genes by hybridizing with its target mRNA. The resulting RNA-RNA duplex might hinder nuclear processing and endogenous mRNA translation and could therefore lead to swift RNA degradation (Bird & Ray, 1991; Frizzie & Huang, 2010). However, this hypothesis could not account for another RNA silencing phenomenon known as co-suppression, where high expression of the transgene is silenced together with the homologous viral gene (Napoli *et al*., 1990; Frizzie & Huang, 2010). A more comprehensive model proposed that PTGS could be triggered by 'RNA over-abundance' (Stam *et al*., 1997). The model suggests that specific degradation of all homologous RNAs is carried out once a critical threshold of RNA accumulation has been reached (Frizzie & Huang, 2010). The concept of 'aberrant

RNAs' was introduced as a result of RNA over-abundance. These aberrant transcripts could act as primers together with a plant encoded RdRp in the synthesis of complementary or antisense RNA, which could lead to RNA degradation (Dougherty & Parks, 1995). However, this model required the presence of a plant encoded RdRp and the events following aberrant RNA production to RNA degradation are unclear. Hence, the production of longer duplex RNA or dsRNA in PTGS became more theoretically plausible. RNase III-type enzymes, also known as Dicer enzymes or DCL, were identified in the processing of dsRNA into siRNAs (Hamiliton & Baulcombe, 1999). This discovery elucidated the steps between dsRNA and guided degradation of homologous RNAs (Frizzie & Huang, 2010).

Other than antisense RNA and co-suppression, complicated transgene insertions were also found to produce dsRNA and hence PTGS. When transgenes are arranged as a direct repeat (DR) or inverted repeat (IR), in which a virus-derived sequence is cloned in sense and antisense orientations and separated by an intron, a dsRNA structure known as hairpin RNA (hpRNA) is formed. This hpRNA activates the plant's silencing machinery, which then processes it into siRNAs that elicit degradation of homologous RNAs (Smith *et al*., 2000; Gaba *et al*., 2010; Frizzie & Huang, 2010).

Although less frequently occurring, gene silencing can also arise from the repression of transcription. Transcriptional gene silencing (TGS) share components and uses similar siRNA-guided processes as PTGS. The difference is that TGS targets DNA sequence that is homologous to the transgene and results in DNA methylation that suppresses transcription (Mette *et al*., 2000; Frizzie & Huang, 2010).

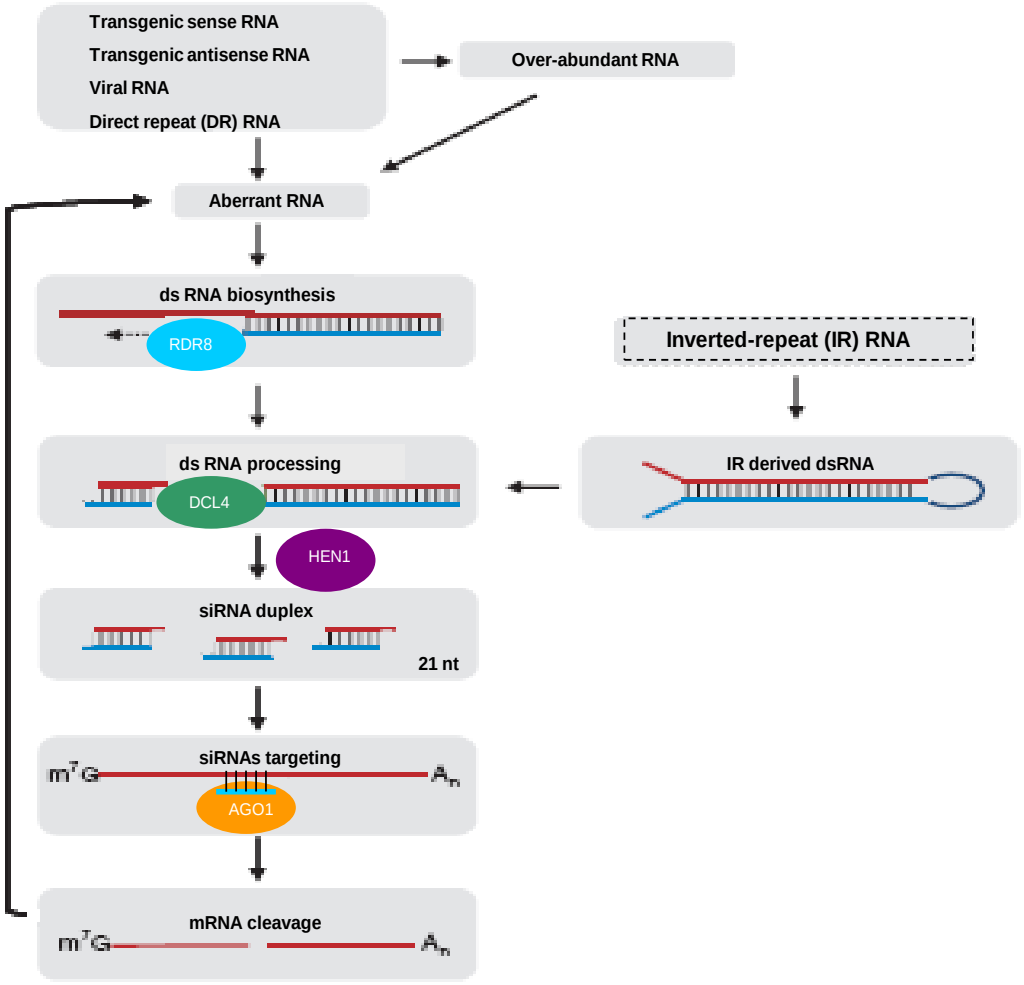



**Figure 1.4** Illustration of the post-transcriptional gene silencing (PTGS) pathway. The pathway is elicited by aberrant RNAs, RNA molecules lacking a polyA tail or 5' capping. They are derived from highly abundant transgenic sense or antisense RNA, viral RNA or truncated transcripts from complicated gene insertions or duplications. The mRNA cleavage products at the end of the pathway can also be perceived as aberrant RNAs and further enhance the pathway. These aberrant RNAs are converted into dsRNA by the RNA-dependent RNA polymerase, RDR6. Dicer enzymes, DCL4, digest dsRNA into 21-nt siRNA duplexes that are then methylated at the 3' terminal nucleotide by a RNA methyltransferase, HEN1. One strand of the siRNA duplex is subsequently incorporated into the RNA-induced silencing complex (RISC) together with Argonaute protein (AGO1). Guided by the complementary 21-nt siRNA, AGO1 cleaves the mRNA target. Genetic engineering can tap into this pathway by expressing a transgenic inverted repeat (IR) RNA (dotted box) (Fizzie & Huang, 2010).

An exciting novel approach related to PTGS uses another class of RNA silencing-related small RNAs, known as microRNAs (miRNAs) (Qu *et al*., 2007; Shepherd *et al*., 2009; Frizzie & Huang, 2010). miRNAs are conserved in plants and best known for their roles in developmental regulation and timing. Although part of the PTGS pathway, the synthesis of miRNA differs from the described siRNA pathways, and therefore the use of miRNA in RNA silencing requires a different transgene design (Frizzie & Huang, 2010). miRNAs are small RNAs derived from transcripts with a distinctive RNA stem-loop secondary structure. Unlike the siRNA pathway, the miRNA pathway does not require a RdRp because portions of the mature, folded transcript are dsRNA. The primary transcripts is processed to produce primary miRNAs (pri-miRNAs). The base of the pri-miRNA is cut to produce another miRNA precursor (pre-miRNA) which is further processed by DCL1 into a miRNA-miRNA duplex (Park *et al*., 2002; Frizzie & Huang, 2010). This duplex is methylated by HEN1 (Yu *et al*., 2005) and its less stable base-pairing enables the unwinding of the duplex which leaves the miRNA strand unbound and rapidly degraded by AGO1 (Frizzie & Huang, 2010). In RNA silencing, transgenes designed to express artificial miRNA targeting sequences were found to be more effective than siRNA (Qu *et al*., 2007; Shepherd *et al*., 2009).

The mechanism of RNA silencing has been successfully applied to the production of transgenic crops with multiple virus resistance. It was shown that transgenes consisting of ~400bp segments of the N gene of Tomato spotted wilt virus (TSWV) conferred resistance to TSWV in transgenic plants through PTGS (Pang *et al*., 1997) but N gene segments linked of 92-235bp did not. However, when transgenic plants expressing transgenes consisting of 218 or 110bp N gene segments were linked to the 720bp green fluorescent protein (GFP) they displayed resistance to TSWV (Pang *et al*., 1997). Further research showed that transgenes consisting of 59 or 24bp similary linked to GFP were not resistant to TSWV, even though the transgene showed PTGS (Jan *et al*., 2000a). Taken together, these findings suggested that N gene segments of 110bp or more could confer resistance when linked to a transcribed DNA (designated ‘silencer’ DNA) that induces PTGS. This observation exposed the possibility of developing multiple virus resistance in transgenic plants by linking viral DNA as a ‘silencer’ to

segments of other viral DNA (Jan *et al*., 2000a). The strategy was demonstrated by Jan *et al.* (2000b) where a single chimeric gene consisting of linked viral segments of TSWV and Turnip mosaic virus (TuMV) conferred multiple resistance in transgenic tobacco via PTGS. Recently, Lin *et al*. (2011) demonstrated that multiple virus resistance in transgenic plants using this approach could be also be achieved by fusing the partial C2 gene of the Tomato leaf curl Taiwan virus (ToLTWCV) to a silencer DNA, the middle half of the nucleocapsid (N) gene of TSWV. Following the successful implementation of this strategy in the development of transgenic plants with multiple resistance, this study aimed to develop transgenic sweet potato with broad virus resistance by linking the coat protein (CP) segments of the four most prevalent viruses infecting sweet potato in KZN to a silencer DNA, the middle half of the nucleocapsid (N) gene of TSWV.

Despite the recent progress made in the field of biotechnology and the commercialization of many virus-resistant transgenic crop plants, transgenic approaches have not resulted in sweet potato cultivars with sustainable resistance to viruses. At least four groups, namely the Kenya Agricultural Research Institute (KARI) in collaboration with Monsanto and the Agricultural Biotechnology Support Project at Michigan State University, the Japan International Research Center for Agriculture, the Center for Plant Biotechnology Research at Tuskegee University and the CIP have attempted to incorporate transgenic resistance to SPFMV in sweet potato (Kreuze, 2002). However, none of these efforts have resulted in the commercialization of a virus-resistant transgenic sweet potato cultivar. Monsanto scientists obtained resistance to SPFMV using the expression of the CP. However, on-farm field trials initiated in Kenya by the KARI/Monsanto group to test the efficacy of their transgenic sweet potato proved unsuccessful. In this cultivar, the high levels of resistance to SPFMV broke down following infection with SPCSV and the plants succumb to severe SPVD (Karyeija *et al*., 2000b; Kreuze *et al*., 2008). When evaluated under field conditions in Kenya, all transgenic sweet potato lines were found susceptible to virus challenge (New Scientist, 2004). A closer examination into science behind the design of the cultivar revealed that the CP segment used in the transgene was taken from an American strain of the virus and no groundwork research was undertaken to determine the relatedness of this

American strain to SPFMV strains indigenous to Kenya (Thomson, 2004). In this instance, the lack of proper genetic variability studies contributed to the failure of transgenic sweet potato cultivar in Kenya. Although CP-mediated resistance to SPFMV was also achieved by Okada *et al*. (2002), the durability of the resistance in the field was not reported.

Other strategies to generate transgenic sweet potato with resistance to SPVD have also been tested. Rice cysteine proteinase inhibitor (*OCI*) mediated resistance to potyviruses is believed to inhibit the viral cysteine proteinase NIa that processes the potyviral polyprotein (Gutierrez-Campos *et al*., 1999). The expression of *OCI* in transgenic plants might also confer resistance to SPCSV, as closteroviruses also encode cysteine proteinases to modify some of their proteins. Sweet potato plants transformed with the *OCI* has been reported to show increased resistance to SPFMV (Cipriani *et al*., 2001). However, the resistance was not effective and typical symptoms of SPVD developed in plants infected with SPCSV and SPFMV (Clark *et al*., 2012).

A RNA silencing approach was also used to target SPCSV and SPFMV simultaneously. A construct was designed to produce transcripts that generate a dsRNA structure that was homologous to the polymerase genes of each virus, thus effectively inducing the RNA silencing defense mechanism against both viruses (Kreuze *et al*., 2008). The study showed that many transgenic lines accumulated only low titers of SPCSV with no symptom development. However, the low titers of SPCSV in transgenic plants were still sufficient to break down the natural high level of resistance to SPFMV, and SPVD developed.

Another key constraint to the sucessful development of transgenic sweet potato with virus resistance is the lack of an efficient and reproducible transformation system for sweet potato. The ability to regenerate a whole plant from isolated plant cells or tissues which have been genetically transformed underpins most plant transformation systems. Therefore, tissue culture and plant regeneration are an integral part of most post-plant transformation strategies, and can often prove to be the most challenging and critical

aspect of a plant transformation system. Instrumental in the success of integrating plant tissue culture into a plant transformation protocol is the development of a rapid and efficient plant regeneration system that delivers a high frequency of regenerable cells that are accessible to gene transfer (Anonymous, 2003).

Studies on the regeneration of sweet potato have predominately focused on the somatic embryogenesis method for plant regeneration (Feng *et al*., 2011). To date, sweet potato somatic embryogenesis has been documented using various explant types including anthers (Tsay & Tseng, 1979), leaves (Liu & Cantliffe, 1984), petioles (Zheng *et al*., 1996), shoots (Liu & Cantliffe, 1984), buds (Sihachakr *et al*., 1997) and meristems (Liu & Cantliffe, 1984; Cheé & Cantliffe, 1988; Liu *et al*., 2001). However, the production of responsive embryogenic tissues is a difficult task that requires considerable time. Once produced, high quality embryogenic tissues must be proliferated and maintained by frequent subculture without loss of their morphogenetic potential (Cheé & Cantliffe, 1988; Liu *et al*., 2001; Feng *et al*., 2011). In addition, production of suitable somatic embryogenic tissues is very commonly cultivar-specific and many sweet potato cultivars were found to be still recalcitrant (González *et al*., 2008; Feng *et al*., 2011). Due to these difficulties, a few studies have focused on organogenesis in sweet potato regeneration with some success (Morán *et al*., 1998; Luo *et al*., 2006; González *et al*., 2008).

In general, a number of reports have been published on the regeneration of sweet potato, using various tissues from different cultivars with varying levels of reproducibility and efficiency. However, there is a severe limitation of genotypes for regeneration and efficiency on the whole is still relatively low (Otani & Shimada, 2002). In addition, sweet potato is considered highly recalcitrant to regeneration and transformation and this has limited its application in genetic engineering technologies (González *et al*., 2008). An efficient and practicable regeneration method of sweet potato is still needed for the plant transformation to meet the requirements of effective genetic improvement of this crop.

Infections by multiple viruses are common occurrences in sweet potato (Loebenstein *et al*., 2009). Therefore, to address the problem of the multiplicity and synergism of sweet potato viruses, future endeavors should develop a strategy that would confer broad-spectrum virus resistance in sweet potato (Lin *et al*., 2007).

## 1.6 REFERENCES


**Abad, J. A., Parks, E. J., New, S. L., Fuentes, S., Jesper, W. & Moyer, J. W. (2007).** First report of *Sweet potato chlorotic stunt virus*, a component of sweet potato virus disease, in North Carolina. *Plant Disease* **91,** 327.

**Adams, I. P., Glover, R. H., Monger, W. A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M. & Boonham, N. (2009).** Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. *Molecular Plant Pathology* **10,** 537–545.

**Agrios, G. N. (2005).** *Plant Pathology.* San Diego: Academic Press, pp. 165-169, 314-315, 724-752.

**Albuquerque, L. C., Inoue-Nagata, A. K., Pinheiro, B., Riberio, S. D., Resende, R. O., Moriones, E. & Navas-Castillo, J. (2011).** A novel monopartite begomovirus infecting sweet potato in Brazil. *Archives of Virology* DOI 10.1007/s00705-011-1016-x.

**Alicia, T., Fenby, N. S., Gibson, R. W., Adipala, E., Vetten, H. J., Foster, G. D. & Seal, S. E. (1999).** Occurrence of two serotypes *of Sweet potato chlorotic stunt virus* in East Africa and their associated differences in coat protein and HSP70 homologue gene sequences. *Plant Pathology* **48,** 718-726.

**Allemann, J., Laurie, S. M., Thiart, S. & Vorster, H. T. (2004).** Sustainable production of root and tuber crops (potato, sweet potato and indigenous potato, cassava) in Southern Africa. *South African Journal of Botany* **70,** 60-66.

**Alvarez, V., Ducasse, D. A., Biderbost, E. & Nome, S. F. (1997).** Sequencing and characterization of the coat protein and 3' non-coding region of a new sweet potato potyvirus. *Archives of Virology* **142,** 1635-1644.

**Anonymous. (2003).** *Plant Tissue Culture.* Dc304.4shared.com/doc/SH7SiPHp/previews.html.

**Aritua, V., Bau, B., Vetten H. J., Adipala E. & Gibson, R. W. (2006).** Incidence of five viruses infecting sweetpotatoes in Uganda; the first evidence of *Sweet potato caulimo-like virus* in Africa. *Plant Pathology* **56,** 324-331.

**Aritua, V., Barg, E., Adipala E., and Gibson, R. W., Lesemann, D. E. & Vetten, H. J. (2009).** Host range, purification, and genetic variability in *Sweet potato chlorotic fleck virus*. *Plant Disease* **93,** 87-93.

**Ateka, E. M., Barg, E., Njeru, R. W., Lesemann, D. E. & Vetten, H. J. (2004).** Further characterization of '*Sweet potato virus 2'*: a distinct species of the genus Potyvirus. *Archives of Virology* **149,** 225-239.

**Ateka, E. M., Barg, E., Njeru, R. W., Thompson, G. & Vetten, H. J. (2007).** Biological and molecular variability among geographically diverse isolates of *Sweet potato virus 2*. *Archives of Virology* **152,** 479-488.

**Atkey, P. T. & Brunt, A. A. (1987).** Electron microscopy of an isometric caulimo-like virus from sweet potato (*Ipomoea batatas*). *Journal of Phytopathology* **118,** 370-376.

**Austin, D. F. (1987).** The taxonomy, evolution and genetic diversity of sweet potatoes and related wild species. In *Exploration maintenance and utilization of sweet potato genetic resources,* in: *Proceedings of the First Planning Conference*, pp. 27-59. Peru: International Potato Center (CIP).

**Banks, G. K., Bedford, I. D., Beitia, F. J., Rodrigues-Cerezo, E. & Markham, P. G. (1999).** A novel geminivirus of *Ipomoea indica* (*Convolvulaceae*) from Southern Spain. *Plant Disease* **83,** 486.

**Barzon, L., Lavezzo, E., Militello, V., Toppo, S. & Palù, G. (2011).** Applications of Next-Generation Sequencing Technologies to Diagnostic Virology. *International Journal of Molecular Sciences* **12,** 7861-7884.

**Baulcombe, D. C. (1996).** Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *The Plant Cell* **8,** 1833-1844.

**Bird, C. R. & Ray, J. A. (1991).** Manipulation of plant gene expression by antisense RNA. *Biotechnology and Genetic Engineering Reviews* **9,** 207-227.

**Bos, L. (1999).** *Plant Viruses: Unique and Intriguing Pathogens, A Textbook of Plant Virology*. Leiden: Backhuys.

**Brown, J. D., Brunt, A. A. & Hugo, S. A. (1988).** Studies on viruses isolated from sweet potato (*Ipomoea batatas*). *Annual Report of Glasshouse Crops Research Institute (1986-1987),* pp. 104-108.

**Brunt, A., Crabtree, K., Dallwitz, M., Gibbs, A. & Watson, L. (1996).** *Viruses of Plants: Descriptions and Lists from VIDE Database*. UK: CAB International, pp. 1216-1229.

**Carey, E. E., Mwanga, R. O. M., Fuentes, S., Kasule, S., Macharia, C., Gichuki, S. T. & Gibson, R. W. (1997).** Sweetpotato viruses in Uganda and Kenya: results of a survey. In *Proceedings of the Sixth Treinnial Symposium of the International Society for Tropical Crops - African Branch*, pp. 22-28.

**Carroll, H. W., Villordon, A. Q., Clark, C. A., La Bonte, D. R. & Hoy, M. W. (2004).** Studies on Beauragard sweetpotato clones naturally infected with viruses. *International Journal of Pest Management* **50,** 101-106.

**Cheé, R. P. & Cantliffe, D.J. (1988).** Selective enhancement of *Ipomoea batatas* Pior. embryogenic and non-embryogenic callus growth and production of embryos in liquid culture. *Plant Cell Tissue Organ Culture* **15,** 149-159.

**Cipriani, G., Fuentes, S., Bello, V., Salazar, L. F., Ghislain, M. & Zhang, D. P. (2001).** Transgene expression of rice cysteine proteinase inhibitors for the development of resistance against sweetpotato feathery mottle virus. In *CIP Program Report 1999-2000*. pp. 267-271. Edited by Fergusson, P., Parrott, S., Sheridan, K., Smith, B. & Stares, J. Lima: International Potato Center.

**Clark, C. A. & Moyer, J. W. (1988).** *Compendium of Sweet Potato Diseases*. USA: APS Press, pp. 49-53.

**Clark, C. A., Davis, J. A., Abad, J. A., Cuellar, W. J., Fuentes, S., Kreuze, J. F., Gibson, R. W., Mukasa, S. B., Tugume, A. K., Tairo, F. D. & Valkonen, J. P. T. (2012).** Sweetpotato viruses : 15 years on understanding and managing complex diseases. *Plant Disease* **96,** 168-185.

**Cohen, J., Franck, A., Vetten, H. J., Lesemann, D. E. & Loebenstein, G. (1992).** Purification and properties of Closterovirus-like particles associated with a

whitefly-transmitted disease of sweet potato. *Annals of Applied Biology* **121,** 257-268.

**Cohen, J., Milgram, M., Antignus, Y., Pearlsman, M., Lachman, O. & Loebenstein, G. (1997).** Ipomoea crinkle leaf curl caused by a whitefly-transmitted gemini-like virus. *Annals of Applied Biology* **131,** 273-282.

**Cohen, J., Gal-On, A. & Loebenstein, G. (2008).** Viral diseases of sweet potato and their control. *Phytoparasitica* **36,** 124-125.

**Colinet, D. & Kummert, J. (1993).** Identification of a *Sweet potato feathery mottle virus* from China (SPFMV-CH) by the polymerase chain reaction with degenerate primers. *Journal of Virological Methods* **45,** 149-159.

**Colinet, D., Kummert, J. & Lepoivre, P. (1994).** The complete nucleotide sequences of the coat protein cistron and the 3' non-coding region of a newly-identified potyvirus infecting sweetpotato, as compared to those of *Sweetpotato feathery mottle virus*. *Archives of Virology* **139,** 327-336.

**Colinet, D., Nguyen, M., Kummer, J. & Lepoivre, P. (1998).** Differentiation among Potyviruses infecting sweet potato based on genus- and virus-specific reverse transcription polymerase chain reaction. *Plant Disease* **82,** 223-229.

**Collinge, D. B., Jorgensen, H. J. L., Lund, O. S. & Lyngkjaer, M. J. (2010).** Engineering pathogen resistance in crop plants: current trends and future prospects. *Annual Review of Phytopathology* **48,** 269-291.

**Cuellar, W. J., De Souza, J., Barrantes, I., Fuentes, S. & Kreuze, J. F. (2011).** Distinct cavemoviruses interact synergistically with *Sweet potato chlorotic stunt virus* (Genus *Crinivirus*) in cultivated sweet potato. *Journal of General Virology* DOI:10.1099/vir.0.029975-0.

**Dasgupta, I., Malathi, V. G. & Mukherjee, S. K. (2003).** Genetic engineering for virus resistance. *Current Science* **84**, 341-354.

**Di Feo, L., Nome, S. F., Biderbost, E., Fuentes, S. & Salazar, L. F. (2000).** Etiology of sweetpotato chlorotic dwarf disease in Argentina. *Plant Disease* **84,** 223-229.

**Domola, M. J., Thompson, G. J., Aveling, T. A. S., Laurie, S. M., Strydom, H. & Van den Berg, A. A. (2008).** Sweet potato viruses in South Africa and the effect of viral infection on storage root yield. *African Plant Protection* **14,** 15-23.

**Doughtery, W. G. & Parks, T. D. (1995).** Transgenes and gene suppression: telling us something new?. *Current Opinion in Cell Biology* **7,** 399-405.

**Du Plooy, C. P. (1986).** Progress and limitations in breeding of sweet potato (*Ipomoea batatas*) in South Africa. *Acta Horticulturae* **194,** 77-82.

**Du Plooy, C. P., Wagner, J. J. & Bosch, S. E. (1996).** Cultivation of sweet potato. In *Vegetable Production in South Africa*, pp. 1-7. Pretoria: ARC Roodeplaat.

**Esbenshade, P. R. & Moyer, J. W. (1982).** Indexing system for *Sweet potato feathery mottle virus* in sweet potato using enzyme-linked immunosorbent assay. *Plant Disease* **66,** 911-913.

**FAO. 2004.** FAO statistics. http://apps.fao.org. Rome, Italy: Food and Agricultural Organization of the United Nations.

**FAOSTAT. 2011.** FAO statistics. http://apps.fao.org. Rome, Italy: Food and Agricultural Organization of the United Nations.

**Feng, G., Yifo, G. & Pinbo, Z. (2000).** Production and deployment of virus-free sweetpotato in China. *Crop Protection* **19,** 105-111.

**Feng, C., Yin, Z., Ma, Y., Zhang, Z., Chen, L., Wang, B., Li, B., Huang, Y. & Wang, Q. (2011).** Cryopreservation of sweetpotato (*Ipomoea batatas*) and its pathogen eradication by cryotherapy. *Biotechnology Advances* **29,** 84-93.

**Fitchen, J. H. & Beachy, R. N. (1993).** Genetically engineered protection against viruses in transgenic plants. *Annual Review of Microbiology* **47,** 739-763.

**Fletcher, J. D., Lewthwaite, S. L., Fletcher, P. J. & Dannock, J. (2000).** Sweetpotato (kumura) virus in disease surveys in New Zealand. In *Proceedings of the International Workshop on Sweetpotato Cultivar Decline Study,* pp. 42-47. Edited by Nakazawa, Y. & Ishiguro, K. Japan: Kyushu National Agricultural Experimental Station (KNAES).

**Flint S. J., Enquist L. W., Krug R. M., Racaniello V. R. & Skalka A. M. (2000).** *Principles of Virology: Molecular Biology, Pathogenesis, and Control*, pp. 597-610. Washington: ASM Press.

**Frizzie, A. & Huang, S. (2010).** Tapping RNA silencing pathways for plant biotechnology. *Plant Biotechnology Journal* **8,** 655-677.

**Fuentes, S.** (**1994).** Preliminary identification of a sweetpotato virus (C-6). *Fitopathologia* **29,** 38.

**Fuentes, S. & Salazar, L. F. (1989).** Identification of sweetpotato [*Ipomoea batatas* (L.) Lam] viruses. *Fitopatologia* **24,** 43.

**Fuentes, S. & Salazar, L. F. (1992).** Identification of a new sweetpotato virus. *Fitopatologia* **27,** 50.

**Fuentes, S. & Salazar, L. F. (2003).** First report of *Sweet potato leaf curl virus* in Peru. *Plant Disease* **87,** 98.

**Fuentes, S., Mayo, M. A., Jolly, C. A., Nakano, M., Querci, M. & Salazar, L. F. (1996).** A novel luteovirus from sweet potato, *Sweet potato leaf speckling virus*. *Annals of Applied Biology* **128**, 491-504.

**Fuchs, M. & Gonsalves, D. (2007).** Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. *Annual Review of Phytopathology* **45,** 173-202.

**Gaba, V., Rosner, A., Maslenin, L., Leibman, D., Singer, S., Kukurt, E., Shiboleth, Y. M. & Gal-On, A. (2010).** Hairpin-based virus resistance depends on the sequence similarity between challenge virus and discrete, highly accumulating siRNA species. *European Journal of Plant Pathology* **128,** 153-164.

**Gamarra, H. A., Fuentes, S., Morales, F. J., Glover, R., Malumphy, C. & Barker, I. (2010).** *Bemisia afer* sensu lato, a vector of *Sweet potato chlorotic stunt virus*. *Plant Disease* **94,** 510-514.

**Gao, F., Gong, Y. F. & Zhang, P. B. (2000).** Production and development of virus-free sweetpotato in China. *Crop Protection* **19,** 105-111.

**Gibb, K. S. & Padavan, A. C. (1993).** Detection of *Sweet potato feathery mottle potyvirus* in sweet potato grown in northern Australia using an efficient and simple assay. *International Journal of Pest Management* **39,** 223-228.

**Gibson, R. W., Mwanga, R. O. M., Kasule, S., Mpembe, I. & Carey, E. E. (1997).** Apparent absence of viruses in most symptomless field-grown sweet potato in Uganda. *Annals of Applied Biology* **130,** 481-490.

**Gibson, R. W., Kaitisha, G. C., Randrianaivoriavony, J. M. & Vetten, H. T. (1998).** Identification of the East African strain of *Sweet potato chlorotic stunt virus*

(SPCSV) as a major component of sweet potato virus disease (SPVD) in southern Africa. *Plant Disease* **82,** 1063.

**Gibson, R. W., Aritua, V., Byamukama, E., Mpembe, I. & Kayongo, J. (2004).** Control strategies for sweet potato virus disease in Africa. *Virus Research* **100,** 115-122.

**González, R. G., Sánchez, D. S., Guerra, Z. Z., Campos, J. M., Quesda, A. L., Valvida, R. M., Arencibia, A. D., Bravo, K. Q. & Caligari, P. D. S. (2008).** Efficient regeneration and *Agrobacterium tumefaciens* mediated transformation of recalcitrant sweet potato (*Ipomoea batatas* L.) cultivars. *Asia Pacific Journal of Molecular Biology and Biotechnology* **16,** 25-33.

**Gutiérrez, D. L. & Valverde, R. A. (2007).** Detection of sweetpotato viruses by NCM-ELISA. *HortScience* **42,** 450-451.

**Gutiérrez-Campos, R., Torres-Acosta, J. A., Saucedo-Arias, L. J. & Gomez-Lim, M. A. (1999).** The use of the cysteine proteinase inhibitors to engineer resistance against potyviruses in transgenic tobacco plants. *Nature Biotechnology* **17,** 1223-1226.

**Gutiérrez, D. L., Fuentes, S. & Salazar, L. F. (2003).** Sweetpotato virus disease (SPVD): distribution, incidente, and effect on sweetpotato yield in Peru. *Plant Disease* **87,** 297-302.

**Haible, D., Kober, S. & Jeske, H. (2006).** Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. *Journal of Virological Methods* **135,** 9-16.

**Hamilton, A. J. & Baulcombe, D. C. (1999).** A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286,** 950-952.

**Hollings, M., Stone, O. M. & Bock, K. (1976).** Purification and properties of *Sweet potato mild mottle*, a whitefly borne virus from sweet potato (*Ipomoea batatas*) in East Africa. *Annals of Applied Biology* **82,** 511-528.

**Hull, R. (2002).** *Matthew's Plant Virology*. San Diego: Academic Press.

**Ishak, J. A., Kreuze, J. F., Johansson, A., Mukasa, S. B., Tairo, F., Abo El-Abbas. & Valkonen, J. P. T. (2003).** Some molecular characteristics of three viruses

from sweet potato virus disease (SPVD) - affected sweet potato plants in Egypt. *Archives of Virology* **148,** 2449-2460.

**Jan, F-J., Fagoaga, C., Pang, S-Z. & Gonsalves, D. (2000a).** A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance. *Journal of General Virology* **81,** 235-242.

**Jan, F-J., Fagoaga, C., Pang, S-Z. & Gonsalves, D. (2000b).** A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *Journal of General Virology* **81,** 2103-2109.

**Jana, R. K. (1982).** Status of sweet potato cultivation in East Africa and its future. In *Sweet Potato: Proceedings of the 1st International Symposium (23- 27 March 1981).* Edited by Villareal, R. L. & Griggs, T. D. China: AVRDC Publications.

**Jericho, C. & Thompson, G. J. (2000).** Detection of sweet potato viruses in South Africa. *African Potato Association Proceedings* **73,** 379-391.

**Jones, R. A. C. & Dwyer, G. I. (2007).** Detection of *Sweetpotato chlorotic fleck virus* and *Sweetpotato feathery mottle virus* - strain O in Australia. *Australian Plant Pathology* **36,** 591-594.

**Joubert, T. G., Ia, G., Klesser, P. & Nel, D. D. (1996).** The value of virus-indexed sweet potato material. In *Vegetable Production in South Africa,* pp. 1-2. Pretoria: ARC Roodeplaat.

**Kado, C. I. & Agrawal H. O. (1972).** *Principles and Techniques in Plant Virology*. New York: Van Nostrand Reinhold Company.

**Kapinga, R., Ndunguru, J., Mulokozi, G. & Tomwegamire, S. (2009).** Impact of common sweet potato viruses on total carotenoids and root yields of an orange-fleshed sweet potato in Tanzania. *Scientia Horticulturae* **122,** 1-5.

**Karuri, H. W., Ateka, E. M., Amata, R., Nyende, A. B. & Muigai, A. W. T. (2009).** Characterization of Kenyan sweet potato genotypes for SPVD resistance and high dry matter content using simple sequence repeat markers. *African Journal of Biotechnology* **8,** 2169-2175.

**Karyeija, R. F., Gibson, R. W. & Valkonen, J. P. T. (1998).** The significance of *Sweet potato feathery mottle virus* in subsistence sweet potato production in Africa. *Plant Disease* **82,** 4-15.

**Karyeija, R. F., Kreuze, J. F., Gibson, R. W. & Valkonen, J. P. T. (2000a).** Synergistic interactions of a potyvirus and a phloem-limited crinivirus in sweet potato plants. *Virology* **269,** 26-36.

**Karyeija, R. F., Kreuze, J. F., Gibson, R. W. & Valkonen, J. P. T. (2000b).** Two serotypes of *Sweetpotato feathery mottle virus* in Uganda and their interaction with resistant sweetpotato cultivars. *Phytopathology* **90,** 1250-1255.

**Kokkinos, C. D. & Clark, C. A. (2006a).** Real-time PCR assays for detection and quantification of sweetpotato viruses. *Plant Disease* **90,** 783-788.

**Kokkinos, C. D. & Clark, C. A. (2006b).** Interactions among *Sweetpotato chlorotic stunt virus* and different potyviruses and potyvirus strains infecting sweetpotato in the United States. *Plant Disease* **90,** 1347-1352.

**Kokkinos, C. D., Clark, C. A., McGregor C. E. & LaBonte, D. R. (2006).** The effect of sweet potato virus disease and its viral components on gene expression levels in sweetpotato. *Journal of the American Society of Horticultural Science* **131,** 657-666.

**Kreuze, J.** (**2002).** *Molecular Studies on the Sweet Potato Virus Disease and its Two Causal Agents*. Uppsala: Swedish University of Agricultural Sciences (Doctoral thesis), pp. 24-33.

**Kreuze, J. & Fuentes, S. (2008).** Sweetpotato viruses. In *Encyclopedia of Virology,* pp. 659-669. Edited by Mahy, B. W. J. & Van Regenmortel, M. H. V. Amsterdam: Academic Press.

**Kreuze, J. F., Karyeija, R. F., Gibson, R. W. & Valkonen, J. P. T. (2000).** Comparisons of coat protein gene sequences show that East African isolates of *Sweet potato feathery mottle virus* form a genetically distinct group. *Archives of Virology* **145,** 567-574.

**Kreuze, J. F., Salmolski, K. I., Lazaro, M. U., Chuquiyuri, W. J. C., Morgan, G. L., Mejla, P. G. C., Ghislain, M. & Valkonen, J. P. T. (2008).** RNA silencing-mediated resistance to a *Crinivirus* (*Closteroviridae*) in cultivated sweet potato

(*Ipomoea batatas* L.) and development of sweetpotato virus disease following co- infection with a potyvirus. *Molecular Plant Pathology* **9,** 589-598.

**Kreuze, K. F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I. & Simon, R. (2009).** Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: A generic method for diagnosis, discovery and sequencing of viruses. *Virology* **388,** 1-7.

**Laurie, L. M. (2001).** Evaluation of sweet potato breeding lines and imported cultivars in southern region of SADC. *Acta Horticulturae* **583,** 91-101.

**Laurie, L. M. (2002).** Overview of breeding and evaluation of orange-fleshed sweet potato in South Africa. http://www.cipotato.org/Vitaa?Proceedings/VITAA-paper-SLAURIE-FINAL-11Feb2002.pdf.

**Laurie, L. M. (2004).** Sweet potato in perspective. In *Guide to Sweet Potato Production in South Africa,* pp. 1-6. Edited by Niederwieser, J. G. Pretoria: ARC- Roodeplaat Vegetable and Ornamental Plant Institute.

**Laurie, L. M. & Niederwieser, J. G. (2004).** The sweet potato plant. In *Guide to Sweet Potato Production in South Africa,* pp. 7-14. Edited by Niederwieser, J. G. Pretoria: ARC- Roodeplaat Vegetable and Ornamental Plant Institute.

**Liao, C. H., Chien, K., Chung, M. L., Chui, R. J. & Han, Y. H. (1979).** A study of a sweetpotato virus disease in Taiwan. I. Sweetpotato yellow spot virus disease. *Journal of Agricultural Research in China* **28,** 127-137.

**Lecoq, H.** (**1998).** Control of plant virus diseases by cross protection. In *Plant Virus Disease Control,* pp. 33-40. Edited by Hadidi, A., Khetarpal, R. K. & Koganezawa, H. USA: APS Press.

**Li, R., Salih, S. & Hurtt, S. (2004).** Detection of Geminiviruses in sweetpotato by polymerase chain reaction. *Plant Disease* **88,** 1347-1351.

**Lin, S-S., Henriques, R., Wu, H-W., Nui, Q-W. & Chua, N-H. (2007).** Strategies and mechanisms of plant virus resistance. *Plant Biotechnology Reports* **1,** 125-134.

**Lindbo, J. A. & Dougherty, W. G. (2005).** Plant pathology and RNAi: A brief history. *Annual Review of Phytopathology* **43,** 191-204.

**Ling, K. S., Jackson, D. M., Harrison, H., Simmons, A. M. & Pesic-VanEsbroeck, Z. (2010).** Field evaluation of yield effects on the USA heirloom sweetpotato cultivars infected by Sweet potato leaf curl virus. *Crop Protection* **29,** 757-765.

**Liu, J. R. & Cantliffe, D. J. (1984).** Somatic embryogenesis and plant regeneration in tissue cultures of sweet potato (*Ipomoea batatas* Pior.). *Plant Cell Reports* **3,** 12-15.

**Liu, J. R., Zhai, H., Wang, Y. & Zhang, D. P. (2001).** Efficient plant regeneration and embryogenic suspension cultures of sweetpotato. *In Vitro Cellular and Developmental Biology* **37,** 564-567.

**Loebenstein, G. & Harpaz, L. (1960).** Virus diseases of sweetpotatoes in Israel. *Phytopathology* **50,** 100-104.

**Loebenstein, G., Fuentes, S., Cohen, J. & Fuentes, S. (2003).** Sweet potato. In *Virus and Virus-like Diseases in Plants of Developing Countries,* pp. 223-246. Edited by Loebenstein, G. & Thottappilly, G. The Netherlands: Kluwer Academic Publishers.

**Loebenstein, G., Thottappilly, G., Fuentes, S. & Cohen, J. (2009).** Viruses and phytoplasma diseases. In *The Sweet Potato,* pp. 114. Edited by Loebenstein, G. & Thottappilly, G. The Netherlands: Springer publications.

**Lomonossoff, G. P. (1995).** Pathogen-derived resistance to plant viruses. *Annual Review of Phytopathology* **33,** 323-343.

**Lotrakul, P., Valverde, R. A. & Clark, C. A. (2003).** Properties of a begomovirus isolated from sweetpotato (*Ipomoea batatas* Lam.) infecting with *Sweet potato leaf curl virus*. *Review Mexicana Fitopatologia* **21,** 128-136.

**Lozano, G., Trenado, H. P., Valverde, R. A. & Navas-Castillo, J. (2009).** Novel begomovirus species of recombinant nature in sweet potato (*Ipomoea batatas*) and *Ipomoea indica*: Taxonomic and phylogenetic implications. *Journal of General Virology* **90,** 2550-2562.

**Luan, Y. S., Zhang, J., Lui, D. M. & Li, W. L. (2007).** Molecular characterization of *Sweet potato leaf curl virus* isolate from China (SPLCV-CN) and its phylogenetic relationship with other members of the Geminiviridae. *Virus Genes* **35,** 379-385.

**Luo, H. R., Santa Maria, M., Benavides, J., Zhang, D. P., Zhang, Y. Z. & Ghislain, M. (2006).** Rapid genetic transformation of sweetpotato (*Ipomoea batatas* (L.) Lam) via organogenesis. *African Journal of Biotechnology* **5,** 1851-1857.

**Mashilo, J. (2009).** *Combined thermotherapy and meristem-tip culture for efficient elimination of feathery mottle virus in sweet potato* (*Ipomoea batatas* L.). South Africa: University of KwaZulu-Natal (Honors thesis).

**Matthews R. E. F. (1992).** *Fundamentals of Plant Virology*. New York: Academic Press, pp. 259.

**McGregor, C. E., Miano, D., LaBonte, D., Hoy, M. & Clark, C. (2009).** The effect of the sequence of infection of the causal agents of sweet potato virus disease on symptom severity and individual virus titres in sweet potato cv. Beauragard. *Journal of Phytopathology* **157,** 514-517.

**Mette, M. F., Aufsatz, W., van den Winden, M. A. & Matzke, A. J. (2000).** Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO Journal* **19,** 5194-5201.

**Miano, D. W., LaBonte, D. R. & Clark, C. A. (2008).** Identification of molecular markers associated with sweet potato resistance to sweet potato virus disease in Kenya. *Eyphytica* **160,** 15-24.

**Milgram, M., Cohen, J. & Loebenstein, G. (1996).** Effect of *Sweet potato feathery mottle virus* and *Sweet potato sunken virus* on sweet potato yields and rates of reinfection of virus-free planting material in Israel. *Phytoparasitica* **24,** 189-93.

**Morán, R., García, R., López, A., Zaldúa, Z., Mena, J., García, M., Armas, R., Somonte, D., Rodríguez, J., Gómez, M. & Pimentel, E. (1998).** Transgenic sweet potato plants carrying the delta-endotoxin gene from *Bacillus thuringiensis* var. *tenebrionis. Plant Science* **139,** 175-184.

**Moyer, J. W. & Salazar, L. F. (1989).** Virus and virus-like diseases of sweet potato. *Plant Disease* **73,** 451-455.

**Moyer, J. W., Abad, J. A., New, J. & Bell, J. (2002).** Isolation, identification and detection of undesribed RNA sweetpotato viruses. *Acta Horticulturae* **583,** 121-127.

**Mukasa, S. B., Rubaihayo, P. R. & Valkonen, J. P. T. (2003).** Incidence of viruses and viruslike diseases of sweet potato in Uganda. *Plant Disease* **87,** 329-335.

**Mukasa, S. B., Rubaihayo, P. R. & Valkonen, J. P. T. (2006).** Interactions between a Crinivirus, an ipomovirus and a potyvirus in coinfected sweet potato plants. *Plant Pathology* **55,** 458-467.

**Mwanga, R. O. M., Yencho, G. C. & Moyer, J. W. (2002).** Dialled analysis of sweetpotatoes for resistance to sweetpotato virus disease. *Euphytica* **128,** 237-249.

**Napoli, C., Lemieux, C. & Jorgensen, R. (1990).** Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2,** 279-289.

**New Scientist. (2004).** Notes, 7 February 2004: 181 pp. 2433.

**Ndunguru, J. & Aloyce, R. C. (2000).** Incidence of sweet potato virus disease in sweet potato grown under different traditional cropping systems in the Lake Victoria zone of Tanzania. *African Potato Association Conference Proceedings* **5,** 405-408.

**Ndunguru, J. & Kapinga, R. (2007).** Viruses and virus-like diseases affecting sweetpotato subsistence farming in Tanzania. *African Journal of Agricultural Research* **2,** 232-239.

**Nome, S. F. (1973).** *Sweetpotato vein mosaic virus* in Argentina. *Phytopathologica Zeitscheif* **77,** 44-45.

**Nome, C. F., Laguna, I. G. & Nome, S. F. (2006).** Cytological alterations produced by *Sweet potato mild speckling virus*. *Journal of Phytopathology* **154,** 504-507.

**Nome, C. F., Nome, S. F., Gusmán, F., Conci, L. & Graciela, L. (2007).** Localization of *Sweet potato chlorotic stunt virus* (SPCSV) in synergistic infection with Potyviruses in sweet potato. *Biocell* **31,** 23-31.

**Nui, J-H., Jian, H., Xu, J-M., Guo, Y-D., & Lui, Q. (2010).** RNAi technology extends its reach: engineering plant resistance against harmful eukaryotes. *African Journal of Biotechnology* **9,** 7573-7582.

**O' Brien, P. J. (1972).** The Sweet Potato: its origin and dispersal. *American Anthropologist* **74,** 342-365.

**Okada, Y., Nishiguchi, M., Saito, A., Kimura, T., Mori, M., Hanada, K., Sakai, J., Matsuda, Y. & Murata, T. (2002).** Inheritance and stability of the virus-resistant gene in the progeny of transgenic sweet potato. *Plant Breeding* **121,** 249-253.

**Opiyo, S. A., Ateka, E. M., Owuor, P. O., Manguro, L. O. A. & Miano, D. W. (2010).** Development of a multiplex PCR technique for the simultaneous detection of *Sweet potato feathery mottle virus* and *Sweet potato chlorotic stunt virus*. *Journal of Plant Pathology* **92,** 363-366.

**Otani, M. & Shimada, T. (2002).** Transgenic sweet potato with agronomically important genes. In *Transgenic Plant and Crops,* pp. 699-716. Edited by G. G. Khachatourians, A. Mchughen, R. Scorza, W. K. Nip, & Y. H. Hui. New York: Marcel Dekker.

**Pang, S-Z., Jan, F-J. & Gonsalves, D. (1997).** Nontarget DNA sequences reduce the transgene length necessary for RNA-mediated tospovirus resistance in transgenic plants. *Proceedings fo the National Academy of Sciences, USA* **94,** 8261-8266.

**Paprotka, T., Bioteux, L. S., Fonseca, M. E. N., Resende, R. O., Jeske, H. & Faria, J. C. (2010).** Genomic diversity of sweet potato geminiviruses in a Brazilian germplasm bank. *Virus Research* **149,** 224-233.

**Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002).** CARPEL FACTORY, a Dicer homolod, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Current Biology* **12,** 1484-1495.

**Parrella, G., De Stradis, A. & Giorgini, M. (2006).** *Sweetpotato feathery mottle virus* is the causal agent of Sweetpotato virus disease (SPVD) in Italy. *Plant Pathology* **55,** 818.

**Perez-Egusquiza, Z., Ward, L. I. & Glover, G. R. G. (2009).** Detection of *Sweet potato virus 2* in sweet potato in New Zealand. *Plant Disease* **93,** 427.

**Peters D. (2003).** Tospoviruses. In *Viruses and Virus-like Diseases of Major Crops in Developing Countries.* Edited by Loebenstein G. & Thottappilly G. USA: Kluwer Academic Publishers.

**Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M. & Tepfer, M. (2008).** Strategies for antiviral resistance in transgenic plants. *Molecular Plant Pathology* **9,** 73-83.

**Qu, J., Ye, J. & Fang, R. (2007).** Artificial microRNA-mediated virus resistance in plants. *Journal of Virology* **81,** 6690-6699.

**Rännäli, M., Czekaj, V., Jones, R. A. C., Fletcher, J. D., Davis, R. I., Mu, L., Dwyer, G. I., Coutts, B. A. & Valkonen, J. P. T. (2008).** Molecular genetic characterization of *Sweet potato virus G* (SPVG) isolates from areas of the Pacific Ocean and southern Africa. *Plant Disease* **92,** 1313-1320.

**Rossel, H. W. & Thottappilly, G. (1988).** Complex virus diseases of sweet potato. In *Exploration, Maintenance, and Utilization of Sweetpotato Genetic Resources, Report of 1st Sweetpotato Planning Conference,* pp. 291-302. Peru: International Potato Centre.

**Sanford, J. C. & Johnston, S. A. (1985).** The concept of pathogen-derived resistance - deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology* **113,** 395-405.

**Schaefers, G. A. & Terry, E. R. (1976).** Insect transmission of sweetpotato disease agents in Nigeria. *Phytopathology* **66,** 642-645.

**Sheffield, F. M. L. (1957).** Virus diseases of sweet potato in East Africa. I. Identification of the viruses and their insect vectors. *Phytopathology* **47,** 582-590.

**Shepherd, D. N., Martin, D. P. & Thomson, J. A. (2009).** Transgenic strategies for developing crops resistant to geminiviruses. *Plant Science* **176,** 1-11.

**Sihachakr, D., Haïcour, R., Cavalcante Aves, J. M., Umboh, I., Nzoghé, D., Servaes, A. & Ducreux, G. (1997).** Plant regeneration in sweet potato (*Ipomoea batatas* L., *Convolvulaceae*). *Euphytica* **96,** 143-152.

**Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. (2000).** Gene expression: total silencing by intron-spliced hairpin RNAs. *Nature* **407,** 319-320.

**Souto, E. R., Sim, J., Chen, J., Valverde, R. A. & Clark, C. A. (2003).** Properties of strains of *Sweet potato feathery mottle virus* and two newly recognized

Potyviruses infecting sweetpotato in the United States. *Plant Disease* **87,** 1226-1232.

**Srisuwan, S., Sihachakr, D. & Siljak-Yakovlev, S. (2006).** The origin and evolution of sweet potato and its wild relatives through cytogenetic approaches. *Plant Science* **171,** 424-433.

**Stam, M., Mol, J. N. M. & Kooter, J. M. (1997).** The silence of genes in transgenic plants. *Annals of Botany* **79,** 3-12.

**Tairo, F., Mukasa, S. R., Jones, R. A. C. Kullaya, A., Rubaihayo, P. R. & Valkonen, J. P. T. (2005).** Unraveling the genetic diversity of the three main viruses involved in sweet potato virus disease (SPVD), and its practical implications. *Molecular Plant Pathology* **6,** 199-211.

**Tairo, F. (2006).** *Molecular Resolution of Genetic Variability of Major Sweet Potato Viruses and Improved Diagnosis of Potyviruses Co-infecting Sweet Potato*. Uppsala: Swedish University of Agricultural Sciences (Masters thesis), pp. 9-36.

**Tairo, F., Jones, R. A. C. & Valkonen, J. P. T. (2006).** Potyvirus complexes in sweetpotato: Occurrence in Australia, serological and molecular resolution, and analysis of the *Sweet potato virus 2* (SPV2) component. *Plant Disease* **90,** 1120-1128.

**Tenllado, F., Llave, C. & Díaz-Ruíz, J. R. (2004).** RNA interference as a new biotechnology tool for the control of virus diseases in plants. *Virus Research* **102,** 85-96.

**Thompson, G. J. & Mynhardt, L. (1986).** A potyvirus infecting sweet potatoes. *Phytophylactica* **18,** 46.

**Thomson, J. A. (2004).** The status of plant biotechnology in Africa. *AgBioForum* **7,** 9-12.

**Trenado, H. P., Lozano, G., Valverde, R. A. & Navas-Castillo, J. (2007).** First report of *Sweetpotato virus G* and *Sweetpotato virus 2* infecting sweetpotato in Spain. *Plant Disease* **91,** 1687.

**Tsay, H. S. & Tseng, M. T. (1979).** Embryoid formation and plantlet regeneration from anther callus of sweet potato. *Botanical Bulletin of Academia Sinica* **20,** 117-122.

**Tugume, A. K., Mukasa, S. B., Kalkkinen, N. & Valkonen, J. P. T. (2010).** Recombinant and selection pressure in Ipomovirus *Sweet potato mild mottle virus* (*Potyviridae*) in wild species and cultivated in sweet potato in the centre of evolution in East Africa. *Journal of General Virology* **91,** 1092-1108.

**Untiveros, M., Fuentes, S. & Salazar, L. F. (2007).** Synergistic interactions between *Sweet potato chlorotic stunt virus* (Crinivirus) with Carla-, Cucumo-, Ipomo- and Potyviruses infecting sweet potato. *Plant Disease* **91,** 669-676.

**Usugi, T., Nakano, M., Shinkai, A. & Hayashi, T. (1991).** Three filamentous viruses isolated from sweetpotato in Japan. *Annual Phytopathology Society of Japan* **57,** 512-521.

**Valverde, R. A., Kokkinos, C. D. & Clark, C. A. (2004a).** Whitefly transmission of sweet potato viruses. *Virus Research* **100,** 123-128.

**Valverde, R. A., Kokkinos, C. D. & Clark, C. A. (2004b).** *Sweet potato leaf curl virus*: Detection by molecular hybridization. *Phytopathology* **94,** S105.

**Van den Berg, A. A. & Laurie, S. M. (2004).** Cultivation. In *Guide to Sweet Potato Production in South Africa,* pp. 15-26. Edited by Niederwieser, J. G. Pretoria: ARC- Roodeplaat Vegetable and Ornamental Plant Institute.

**Van Jaarsveld, P. J., Faber, M., Tanumihardjo, S. A., Nestel, O., Lombard, C. J. & Benadé, A. J. S. (2005).** ß- carotene-rich orange- fleshed sweetpotato improved the vitamin A status of primary school children assessed with the modified- relative- dose- response test. *American Journal of Clinical Nutrition* **81,** 1080-1087.

**Voller A., Bartlett A., Bidwell D. E., Clark M. F. & Adams A. N. (1976).** The detection of viruses of enzyme-linked immunosorbent assay (ELISA). *Journal of General Virology* **33,** 165-167.

**Wambugu, F. M. (2003).** Development and transfer of genetically modified virus-resistant sweet potato for subsistence farmers in Kenya. *Nutritional Reviews* **61,** s110-s113.

**Wang M. & Gonsalves D. (1990).** ELISA detection of various *Tomato spotted wilt virus* isolates using specific antisera to structural proteins of the virus. *Plant Disease* **74,** 154-158.

**Wang, L-Y., Chen, K-C., Chen, T-C. & Yeh, S-D. (2007).** Nucleotide sequence analysis and detection application of the coat protein gene of *Sweet potato latent virus* isolated from central Taiwan. *Plant Pathology Bulletin* **16,** 141-148.

**Wang, Q. C. & Valkonen, J. P. T. (2008).** Elimination of two viruses which interact synergistically from sweetpotato by shoot tip culture and cryotherapy. *Journal of Virological Methods* **154,** 135-145.

**Wang, Q., Zhang, L., Wang, B., Yin, Z., Feng, C. & Wang Q. (2010).** Sweetpotato viruses in China. *Crop Protection* **29,** 110-114.

**Winter, S., Purac, A., Leggett, F., Frison, E. A., Rossel, H. W. & Hamilton, R. I. (1992).** Partial characterization and molecular cloning of a closterovirus from sweet potato infected with the sweet potato virus disease complex from Nigeria. *Phytopathology* **82,** 869-875.

**Woolfe, J. A. (1992).** *Sweet Potato- an Untapped Food Resource*. New York: Cambridge University Press.

**Wu, Q., Luo, Y., Lu, R., Lau, N., Lai, E. C., Li, W. X. & Ding, S. W. (2010).** Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proceeding of the National Academy of Sciences in USA* **107,** 1606–1611.

**Yang, C. L., Xing, J. Y. & Wu, J. Y. (1995).** The types of main sweetpotato viruses in China and productive program of virus-free plantlets. *Jiangshu Agricultural Science* **6,** 35-36.

**Yao, D. J. E. & Hortense, A. D. (2005).** Detection and distribution of *Sweetpotato feathery mottle virus* in sweetpotato using membrane immunobinding assay. *African Journal of Biotechnology* **4,** 717-723.

**Yamasaki, S., Sakai, J., Kamisoyama, S., Goto, H., Okuda, M. & Hanada, K. (2009).** Control of russet crack disease in sweetpotato plants using a protective mild strain of *Sweet potato feathery mottle virus*. *Plant Disease* **93,** 190-194.

**Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R. W., Steward, R. & Chen, X. (2005).** Methylation as a crucial step in plant microRNA biogenesis. *Science* **307,** 932-935.

**Zhang, S. C. & Ling, K-S. (2011).** Genetic diversity of sweet potato begomoviruses in the United States and identification of a recombinant between *Sweet potato leaf curl virus* and *Sweet potato leaf curl Georgia virus*. *Archives of Virology* DOI 10.1007/s00705-011-0930-2.

**Zheng, Q., Dessai, A. P. & Prakash, C. S. (1996).** Rapid and repetitive plant regeneration in sweetpotato via somatic embryogenesis. *Plant Cell Reports* **15,** 381-385.

**Ziegler, R. S. (2001).** Agricultural biotechnology, reducing poverty in developing countries. *Plant Disease* **85,** 568-579.

# CHAPTER TWO

# IDENTIFICATION AND DISTRIBUTION OF VIRUSES INFECTING SWEET POTATO (*Ipomoea batatas* L.) IN KWAZULU-NATAL (KZN)

## 2.1 ABSTRACT

A survey to determine the occurrence and distribution of viruses infecting sweet potato (*Ipomoea batatas* Lam.) was conducted in major sweet potato-growing areas in KwaZulu-Natal (KZN). A total of 84 symptomatic vine samples were collected and graft inoculated onto universal indicator plants, *Ipomoea setosa* Ker. and *Ipomoea nil* Lam. Six weeks post inoculation, typical sweet potato virus-like symptoms of chlorotic flecking, severe leaf deformation, stunting, chlorotic mosaic, and distinct interveinal chlorotic patterns were observed on indicator plants. Under the transmission electron microscope (TEM), negatively stained preparations of crude leaf sap and ultra-thin sections from symptomatic grafted *I. setosa* plants revealed the presence of elongated flexuous particles and pinwheel type inclusion bodies that are characteristic to the cytopathology of potyviruses. Symptomatic leaf samples from graft-inoculated *I. setosa* and *I. nil* were assayed for Sweet potato feathery mottle virus (SPFMV), Sweet potato mild mottle virus (SPMMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato chlorotic fleck virus (SPCFV), Sweet potato virus G (SPVG), Sweet potato mild speckling virus (SPMSV), Sweet potato caulimo-like virus (SPCaLV), Sweet potato latent virus (SPLV), Cucumber mosaic virus (CMV), and Sweet potato C-6 virus (C-6) using the nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA). The majority of leaf samples (52%) tested positive for virus disease and showed the occurrence of SPFMV, SPMMV, SPCSV, SPCFV, SPVG, SPMSV, and SPCaLV. Of these 7 viruses, the most frequently detected were SPFMV (39%), SPVG (30%), followed by SPCSV (13%) and SPMMV (12%). SPCaLV and SPCFV at 10% and SPMSV at 7% were found exclusively in samples collected from one area. SPFMV, SPVG, SPCSV, and SPMMV were identified as the most prevalent viruses infecting sweet potato in KZN.

## 2.2 INTRODUCTION

Sweet potato (*Ipomoea batatas* Lam.) is an important staple crop for many small-scale farmers in Africa. Its high nutritional value coupled to its good quality performance under marginal and low input conditions; give it a unique role in combating food shortage and malnutrition in Africa (Wambugu, 2003). In South Africa (SA), with an annual production of 62 688 tons, the sweet potato industry is relatively small when compared to that of other countries in Africa, (FAOSTAT, 2011). However, large quantities of sweet potato is produced and sold by the informal sector, which is not reflected in the official figures (Alleman *et al*., 2004; Laurie, 2004). Therefore, the total production has been re-estimated at 120 000 tons per annum (Alleman *et al*., 2004). Sweet potato is produced in all provinces of SA with the major production areas being Limpopo, Mpumalanga, Western and Eastern Cape, KwaZulu-Natal (KZN) and Free State provinces (Du Plooy *et al*., 1996; Laurie, 2004). Majority of sweet potato farming in KZN occurs at a subsistence level where resource-poor farmers prize the crop as a reliable food source that provides durable household security even when other crops fail.

Virus infections have been identified as the second most important biotic constraint [after insects (weevils)] to sweet potato production (Jannson & Raman, 1991). Infections with viruses may result in zero yield of marketable tubers, depending on the sweet potato cultivar and whether the infection comprises of single or multiple viruses (Njeru *et al*., 2004). In SA, a progressive deterioration of the yield and quality of sweet potato crops has been attributed to virus infection (Laurie, 2002). This phenomenon, commonly referred to as 'cultivar decline', has been experienced by many sweet potato farmers across SA and particularly in KZN (Clark *et al*., 2002; Laurie, 2002). It is known that farmers in KZN normally retain vine cuttings as propagation material for the next growing season, and this may provide a means to perpetuate systemic viral disease leading to cultivar decline.

From a viral disease control perspective, knowledge of the distribution and diversity of viruses infecting clonally propagated sweet potato in KZN is essential. However,

research on viral disease of sweet potato in KZN is limited. A survey of sweet potato viruses in SA in 2003 (Domola *et al*., 2008), did not cover all major production areas in KZN with sampling been done in one area and only a relatively small number of samples being analysed. As a result, a low incidence of viral disease was reported in sweet potato from KZN. Therefore, more detailed and recent information on the identity and relative importance of viruses infecting sweet potato in KZN is needed. This study was conducted to provide comprehensive information on the identity, distribution and relative importance of viruses infecting sweet potato in all major sweet potato growing areas in KZN.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Field survey

A field survey for virus infection was conducted between January 2007 and March 2009 in nine major sweet potato growing areas in KZN (Fig. 2.1). These areas included: a commercial sweet potato field in Weenen, research-based fields growing commercial sweet potato cultivars in Pietermaritzburg (University of KwaZulu-Natal) and Cedara (Plant Protection Institute, Agricultural Research Council), and six rural areas growing both commercial cultivars and local varieties on a small-scale. The small-scale fields included: Umbumbulu, areas in the South Coast of KZN (Umfume and Umphambanyomi River), Bergville, Makatini and Msinga. Sweet potato fields in each area were inspected and samples were collected along two diagonals in each field. A total of 84 vine samples from 19 fields in the nine sweet potato growing areas were collected from sweet potato plants exhibiting virus-like symptoms and/or yield decline. Vines were obtained from the different commercial and local varieties grown by the farmers. The most common commercial cultivars were A15, A40, A56, Beauregard, 199062.1, and Old Jewel. Local varieties were tentatively named Landrace 1, 2 and 3 respectively. Vine samples were maintained at 25$^{o}$C in an insect-proof greenhouse in the Controlled Environment Research Unit (CERU) at the University of KwaZulu-Natal (UKZN-PMB). Once vines were established, samples were assayed for virus infection by indexing onto

*Ipomoea setosa* Ker. and *Ipomoea nil* Lam., transmission electron microscopy (TEM), and nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA).


**Figure 2.1** Map of KwaZulu-Natal (KZN) showing locations of sweet potato (*Ipomoea batatas* Lam.) production areas sampled. Areas surveyed include: 1 = Pietermaritzburg, 2 = Cedara, 3 = Weenen, 4 = Umbumbulu, 5 = Umfume, 6 = Umphambanyomi River, 7 = Bergville, 8 = Makatini and 9 = Msinga (adapted from www.thesafaricompany.co.za/map_kwazulu_natal.htm).

### 2.3.2 Virus indexing

Virus accumulation in many sweet potato cultivars is low and direct virus detection from sweet potato field samples is unreliable (Karyeija *et al*., 2000). Therefore, grafting onto *I. setosa* or *I. nil*, nearly universal indicator plants for sweet potato viruses, is used to boost virus titer and leaves of grafted *I. setosa* or *I. nil* are subsequently used in virus testing (Mukasa *et al*., 2003; Tairo *et al*., 2006). A section of each sampled vine was virus-indexed by grafting onto three week-old indicator plants, *I. setosa* and *I. nil*, using the crown-cleft grafting method (Fig. 2.2) described by De Bokx (1972). Each sample was grafted onto at least three indicator plants. The method entailed cutting a 5cm section containing at least one node from the vine sample and shaping the base of the section into a wedge (scion). The apex of the indicator plant was cut off to create a rootstock containing at least three leaves. The scion was then inserted into a 1.5cm lateral slit in the rootstock stem. The graft junction was secured with parafilm and cheesecloth. Healthy non-grafted indicator plants were maintained as negative controls. All plants were kept at 20-25$^{o}$C in the Jolley Roger tunnel (CERU, UKZN-PMB), under 80% relative humidity, and fertilized weekly with 3:1:3 (nitrogen: phosphate: potassium). Symptoms were recorded six weeks after grafting and symptomatic plants were assayed for virus infection using TEM, and NCM-ELISA.

**Figure 2.2** Crown-cleft grafting of sweet potato (*Ipomoea batatas* L.) field samples onto indicator plant, *Ipomoea setosa* Ker.

### 2.3.3 Transmission electron microscopy

To verify the presence of viruses in symptomatic indicator plants, crude leaf sap and embedded infected leaf tissues were viewed under the TEM. Leaf material taken from healthy indicator plants was used as a control.

#### *2.3.3.1 Leaf-dip method*

A small section of infected leaf material was cut into a 5mm by 5mm section. The crude virus sap was prepared by macerating a leaf section in a drop of distilled water. Formvar-coated grids were then placed on the crude sap and allowed to stand for 30s. Excess liquid was removed from the grid using the edge of a filter paper wedge. Grids were negatively stained with 3% (w/v) uranyl acetate for 10s. Excess stain was slowly removed with the edge of a filter paper wedge. The grids were then viewed under a Jeol 100 CX TEM.

#### *2.3.3.2 Tissue embedding and ultra-thin sectioning*

A standard procedure provided by the Electron Microscopy Centre, University of KwaZulu-Natal (PMB) was followed. Leaf slices (2mm x 2mm) from symptomatic graft-inoculated *I. setosa* plants were prefixed for 48h in 3% (v/v) glutaraldehyde in 0.05M sodium cacodylate buffer (pH 6.88), washed twice in that buffer and then postfixed with 2% (v/v) osmium tetroxide in 0.05M sodium cacodylate buffer (pH 6.88) for 1.5h. After dehydration with a graded ethanol series (10-100%) for a minimum of 10min per solution, the samples were imbedded in Epon-Araldite resin. Ultra-thin sectioning was performed with glass knives mounted on a microtome. Sections were placed on 200-mesh copper grids, stained with 2% uranyl acetate and viewed under a Jeol 100 CX TEM. All images were captured using a digital MegiViewBIII camera.

### 2.3.4 Nitrocellulose membrane enzyme-linked immunosorbent assay

Symptomatic leaf samples from graft-inoculated *I. setosa* and *I. nil* were assayed for Sweet potato feathery mottle virus (SPFMV), Sweet potato mild mottle virus (SPMMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato chlorotic fleck virus (SPCFV), Sweet potato virus G (SPVG), Sweet potato mild speckling virus (SPMSV), Sweet potato caulimo-like virus (SPCaLV), Sweet potato latent virus (SPLV), Cucumber mosaic virus (CMV), and Sweet potato C-6 virus (C-6) using standard NCM-ELISA kits obtained from the International Potato Centre (CIP), Lima, Peru. In addition to polyclonal antisera to the above 10 viruses and goat anti-rabbit conjugated antibody, the kit also contained membrane strips pre-spotted with sap from virus-positive (positive control) and healthy control plants (negative control). To test leaf samples with NCM-ELISA, the protocol was followed according to the manufacturers' instructions and visual assessment for varied degrees of purple colour development on the blot was used to identify virus-positive samples.

## 2.4 RESULTS

### 2.4.1 Field assessment of virus disease

The vast majority of samples collected from sweet potato growing areas in KZN exhibited a diverse array of symptoms characteristic to virus infection. Symptoms observed in field samples from areas growing commercial cultivars (Weenen, PMB and Cedara) were not severe, and predominately consisted of leaf distortion; leaf curl and mild mosaic (Fig. 2.3). However, symptoms observed on field samples taken from the rural fields (Umbumbulu, Umfume, Umphambanyomi River, Bergville, Makatini and Msinga) were more severe and consisted of mottling, leaf distortion, yellowing, vein clearing, mosaic, purpling, chlorosis and stunting (Fig. 2.4). The frequency of each symptom varied with each cultivar, but local varieties exhibited a more severe combination of symptoms.


**Figure 2.3** Symptoms observed in field samples from areas growing commercial cultivars (Weenen, Pietermaritzburg and Cedara). (A-B) leaf deformation on cv. A40 and cv. A15 respectively, (C) leaf curl on cv. A15 and (D) mosaic on cv. A56.


**Figure 2.4** Symptoms observed in field samples from rural areas (Umbumbulu, Umfume, Umphambanyomi River, Bergville, Makatini and Msinga). (A) Yellowing, mosaic and chlorosis, (B) vein clearing, (C) mottling and purpling on local varieties, and (D) stunting and leaf deformation on cv. A15.

### 2.4.2 Virus indexing

Typical sweet potato virus-like symptoms were observed on grafted *I. setosa* and *I. nil* plants. Indicator plants grafted with samples from Weenen showed a unique combination of chlorotic flecking followed by severe leaf deformation and stunting whilst plants grafted with samples from the remaining eight areas showed leaf deformation, chlorotic mosaic, and distinct interveinal chlorotic patterns (Fig. 2.5). Many of these symptoms resembled those characteristic to Sweet potato virus disease (SPVD).

### 2.4.3 Transmission electron microscopy

Under the TEM, negatively stained preparations of crude leaf sap from symptomatic grafted *I. setosa* plants revealed the presence of elongated flexuous particles. The flexuous particles were easily detected and showed characteristic morphology of potyviruses (Fig. 2.6). No virus particles were observed in crude sap samples taken from healthy control *I. setosa* plants. In negatively stained ultra-thin sections of infected leaf material, pinwheel type inclusions bodies, typical of Potyvirus infection, were readily detected (Fig. 2.7A). No comparable structures were found in leaf sections of healthy control *I. setosa* plants (Fig. 2.7B).


**Figure 2.5** Symptoms observed on *Ipomoea setosa* plants grafted with symptomatic field samples. (A) Chlorotic flecking progessed into (B) severe leaf deformation and stunting in plants grafted with field samples from Weenen. (C) Distinct interveinal chlorotic patterns and (D) leaf deformation and chlorotic mosaic observed in plants grafted with samples from the remaining eight areas surveyed.

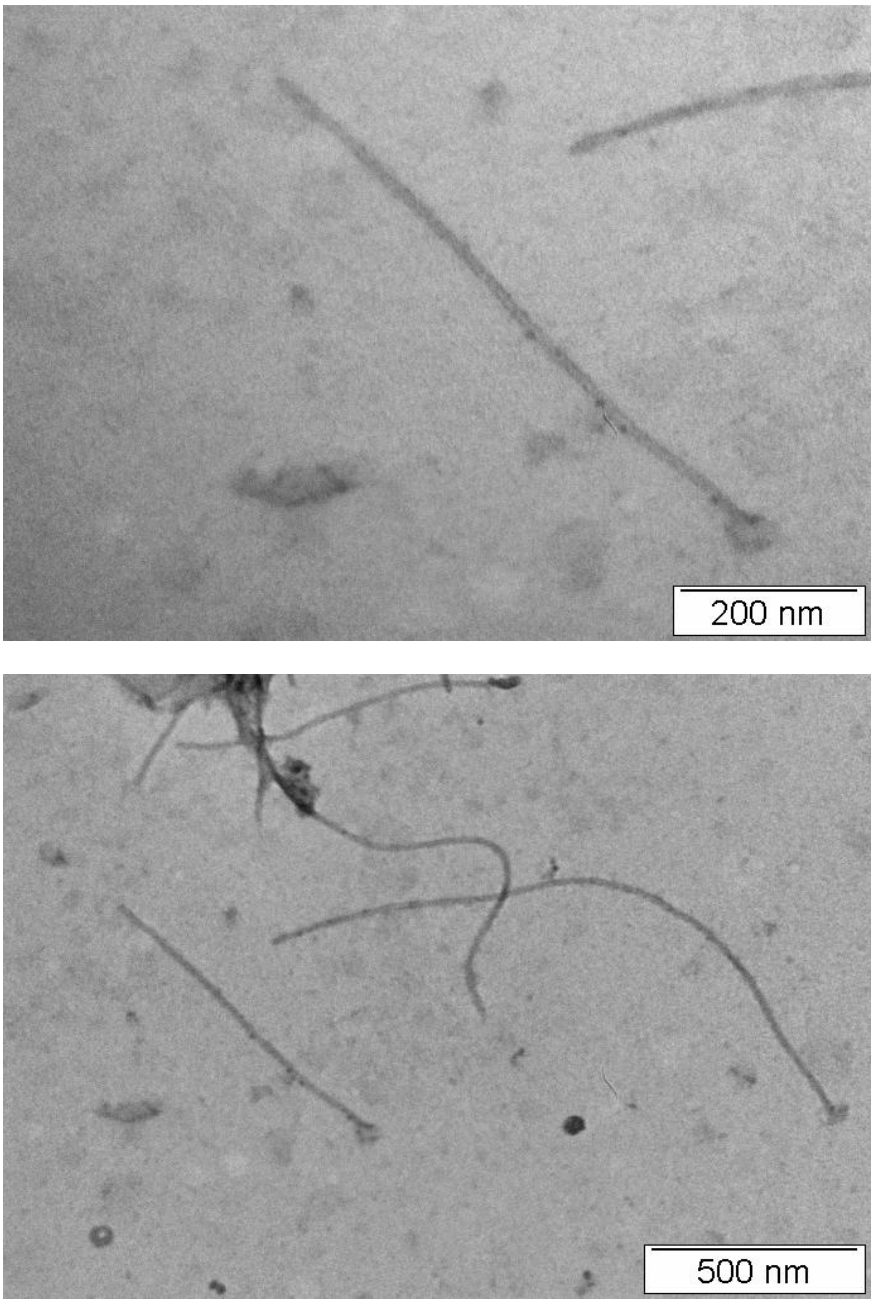



**Figure 2.6** Transmission electron micrographs of negatively stained crude leaf sap from symptomatic *Ipomoea setosa* Ker. plants showing Potyvirus-like particles.

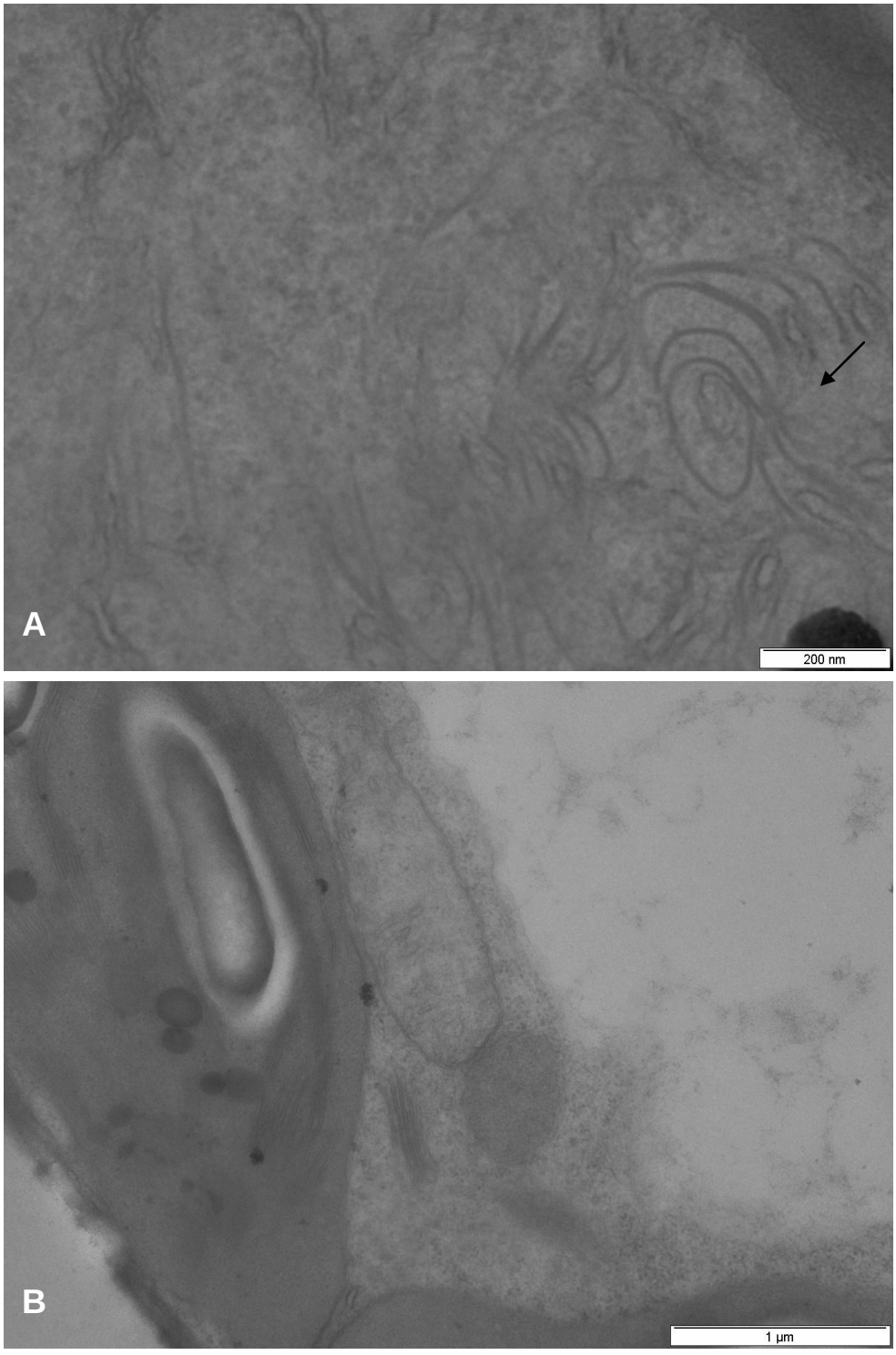



**Figure 2.7** Transmission electron micrographs of negatively stained ultra-thin leaf sections from (A) a infected *Ipomoea setosa* Ker. plant showing typical potyvirus pinwheel inclusion bodies and (B) healthy uninoculated *I. setosa* plant.

### 2.4.4 Nitrocellulose membrane enzyme-linked immunosorbent assay

A typical NCM-ELISA reaction is depicted in Fig 2.8. A total of seven out of the 10 viruses were detected in samples from 84 symptomatic sweet potato plants collected from the nine areas surveyed in KZN. These viruses include: SPFMV, SPVG, SPCSV, SPMMV, SPCaLV, SPCFV, and SPMSV. As seen in Table 2.2, the slight majority (52%) of samples reacted positively to antisera to multiple viruses or SPFMV alone. The most frequently detected virus was SPFMV that was present in 39% of samples. SPFMV was detected as a single infection at low incidence in PMB, but most commonly detected in multiple or dual infections with SPVG, SPCSV and SPMMV. SPVG was the second most frequently detected virus with 30% of all samples reacting positive, followed by SPCSV (13%) and SPMMV (12%). SPCaLV (10%), SPCFV (10%), and SPMSV (7%) were found exclusively in samples collected from Weenen. SPLV, CMV and C-6 virus were not detected in any samples and all samples from Cedara tested negative for virus infection. The incidence, distribution and diversity of viral disease in sweet potato were significantly lower in areas growing commercial cultivars than in rural areas growing both commercial cultivars and local varieties. All samples from local sweet potato varieties (local landraces) tested positive for the presence of two or multiple viruses.

**Table 2.1** Serological detection of viruses in sweet potato (*Ipomoea batatas* Lam.) samples collected from surveyed areas in KwaZulu Natal (KZN)

| Area surveyed | Cultivar | Total number of samples | Number of samples testing positive for[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | SPFMV | SPVG | SPCSV | SPMMV | SPCFV | SPCaLV | SPMSV | SPLV | CMV | C-6 |
| *Commercial areas* | | | | | | | | | | | | |
| Pietermaritzburg | A40 | 4 | -[b] | - | - | - | - | - | - | - | - | - |
| | A56 | 4 | - | - | - | - | - | - | - | - | - | - |
| | A15 | 2 | + | - | - | - | - | - | - | - | - | - |
| | Old Jewel | 2 | - | - | - | - | - | - | - | - | - | - |
| | Beauregard | 2 | + | - | - | - | - | - | - | - | - | - |
| Total | | 14 | 4/14 | 0/14 | 0/14 | 0/14 | 0/14 | 0/14 | 0/14 | 0/14 | 0/14 | 0/14 |
| Cedara | A40 | 5 | - | - | - | - | - | - | - | - | - | - |
| | A56 | 3 | - | - | - | - | - | - | - | - | - | - |
| | A15 | 6 | - | - | - | - | - | - | - | - | - | - |
| | 1990062.1 | 5 | - | - | - | - | - | - | - | - | - | - |
| | Beauregard | 2 | - | - | - | - | - | - | - | - | - | - |
| Total | | 21 | 0/21 | 0/21 | 0/21 | 0/21 | 0/21 | 0/21 | 0/21 | 0/21 | 0/21 | 0/21 |
| Weenen | A40 | 2 | - | - | - | - | + | +++ | - | - | - | - |
| | A56 | 2 | - | - | - | - | + | +++ | ++ | - | - | - |
| | 1990062.1 | 2 | - | - | - | - | + | +++ | ++ | - | - | - |
| | Beauregard | 2 | - | - | - | - | + | + | ++ | - | - | - |
| Total | | 8 | 0/8 | 0/8 | 0/8 | 0/8 | 8/8 | 8/8 | 6/8 | 0/8 | 0/8 | 0/8 |
| *Rural areas* | | | | | | | | | | | | |
| Umbumbulu | A40 | 2 | - | - | - | - | - | - | - | - | - | - |
| | A56 | 2 | - | - | - | - | - | - | - | - | - | - |
| | Local landrace 1 | 2 | +++ | ++ | - | - | - | - | - | - | - | - |
| | Local landrace 2 | 2 | +++ | ++ | - | + | - | - | - | - | - | - |
| | Local landrace 3 | 2 | - | ++ | - | + | - | - | - | - | - | - |
| Total | | 10 | 4/10 | 6/10 | 0/10 | 4/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |

*Table 2.1 continued…*

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Umfume | A40 | 5 | ++ | +++ | + | - | - | - | - | - | - | - |
| | 1990062.1 | 2 | - | - | - | - | - | - | - | - | - | - |
| Total | | 7 | 5/7 | 5/7 | 5/7 | 0/7 | 0/7 | 0/7 | 0/7 | 0/7 | 0/7 | 0/7 |
| Umphambanyomi River | A40 | 4 | - | - | - | - | - | - | - | - | - | - |
| | A56 | 2 | + | ++ | - | - | - | - | - | - | - | - |
| | Local landrace 1 | 3 | + | ++ | - | + | - | - | - | - | - | - |
| | Local landrace 2 | 3 | + | ++ | - | + | - | - | - | - | - | - |
| Total | | 12 | 8/12 | 8/12 | 0/12 | 6/12 | 0/12 | 0/12 | 0/12 | 0/12 | 0/12 | 0/12 |
| Bergville | Local landrace 1 | 2 | ++ | ++ | - | - | - | - | - | - | - | - |
| | Local landrace 2 | 2 | ++ | ++ | - | - | - | - | - | - | - | - |
| Total | | 4 | 4/4 | 4/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| Makatini | A40 | 2 | ++ | - | + | - | - | - | - | - | - | - |
| | 1990062.1 | 2 | ++ | - | + | - | - | - | - | - | - | - |
| | Beauregard | 2 | ++ | - | + | - | - | - | - | - | - | - |
| Total | | 6 | 6/6 | 0/6 | 6/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Msinga | Local landrace 1 | 2 | ++ | ++ | - | - | - | - | - | - | - | - |
| Total | | 2 | 2/2 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| **TOTAL (%)**[c] | | **84(52)**[d] | **33(39)** | **25(30)** | **11(13)** | **10(12)** | **8(10)** | **8(10)** | **6(7)** | **0(0)** | **0(0)** | **0(0)** |

[a] Viruses detected by nitrocellulose membrane enzyme-linked immunosorbent assay.
[b] Visual assessment of colour intensity as - = no colour change (negative reaction), + = weak positive, ++ = intermediate positive and +++ = strong positive reactions.
[c] Figures in parenthesis give the incidence in percentage.
[d] Percentage of samples testing positive for virus infection.

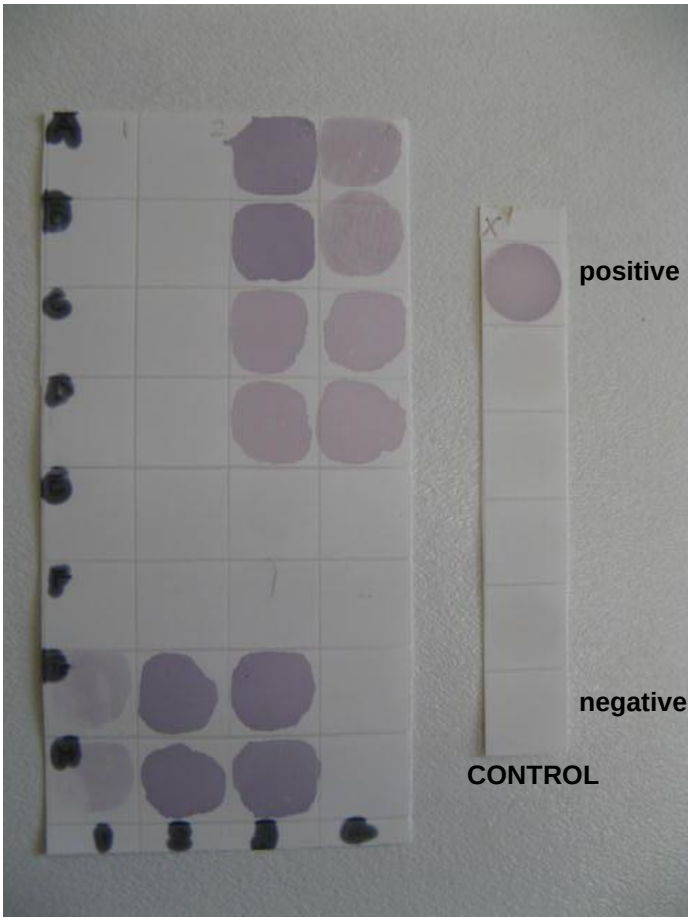



**Figure 2.8** Nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) detection of Sweet potato feathery mottle virus (SPFMV) in sweet potato (*Ipomoea batatas* Lam.) samples collected from areas surveyed in KwaZulu-Natal (KZN). NCM control strips pre-spotted with sap from virus-positive (positive control) and healthy plants (negative control) were included.

## 2.5 DISCUSSION

This is the first comprehensive survey of viruses infecting sweet potato in all major sweet potato-growing areas in KZN. Results indicate a diverse and increased array of viruses infecting sweet potato in KZN.

A prominent feature of the results was the high incidence of viral disease in rural areas of KZN, where sweet potato is grown at a small-scale by resource-poor farmers. A high level of viral incidence was initially speculated due to the observation of distinct and severe field symptoms on sweet potato grown in these rural areas (Fig. 2.4). Speculations were confirmed by subsequent indexing of symptomatic vine samples onto indicator plants (Fig. 2.5 C/D) and serological testing (Fig. 2.8, Table 2.2). The rural areas surveyed in this study are located at relatively low altitudes with high rainfall and warm climate. Farmers in these areas often grow sweet potato throughout the year and tend to favour the cultivation of their own local varieties over commercial cultivars. Cultivation of these local varieties may perpetuate virus disease by ensuring a continuous reservoir for viruses and their vectors (Aritua *et al*., 2007). Warm humid climates have been reported to favour virus disease development by influencing crop growth and virus multiplication (Thresh *et al*., 2003). In addition, data of Sheffield (1957) suggested that virus incidence is negatively correlated with altitude. This was confirmed in a field survey of virus disease conducted in Rwanda, which reported a greater frequency of virus detection in samples from low altitude zones (Njeru *et al*., 2008). Therefore, the high incidence of viral disease in rural areas of KZN could be attributed to the low altitude zone, favourable climatic conditions and the continuous cultivation of infected local varieties.

In contrast, a low level of disease observed in Weenen, Cedara and PMB was reflected in the less pronounced symptoms observed in the field (Fig. 2.3) and in virus-indexed indicator plants (Fig. 2.5A/B). Serological testing confirmed a lower level of virus disease incidence (Table 2.2). These areas are of a higher altitude and cooler climate than rural areas surveyed in KZN. Sweet potato farming in these areas focuses solely

on the cultivation of commercial cultivars during a few months of the year. Therefore, the interrupted supply of reservoir hosts plants for virus and vector coupled to the unfavourable altitude and climatic conditions may have provided an unsuitable environment for the development of high levels of virus disease in sweet potato grown in these areas.

Of the 10 viruses assayed using NCM-ELISA, SPFMV, SPVG, SPCSV, SPMMV were identified as the most prevalent viruses infecting sweet potato in KZN (Table 2.2). SPFMV was the most commonly occurring virus, detected in 39% of samples assayed. This is consistent with previous reports that SPFMV occurs wherever sweet potato is grown (Moyer & Salazar, 1989; Karyeija *et al*., 1998; Kreuze & Fuentes, 2008).

Based on the findings of similar field surveys conducted in Uganda (Aritua *et al*., 2007), Kenya (Ateka *et al*., 2004) and Rwanda (Njeru *et al*., 2008), it was anticipated that SPCSV, being a common accomplice to SPFMV in the SPVD complex, would be found as the second most commonly occurring virus infecting sweet potato in KZN. However, this was not the case. Instead, the relatively new SPVG was the second most commonly occurring virus, detected in 30% of samples assayed from KZN. SPVG was found exclusively in mixed infection that consistently involved SPFMV and rarely SPMMV and/or SPCSV (Table 2.2). The tight association between SPVG and SPFMV could be attributed to the fact that being members of the genus *Potyvirus,* they share the aphid as their insect vector, and that this commonality may benefit each other through some shared function (Ateka *et al*., 2004). The fact that the newly described SPVG was frequently detected in KZN as well as in some provinces in SA (Domola *et al*., 2008) suggests that the virus may now be widespread in SA.

SPCSV and SPMMV accounted for 13% and 14% of infections, ranking them as the third and fourth most prevalent virus infecting sweet potato in KZN (Table 2.2). As described by Ateka *et al*. (2004), a higher incidence of SPVG and SPFMV as compared to SPCSV and SPMMV could be due to the relative abundance of their aphid vectors over the whitefly vectors of SPCSV and SPMMV. Although less frequently occurring,

SPMMV and SPCSV were always detected in multiple infections with SPFMV and SPVG. The results presented here differ from previous surveys in that SPCSV seemed always present in multiple infections with SPFMV to cause the SPVD complex and apparently also SPMMV (Tairo *et al*., 2005). SPVD is the most devastating viral disease affecting sweet potatoes worldwide (Kokkinos *et al*., 2006; Miano *et al*., 2008). The results presented in this survey suggest that SPVG is also able to synergize with SPCSV, SPFMV and SPMMV and hence potentially contribute to a new SPVD complex that encompasses all four viruses.

Single infections were rarely detected in plants and consisted primarily of SPFMV. Instead, multiple infections (double, triple, and quadruple) were commonly detected. In this regard, results are similar to, but more extreme, than those of Mukasa *et al*. (2003) in Uganda and Ndunguru & Kapinga (2007) in Tanzania. Infection of plants by multiple viruses is a common phenomenon (Hull, 2001) and the occurrence of multiple infections in sweet potato has been well documented (Schaefers & Terry, 1976; Di Feo *et al*., 2000; Mukasa *et al*., 2006; Kreuze & Fuentes, 2008). The incidence of single infection by SPFMV was localized to samples from PMB (Table 2.2). This type of infection induces mild or no symptoms in sweet potato cultivars (Gibson *et al*., 1997; Njeru *et al*., 2004). Consequently, growers may have selected asymptomatic SPFMV-infected cuttings as propagation material for the next crop, thus maintaining the virus in sweet potato crops grown in PMB.

The distinctive appearance of chlorotic flecking, severe leaf deformation and stunting (Fig. 2.5A/B) was observed only in indicator plants grafted with field samples from Weenen when compared to the symptoms of distinct interveinal chlorotic patterns (Fig. 2.5C) and leaf deformation and chlorotic mosaic (Fig. 2.5D) observed on leaves of plants that had been grafted with samples from the remaining eight areas surveyed. The presence of SPMSV, SPCFV and SPCaLV was detected exclusively in sweet potato crops grown in Weenen. This unique combination of virus disease could be responsible for distinctive symtpom development in plants grafted with the samples from Weenen. SPMSV and SPCFV have a narrow distribution and are rare, which is in

agreement with a previous report from SA (Domola *et al*., 2008) and other East African countries (Mukasa *et al*., 2003; Ateka *et al*., 2004; Nome *et al*., 2006). By contrast, after its first isolation from Puerto Rico in 1987 (Atkey & Brunt, 1987), SPCaLV has only been confirmed in Uganda (Aritua *et al*., 2007), Madeira, New Zealand, Papua New Guinea, Solomon Islands (Atkey & Brunt, 1987), and China (Gao *et al*., 2000). Its identification in this study is therefore only the second confirmed report of its occurrence in Africa and the first of its occurrence in SA. The exclusive presence of these viruses in samples from Weenen represents an interesting dynamic in the diversity of viruses infecting sweet potato in KZN and justifies the need for further confirmatory testing. SPLV, CMV and C-6 virus rarely occur on sweet potato in Africa and were not detected in this study (Ateka *et al*., 2004; Ndunguru & Kapinga, 2007)

The occurrence of potyvirus infection in graft-inoculated *I. setosa* was seen under the TEM (Fig. 2.6). Furthermore, pinwheel inclusion structures that are distinctive features of the cytopathology of potyviruses were readily seen in sections of infected *I. setosa* (Nome *et al*., 2006; Fig. 2.7A). Therefore, based on particle characteristics and the presence of pinwheel inclusions in plant cells, the presence of potyvirus infection was confirmed.

In a survey of sweet potato infecting viruses in SA, Domola *et al*. (2008) identified the presence of SPFMV, SPVG and *Sweet potato virus 2* (SPV2) at low incidences in one sampling area in KZN. This present survey reports the identification of several viruses in 52% of total samples tested from nine sampling areas in KZN (Table 2.2). An evolving cropping system and population dynamics of virus vectors in KZN may have greatly impacted the disease dynamics over the years resulting in an increase in virus incidence. Reports of similar upsurges of viral disease in sweet potato have been recorded in Rwanda (Njeru *et al*., 2008), Kenya (Ateka *et al*., 2004), and Uganda (Aritua *et al*., 2007) and therefore there is a need for continuous updated information on virus identity and distribution. Such information is crucial for the development of virus-resistant cultivars. Future strategies in resistance should give priority to SPFMV, SPVG,

SPCSV and SPMMV as they are the viruses known to interact synergistically and show the highest incidence in sweet potato in KZN.

## 2.6 REFERENCES


**Allemann, J., Laurie, S. M., Thiart, S. & Vorster, H. T. (2004).** Sustainable production of root and tuber crops (potato, sweet potato and indigenous potato, cassava) in Southern Africa. *South African Journal of Botany* **70,** 60-66.

**Aritua, V., Bau, B., Vetten H. J., Adipala E. & Gibson, R. W. (2007).** Incidence of five viruses infecting sweetpotatoes in Uganda; the first evidence of *Sweet potato caulimo-like virus* in Africa. *Plant Pathology* **56,** 324-331.

**Ateka, E. M., Barg, E., Njeru, R. W., Lesemann, D. E. & Vetten, H. J. (2004).** Further characterization of '*Sweet potato virus 2'*: a distinct species of the genus Potyvirus. *Archives of Virology* **149,** 225-239.

**Atkey, P. T. & Brunt, A. A. (1987).** Electron microscopy of an isometric caulimo-like virus from sweet potato (*Ipomoea batatas*). *Journal of Phytopathology* **118,** 370-376.

**Clark, C. A, Valverde, R. A., Fuentes, S., Salazar, L. F. & Moyer, J. W. (2002).** Research for improved management of sweet potato pests and diseases: cultivar decline. *Acta Horticulturae* **583,** 103-113.

**Di Feo, L., Nome, S. F., Biderbost, E., Fuentes, S. & Salazar, L. F. (2000).** Etiology of sweetpotato chlorotic dwarf disease in Argentina. *Plant Disease* **84,** 223-229.

**Domola, M. J., Thompson, G. J., Aveling, T. A. S., Laurie, S. M., Strydom, H. & Van den Berg, A. A. (2008).** Sweet potato viruses in South Africa and the effect of viral infection on storage root yield. *African Plant Protection* **14,** 15-23.

**De Bokx, J. A. (1972).** Graft and mechanical transmission. In *Viruses of Potatoes and Seed-potato Production.* Edited by De Bokx, J. A. Wageningen: Centre for Agricultural Publishing and Documentation, pp. 26-35.

**Du Plooy, C. P., Wagner, J. J. & Bosch, S. E. (1996).** Cultivation of sweet potato. In *Vegetable Production in South Africa*, pp. 1-7. Pretoria: ARC Roodeplaat.

**FAOSTAT. 2011.** FAO statistics. http://apps.fao.org. Rome, Italy: Food and Agricultural Organization of the United Nations.

**Gao, F., Gong, Y. F. & Zhang, P. B. (2000).** Production and development of virus-free sweetpotato in China. *Crop Protection* **19,** 105-111.

**Gibson, R. W., Mwanga, R. O. M., Kasule, S., Mpembe, I. & Carey, E. E. (1997).** Apparent absence of viruses in most symptomless field-grown sweet potato in Uganda. *Annals of Applied Biology* **130,** 481-490.

**Hull, R. (2001).** *Matthew's Plant Virology*. San Diego: Academic Press.

**Jannson, R. K. & Raman, K. V. (1991).** Sweet potato pest management: a global review. In *Sweet Potato Pest Management: A Global Perspective.* Edited by Jannson, R. K. & Raman, K. V. Oxford: Westview Press.

**Karyeija, R. F., Gibson, R. W. & Valkonen, J. P. T. (1998).** The significance of *Sweet potato feathery mottle virus* in subsistence sweet potato production in Africa. *Plant Disease* **82,** 4-15.

**Karyeija, R. F., Kreuze, J. F., Gibson, R. W. & Valkonen, J. P. T. (2000).** Two serotypes of *Sweetpotato feathery mottle virus* in Uganda and their interaction with resistant sweetpotato cultivars. *Phytopathology* **90,** 1250-1255.

**Kokkinos, C. D., Clark, C. A., McGregor C. E. & LaBonte, D. R. (2006).** The effect of sweet potato virus disease and its viral components on gene expression levels in sweetpotato. *Journal of the American Society of Horticultural Science* **131,** 657-666.

**Kreuze, J. & Fuentes, S. (2008).** Sweetpotato viruses. In *Encyclopedia of Virology,* pp. 659-669. Edited by Mahy, B. W. J. & Van Regenmortel, M. H. V. Amsterdam: Academic Press.

**Laurie, L. M. (2002).** Overview of breeding and evaluation of orange-fleshed sweet potato in South Africa. http://www.cipotato.org/Vitaa?Proceedings/VITAA-paper-SLAURIE-FINAL-11Feb2002.pdf.

**Laurie, L. M. (2004).** Sweet potato in perspective. In *Guide to Sweet Potato Production in South Africa,* pp. 1-6. Edited by Niederwieser, J. G. Pretoria: ARC- Roodeplaat Vegetable and Ornamental Plant Institute.

**Miano, D. W., LaBonte, D. R. & Clark, C. A. (2008).** Identification of molecular markers associated with sweet potato resistance to sweet potato virus disease in Kenya. *Euphytica* **160,** 15-24.

**Moyer, J. W. & Salazar, L. F. (1989).** Virus and virus-like diseases of sweet potato. *Plant Disease* **73,** 451-455.

**Mukasa, S. B., Rubaihayo, P. R. & Valkonen, J. P. T. (2003).** Incidence of viruses and viruslike diseases of sweet potato in Uganda. *Plant Disease* **87,** 329-335.

**Mukasa, S. B., Rubaihayo, P. R. & Valkonen, J. P. T. (2006).** Interactions between a Crinivirus, an ipomovirus and a potyvirus in coinfected sweet potato plants. *Plant Pathology* **55,** 458-467.

**Ndunguru, J. & Kapinga, R. (2007).** Viruses and virus-like diseases affecting sweetpotato subsistence farming in Tanzania. *African Journal of Agricultural Research* **2,** 232-239.

**Njeru, R. W., Mburu, M. W., Cheramgoi, E. C., Gibson, R. W., Kiburi, Z. M., Obudho, E. & Yobera, D. (2004).** Studies on the physiological effects of viruses on sweet potato yield in Kenya. *Annals of Applied Biology* **145,** 71-76

**Njeru, R. W., Bagabe, M. C., Nkezabahizi, D., Kayiranga, D., Kajuga, J., Butare, L. & Ndirigue, J. (2008).** Viruses infecting sweet potato in Rwanda: occurrence and distribution. *Annals of Applied Biology* **153,** 215-221.

**Nome, C. F., Laguna, I. G. & Nome, S. F. (2006).** Cytological alterations produced by *Sweet potato mild speckling virus*. *Journal of Phytopathology* **154,** 504-507.

**Schaefers, G. A. & Terry, E. R. (1976).** Insect transmission of sweetpotato disease agents in Nigeria. *Phytopathology* **66,** 642-645.

**Sheffield, F. M. L. (1957).** Virus diseases of sweet potato in East Africa. I. Identification of the viruses and their insect vectors. *Phytopathology* **47,** 582-590.

**Tairo, F., Mukasa, S. R., Jones, R. A. C. Kullaya, A., Rubaihayo, P. R. & Valkonen, J. P. T. (2005).** Unraveling the genetic diversity of the three main viruses involved in sweet potato virus disease (SPVD), and its practical implications. *Molecular Plant Pathology* **6,** 199-211.

**Tairo, F., Jones, R. A. C. & Valkonen, J. P. T. (2006).** Potyvirus complexes in sweetpotato: Occurrence in Australia, serological and molecular resolution, and analysis of the *Sweet potato virus 2* (SPV2) component. *Plant Disease* **90,** 1120-1128.

**Thresh, J. M., Fargette, D. & Jeger, M. J. (2003).** Epidemiology of tropical plant viruses. In *Viruses and Virus-like Diseases of Major Crops in Developing Countries*, pp. 223-226. Edited by Loebenstein G. & Thottappilly G. USA: Kluwer Academic Publishers.

**Wambugu, F. M. (2003).** Development and transfer of genetically modified virus-resistant sweet potato for subsistence farmers in Kenya. *Nutritional Reviews* **61,** s110-s113.

# CHAPTER THREE

# MOLECULAR RESOLUTION OF THE GENETIC VARIABILITY OF MAJOR VIRUSES INFECTING SWEET POTATO (*Ipomoea batatas* L.) IN KWAZULU-NATAL (KZN)

## 3.1 ABSTRACT

The genetic variability of the three major viruses infecting sweet potato (*Ipomoea batatas* Lam.) in KwaZulu-Natal (KZN), South Africa was determined in this study. A total of 16 virus isolates originating from three different locations (Umbumbulu, Umfume and Umphambanyomi River) in KZN were analyzed. These comprised of 10 isolates of Sweet potato feathery mottle virus (SPFMV), five isolates of Sweet potato virus G (SPVG) and one isolate of Sweet potato chlorotic stunt virus (SPCSV). The phylogenetic relationships of the SPFMV, SPVG and SPCSV isolates from KZN relative to isolates occurring in SA and different parts of the world were assessed. The division of SPFMV into four genetic groups (strains) according to the phylogenetic analysis of coat protein encoding sequences revealed mixed infections of the O (ordinary) and C (common) strains in sweet potato crops from KZN. All SPFMV isolates showed close lineage with isolates from South America, East Asia and Africa. The SPVG isolates showed high relatedness to each other and close lineage with other isolates, especially those from China and Egypt. Analysis of the partial sequence of the Heat shock protein 70 homologue (*Hsp70*h) gene indicated that the SPCSV isolate from KZN belongs to the West African (WA) strain group of SPCSV and showed close relatedness to an isolate from Argentina. The knowledge of specific viral diversity is essential in developing effective control measures against sweet potato viruses in KZN.

## 3.2 INTRODUCTION

The strategy of controlling viral diseases in sweet potato (*Ipomoea batatas* Lam.), or any other crop, requires a comprehensive knowledge of the viruses involved (Kreuze, 2002). Knowledge of the population genetic variability and strain distribution of viruses is

required for an understanding of their molecular epidemiology, which will assist in the developing effective control measures. The genetic variability of viruses affects both their detection and control. Knowledge of intraspecific viral diversity is also required to determine whether available serological or molecular based detection methods are able to detect different virus isolates of diverse geographical origin (Tairo *et al*., 2005). Similarly, engineered mechanisms of virus resistance are effective only against viral isolates with a high degree of sequence homology to the transgene (Fuchs & Gonsalves, 2007). As Sweet potato feathery mottle virus (SPFMV), Sweet potato virus G (SPVG), and Sweet potato chlorotic stunt virus (SPCSV) have been identified as the major viruses infecting sweet potato in KwaZulu-Natal (KZN), knowledge of the relatedness of their sequences is essential for the successful implementation of genetic engineering resistance strategies.

SPFMV and SPVG are typical members of the genus *Potyvirus* containing a single-stranded, 3'-polyadenylated RNA genome with a single open reading frame that is translated into a large polyprotein (Van Regemortel *et al*., 2000). Virus-encoded proteinases process the large polyprotein into ten mature proteins. The coat protein (CP) produced from the proximal end of the polyprotein encapsidates the viral genome and is an important determinant of aphid transmission (Dougherty & Carrington, 1988). The potyvirus CP is extensively used to infer phylogenetic relationships among viral isolates (Colinet *et al*., 1998; Tairo *et al*., 2005; Rännäli *et al*., 2008; Rännäli *et al*., 2009). Comparing CP gene sequences has become a useful means for studying the taxonomy of viruses belonging to the *Potyvirus* genus and also to differentiate closely related strains or isolates (Tairo, 2006).

Phylogenetic analysis of CP-encoding sequences has differentiated SPFMV into four strains: common (C), East African (EA), ordinary (O) and russet crack (RC) (Kreuze *et al*., 2000). Isolates of strains RC, O and EA are closely related to each other and are phylogenetically distant from strain C (Tairo *et al*., 2005). Strains RC, O and C are distributed worldwide, whereas isolates of the EA strain have been largely restricted to countries in East Africa (Kreuze *et al*., 2000; Mukasa *et al*., 2003). However, recent

reports have detected isolates of the EA strain outside East Africa in Spain (Valverde *et al*., 2004), Vietnam (Ha *et al*., 2008), Peru (Untiveros *et al*., 2008) and Easter Island (Rännäli *et al*., 2009).

SPCSV is a member of the genus *Crinivirus* in the family *Closteroviridae* (Winter *et al*., 1992). It has a bipartite single-stranded RNA genome consisting of RNA-1 and RNA-2. The Closteroviridae gene array is located on RNA-2 and comprises a putative small hydrophobic protein (SHP), a Heat shock protein homologue (*Hsp70*h), the major CP and a divergent copy of the CP referred to as minor CP (Kreuze *et al*., 2002; Aritua *et al*., 2008). The *Hsp70*h is the hallmark gene of the family *Closteroviridae* which, due to its high degree of conservation, has been traditionally used for phylogenetic analysis of members of this family (Alicia *et al*., 1999; Kreuze *et al*., 2002; Aritua *et al*., 2008; Cuellar *et al*., 2011). Initial studies using monoclonal antibodies to SPCSV CP distinguished two SPCSV strains: West African (WA) and East African (EA) (Vetten *et al*., 1996; Alicia *et al*., 1999). A similar division into the two genetic strains was revealed by phylogenetic analysis of the CP and partial *Hsp70*h gene sequences (Alicia *et al*., 1999). Outside West Africa, strain WA seems to have a wide geographical distribution, occurring in Egypt (Ishak *et al*., 2003), Israel (Cohen *et al*., 1992), Australia (Tairo *et al*., 2005), and America (Di Feo *et al*., 2000, Fenby *et al*., 2002, Abad *et al*., 2006). Isolates of the EA strain have been largely restricted to countries in East Africa, with only one isolate from Peru being reported from outside Africa (Gutièrrez *et al*., 2003).

Viruses infecting sweet potato have been identified and partially characterized at a molecular level, especially in East Africa, Australasia, Peru and the United States (Mukasa *et a*l., 2003; Souto *et al*., 2003; Tairo *et al*., 2005; Untiveros *et al*., 2008). However, such molecular characterization has received much less attention in South Africa (Rännäli *et al*., 2009). The aim of this study was to determine the molecular variability among the three major viruses (SPFMV, SPVG and SPCSV) infecting sweet potato in KZN.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Virus isolates

Collection of samples and subsequent identification of the most prevalent viruses infecting sweet potato (*Ipomoea batatas* Lam.) in KwaZulu-Natal (KZN) was previously described in Chapter 2 and the major three viruses where chosen for this study. A total of 10 isolates of SPFMV and five isolates of SPVG originating from one location in Umbumbulu (UMBUM) and Umfume (UMFUME), and three locations in Umphambanyomi River (UMP1, UMP2, UMP3) respectively and one isolate of SPCSV from Umfume were used in this study. All isolates were maintained at 25$^{o}$C in the original host plant and propagated into indicator plants, *Ipomoea setosa* Ker. and *Ipomoea nil* Lam. using the crown-cleft grafting method described in section 2.3.2. All plants were kept at a constant temperature of 20-25$^{o}$C in an insect-proof greenhouse in the Controlled Environment Research Unit (CERU) at the University of KwaZulu-Natal (UKZN-PMB) for the duration of the study.

### 3.3.2 RNA extraction and RT-PCR

#### *3.3.2.1 Total plant RNA isolation*

Total RNA was extracted from symptomatic *I. setosa* leaf material using the SV Total RNA Isolation System (Promega, USA) according to manufacturers' instructions. Fresh leaf material was ground into a fine powder in an microfuge tube containing liquid nitrogen using a plastic drill bit. Total RNA was also extracted from leaf samples of uninfected healthy control plants. All RNA preparations were stored at -80$^{o}$C.

#### *3.3.2.2 RT-PCR of total plant RNA*

Total RNA from infected leaf material of *I. setosa* was used as a template for first strand synthesis in the reverse transcription (RT) procedure. RT was performed using the

Reverse Transcription System (Promega, USA) according to manufacturers' instructions. cDNA was synthesized on 3µg of total RNA using an oligo-$dT_{15}$ primer in a 20µl reaction. Negative controls consisted of DEPC-treated water and total RNA extracted from an uninfected healthy control plant. PCR was performed using the GoTaq® PCR Core System II (Promega, USA) according to manufacturers' instructions using primers specific to SPFMV, SPVG and SPCSV. An amplified 323bp fragment from positive control plasmid DNA, provided in the kit, was used as the positive control reaction.

*SPFMV*

Strain specific primer pairs were used in the PCR amplification of SPFMV. SPFMV strains RC, O and EA were amplified using the forward primer FM1360-F, whereas strain C was amplified using the forward primer SPVC474-F (Tairo *et al*., 2006). The reverse primer 10820-R (Tairo *et al*., 2005) was used with both forward primers. The amplified fragments were expected to be 1356bp in size for strains RC, O and EA and 1335bp for strain C. The amplicons consisted of 187bp (strains RC, O and EA) and 172bp (strain C) of the RNA-dependent RNA polymerase (NIb) - encoding region, the entire coat protein (CP) - encoding region (945bp in strains RC, O and EA and 939bp in strain C), and 224bp of the 3'-nontranslated (3'NTR) region (Rännäli *et al*., 2009). Amplification was performed in an automated thermal cycler programmed according to the parameters described by Rännäli *et al*. (2009): one cycle of initial denaturation of 98$^{o}$C for 10s and 40 cycles of amplification with 10s of denaturation at 98$^{o}$C, 20s of annealing at 59$^{o}$C (primer FM1360-F), or 57$^{o}$C (primer SPVC474-F) and 45s of extension at 72$^{o}$C followed by a final extension for 10min at 72$^{o}$C.

*SPVG*

The primer pair SPVG1-F and SPVG3-R (Rännäli *et al*., 2008) was used in the PCR amplification of SPVG. The amplified fragment of 1285bp consisted of 161bp of the NIb - encoding region, the entire CP - encoding region (1068bp), and 51bp of the 3' NTR region. Amplification was done according to parameters defined by Rännäli *et al*.

(2008): one cycle at 98$^{o}$C for 1min, followed by 40 cycles of 10s at 98$^{o}$C, 20s at 63$^{o}$C and 45s at 72$^{o}$C followed by final extension for 10min at 72$^{o}$C.

*SPCSV*

The primer pair CL43U-F and CL43L-R (Winter *et al*., 1997) was used in the PCR amplification of SPCSV. An amplification product of 486bp consisting of the Heat shock protein-70 homologue (*Hsp70*h) gene was expected. Parameters for amplification consisted of one cycle at 95$^{o}$C for 2min, followed by 35 cycles of 1min at 95$^{o}$C, 30s at 50$^{o}$C and 1min at 72$^{o}$C followed by final extension for 10min at 72$^{o}$C.

#### *3.3.2.3 Analysis of RT-PCR products by agarose gel electrophoresis*

Amplified products of the RT-PCR was examined on a 1% (w/v) agarose gel prepared in 1× TAE buffer (Promega, USA) and stained with ethidium bromide (1µl/ml). The GeneRuler$^{TM}$ 100bp DNA ladder (Fermentas), or O' RangeRuler$^{TM}$ 100bp + 500bp DNA ladder (Fermentas) was used as the molecular weight markers (MWM). Electrophoresis was done at 100V for 90min. DNA bands were visualized under an ultraviolet (UV) transilluminator and photographed using the G-Box gel documenting system (Syngene, Vacutec).

### 3.3.3 Cloning and sequencing

RT-PCR products were purified from the agarose gel using the MiniElute Gel Extraction Kit (Qiagen, USA). The cloning of the RT-PCR products was done using the TA Cloning® Kit according to manufacturers' instructions with minor modifications (Invitrogen, CA). RT-PCR products were ligated to the plasmid cloning vector pCR®2.1 and transformed into TOP10F competent *E.coli* One Shot® cells according to manufacturers' instructions (Invitrogen, CA). Recombinant clones were selected on Luria Bertani (LB) plates containing 50µg/ml kanamycin and 40mg/ml X-Gal (Promega, USA).

**Table 3.1** Primers used for the amplification of Sweet potato feathery mottle virus (SPFMV), Sweet potato virus G (SPVG), and Sweet potato chlorotic stunt virus (SPCSV)

| Virus | Primer name | Sequence | Expected size (bp) | Reference |
|---|---|---|---|---|
| SPFMV, strains RC, O and EA | | | | |
| Forward | FM1360-F | 5’ GACCAAGCCCCATACAATGA 3’ | | Tairo *et al*. (2006) |
| Reverse | 10820-R | 5’ GGCTCGATCACGAACCAAAA 3’ | 1 356 | Tairo *et al*. (2005) |
| SPFMV, strain C | | | | |
| Forward | SPVC474-F | 5’ CAGTGAACTAGCACGAAC 3’ | | Tairo *et al*. (2006) |
| Reverse | 10820-R | 5’ GGCTCGATCACGAACCAAAA 3’ | 1 335 | Tairo *et al*. (2005) |
| SPVG | | | | |
| Forward | SPVG1-F | 5’ TATACCGCGGAAAAGCACCCTACATAGCT 3’ | | |
| Reverse | SPVG3-R | 5’ TATATGAGCTCACTGAAGGCGAAACTGAA 3’ | 1 285 | Rännäli *et al*. (2009) |
| SPCSV | | | | |
| Forward | CL43U-F | 5’ ATCGGCGTATGTTGGTGGTA 3’ | | |
| Reverse | CL43L-R | 5’ GCAGCAGAAGGCTCGTTTAT 3’ | 486 | Winter *et al*. (1997) |

Plasmid DNA of the independently transformed bacterial cells was purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). Each colony was transferred aseptically into tubes containing 2ml of LB broth containing 50µg/ml kanamycin. Recombinant clones were identified by *EcoR*I (BioLabs, New England) restriction endonuclease digestion at 37$^{o}$C for 2h. Four positively identified recombinant clones for each of the 16 transformations (5 SPFMV strain C isolates, 5 SPFMV strain O/RC/EA isolates, 5 SPVG isolates and 1 SPCSV isolate) were selected and sent to Inqaba Biotech[1] for sequencing with the M13 forward and reverse primers.

### 3.3.4 Phylogenetic analysis

The phylogenetic status of SPFMV, SPVG and SPCSV isolates from KZN relative to isolates occurring in SA and different parts of the world was assessed using the MEGA software package version 4.0 (Tamura *et al*., 2007). The sequence data reported in this study were deposited into GenBank (Benson *et al*., 1996). The details of the sequences used in the analysis are presented (Table 3.2.). The BLAST program (Altschul *et al*., 1990) was used to obtain related SPFMV, SPVG and SPCSV sequences from different geographical locations. These sequences were obtained from the public database of the National Centre for Biotechnology Information (NCBI) (www.ncbi.nml.nih.gov). Sequence alignments and phylogenetic analyses were done using the UPGMA method in the MEGA4 software package. The bootstrap test was conducted using 1,000 replicates and evolutionary distances were computed using the Jukes-Cantor method.

[1] Inqaba Biotechnical Industries (Pty) Ltd, P.O. Box 14356, Hatfield 0028, SA

**Table 3.2** Isolates of Sweet potato feathery mottle virus (SPFMV), Sweet potato virus G (SPVG) and Sweet potato chlorotic stunt virus (SPCSV) used in this study

| Virus species/ Isolate | Strain[a] group | Geographical origin (region/country) | Accession no. | Reference |
|---|---|---|---|---|
| SPFMV | | | | |
| Aus142-AC | C | Australia | AM050891 | Tairo *et al*. (2005) |
| Aus55-4C | C | Australia | AM050892 | Tairo *et al*. (2006) |
| Aus5c | C | Australia | AJ781779 | Tairo *et al*. (2005) |
| C | C | United States | S43451 | Abad & Moyer (1992) |
| CY1 | C | Taiwan | EF492048 | Wang *et al*. (2007)[b] |
| CTB3-1 | C | South Africa | EU809485 | Rännäli *et al*. (2009) |
| Ita1 | C | Italy | AM076411 | Parrella *et al*. (2006) |
| MDI/1 | C | South Africa | AY459601 | Ateka *et al*. (2003)[b] |
| NZ1-1 | C | New Zealand | EU809483 | Rännäli *et al*. (2009) |
| ORI-2 | C | Moorea | EU809486 | Rännäli *et al*. (2009) |
| PS1B-4 | C | Easter Island | EU829499 | Rännäli *et al*. (2009) |
| Sor | C | Uganda | AJ539129 | Mukasa *et al*. (2003) |
| UMBUM-C | C | SA, KwaZulu Natal | JQ073717 | This study |
| UMFUME-C | C | SA, KwaZulu Natal | JQ073713 | This study |
| UMP1-C | C | SA, KwaZulu Natal | JQ073714 | This study |
| UMP2-C | C | SA, KwaZulu Natal | JQ073715 | This study |
| UMP3-C | C | SA, KwaZulu Natal | JQ073716 | This study |
| ZIMB1-2 | C | Zimbabwe | EU809510 | Rännäli *et al*. (2009) |
| 6 | C | Argentina | U9925 | Alvarez *et al*. (1997)[b] |
| 25/4A | C | Kenya | AY523543 | Ateka *et al*. (2004)[b] |
| 3817-2 | C | Tahiti | EU809495 | Rännäli *et al*. (2009) |
| CH2 | EA | Peru | EU021067 | Untiveros *et al*. (2008) |
| Mis1 | EA | Tanzania | AJ781783 | Tairo *et al*. (2005) |
| M2-44 | EA | Peru | EU021069 | Untiveros *et al*. (2008) |
| NAM 1 | EA | Uganda | AJ010704 | Kreuze *et al*. (2000) |
| Putisrabe | EA | Madagascar | AY459597 | Ateka *et al*. (2003)[b] |
| PS1B-1 | EA | Easter Island | EU809496 | Rännäli *et al*. (2009) |
| PS1B-2 | EA | Easter Island | EU809497 | Rännäli *et al*. (2009) |
| PS3A-1 | EA | Easter Island | EU809500 | Rännäli *et al*. (2009) |
| RUK | EA | Uganda | AJ010707 | Kreuze *et al*. (2000) |
| Zam1 | EA | Zambia | AY523552 | Ateka *et al*. (2004)[b] |
| ZIMB1-1 | EA | Zimbabwe | EU809509 | Rännäli *et al*. (2009) |
| Arua10a | O | Uganda | AY459595 | Ateka *et al*. (2003)[b] |
| BAU | O | Niger | AJ010699 | Kreuze *et al*. (2000) |
| CH | O | China | Z98942 | Colinet *et al*. (1998) |
| CTB3-3 | O | South Africa | EU809504 | Rännäli *et al*. (2009) |
| CY2 | O | Taiwan | EF492049 | Wang *et al*. (2007)[b] |
| K2 | O | Korea | AF015541 | Ryu *et al*. (1998) |
| O | O | Japan | D16664 | Mori *et al*. (1994) |
| OR2-2 | O | Moorea | EU809490 | Rännäli *et al*. (2009) |
| UMBUM-O | O | SA, KwaZulu Natal | JQ073712 | This study |
| UMFUME-O | O | SA, KwaZulu Natal | JQ073708 | This study |
| UMP1-O | O | SA, KwaZulu Natal | JQ073709 | This study |
| UMP2-O | O | SA, KwaZulu Natal | JQ073710 | This study |

*Table 3.2 continued…*

| | | | | |
|---|---|---|---|---|
| UMP3-O | O | SA, KwaZulu Natal | JQ073711 | This study |
| 115/1s | O | Kenya | AY523540 | Ateka *et al*. (2004)[b] |
| 3817-1 | O | Tahiti | EU809494 | Rännäli *et al*. (2009) |
| 3900 | O | Tubuai | EU809484 | Rännäli *et al*. (2009) |
| 5 | O | Argentina | U96624 | Alvarez *et al*. (1997)[b] |
| Aus6 | RC | Australia | AJ781777 | Tairo *et al*. (2005) |
| Fio | RC | Peru | EU021065 | Untiveros *et al*. (2008) |
| K1 | RC | Korea | AF015540 | Ryu *et al*. (1998) |
| KmtMil | RC | Peru | EU021066 | Untiveros *et al*. (2008) |
| M2-41 | RC | Peru | EU021064 | Untiveros *et al*. (2008) |
| RC | RC | United States | S43450 | Abad & Moyer (1992) |
| S | RC | Japan | D86371 | Sakai *et al*. (1997) |
| SPVG | | | | |
| CH | - | China | Z83314 | Colinet *et al*. (1994) |
| CH2 | - | China | X76944 | Colinet *et al*. (1998) |
| CTB3 | - | South Africa | EU220756 | Rännäli *et al*. (2008) |
| Egypt1 | - | Egypt | AJ515380 | Ishak *et al.* (2003) |
| HawA2 | - | Hawaii | EU220753 | Rännäli *et al*. (2008) |
| Henan | - | China | DQ399861 | Zhang *et al*. (2004) |
| Hua2 | - | Peru | EU218528 | Untiveros *et al*. (2008) |
| JAAC | - | Java | EU220755 | Rännäli *et al*. (2008) |
| NZ-4 | - | New Zealand | EU220758 | Rännäli *et al*. (2008) |
| Oita | - | Japan | AB435072 | Yamasaki *et al.* (2009) |
| PS1B | - | Easter Island | EU220754 | Rännäli *et al*. (2008) |
| SP1 | - | New Zealand | EF514221 | Rännäli *et al*. (2008) |
| Sichuan-1 | - | China | EU218531 | Qin *et al.* (2010)[b] |
| UMBUM-VG | - | SA, KwaZulu Natal | JQ073704 | This study |
| UMFUME-VG | - | SA, KwaZulu Natal | JQ073707 | This study |
| UMP1-VG | - | SA, KwaZulu Natal | JQ073705 | This study |
| UMP2-VG | - | SA, KwaZulu Natal | JQ073706 | This study |
| UMP3-VG | - | SA, KwaZulu Natal | JQ073703 | This study |
| Zimb1C | - | Zimbabwe | EU220752 | Rännäli *et al*. (2008) |
| Zimb1H | - | Zimbabwe | EU220751 | Rännäli *et al*. (2008) |
| 3900A | - | Tubuai | EU220759 | Rännäli *et al*. (2008) |
| 3817 | - | Tahiti | EU220757 | Rännäli *et al*. (2008) |
| SPCSV | | | | |
| Bag | EA | Tanzania | AJ783445 | Tairo *et al*. (2005) |
| Bkb1 | EA | Tanzania | AJ783446 | Tairo *et al*. (2005) |
| EA2 | EA | Uganda | AJ428555 | Kreuze *et al*. (2002) |
| Kiboga3d | EA | Uganda | DQ864360 | Aritua *et al*. (2008) |
| Madagascar | EA | Madagascar | AJ278651 | Fenby *et al*. (2002) |
| Mis1 | EA | Tanzania | AJ783447 | Tairo *et al*. (2005) |
| S2EA-4a | EA | Uganda | AJ010921 | Alicai *et al*. (1999) |
| S2EA-11a | EA | Uganda | AJ010914 | Alicai *et al*. (1999) |
| S2EA-13a | EA | Uganda | AJ010916 | Alicai *et al*. (1999) |
| S2EA-4a | EA | Uganda | AJ010921 | Alicai *et al*. (1999) |
| S2EA-19a | EA | Uganda | AJ010920 | Alicai *et al*. (1999) |
| S2EA-22a | EA | Uganda | AJ010918 | Alicai *et al*. (1999) |

*Table 3.2 continued…*

| | | | | |
|---|---|---|---|---|
| S2EA-25a | EA | Uganda | AJ010923 | Alicai *et al*. (1999) |
| S2EA-39a | EA | Uganda | AJ010925 | Alicai *et al*. (1999) |
| S2EA-41a | EA | Uganda | AJ010927 | Alicai *et al*. (1999) |
| Tar2 | EA | Tanzania | AJ783448 | Tairo *et al*. (2005) |
| TZ297 | EA | Tanzania | DQ864372 | Aritua *et al*. (2008) |
| TZ3 | EA | Tanzania | DQ864371 | Aritua *et al*. (2008) |
| Ung2 | EA | Tanzania | AJ783449 | Tairo *et al*. (2005) |
| Zambia1 | EA | Zambia | DQ864374 | Aritua *et al*. (2008) |
| 13-1S | EA | Kenya | DQ864364 | Aritua *et al*. (2008) |
| 15-3S | EA | Kenya | DQ864365 | Aritua *et al*. (2008) |
| 84-5S | EA | Kenya | DQ864369 | Aritua *et al*. (2008) |
| 115-2S | EA | Zambia | DQ864366 | Aritua *et al*. (2008) |
| B4 | WA | Spain | EF667069 | Valverde *et al*. (2004) |
| Cordoba | WA | Argentina | AY729021 | Nome *et al*. (2004)[b] |
| Egypt1 | WA | Egypt | AJ515381 | Ishak *et al*. (2003) |
| KZN | WA | SA, KwaZulu Natal | JQ073702 | This study |
| Nigeria1 | WA | Nigeria | AJ278652 | Fenby *et al*. (2002) |
| Nigeria2 | WA | Nigeria | AJ278653 | Fenby *et al*. (2002) |
| USA | WA | United States | AF260321 | Sim *et al*. (2000)[b] |

[a] Common (C), East African (EA), ordinary (O) and russet crack (RC) strains of SPFMV. East African (EA) and West African (WA) strains of SPCSV.
[b] Unpublished reference.

## 3.4 RESULTS

### 3.4.1 RT-PCR of total plant RNA

RT-PCR amplification products of the expected size were obtained using primers specific to strains RC, O and EA of SPFMV, strain C of SPFMV, SPVG, and SPCSV. The positive control yielded an expected 323bp band whilst negative controls showed no products (Fig. 3.1).

### 3.4.2 Phylogenetic analysis

#### *3.4.2.1 SPFMV*

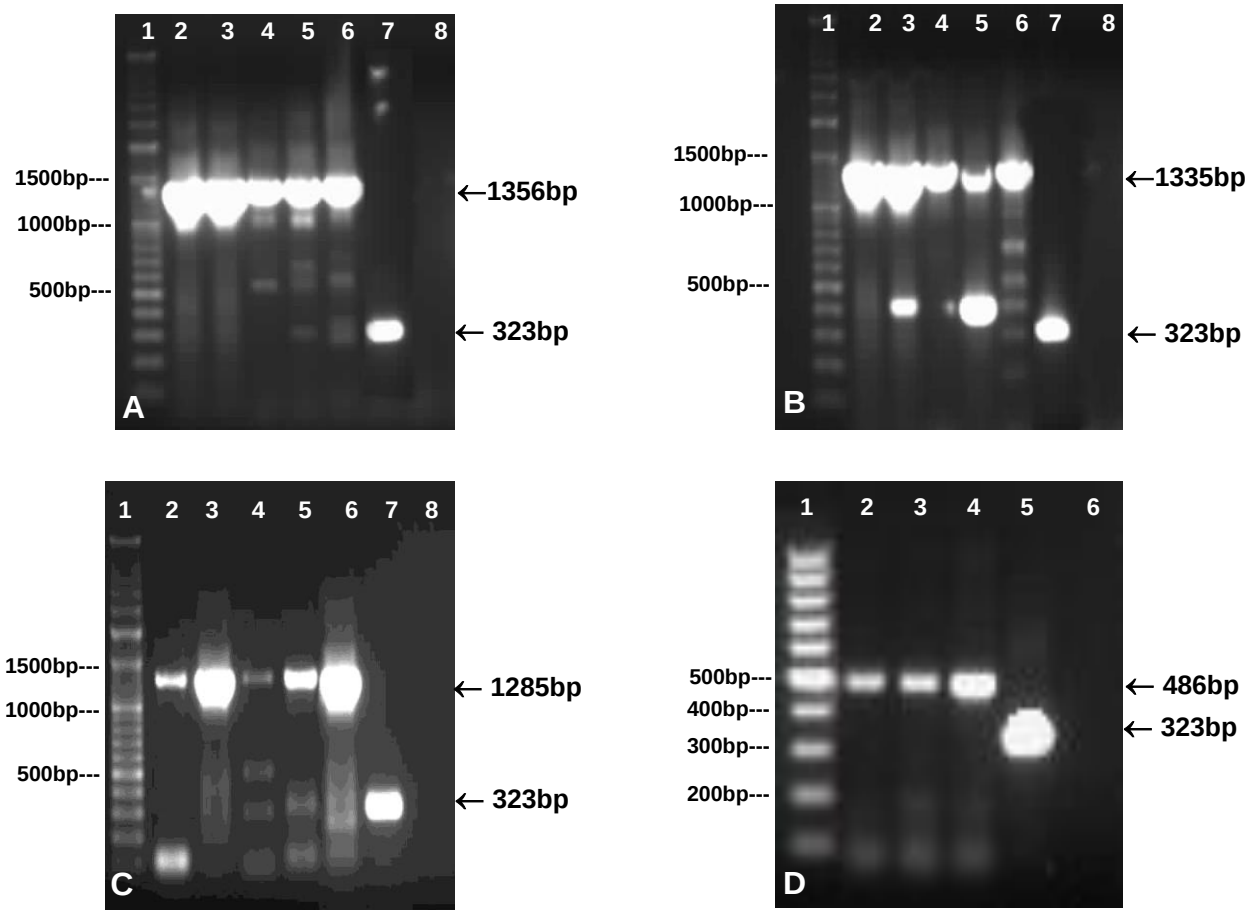



**Figure 3.1** Agarose gels showing the reverse-transcription polymerase chain reaction (RT-PCR) products of total plant RNA extracted from symptomatic *Ipomoea setosa* Ker., infected with (A) Sweet potato feathery mottle virus (SPFMV) strains O, RC and EA, (B) Sweet potato feathery mottle virus (SPFMV) strains C, (C) Sweet potato virus G (SPVG), and (D) Sweet potato chlorotic stunt virus (SPCSV). Lanes 1 shows the 100bp + 500bp or 100bp molecular weight marker. (A-C) Lanes 2-6 show the amplification products of isolates from Umbumbulu, Umfume and Umphambanyomi River (3 samples) respectively. (D) Lanes 2-4 show the amplification products of an SPCSV isolate from Umfume. Lane 7 (A-C) and Lane 5 (D) show the 323bp product of the positive control reaction. Lane 8 (A-C) and Lane 6 (D) show the negative control reaction which contains water.

Phylogenetic analysis using the CP nt sequences of SPFMV isolates described in this study and 47 CP sequences of SPFMV available in GenBank, grouped SPFMV isolates into four distinct clusters corresponding to the four SPFMV strains: O, RC, EA and C (Fig. 3.2). The analysis placed the KZN SPFMV strains described in this study into SPFMV group O and SPFMV group C. Strain C isolates from KZN showed higher relatedness to each other than to the UMP1-C isolate. Based on the phylogenetic topology, all SPFMV isolates from KZN showed close lineage with isolates from South America (Easter Island [PS1B-4], Moorea [OR1-2, OR2-2], and Tahiti [3817-1]), East Asia (Taiwan [CY1, CY2] and Japan [O]) and Africa (South Africa [CTB3-3, CTB3-1, MD1/1], Zimbabwe [ZIMB1-2] and Uganda [Arua10a]). Strain C isolates showed close lineage with European isolates from Argentina [6] and Italy [Ita1].

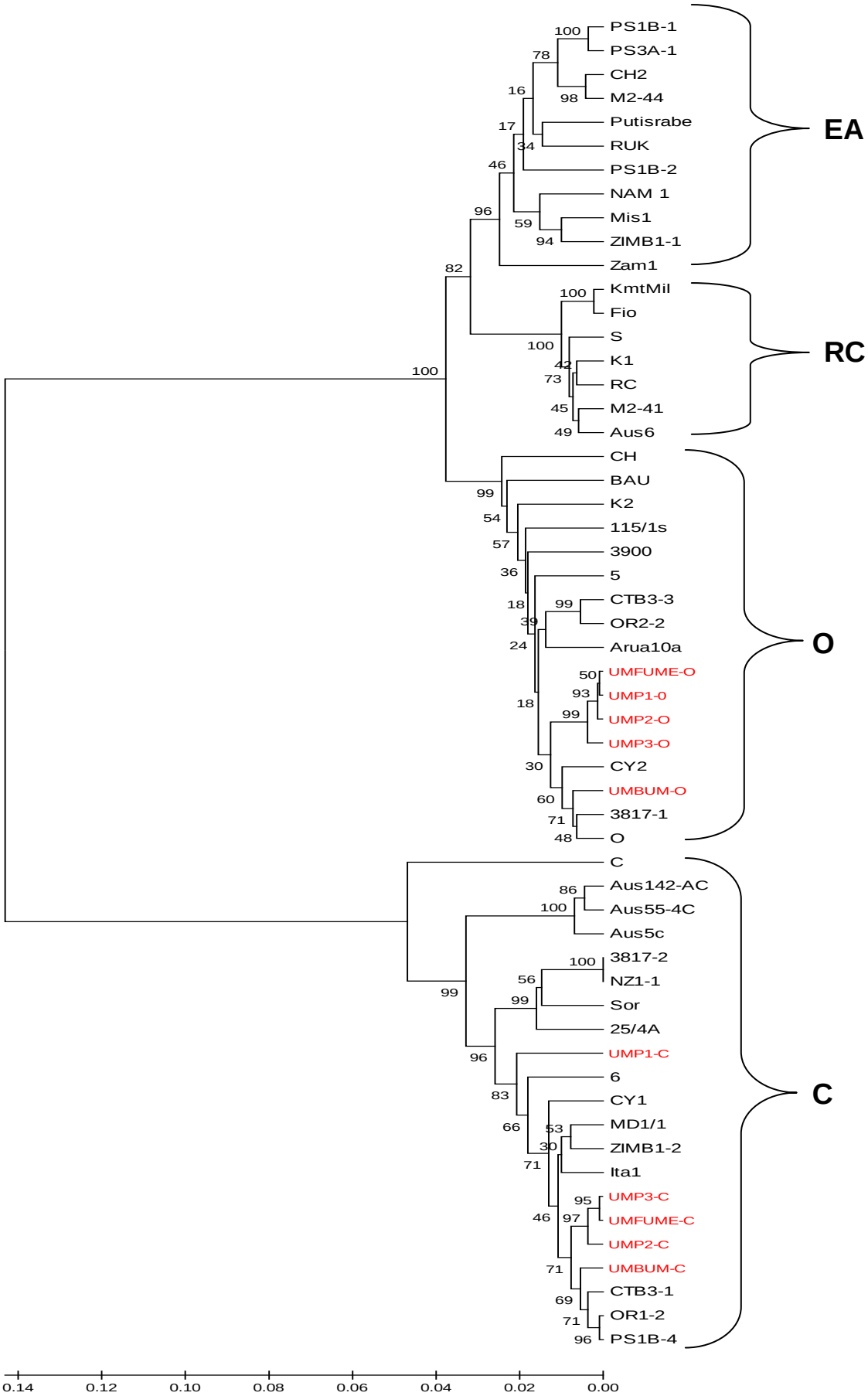
#### *3.4.2.2 SPVG*

Phylogenetic analysis was done using the CP nt sequences of SPVG isolates described in this study and 17 CP sequences of SPVG available in GenBank (Fig. 3.3). Results indicate that SPVG isolates from Umbumbulu (UMBUM-VG) and Umphambanyomi River (UMP2-VG) showed high sequence divergence forming a distinct clade. Isolates from Umfume (UMFUME-VG) and Umphambanyomi River (UMP1-VG, UMP3-VG) showed high relatedness to each other and close lineage with other isolates, especially those from China (CH, Henan) and Egypt (Egypt-1).

#### *3.4.2.3 SPCSV*

The analysis of the phylogenetic relationships of the *Hsp70h* gene from a SPCSV isolate from KZN with 29 other sequences of SPCSV available in GenBank, grouped SPCSV isolates into two distinct clusters corresponding to the EA and WA SPCSV strain groups (Fig. 3.4). The SPCSV isolate described in this study (KZN) was placed in the strain group WA and showed close relatedness to an isolate from Argentina (Cordoba).

**Figure 3.2** Phylogenetic analysis of the Sweet potato feathery mottle virus (SPFMV) coat protein gene encoding sequences. The clusters containing virus isolates belonging to the four strains of SPFMV are indicated: EA (East African), RC (Russet Crack), O (Ordinary), C (Common). SPFMV isolates from Umbumbulu (UMBUM-O/C), Umfume (UMFUME-O/C) and Umphambanyomi River (UMP1-O/C, UMP2-O/C, UMP3-O/C) in KwaZulu-Natal (KZN) are indicated in red. The scale bar indicates evolutionary distances. The bootstrap values of 1000 replicates are shown.


PS1B-1
PS3A-1
CH2
M2-44
Putisrabe
RUK
PS1B-2
NAM 1
Mis1
ZIMB1-1
Zam1
KmtMil
Fio
S
K1
RC
M2-41
Aus6
CH
BAU
K2
115/1s
3900
5
CTB3-3
OR2-2
Arua10a
UMFUME-O
UMP1-0
UMP2-O
UMP3-O
CY2
UMBUM-O
3817-1
O
C
Aus142-AC
Aus55-4C
Aus5c
3817-2
NZ1-1
Sor
25/4A
UMP1-C
6
CY1
MD1/1
ZIMB1-2
Ita1
UMP3-C
UMFUME-C
UMP2-C
UMBUM-C
CTB3-1
OR1-2
PS1B-4
EA
RC
O
C
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0.12
0.10
0.08
0.06
0.04
0.02
0.00

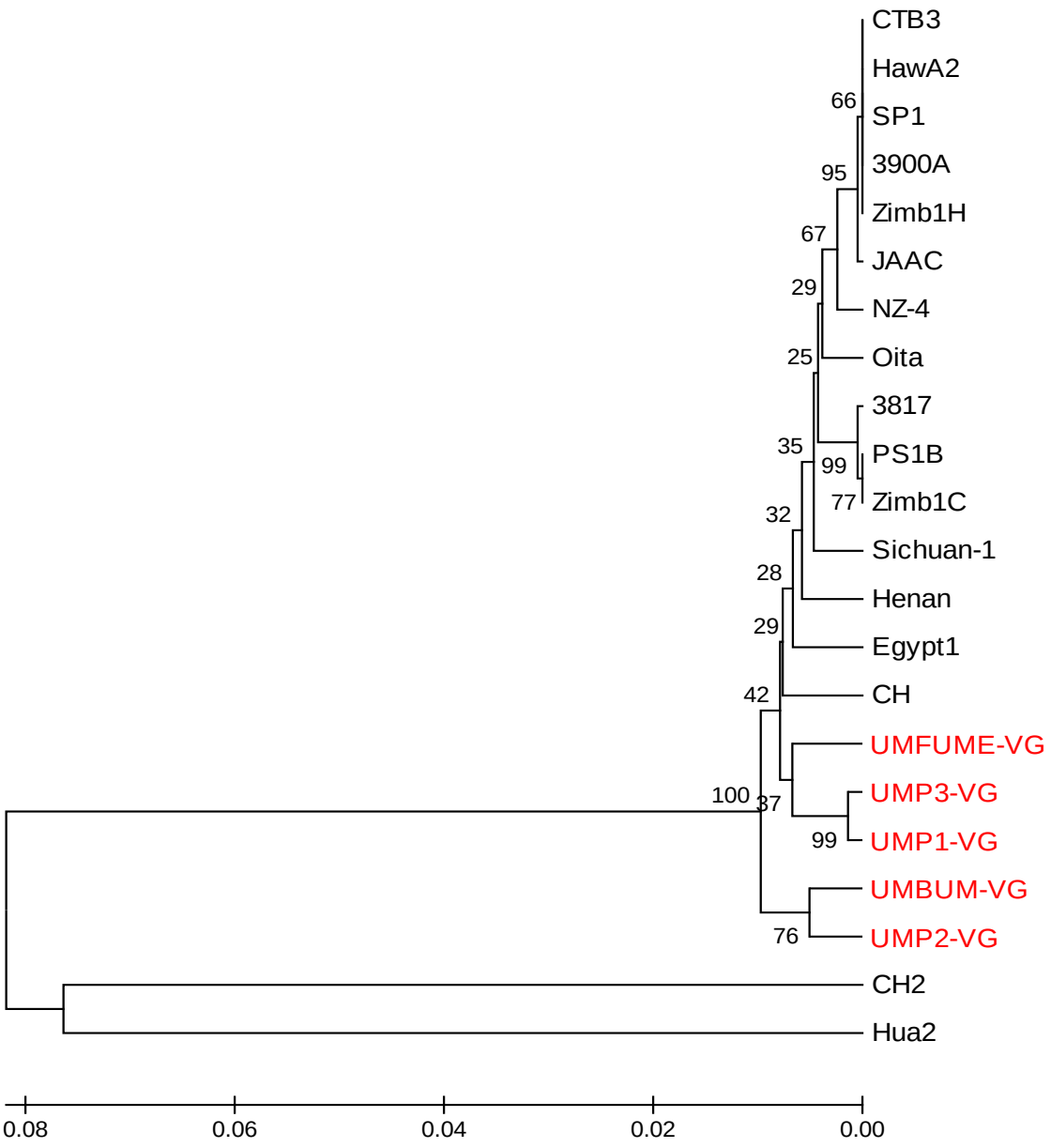


**Figure 3.3** Phylogenetic analysis of the Sweet potato virus G (SPVG) coat protein encoding sequences. SPVG isolates from Umbumbulu (UMBUM-VG), Umfume (UMFUME-VG) and Umphambanyomi River (UMP1-VG, UMP2-VG, UMP3-VG) in KwaZulu-Natal (KZN) are indicated in red. The scale bar indicates evolutionary distances. The bootstrap values of 1000 replicates are shown.


**Figure 3.4** Phylogenetic analysis of the Sweet potato chlorotic stunt virus (SPCSV) Heat shock protein-70 homologue (*Hsp70*h) gene encoding sequences. The clusters containing virus isolates belonging to the two strains of SPCSV are indicated: EA (East African) and WA (West African). The SPCSV isolate from KwaZulu-Natal (KZN) is indicated in red. The scale bar indicates evolutionary distances. The bootstrap values of 1000 replicates are shown.

## 3.5 DISCUSSION

This study was the first attempt to classify sweet potato viruses from KZN at a molecular level. Although a few SPFMV (Rännäli *et al*., 2009) and SPVG (Rännäli *et al*., 2008) sequences from SA have been incorporated into characterization studies, this study presents the first nucleotide sequence and phylogenetic analysis of SPCSV from SA.

Molecular analysis of the SPFMV population in KZN by phylogenetic analysis of CP encoding sequences (Fig. 3.2) indicated the mixed presence of strains O and C in all samples analyzed. This result is in agreement with a previous report on the genetic structure of SPFMV population from SA, where the combined presence of strains C, O and RC were revealed in the sweet potato samples from Cape Town, SA (Rännäli *et al*., 2009). In terms of the worldwide genetic structure of SPFMV, results are also consistent with reports that strains C, O and RC are widely distributed in sweet potato growing areas and strain EA is restricted to East African countries (Kreuze *et al*., 2000; Tairo *et al*., 2005; Rännäli *et al*., 2009). The phylogenetic analysis also highlighted how distant strain C is from all other isolates of SPFMV. This report together with the analysis of other reports (Kreuze *et al*., 2000; Tairo *et al*., 2005; Untiveros *et al*., 2008; Rännäli *et al*., 2009; Tugume *et al*., 2010) provides ample evidence to verify that this deviated branching pattern of SPFMV strain C is a firmly based distinguishing feature in the phylogenetic analysis of SPFMV. The analysis reported in this study supports the proposals by Moyer *et al*. (1980) and Tairo *et al*. (2005) in the naming of SPFMV strain C as a distinct species, Sweet potato virus C (SPVC).

The SPFMV strains from KZN showed close relation to each other and to previously determined sequences of SPFMV strain C (MD1/1 and CTB3-1) and O (CTB3-3) isolates from SA (Fig. 3.2). The small genetic variability among SPFMV isolates from SA could prove useful in the development and deployment of a transgenic virus resistant sweet potato cultivar that could be viable against homogenous SPFMV isolates across SA. However, further sequence data is needed to expand our knowledge of the genetic variability of SPFMV in SA.

The tree topology resulting from the phylogenetic analysis of SPVG (Fig. 3.3) is consistent with that reported by Rännäli *et al*. (2008). Analysis confirmed the worldwide distribution of SPVG and confirmed the presence of a large homogenous global population of SPVG isolates (Rännäli *et al*., 2008) that included the UMP1-VG, UMP2-VG and UMFUME-VG isolates from KZN. The exceptions were the isolates from China (CH2) and Peru (Hua2) which branched into a distinct clade. As mentioned by Rännäli *et al*. (2008), these isolates are thought to represent a divergent genetic lineage of SPVG. The limited genetic variability among the remaining SPVG isolates could imply that the same SPVG strain was distributed to different geographical locations (Rännäli *et al*., 2008). This could occur by plant material exchanges and/or introduction of efficient vectors which dispersed a homogenous population of SPVG into distinct geographical regions including SA. Although the KZN SPVG isolates form a cluster with the homologous SPVG isolates, the UMBUM-VG and UMP3-VG isolates formed a distinct clade within the cluster. The grouping of these isolates apart from the rest may reflect the making of a divergent lineage within the SPVG group. To confirm this speculation, more SPVG isolates from the rest of the African continent need to be characterized.

Phylogenetic analysis of the *Hsp70*h nt sequences placed the SPCSV isolate from KZN into the WA strain group, along with other isolates previously characterized as WA strains (Fig. 3.4) (Tairo *et al*., 2005; Aritua *et al*., 2008). The deviated branching topology is consistent with previous reports (Ishak *et al*., 2003; Tairo *et al*., 2005; Aritua *et al*., 2008). The intraspecific phylogeny of SPCSV emphasizes the uniqueness of the EA strain and its geographical restricted incidence. This study presents the first sequence data of a SPCSV isolate from SA. The addition of an SPCSV isolate from KZN, increases the genetic diversity and global distribution of the WA strain group, particularly because it shows high similarity with the divergent Cordoba isolate from Argentina. West Africa has traditionally been more significant for world trade than East Africa and this may explain the wider distribution of the WA strain in geographically distinct areas (Aritua *et al*., 2008). However, no definite conclusions on the phylogeny of the SPCSV isolate from KZN can be made as a result of the determination of only a

single SPCSV sequence. Therefore, these results are considered preliminary until further isolates are cloned and sequenced.

The information generated in this study is the first step in the development of engineered resistance to local strains of SPFMV, SPVG and SPCSV. It is needed to ensure that resistance is sufficiently comprehensive to adequately cover the diversity, if any, of local virus strains. This is of particular importance in terms of genetically variable virus strains being able to overcome strain-specific resistance in an introduced transgenic cultivar, hence nullifying considerable time consuming and costly efforts in resistance development. Fortunately, the low sequence diversity among SPFMV and SPVG isolates in KZN is beneficial for their control using engineered resistance. The data of low genetic variability provided by this study will be pertinent to the design of a transgenic resistance sweet potato cultivar that will be effective in all locations in KZN and possibly in SA.

## 3.6 REFERENCES


**Abad, J. A. & Moyer, J. W. (1992).** Detection and distribution of sweetpotato feathery mottle virus in sweetpotato by in vitro-transcribed RNA probes (riboprobes), membrane immunobinding assay, and direct blotting. *Phytopathology* **82,** 300-305.

**Abad, J. A, Parks, Parks, E. J., Speck, J. L., New, S. L. & Moyer, J. W. (2006).** Phylogenetic analysis of two *Sweet potato chlorotic stunt virus* (SPCSV) (genus Crinivirus) isolates from North Carolina. *Phytopathology* **96**, S1.

**Alicia, T., Fenby, N. S., Gibson, R. W., Adipala, E., Vetten, H. J., Foster, G. D. & Seal, S. E. (1999).** Occurrence of two serotypes *of Sweet potato chlorotic stunt virus* in East Africa and their associated differences in coat protein and HSP70 homologue gene sequences. *Plant Pathology* **48,** 718-726.

**Altschul S. F., Gish W., Miller W., Myers E. W., & Lipman D. J. (1990).** Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

**Aritua V., Barg, E., Adipala, E., Gibson., R. W., & Vetten, H. J. (2008).** Further evidence for limited genetic diversity among East African isolates of *Sweet potato chlorotic stunt virus*. *Journal of Phytopathology* **156**, 181-189.

**Benson, D. A., Boguski M., Lipman D. J., & Ostell J. (1996).** GenBank. *Nucleic Acids Research* **24**, 1-5.

**Cohen, J., Franck, A., Vetten, H. J., Lesemann, D. E. & Loebenstein, G. (1992).** Purification and properties of Closterovirus-like particles associated with a whitefly-transmitted disease of sweet potato. *Annals of Applied Biology* **121,** 257-268.

**Colinet, D., Kummert, J. & Lepoivre, P. (1994).** The complete nucleotide sequences of the coat protein cistron and the 3' non-coding region of a newly-identified potyvirus infecting sweetpotato, as compared to those of *Sweetpotato feathery mottle virus*. *Archives of Virology* **139,** 327-336.

**Colinet, D., Nguyen, M., Kummer, J. & Lepoivre, P. (1998).** Differentiation among Potyviruses infecting sweet potato based on genus- and virus-specific reverse transcription polymerase chain reaction. *Plant Disease* **82,** 223-229.

**Cuellar, W. J., De Souza, J., Barrantes, I., Fuentes, S. & Kreuze, J. F. (2011).** Distinct cavemoviruses interact synergistically with *Sweet potato chlorotic stunt virus* (Genus *Crinivirus*) in cultivated sweet potato. *Journal of General Virology* DOI:10.1099/vir.0.029975-0.

**Di Feo, L., Nome, S. F., Biderbost, E., Fuentes, S. & Salazar, L. F. (2000).** Etiology of sweetpotato chlorotic dwarf disease in Argentina. *Plant Disease* **84,** 223-229.

**Dougherty, W. G. & Carrington, J. C. (1988).** Expression and function of potyviral gene products. *Annual Review of Phytopathology* **26,** 123-143.

**Fenby, N., Foster, G, Gibson, R. W. & Seal, S. (2002).** Partial sequence of HSP70 homologue gene shows diversity between West African and East African isolates of *Sweet potato chlorotic stunt virus*. *Tropical Agriculture* **79**, 26-30.

**Fuchs, M. & Gonsalves, D. (2007).** Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. *Annual Review of Phytopathology* **45,** 173-202.

**Gutiérrez, D. L., Fuentes, S. & Salazar, L. F. (2003).** Sweetpotato virus disease (SPVD): distribution, incidente, and effect on sweetpotato yield in Peru. *Plant Disease* **87,** 297-302.

**Ha, C., Revill, P., Harding, R. M., Vu, M. & Dale, J. L. (2008).** Identification and sequence analysis of Potyviruses infecting crops in Vietnam. *Archives of Virology* **153**, 45-60.

**Ishak, J. A., Kreuze, J. F., Johansson, A., Mukasa, S. B., Tairo, F., Abo El-Abbas. & Valkonen, J. P. T. (2003).** Some molecular characteristics of three viruses from sweet potato virus disease (SPVD) - affected sweet potato plants in Egypt. *Archives of Virology* **148,** 2449-2460.

**Kreuze, J. F., Karyeija, R. F., Gibson, R. W. & Valkonen, J. P. T. (2000).** Comparisons of coat protein gene sequences show that East African isolates of *Sweet potato feathery mottle virus* form a genetically distinct group. *Archives of Virology* **145,** 567-574.

**Kreuze, J.** (**2002).** *Molecular Studies on the Sweet Potato Virus Disease and its Two Causal Agents*. Uppsala: Swedish University of Agricultural Sciences (Doctoral thesis), pp. 24-33.

**Kreuze, J. F., Savenkov, E. I. & Valkonen, J. P. T. (2002).** Analysis of complete genomic sequence and subgenomics RNAs of *Sweet potato chlorotic stunt virus* reveals several new features of the genus Crinivirus. *Journal of Virology* **76**, 9260-9270.

**Mori, M., Usugi, T., Hayashi, T. & Nishiguchi, M. (1994).** Nucleotide sequence at the 3'- terminal region of *Sweet potato feathery mottle virus* (ordinary strain, SPFMV-O) RNA. *Bioscience, Biotechnology and Biochemistry* **58**, 965-967.

**Moyer, J. W., Cali, B. B., Kennedy, G. G. & Abu-Ghadir, M. F. (1980).** Identification of *Sweet potato feathery mottle virus* strains in North Carolina. *Plant Disease* **64**, 762-764.

**Mukasa, S. B., Rubaihayo, P. R. & Valkonen, J. P. T. (2003).** Incidence of viruses and viruslike diseases of sweet potato in Uganda. *Plant Disease* **87,** 329-335.

**Parrella, G., De Stradis, A. & Giorgini, M. (2006).** *Sweetpotato feathery mottle virus* is the causal agent of Sweetpotato virus disease (SPVD) in Italy. *Plant Pathology* **55,** 818.

**Rännäli, M., Czekaj, V., Jones, R. A. C., Fletcher, J. D., Davis, R. I., Mu, L., Dwyer, G. I., Coutts, B. A. & Valkonen, J. P. T. (2008).** Molecular genetic characterization of *Sweet potato virus G* (SPVG) isolates from areas of the Pacific Ocean and southern Africa. *Plant Disease* **92,** 1313-1320.

**Rännäli, M., Czekaj, V., Jones, R. A. C., Fletcher, J. D., Davis, R. I., Mu, L. & Valkonen, J. P. T. (2009).** Molecular genetic characterization of *Sweet potato feathery mottle virus* (SPFMV) isolates from Easter Island, French Polynesia, New Zealand, Southern Africa. *Plant Disease* **93,** 933-939.

**Ryu, K. H., Kim, S. J. & Park, W. M. (1998).** Nucleotide sequence analysis of the coat protein genes of two Korean isolates of *Sweet potato feathery mottle potyvirus*. *Archives of Virology* **143**, 557-562.

**Sakai, J., Mori, M., Morishita, T., Tanaka, M., Hanada, K., Usugi, T. & Nishiguchi, M. (1997).** Complete nucleotide sequence and genome organization of *Sweet potato feathery mottle virus* (S strain) genomic RNA: the large coding region of the P1 gene. *Archives of Virology* **142**, 1553-1562.

**Souto, E. R., Sim, J., Chen, J., Valverde, R. A. & Clark, C. A. (2003).** Properties of strains of *Sweet potato feathery mottle virus* and two newly recognized Potyviruses infecting sweetpotato in the United States. *Plant Disease* **87,** 1226-1232.

**Tairo, F., Mukasa, S. R., Jones, R. A. C. Kullaya, A., Rubaihayo, P. R. & Valkonen, J. P. T. (2005).** Unraveling the genetic diversity of the three main viruses involved in sweet potato virus disease (SPVD), and its practical implications. *Molecular Plant Pathology* **6,** 199-211.

**Tairo, F. (2006).** *Molecular Resolution of Genetic Variability of Major Sweet Potato Viruses and Improved Diagnosis of Potyviruses Co-infecting Sweet Potato*. Uppsala: Swedish University of Agricultural Sciences (Masters thesis), pp. 9-36.

**Tairo, F., Jones, R. A. C. & Valkonen, J. P. T. (2006).** Potyvirus complexes in sweetpotato: Occurrence in Australia, serological and molecular resolution, and

analysis of the *Sweet potato virus 2* (SPV2) component. *Plant Disease* **90,** 1120-1128.

**Tamura, K., Dudely, J., Nel, M. & Kumar, S. (2007).** MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24,** 1695-1599.

**Tugume, A. K., Mukasa, S. B., Kalkkinen, N. & Valkonen, J. P. T. (2010).** Recombinant and selection pressure in Ipomovirus *Sweet potato mild mottle virus* (*Potyviridae*) in wild species and cultivated in sweet potato in the centre of evolution in East Africa. *Journal of General Virology* **91,** 1092-1108.

**Untiveros, M., Fuentes, S. & Kreuze, J. (2008).** Molecular variability of *Sweet potato feathery mottle virus* and other Potyviruses infecting sweet potato in Peru. Archives of Virology **153,** 473-483.

**Valverde, R. A., Kokkinos, C. D. & Clark, C. A. (2004).** Whitefly transmission of sweet potato viruses. *Virus Research* **100,** 123-128.

**Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGreoch, D. J., Pringle, C. R. & Wickner, R. B. (2000).** Potyviridae. In *Virus Taxonomy : Classification and Nomenclature of Viruses,* pp. 706-723. San Deigo: Academic Press.

**Vetten, H. J., Hoyer, U., Maiss, E., Lesseman, D. E. & Jelkmann, W. (1996).** Serological detection and discrimination of geographical diverse isolates of sweet potato sunken vein closteovirus. *Phytopathology* **86,** S100.

**Winter, S., Purac, A., Leggett, F., Frison, E. A., Rossel, H. W. & Hamilton, R. I. (1992).** Partial characterization and molecular cloning of a closterovirus from sweet potato infected with the sweet potato virus disease complex from Nigeria. *Phytopathology* **82,** 869-875.

**Winter, S., Korbler, M. & Kollner, U. (1997).** Detection and differentiation of sweet potato closteroviruses by RT-PCR and single-stranded conformational polymorhisms. In *Diagnosis and Identification of Plant Pathogens, Proceedings of the 4$^{th}$ International Symposium of the European Foundation for Plant Pathology.* Dordoecht: Kluwer Academic Publishers, pp. 473-475.

**Yamasaki, S., Sakai, J., Kamisoyama, S., & Hanada, S. (2009).** Identification of Sweet potato virus G isolated from sweet potato in Japan. *Nippon Shokubutsu Byori Gakkaiho* **75,** 102-108.

# CHAPTER FOUR

# GENETIC ENGINEERING OF TRANSGENIC SWEET POTATO (*Ipomoea batatas* L.) WITH BROAD VIRUS RESISTANCE

## 4.1 ABSTRACT

Multiple virus infections of Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato virus G (SPVG) and Sweet potato mild mottle virus (SPMMV) cause a devastating synergistic disease complex of sweet potato (*Ipomoea batatas* Lam.) in KwaZulu-Natal (KZN). In order to address the problem of the multiplicity and synergism of sweet potato viruses in KZN, this study aimed to develop transgenic sweet potato cv. Blesbok with broad virus resistance. An efficient and reproducible plant regeneration protocol for sweet potato (*Ipomoea batatas* Lam.) cultivar Blesbok was also developed in this study. The effect of different hormone combinations and type of explants on shoot regeneration was evaluated in order to optimize the regeneration protocol. Coat protein (CP) gene segments of SPFMV, SPCSV, SPVG and SPMMV were fused to a silencer DNA, the middle half of the nucleocapsid (N) gene of Tomato spotted wilt virus (TSWV) and used as a chimeric transgene in a sense orientation to induce gene silencing in the transgenic sweet potato. Transformation of apical tips of sweet potato cv. Blesbok was achieved by using *Agrobacterium tumefaciens* strain LBA4404 harboring a modified binary vector pGA482G carrying the plant expressible neomycin phosphotransferase ll gene (*npt*ll), the bacterial gentamycin-(3)-N-acetyl-transferase gene and the expression cassette. A total of 24 putative transgenic plants (8%) were produced from the transformed apical tips via *de novo* organogenesis and regeneration into plants under 50mg/L kanamycin and 200 mg/L carbenicillin selection. Polymerase chain reaction (PCR) and Southern blot analyses showed that six of the 24 putative transgenic plants were transgenic with two insertion loci and that all plants were derived from the same transgenic event. The six transgenic sweet potato plants were challenged by graft inoculation with SPFMV, SPCSV, SPVG and SPMMV- infected *Ipomoea setosa* Ker. Although virus presence was detected using Nitrocellulose enzyme-linked immunosorbent assay (NCM-ELISA),

all transgenic plants displayed delayed and milder symptoms, of chlorosis and mottle of lower leaves when compared to the untransformed control plants. These results warrant further investigation under field conditions.

## 4.2 INTRODUCTION

Sweet potato (*Ipomoea batatas* Lam.) is an important food staple that provides food security among the poor and rural households in South Africa (SA). The crop is favoured by resource-poor farmers due to its good performance under adverse farming conditions, peak productivity in small farming areas and high carbohydrate and vitamin content (Naylor *et al*., 2004). However, the common practice of cutting and propagating vines in newly planted fields has resulted in the rampant spread and build-up of virus disease which has subsequently led to a discernable decline in tuber yield and/or quality over time (Clark *et al*., 2002; Domola *et al*., 2008). As a result, the incidence of viral disease in major sweetpotato growing provinces in SA has hindered the successful cultivation and production of this important crop. Moreover, the problem is exacerbated by the frequent occurrence of multiple viruses infecting sweet potato in SA (Domola *et al*., 2008).

Infection of sweet potato by multiple viruses is a common occurrence and often results in a viral synergism which appears as a more severe disease than the sum effect of infection with each virus alone (Mukasa *et al*., 2006; Untiveros *et al*., 2007). The most devastating viral synergistic disease affecting sweet potatoes worldwide is sweet potato virus disease (SPVD) (Kokkinos *et al*., 2006; Miano *et al*., 2008) and is caused by the interaction between Sweet potato feathery mottle virus (SPFMV) and Sweet potato chlorotic stunt virus (SPCSV). The occurrence of SPVD and other viral co-infections has been reported in almost every sweet potato growing area. The survey for sweet potato-infecting viruses presented in this study demonstrated that Sweet potato virus G (SPVG) and Sweet potato mild mottle virus (SPMMV) are also able to form synergistic associations with SPCSV and SPFMV to form a new SPVD complex that is commonly detected in sweet potato grown in KwaZulu-Natal (KZN).

The frequent occurrence of mixed synergistic viral complexes has complicated efforts to control viral diseases in sweet potato. Currently, no single management tool is available that provides adequate control against the natural viral complexes that infect sweet potato. The use of healthy planting material, phytosanitation and cultural measures have been used with minimal success due to difficulties encountered when trying to integrate these strategies into the subsistence production systems practiced by resource-poor farmers (Gibson *et al*., 2004; Nyaboga *et al*., 2008). Little success has also been reported in the breeding for virus resistance in sweet potato cultivars (Gibson *et al*., 1998; Gibson *et al*., 2004; Karuri *et al*., 2009). Factors such as virus variation, time and expenditure required has mired conventional breeding efforts (Lomonosoff, 1995). Moreover, genetic sources of resistance are scarce and the incorporation of such resistance from the wild diploid species into polyploid sweet potato is a complicated task (Kreuze, 2002).

Alternate strategies for obtaining virus resistance through biotechnology-based strategies have the potential to serve as valuable interventions in sweet potato virus control (Kreuze, 2002). Most of theses strategies are based on the concept of 'pathogen-derived resistance' (PDR), which proposes that pathogen resistance genes may be developed from the pathogen's own genetic material (Sanford & Johnston, 1985). PDR for plant viruses can be mediated by the expression of RNA (RNA-mediated PDR) (Shepherd *et al*., 2009; Collinge *et al*., 2010). The post-transcriptional gene silencing (PTGS) process, also known as RNA interference (RNAi) or RNA silencing, is the mechanism of RNA-mediated PDR (Tenllado *et al*., 2004; Lindbo & Dougherty, 2005; Fuchs & Gonsalves, 2007). PTGS is a specific RNA degradation mechanism of any organism that breaks down abberant, excess or foreign RNA intracellulary in a homology-dependent manner (Dasgupta *et al*., 2003). Induction of PTGS using a viral transgene enables a specific degradation of the genome of the invading virus and those that have high sequence homology with the virus transgene, resulting in a resistance phenotype (Fuchs & Gonsalves, 2007).

Current genetic engineering efforts have been used to develop transgenic sweet potato with virus resistance using the coat protein gene of SPFMV and/or SPCSV (Newell *et al*., 1995; Gama *et al*., 1996; Otani *et al*., 1998; Okada *et al*., 2002; Kreuze *et al*., 2008). However, given the multiplicity of viruses occurring under field conditions, this approach has had limited success. In addition, sweet potato is considered highly recalcitrant to regeneration and transformation and this has limited its application in genetic engineering technologies (González *et al*., 2008). Therefore, to address the problem of the multiplicity and synergism of sweet potato viruses, future endeavors should develop an efficient and practicable regeneration method of sweet potato that could easily be incorporated into a plant transformation system to produce transgenic sweet potato with broad virus resistance.

The mechanism of RNA silencing has been successfully applied in the production of transgenic crops with multiple virus resistance. A previous study by Jan *et al.* (2000), indicated that a single chimeric gene consisting of linked viral segments of Tomato spotted wilt virus (TSWV) and Turnip mosaic virus (TuMV) can confer multiple resistance in transgenic tobacco via PTGS. This strategy demonstrated that multiple virus resistance in transgenic plants can be induced by using viral DNA as a 'silencer' and linking it to DNA segments derived from other viruses. Recently, Lin *et al*. (2011) demonstrated that multiple virus resistance in transgenic plants using this approach could also be achieved by fusing the partial C2 gene of the Tomato leaf curl Taiwan virus (ToLTWCV) to a silencer DNA, the middle half of the nucleocapsid (N) gene of TSWV.

Following the successful implementation of this strategy in the development of transgenic plants with multiple resistance, this study aimed to develop transgenic sweet potato cv. Blesbok with broad virus resistance. To achieve this, an efficient and reproducible method for the regeneration of sweet potato cv. Blesbok is reported. The effect of different hormone combinations and explant types on shoot regeneration efficiency was studied in order to determine an efficient protocol for optimal plant regeneration. Coat protein (CP) segments of the four most prevalent viruses infecting

sweet potato in KZN were fused to a silencer DNA, the middle half of the nucleocapsid (N) gene of TSWV and introduced into sweet potato via *Agrobacterium*-mediated transformation. Transgenic sweet potato plants showing varying levels of resistance to SPFMV and SPVG were obtained by PTGS.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Plant materials

Sweet potato cultivar 'Blesbok', a popular variety in South Africa (SA), was obtained from Dr P. Shanahan[2] and used in this study. Vine cuttings of virus-free plants were potted in sterilized potting medium and maintained at 25$^{o}$C in an insect-proof greenhouse in the Controlled Environment Research Unit (CERU) at the University of KwaZulu-Natal (UKZN-PMB). These plants were fertilized weekly with 3:1:3 (nitrogen: phosphate: potassium) and used as stock plants for the establishment of virus-free *in vitro* plantlets. Stock plants were maintained in the greenhouse for up to one year and were kept in highly vegetative state by constant cutting back of stems.

### 4.3.2 Establishment of *in vitro* plantlets

To establish *in vitro* cultures, shoot tips 5cm in length were excised from greenhouse-grown stock plants and rinsed in sterile distilled water. The shoot tips were cut down to 3cm in length and immersed in 70% (v/v) ethanol for 2min. Shoots were surface-sterilized in dilute sodium hypochlorite (5%) solution for 20min and then rinsed three times in sterile distilled water. They were cultured in 9cm Petri dishes containing 25ml of shoot induction medium (SIM) consisting of a basic medium (BM) supplemented with 1mg/L 6-benzylaminopurine (BAP, Sigma-Aldrich, UK) and 0.01mg/L naphthalene acetic acid (NAA, Sigma-Aldrich, UK). The BM consisted of Murashige & Skoog (1962) inorganic medium (MS, Sigma-Aldrich, UK) supplemented with 30% (w/v) sucrose,

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myoinositol (100mg/L), thiamine (0.1mg/L), pyroxidine (0.5mg/L), nicotinitic acid (0.5mg/L), glycine (2mg/L) and 0.6% (w/v) agar. The pH was adjusted to 5.8 with NaOH prior to autoclaving at 121$^{o}$C for 20min. The cultures were grown in a growth room at 25 +/- 2$^{o}$C under a 16h photoperiod with a light intensity of 54µEs$^{-1}$m$^{-2}$ provided by white fluorescent tubes. After 3-4 weeks, excess callus was removed and the elongating shoot tips were cultured in glass culture vessels (Sigma-Aldrich, UK) containing 50ml of root induction medium (RIM) which consisted of BM supplemented with 1mg/L NAA for root development. After 2 weeks, plantlets were singly cultured in Magenta GA7 vessels (Sigma-Aldrich, UK), each containing 100ml of hormone-free BM. These *in vitro* plantlets were maintained in the same environmental conditions as the starting dishes and were used to provide axenic explant material for propagation and ensuing studies.

### 4.3.3 Optimization of regeneration protocol for sweet potato cv. Blesbok

For rapid and efficient shoot regeneration of sweet potato cv Blesbok, an optimum hormone combination and explant type was determined. In this experiment, four different explant types (leaf discs, stem portions, axillary buds and apical tips) were subjected to 36 differing hormone concentrations of auxin (NAA) and cytokinin (BAP).

Leaf (lamina with petiole, 1-1.5cm), stem (3cm), axillary buds (stem with bud, 2cm) and apical shoots (2cm) were excised from the apical portions of five week old *in vitro* stock plants and surface sterilized as previously mentioned. Leaf explants were cut into 1cm$^{2}$ discs and stem portions were cut transversely into 1cm sections. Shoot tips from apical shoots and axillary buds were trimmed to 3-5mm long with four to five primordia. Stem sections, leaf discs, apical shoots and axillary buds were cultured in 9cm Petri dishes containing 25ml BM supplemented with 36 hormone combinations of NAA (0, 0.01, 0.1, 0.2, 0.5, and 1mg/L) and BAP (0, 0.01, 0.1, 0.2, 0.5, and 1mg/L) and grown in the growth room under the conditions described above. After 3-4 weeks of culture, emerging shoots were counted and the percentage shoot generation was computed as the proportion of explants showing shoots, multiplied by 100. Surviving shoots were rooted in root induction medium (RIM) supplemented with 1mg/L NAA and fully

regenerated in hormone-free BM as previously described in section 4.3.2. The regenerated plantlets with fully developed roots were transferred to 8cm plastic pots filled with sterilized seedling mix (Growmor, Cato Ridge), and placed in a growth chamber at 25 +/- 2$^{o}$C and 80% relative humidity (RH) under a 16h photoperiod. After 2 weeks, plants were transferred to bigger pots (20cm) and placed in the greenhouse for further development of tuberous roots and morphological observations, including plant stature, growth habit, leaf size, shape and colour, and rooting capacity.

### 4.3.4 Molecular cloning

#### *4.3.4.1 Construction of segmented transgene and integration into plant expression vector*

The coat protein (CP)- encoding regions of Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato virus G (SPVG) and Sweet potato mild mottle virus (SPMMV) were chosen as target genes in the design of a segmented gene construct. The CP represents a conserved region in viruses and thus increased the probability of engineering resistance to different virus strains. To create the construct, individual fragments of the CP genes of SPFMV, SPCSV, SPVG and SPMMV isolates were amplified by RT-PCR with virus-specific primers containing restriction sites for *Xba*I, *Xho*I, *Sal*I, *Sma*I, and *Apa*I. Total RNA extraction and RT-PCR procedures, parameters and product analysis were performed as previously described in Chapter 3, section 3.3.2, with the annealing temperature for all primer combinations amended to 60$^{o}$C. Each primer pair was flanked by unique restriction sites and the SPFMV-F primer, used for the amplification of the first segment in the construct, was designed to contain a stop codon (TAA) to ensure the production of untranslatable transgene transcripts. Details of the primer sequences and combinations used in the amplification is given in Table 4.1. PCR products corresponding to the CP segments of SPFMV, SPCSV, SPVG and SPMMV were purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen, USA). The individual segments were then digested with their flanking restriction enzymes (Table 4.1) and sequentially ligated in the sense

orientation to the plant expression silencing vector pEPJ86-m/2N (Jan, 1998; kindly provided by Dennis Gonsalves[3]) that had been digested with the same restriction enzymes.

The pEPJ86-m/2N vector is a derivative of the plant expression vector pEPJ86 and has an expression cassette that contains the 35S enhancer, promoter and terminator sequences of Cauliflower mosaic virus (CaMV), the 5' untranslated region of the CP gene of Alfalfa mosaic virus (AIMV) and the middle half of the nucleocapsid protein (NP) gene of Tomato spotted wilt virus (TSWV) which will act as the 'silencer' DNA. The entire expression cassette is flanked by recognition sites specific to the restriction enzymes *Hind*lll and *Kpn*l (Jan, 1998). All ligation and digestion reactions were purified using the MiniElute Reaction Cleanup Kit (Qiagen, USA). After each sequential ligation of the individual segments to pEPJ86-m/2N, ligation reactions were transformed into *E. cloni*® 10GF' chemically competent cells according to manufacturers' instructions (Lucigen, USA). Recombinant clones were selected on YT plates containing 200mg/L ampicillin and 40mg/ml X-Gal (Promega, USA).

Ten white colonies for each SPFMV, SPCSV, SPVG and SPMMV reaction were selected for plasmid isolation and confirmation of transgene integration using PCR. Plasmid DNA of the independently transformed bacterial cells was purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). PCR was performed to verify the integration of each segment in pEPJ86-m/2N using the specific primers outlined in Table 4.1. After confirming the presence of the entire segmented construct in the plant expression cassette of pEPJ86-m/2N, the resulting plasmid was designated pEPJ86-m/2N-SPVD.

[3] Dennis Gonsalves, US Pacific Basin Agricultural Research Center, US Department of Agriculture, 64 Nowelo Street, Hilo, HI 96720, USA.

**Table 4.1** Primers used for the engineering of a segmented gene construct containing the partial coat protein (CP) sequences of Sweet potato feathery mottle virus (SPFMV), Sweet potato virus G (SPVG), Sweet potato chlorotic stunt virus (SPCSV), and Sweet potato mild mottle virus (SPMMV)

| Virus | Primer name | Sequence | Expected size (bp) | Restriction enzymes |
|---|---|---|---|---|
| SPFMV | SPFMV-F | 5' GACGtctaga**TAA**CACTTCAGTGACGTTGCTGA 3' | | |
| | SPFMV-R | 5' TATTctcgagGCACACCCCTCATTCCTAAG 3' | 319 | *Xba*I-*Xho*I |
| SPCSV | SPCSV-F | 5' TGAGctcgagAACGATAATCTACGAAGACGTTCC 3' | | |
| | SPCSV-R | 5' AGATgtcgacGCGTAGAAACCTGCAAAAATCT 3' | 266 | *Xho*I-*Sal*I |
| SPVG | SPVG-F | 5' CAATgtcgacAGGATCAAGGCATACATGCC 3' | | |
| | SPVG-R | 5' ACGTcccgggTTCCGTCCAAACCAAACAA 3' | 213 | *Sal*I-*Sma*I |
| SPMMV | SPMMV-F | 5' TACTcccgggGCCAGCATGGATTGTCAAT 3' | | |
| | SPMMV-R | 5' CGGAgggcccTCACCTGCCTCAAAGTTGG 3' | 168 | *Sma*I-*Apa*I |

The translational stop codon is in bold letters.

Recognition sites for restriction enzymes are underlined.

Letters in upper case represent CP sequence.

#### *4.3.4.2 Construction of plant transformation vector and transformation into Agrobacterium strain LBA4404*

The expression cassette was digested out of pEPJ86-m/2N-SPVD with *Hind*III and *Kpn*I and ligated into the *Hind*III and *Kpn*I sites of the binary plant transformation vector pGA482G (Jan, 1998; also kindly provided by Dennis Gonsalves) that had been digested with the same enzymes. The resulting plasmid was designated pGA482G-SPVD (Fig. 4.1). The pGA482G vector is a derivative of the plant transformation vector pGA482 described by An *et al*. (1988). The ordinary binary vector pGA482 contains the left and right T-DNA borders of pTi37 which flank the plant expressible neomycin phosphotransferase II gene (*npt*II), restriction enzyme polylinker, and the bacteriophage lamda cos site. To improve the use of the vector, the bacterial gentamycin-(3)-N-acetyl-transferase gene was cloned into the *Sal*I site located outside the T-DNA region, generating pGA482G (Jan, 1998; Pang *et al*., 2000).

Ligation reactions were transformed into *E. cloni*® 10GF' electrocompetent cells according to manufacturers' instructions (Lucigen, USA) using the MicroPulser™ Electroporation Apparatus (BIO-RAD, RSA). Recombinant clones were selected on YT plates containing 50mg/L gentamycin and 40mg/ml X-Gal (Promega, USA). Ten white colonies were selected for plasmid isolation and the recombinant binary vector pGA482G-SPVD was verified using PCR. Plasmid DNA of the independently transformed bacterial cells was purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). PCR was performed to verify the integration of each segment in pGA482G-SPVD using the specific primers outlined in Table 4.1. Plasmid DNA of the pGA482G-SPVD was electroporated into electrocompetent cells of the *Agrobacterium tumefaciens* strain LBA4404 (kindly provided by Stephanie Stewart[4]) using the MicroPulser™ Electroporation Apparatus (BIO-RAD, RSA). Recombinant clones were selected on Luria Broth (LB) plates containing 50mg/L gentamycin and 50mg/L rifampicin.

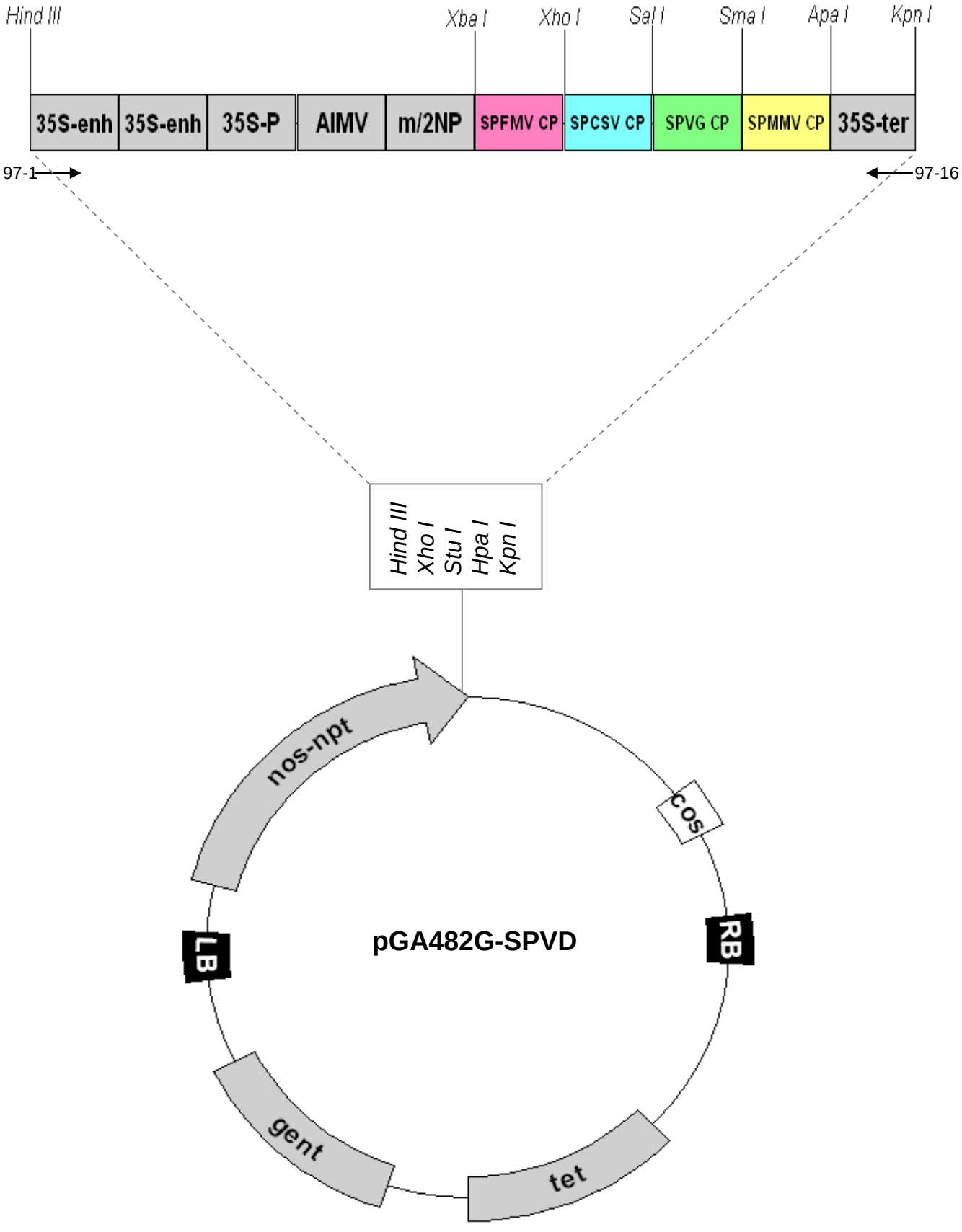
[4] Stephanie Stewart, Senior Technician, Department of Botany, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Private Bag x01, Scottsville 3209, SA.

Ten white colonies were selected for plasmid DNA isolation and the presence of the recombinant binary vector pGA482G-SPVD was verified using PCR. Plasmid DNA of the independently transformed bacterial cells was purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). PCR was performed to verify the integration of each segment in pGA482G-SPVD DNA using the specific primers outlined in Table 4.1 and the primers 97-1 (5' TGCGCAAGCTTGTTAATTCAATTGAGACTT TTCAAC 3') and 97-16 (5' AGAGATAGATTTGTAAGAG 3'). The former primer is identical to the enhancer of the 35S CaMV promoter and the latter is complementary to 5' end of the 35S terminator (Fig. 4.1; Jan, 1998). The engineered strains of *Agrobacterium* were maintained and used for plant transformation.

### 4.3.5 Plant transformation and regeneration

A single bacterial colony of *A. tumefaciens* strain LBA4404 harboring the binary vector pGA482G-SPVD was used to inoculate 5ml of LB broth that contained 50mg/L rifampicin and 50 mg/L gentamycin. The culture was grown in an incubator with 220rpm shaking at 28$^{o}$C until an optical density of 0.8-1 was reached at 600nm. The culture was then transferred to 25ml LB that contained 50mg/L rifampicin, 50 mg/L gentamycin and 200μM acetosyringone and grown in an incubator with 220rpm shaking at 28$^{o}$C overnight. Bacterial cells of overnight cultures were spun at 8000rpm for 20min at 25$^{o}$C and resuspended in 25ml of liquid Murashige & Skoog (1962) medium (MS, Sigma-Aldrich, UK) containing 200μM acetosyringone.

**Figure 4.1** Schematic representation of the recombinant binary vector pGA482G-SPVD. The locations of the important genetic elements within the integrated expression cassette are indicated: the segmented gene construct consists of partial coat protein (CP) genes of Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato virus G (SPVG) and Sweet potato mild mottle virus (SPMMV). The entire segmented construct is driven by a double-enhanced (35S-enh) CaMV 35S promoter (35S-P), the 5' untranslated region of the CP gene of Alfalfa mosaic virus (AIMV), the middle half of the nucleocapsid protein (m/2NP) gene of Tomato spotted wilt virus (TSWV), and 35S terminator (35S-ter). Genetic elements within the binary vector are also indicated: LB, left border; RB, right border; nos-npt, plant expressible neomycin phosphotransferase ll gene (*npt*ll); cos, bacteriophage lamda cos site; tet, bacterial tetracycline resistance gene; and gent, bacterial gentamycin-(3)-N-acetyl-transferase gene. The major part of the expression cassette can be amplified using primers 97-1 and 97-16 (black arrows).


Hind III
Xba I
Xho I
Sal I
Sma I
Apa I
Kpn I
35S-enh
35S-enh
35S-P
AIMV
m/2NP
SPFMV CP
SPCSV CP
SPVG CP
SPMMV CP
35S-ter
97-1
97-16
Hind III
Xho I
Stu I
Hpa I
Kpn I
nos-npt
cos
LB
RB
pGA482G-SPVD
gent
tet

Apical shoots (3-5mm long) with four to five primordia were excised from the apical portions of five week old *in vitro* stock plants and surface sterilized as previously mentioned in Chapter 4, section 4.3.2 and used as explants in transformation experiments. Explants were submerged into the MS liquid suspension containing the *A. tumefaciens* strain LBA4404 harboring the binary vector pGA482G-SPVD and kept in the dark at 28$^{o}$C without agitation for 10min. After this co-culture step, explants were blotted onto sterile filter paper and cultured in 9cm Petri dishes containing 25ml of shoot induction medium (SIM) consisting of a basic medium (BM) supplemented with 1mg/L 6-benzylaminopurine (BAP, Sigma-Aldrich, UK), 0.01mg/L naphthalene acetic acid (NAA, Sigma-Aldrich, UK), 50mg/L kanamycin and 200mg/L carbenicillin. The pH was adjusted to 5.8 with NaOH prior to autoclaving at 121$^{o}$C for 20min. The cultures were grown in a growth room at 25 +/- 2$^{o}$C under a 16h photoperiod with a light intensity of 54µEs$^{-1}$m$^{-2}$ provided by white fluorescent tubes. Cultures were transferred to new medium every 3-4 weeks. Regeneration of putative transgenic plants was achieved following organogenesis. Excess callus from regenerating shoots was removed and elongating shoot tips were cultured in glass culture vessels (Sigma-Aldrich, UK) containing 50ml of root induction medium (RIM) supplemented with 1mg/L NAA and 25mg/L kanamycin for root development and plantlet maturation. After 2-4 weeks, plantlets were singly cultured in Magenta GA7 vessels (Sigma-Aldrich, UK), each containing 100ml of hormone and antibiotic-free BM, for elongation of plantlets. The regenerated plantlets with fully developed roots were transferred to 8cm plastic pots filled with sterilized seedling mix (Growmor, Cato Ridge), and placed in a growth chamber at 25 +/- 2$^{o}$C and 80% relative humidity (RH) under a 16h photoperiod. After 2 weeks, plants were transferred to bigger pots (20cm) and placed in the greenhouse for characterization.

### 4.3.6 Molecular characterization of putative transgenic plants

Total genomic DNA was isolated from fresh leaf tissue from putative transgenic and untransformed (control) plants using the ZR Plant/Seed DNA Miniprep$^{TM}$ kit (Zymo Research, SA). The DNA was then used for PCR and Southern blot analysis to confirm transgenic events.

#### *4.3.6.1 Detection of transgenic events by PCR analysis*

PCR analysis was used for rapid initial detection of putative transgenic plants. Primers (Table 4.1) complementary to the individual transgene segments of SPFMV, SPCSV, SPMMV and SPVG were used to detect the integrity of the transgene construct in plants. In addition, the primers NPTll-F (5' GATGCGCTGCGAATCGGGAGCG 3') and NPTII-R (5' GGAGAGGCTATTCGGCTATGAC 3') described by Yi *et al*. (2006) were used to detect a 715bp fragment of the integrated *npt*ll gene. PCR procedures and parameters were performed as previously described with the annealing temperature for the NPTll-F and R primer combination amended to 55$^{o}$C.

#### *4.3.6.2 Detection of transgene integration by Southern blot analysis*

For Southern blot analysis, total plant DNA (20µg) from putative transgenic plants was digested overnight at 37$^{o}$C with restriction endonuclease *Hind*III. PGA482G-SPVD DNA digested with *Hind*III was used as a positive control. In addition, undigested genomic DNA from the T7 transgenic plant was also included as a positive control to prove stable integration. A negative control consisted of untransformed non-transgenic genomic plant DNA. Similarly, a second negative control consisting of digested untransformed genomic DNA spiked with *Hind*III-digested PGA482G DNA was used to prove the authenicity of the transgene. DNA fragments were separated by electrophoresis at 40V for 3h in a 1.2% agarose gel and blotted onto positively charged nylon membranes (Roche, Germany) using the VacuGene XL Vacuum blotting System (GE Healthcare Bio-Sciences, Sweden). DNA fragments were fixed to the membrane by UV cross-

linking for 2-3min (Stratalinker, Stratagene, CA). The hybridization probe corresponding to the SPFMV segment was used for the detection of the transgene and generated and labeled with alkali-labile digoxigenin (DIG-dUTP) in PCR using the PCR DIG Probe Synthesis kit (Roche, Germany). The probe was developed by PCR amplification of the SPFMV segment with the primer pair outlined in Table 4.1 and the pEPJ86-m/2N-SPVD as the template. The labeling efficiency of the PCR-labeled probe was determined by electrophoresis in a 1% (w/v) agarose gel. Hybridization of the probe to membrane and subsequent chemiluminescence detection by enzyme immunoassay was performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) according to manufacturer's instructions. Membranes were exposed to Lumi-Film Chemiluminescent Detection Film (Roche, Germany) and film was developed using standard procedures.

### 4.3.7 Greenhouse evaluation of transgenic plants for virus resistance

Transgenic plants were assayed for virus resistance according to the method described by Okada *et al*. (2002). Vine samples infected with SPFMV, SPCSV, SPVG and SPMMV were graft-inoculated onto three week-old indicator plants, *Ipomoea setosa* Ker. using the crown-cleft grafting described in Chapter 2, section 2.3.2. Two to three weeks post inoculation, typical virus induced symptoms were observed on growing leaves of indicator plants. Five apical cuttings from each of the six transgenic plants and two control plants were rooted in sterilized potting medium. All plants were kept at 25$^{o}$C in an insect-proof growth room in the Controlled Environment Research Unit (CERU) at the University of KwaZulu-Natal (UKZN-PMB), under 80% relative humidity, and fertilized weekly with 3:1:3 (nitrogen: phosphate: potassium). Approximately four weeks after planting, each clone was graft-inoculated with vine cuttings from the virus-infected *I. setosa*.

Plants were maintained in the growth room for a period of twelve weeks to allow for infection of the viruses to transverse through the graft and progress into the transgenic sweet potato plant (Usugi *et al*., 1990). Twelve weeks post inoculation, both the

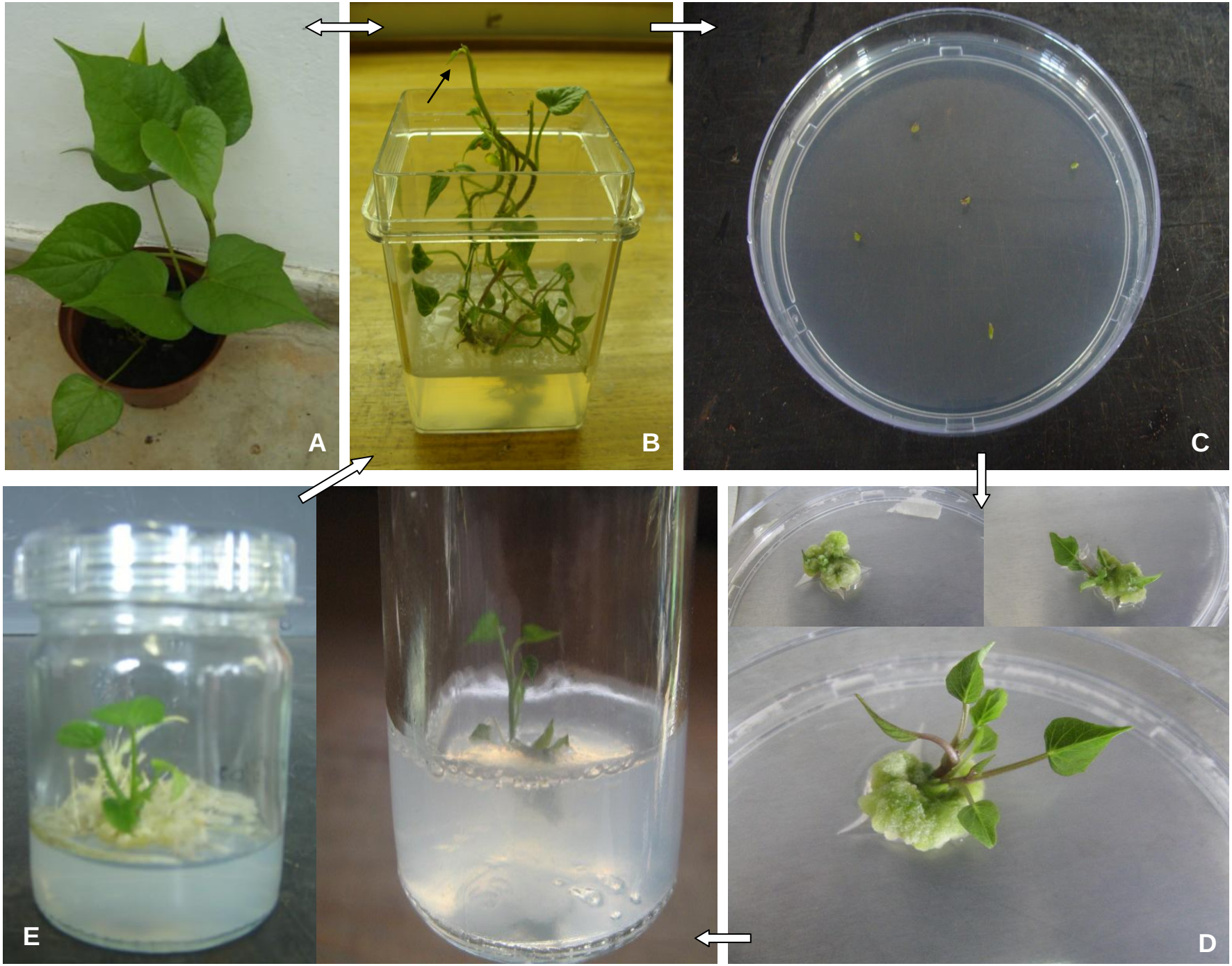
inoculated transgenic plants and corresponding indicator plants were monitored for symptom development and assayed for SPFMV, SPCSV, SPVG and SPMMV by nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) as previously described in Chapter 2, section 2.3.4.

## 4.4 RESULTS

### 4.4.1 Optimization of regeneration protocol for sweet potato cv. Blesbok

The highest percentage shoot regeneration was obtained from apical shoot explants generated on 0.01mg/L NAA + 1mg/L BAP. Greenhouse-grown stock plants (Fig. 4.2A) provided the apical shoot explants needed for the establishment of *in vitro* stock plantlets (Fig. 4.2B). Apical shoots derived from the *in vitro* stock plantlets were cultured on SIM (Fig. 4.2C). Responsive apical shoots exhibited a swelling at the base that developed into callus 2-3 weeks of incubation. At 3-4 weeks of culture, *de novo* organogenesis regeneration of shoots from callus was observed (Fig. 4.2D). Organogenic shoots were able to regenerate roots on RIM after a further 3-4 weeks of culture (Fig. 4.2E). Plantlets were fully regenerated on hormone-free BM (Fig. 4.2B) and then in sterilized soil (Fig. 4.2A). Once regenerated plants were transferred to soil, they showed a 100% survival rate. The plants grew normally (Fig. 4.2A) and rapidly in 20cm pots and produced storage roots after 3 months of cultivation in the greenhouse. Plants derived from tissue culture were phenotypically indistinguishable from donor plants and showed no obvious variations in leaf shape, plant stature and root formation.

**Figure 4.2** Schematic illustration of plant regeneration system for sweet potato (*Ipomoea batatas* Lam.) cv. Blesbok. (A) Greenhouse-grown stock plant in pot provides starting material for the establishment of *in vitro* stock cultures. (B) *in vitro* stock plants provided apical shoots (black arrow) for culture. (C) Apical shoots explants cultured on shoot induction medium (SIM) gave rise to the (D) *de novo* organogenic regeneration of shoots from callus after 2 (top left), 3 (top right) and 4 (bottom) weeks of culture. (E) Organogenic shoots (right) were able to regenerate roots (left) on root induction medium (RIM) after a further 3-4 weeks of culture. Plantlets were fully regenerated on hormone-free basal medium (BM) and then in sterilized soil.


A
B
C
D
E

### 4.4.2 Molecular cloning

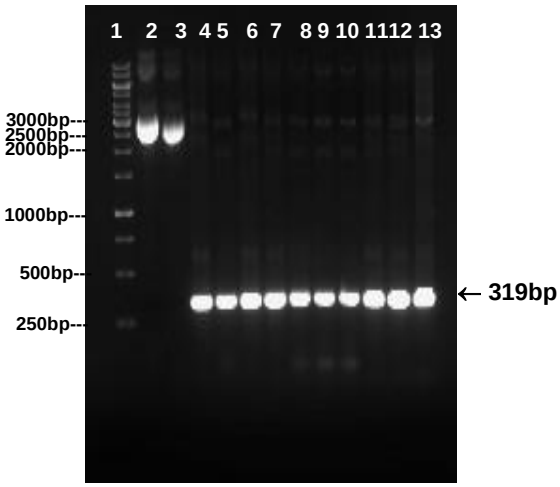
PCR analysis of plasmid DNA extracts from transformed *E.cloni*® and *A. tumefaciens* strain LBA4404 confirmed the integration of CP segments of SPFMV, SPCSV, SPVG and SPMMV in plant expression and transformation vectors. The analysis confirmed the successful integration of segmented gene constructs in pEPJ86-m/2N-SPVD (Fig. 4.3) and pGA482G-SPVD (Fig. 4.4). The presence of recombinant pGA482G-SPVD DNA in transformed *A. tumefaciens* strain LBA4404 was also confirmed using primers specific to each transgene segment (Fig. 4.5) and primers specific to the expression cassette (Fig. 4.6).

### 4.4.3 Plant transformation and regeneration

The sweet potato regeneration system described in section 4.4.1 was used in the transformation procedure. Overall, the regeneration of transformed plants was significantly slower due to the presence of selective antibiotics. Callus formation started after three weeks on those explants that survived repeated subculture and residual contamination by *A. tumefaciens*. Organogenic shoots formed on SIM after one month (Fig. 4.7A) and were allowed to elongate for a further 3 weeks (Fig. 4.7B) before being transferred to RIM. The first regenerative roots formed, appeared after 1.5 months on RIM and were transferred to BM without antibiotics to allow development into mature plantlets (Fig. 4.7C). The first fully developed plants (Fig. 4.7D) were obtained four months from the beginning of the transformation procedure.

In this study, performed with ~300 explants, 24 independently transformed plants were regenerated in the presence of kanamycin. All 24 plants (~8%) rooted well, indicating their transgenic nature due to the expression of the *npt*II gene. This resistance to kanamycin indicated that regenerated plants were putative transgenic transformants. All putative transgenic plants appeared normal in morphology and development.

**Figure 4.3** Polymerase chain reaction (PCR) analysis used to confirm the integration of (A) Sweet potato feathery mottle virus (SPFMV), (B) Sweet potato chlorotic stunt virus (SPCSV), (C) Sweet potato virus G (SPVG) and (D) Sweet potato mild mottle virus (SPMMV) coat protein (CP) gene segments in pEPJ86-m/2N-SPVD. Lane 1 contains the O' GeneRuler$^{TM}$ 1kb DNA ladder. (A) SPFMV: Lane 2 contains pEPJ86-m/2N + SPFMV CP DNA and Lane 3 contains pEPJ86-m/2N DNA only. Lanes 4-13 contains PCR products of plasmid DNA isolated from 10 recombinant clones. (B) SPCSV: Lane 2 contains pEPJ86-m/2N DNA only, Lane 3 contains pEPJ86-m/2N + SPFMV CP DNA and Lane 4 contains pEPJ86-m/2N + SPFMV CP + SPCSV CP DNA. Lanes 5-14 contains PCR products of plasmid DNA isolated from 10 recombinant clones. (C) SPVG: Lane 2 contains pEPJ86-m/2N + SPFMV CP DNA, Lane 3 contains pEPJ86-m/2N + SPFMV CP + SPCSV CP DNA and Lane 4 contains pEPJ86-m/2N + SPFMV CP + SPCSV CP + SPVG CP DNA. Lane 5 contains the O' GeneRuler$^{TM}$ 100bp DNA ladder. Lanes 6-15 contains PCR products of plasmid DNA isolated from 10 recombinant clones. (D) SPMMV: Lane 2 contains pEPJ86-m/2N DNA only, Lane 3 is blank, Lane 4 contains pEPJ86-m/2N + SPFMV CP DNA, Lane 5 contains pEPJ86-m/2N + SPFMV CP + SPCSV CP DNA, Lane 6 contains pEPJ86-m/2N + SPFMV CP + SPCSV CP + SPVG CP DNA and Lane 7 contains pEPJ86-m/2N + SPFMV CP + SPCSV CP + SPVG CP + SPMMV DNA. Lane 8 contains the O' GeneRuler$^{TM}$ 100bp DNA ladder. Lanes 9-18 contains PCR products of plasmid DNA isolated from 10 recombinant clones.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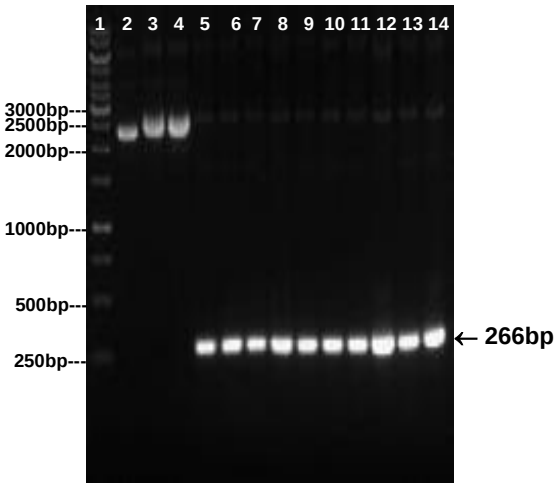
2500bp---
2000bp---
1000bp---
500bp---
250bp---
← 319bp

(A)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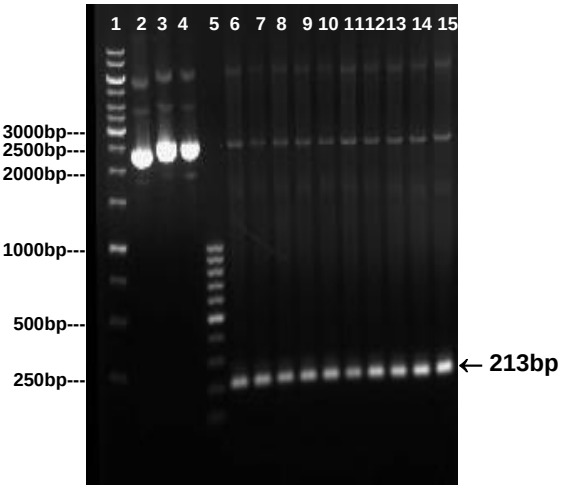
2500bp---
2000bp---
1000bp---
500bp---
250bp---
← 266bp

(B)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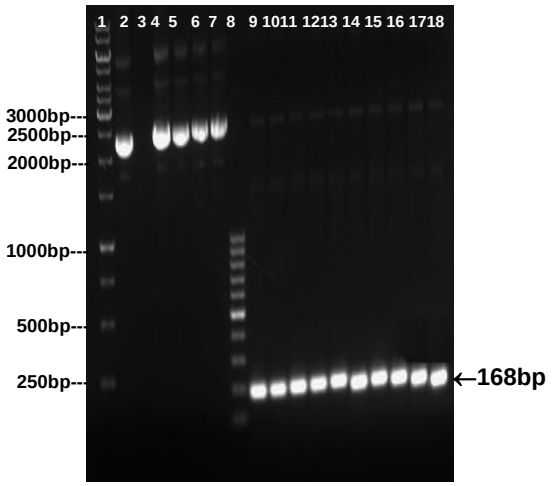
2000bp---
1000bp---
500bp---
250bp---
← 213bp

(C)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
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500bp---
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←168bp

(D)

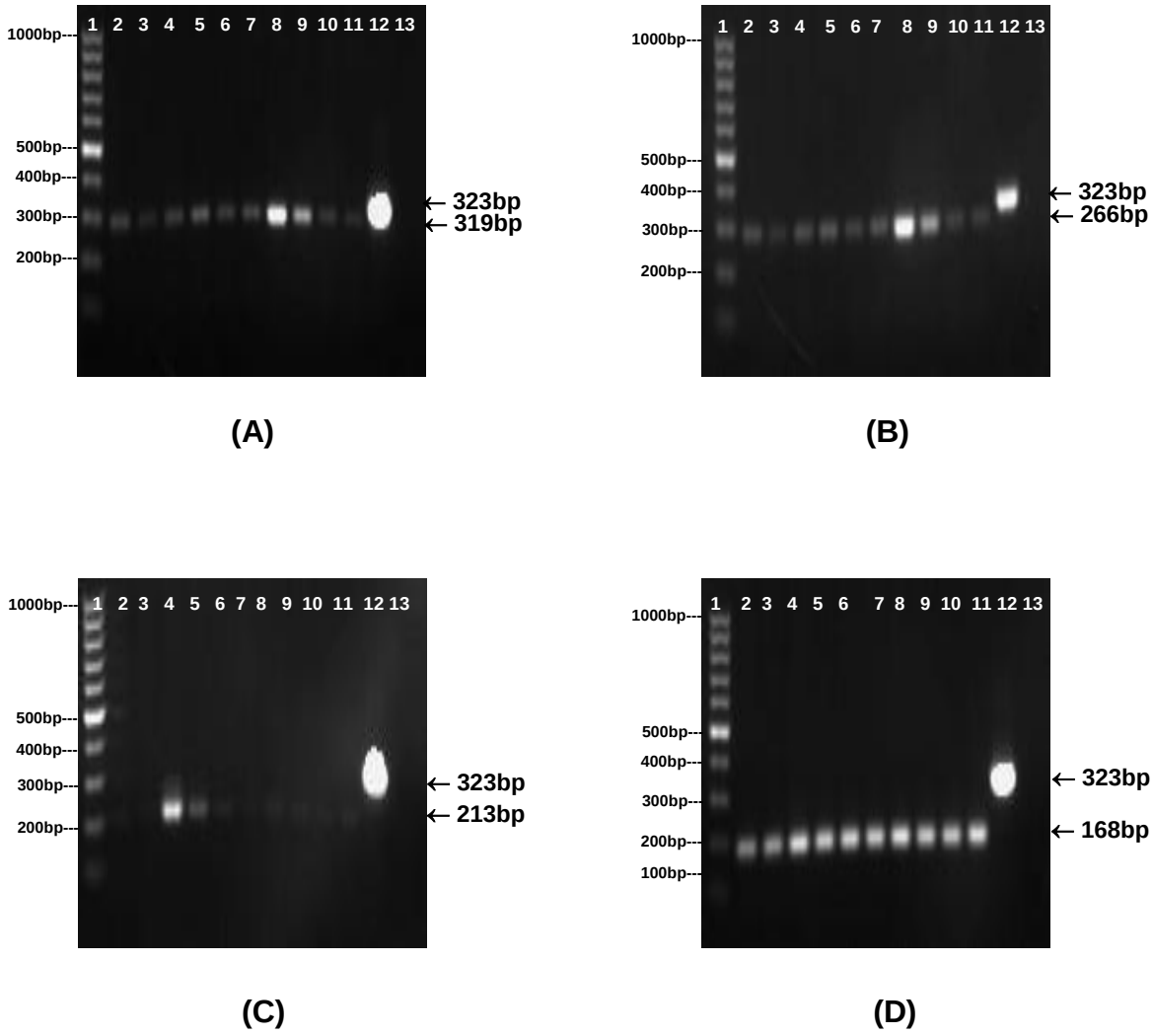



**Figure 4.4** Polymerase chain reaction (PCR) analysis used to confirm the integration of (A) Sweet potato feathery mottle virus (SPFMV), (B) Sweet potato chlorotic stunt virus (SPCSV), (C) Sweet potato virus G (SPVG) and (D) Sweet potato mild mottle virus (SPMMV) coat protein (CP) gene segments in pGA482G-SPVD. Lanes 1 contains the O' GeneRuler$^{TM}$ 100bp DNA ladder. Lanes 2-11 contains PCR products of plasmid DNA isolated from 10 recombinant clones. Lane 12 shows the 323bp product of the positive control reaction and Lane 13 shows the negative control reaction which contains product of pGA482G DNA.

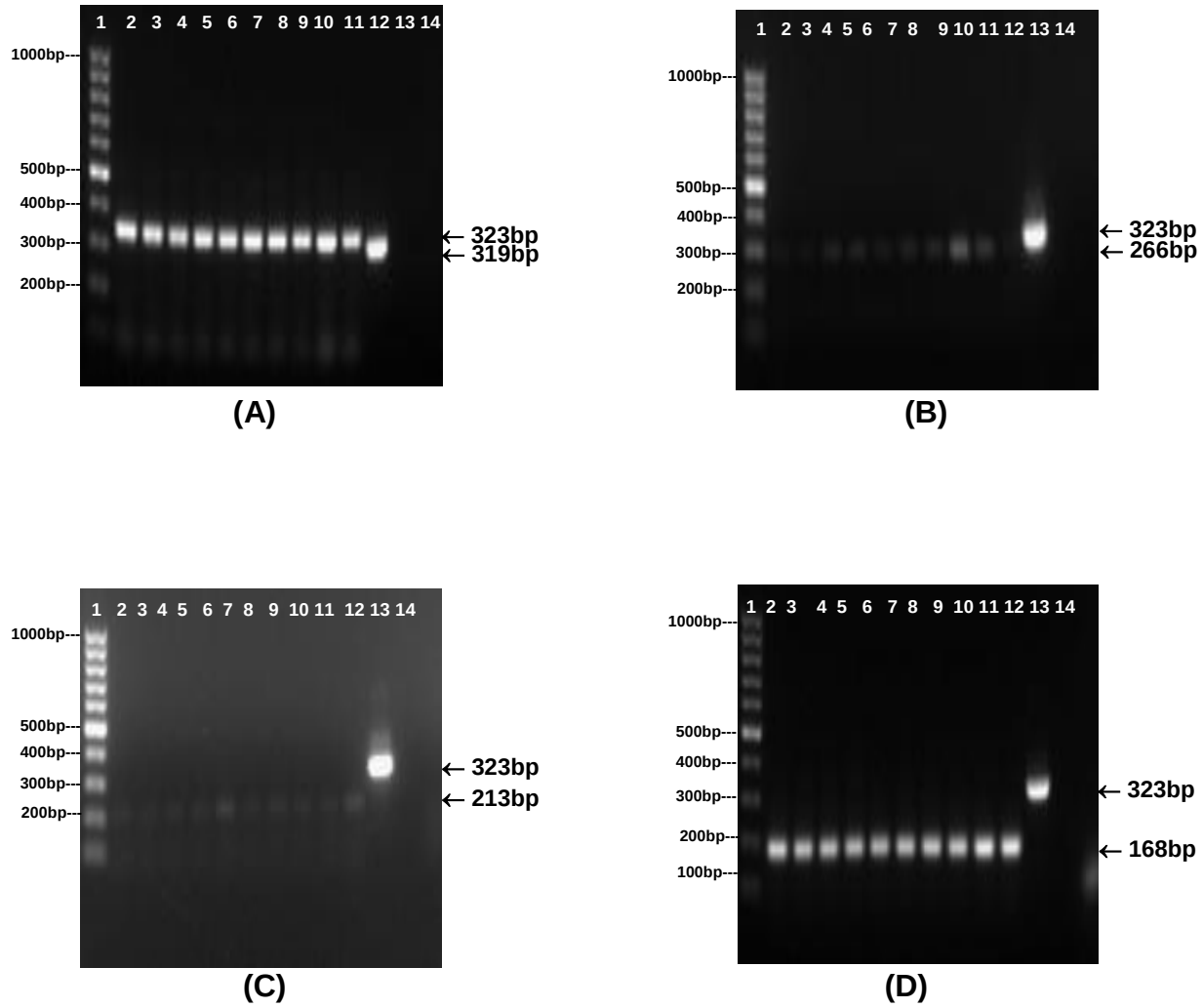



**Figure 4.5** Polymerase chain reaction (PCR) analysis used to confirm the transformation of *Agrobacterium tumefaciens strain* LBA4404 with pGA482G-SPVD containing (A) Sweet potato feathery mottle virus (SPFMV), (B) Sweet potato chlorotic stunt virus (SPCSV), (C) Sweet potato virus G (SPVG) and (D) Sweet potato mild mottle virus (SPMMV) coat protein (CP) gene segments. Lanes 1 contains the O' GeneRuler$^{TM}$ 100bp DNA ladder. Lanes 2-11 contains PCR products of plasmid DNA isolated from 10 recombinant clones. Lane 12 shows the 323bp product of the positive control reaction and Lanes 13 and 14 show the negative control reactions which contain product of pGA482G DNA and water respectively.

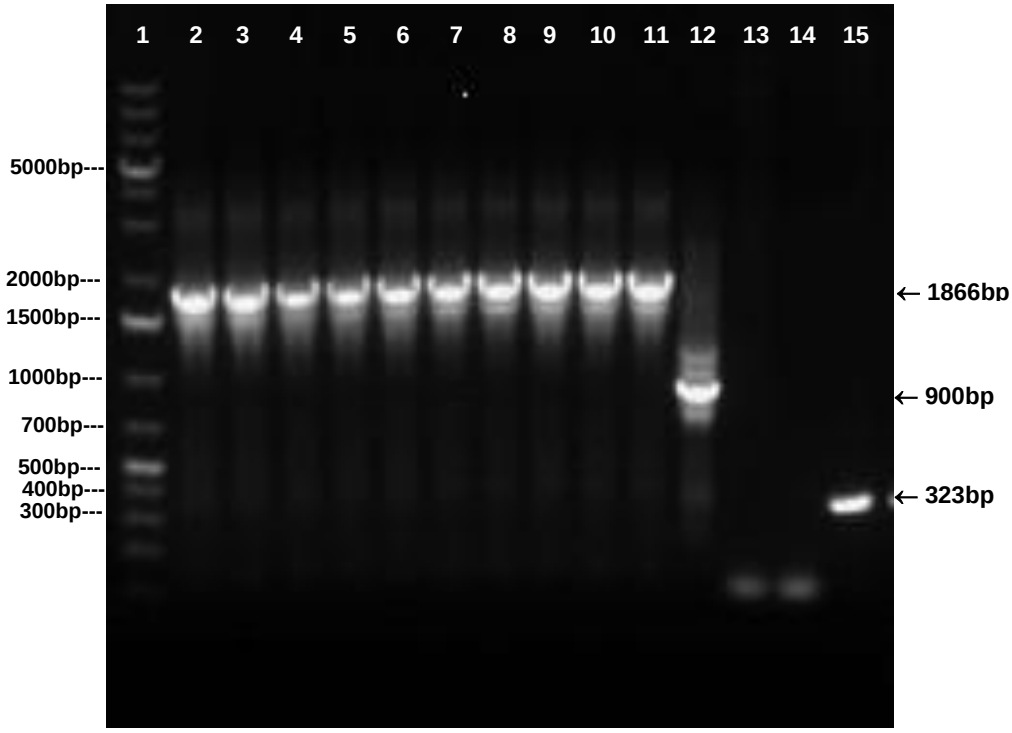



**Figure 4.6** Polymerase chain reaction (PCR) analysis used to confirm the transformation of *Agrobacterium tumefaciens strain* LBA4404 with pGA482G-SPVD containing the entire recombinant expression cassette. Lane 1 contains the O' GeneRuler$^{TM}$ 1kb plus DNA ladder. Lanes 2-11 contains the ~1,866bp PCR products of pGA482G-SPVD DNA isolated from 10 recombinant clones. Lanes 12 and 13 show the products of pEPJ86-m/2N (~900bp expression cassette) and pGA482G DNA respectively. Lane 14 shows the negative water control reaction and Lane 15 shows the 323bp product of the positive control reaction. Primers 97-1 and 97-16 (Fig. 4.1) were used to amplify the major part of the corresponding expression cassette. Two bands were obtained since the primer 97-1 can bind to both the enhancers of the 35S promoter.

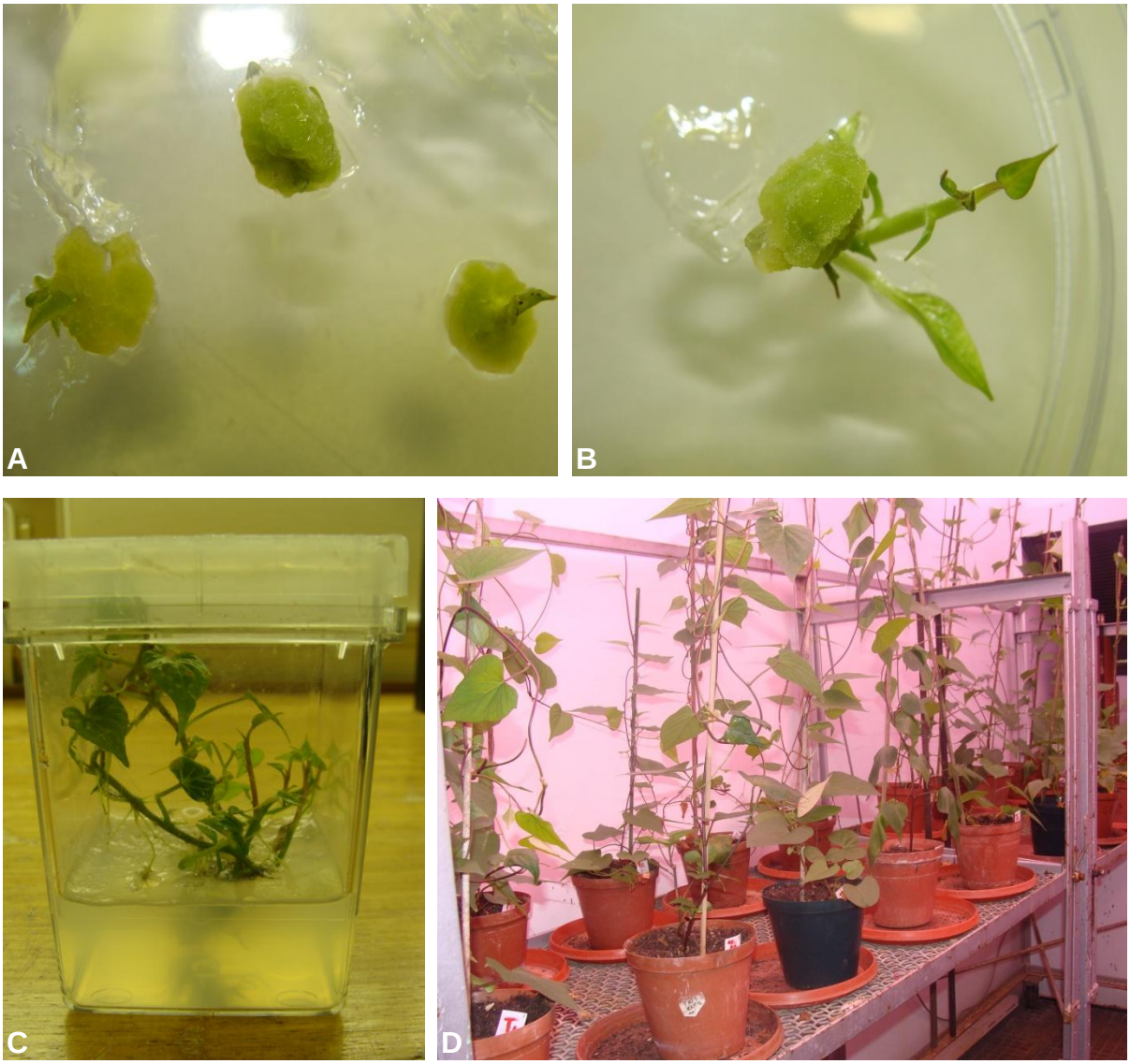



**Figure 4.7** Regeneration of transgenic sweet potato (*Ipomoea batatas* Lam.) cv. Blesbok. (A) Organogenic regeneration of shoots from callus after one month of culture. (B) Shoot elongation after three weeks of culture. (C) Fully regenerated plantlet on antibiotic-free basal medium (BM). (D) Fully developed plants obtained four months from the beginning of the transformation procedure.

### 4.4.4 Molecular characterization of putative transgenic plants

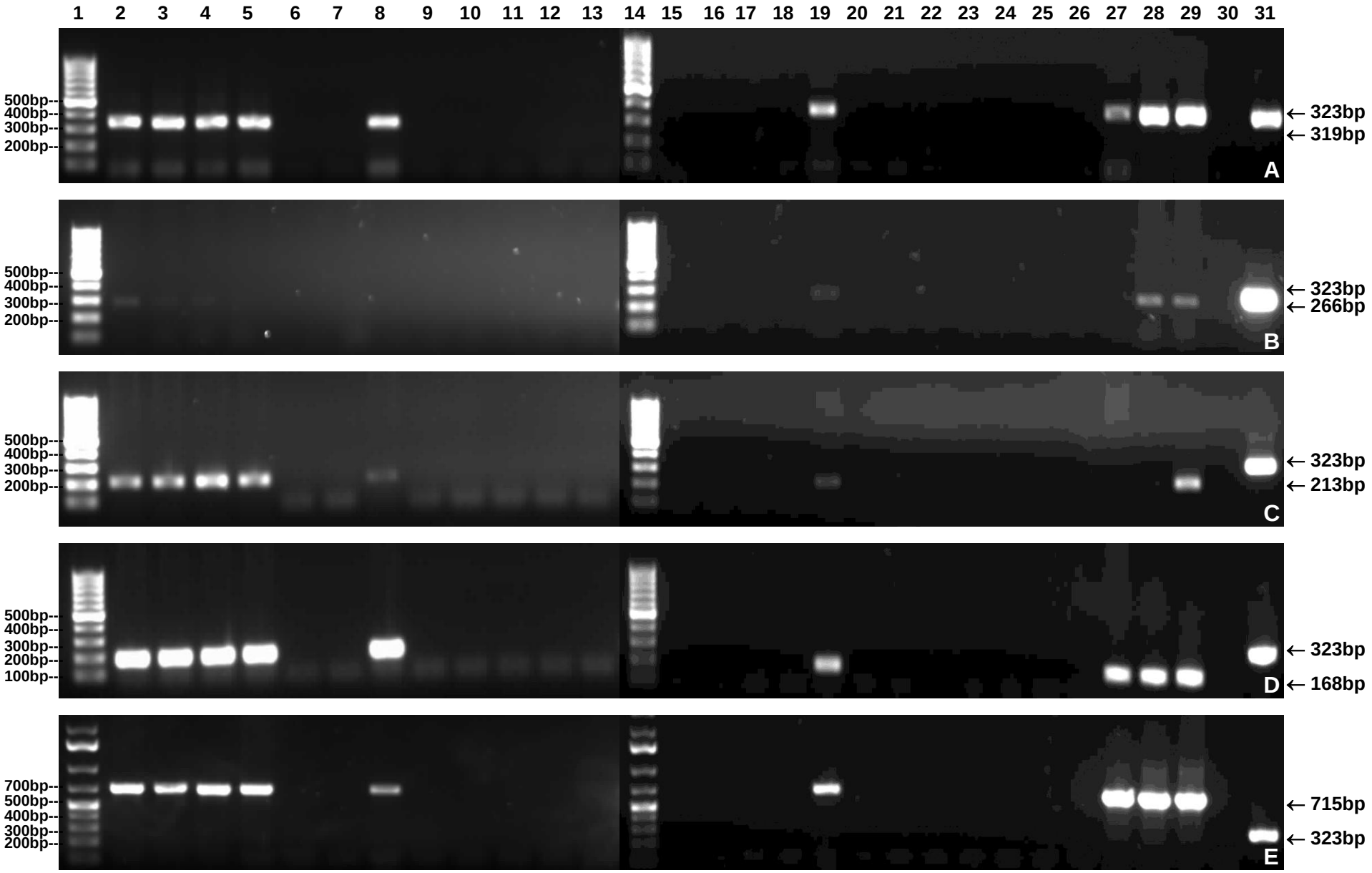
#### *4.4.4.1 Detection of transgenic events by PCR analysis*

PCR analysis confirmed the presence of each segment of the transgene in six of the 24 putative transgenic plants (Fig. 4.8). The size of each amplified product was consistent with that of the original inserts of the CP genes from SPFMV (319bp), SPCSV (266bp), SPVG (213bp) and SPMMV (168bp). In addition, the expected band of 715bp from the coding region of the *npt*II gene was found in all six plants. No PCR products were observed in non-transgenic control plants.

#### *4.4.4.2 Detection of transgene integration by Southern blot analysis*

Southern blot analysis was performed to confirm integration and copy number for the six putative transgenic plants determined by PCR amplification. The results revealed the presence of two hybridization signals specific to the transgene segment in all transformed plants (Fig. 4.9). No signal was detected in both negative control reactions, hence confirming the authenicity of the trangene. An estimated 16366bp band was observed for the positive plasmid control. Undigested genomic DNA from a transformed plant yielded a hybridization signal which confirmed stable integration of the transgene in the genome of the transgenic plants. All six transgenic plants showed an identical hybridization pattern and two insertion loci. This identifical profile would indicate that all transgenic plants are not independent transformation events and are probably clones of each other.

**Figure 4.8** Detection of transgenic events by polymerase chain reaction (PCR) analysis of the (A) Sweet potato feathery mottle virus (SPFMV), (B) Sweet potato chlorotic stunt virus (SPCSV), (C) Sweet potato virus G (SPVG), (D) Sweet potato mild mottle virus (SPMMV) coat protein (CP) gene segments and (E) *npt*II gene in putative transgenic sweet potato (*Ipomoea batatas* Lam.) plants. Lanes 1 and 14 contain the O' GeneRuler$^{TM}$ 100bp DNA ladder in (A-D) and the O' GeneRuler$^{TM}$ 1kb plus DNA ladder in (E). Lanes 2-13 and 15-26 contain PCR products from DNA extracted from the 24 putative transgenic plants. Lanes 27-29 in (A, D, E) and Lanes 28-29 in (B) and Lane 29 in (C) show the PCR products of pGA482G-SPVD DNA. Lane 27 in (B) and Lanes 27-28 in (C) are blank. Lane 30 contains the PCR product from DNA extracted from untransformed control plants. Lane 31 shows the 323bp product of the PCR positive control reaction.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
500bp--
400bp--
300bp--
200bp--
← 323bp
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A
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← 323bp
← 213bp
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100bp--
← 323bp
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D
700bp--
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400bp--
300bp--
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← 715bp
← 323bp
E

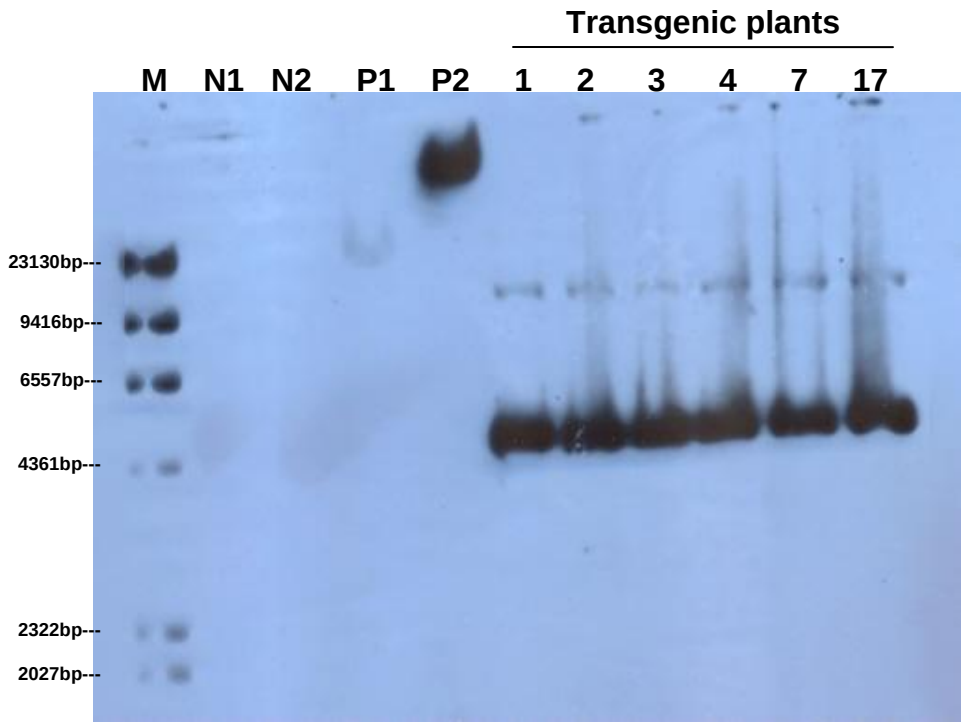


**Figure 4.9** Southern blot analyses of *Hind*III-digested total DNA from transgenic sweet potato (*Ipomoea batatas* Lam.) plants hybridized with a probe corresponding to the Sweet potato feathery mottle virus (SPFMV) gene segment of the transgene. M = DIG labeled DNA molecular weight marker; N1 = untransformed non-transgenic genomic plant DNA; N2 = digested untransformed genomic DNA spiked with *Hind*III-digested PGA482G DNA; P1 = digested PGA482G-SPVD DNA (~ 16366bp); P2 = undigested genomic DNA from a transgenic plant; 1, 2, 3, 4, 7, 17 = transgenic sweet potato plants of cultivar Blesbok.

### 4.4.5 Greenhouse evaluation of transgenic plants for virus resistance

At ten weeks post inoculation, all non-transgenic control plants displayed mild chlorosis and mosaic whilst all transgenic plants remained symptomless. Two weeks later, symptoms on non-transgenic control plants progressed into severe mosaic, mottle, leaf strap, vein clearing, crinkling and leaf curl (Fig. 4.10A/B). In comparison to the control plants, all transgenic plants displayed a good level of resistance in the form of delayed and attenuated symptoms of chlorosis and mottle (Fig. 4.10A/C). Transgenic plants T7 and T17 displayed a milder level of disease than transgenic plant T1, T2, T3, and T4 (Fig. 4.11). The milder symptoms were mainly observed in lower leaves, whilst many newly emerged leaves remained non-symptomatic. This would indicate that the spread of viruses from initially infected leaves may have been inhibited.

The presence of SPFMV and SPVG was confirmed by NCM-ELISA in all transgenic, non-transgenic plants as well as their corresponding graft-inoculated *I. setosa* plants (Fig. 4.12). Reactions for non-transgenic control plants and corresponding graft-inoculated *I. setosa* were strong and indicated a high level of virus accumulation in both the control and indicator plants. Transgenic plants T1, T2, T3, and T4 displayed a weak positive reaction whilst their corresponding graft-inoculated *I. setosa* displayed stronger positive reactions. This would indicate a low level of virus accumulation in these transgenic plants and a subsequent build up of virus levels in indicator plants. Reactions for transgenic plants T7 and T17 and their corresponding graft-inoculated *I. setosa* plants were weak and this indicated a low level of virus accumulation in both the transgenic and indicator plants. The presence of SPMMV and SPCSV was not detected by NCM-ELISA in all transgenic, non-transgenic plants and corresponding graft-inoculated *I. setosa* plants. Therefore, graft-inoculation with SPMMV and SPCSV was not successful.

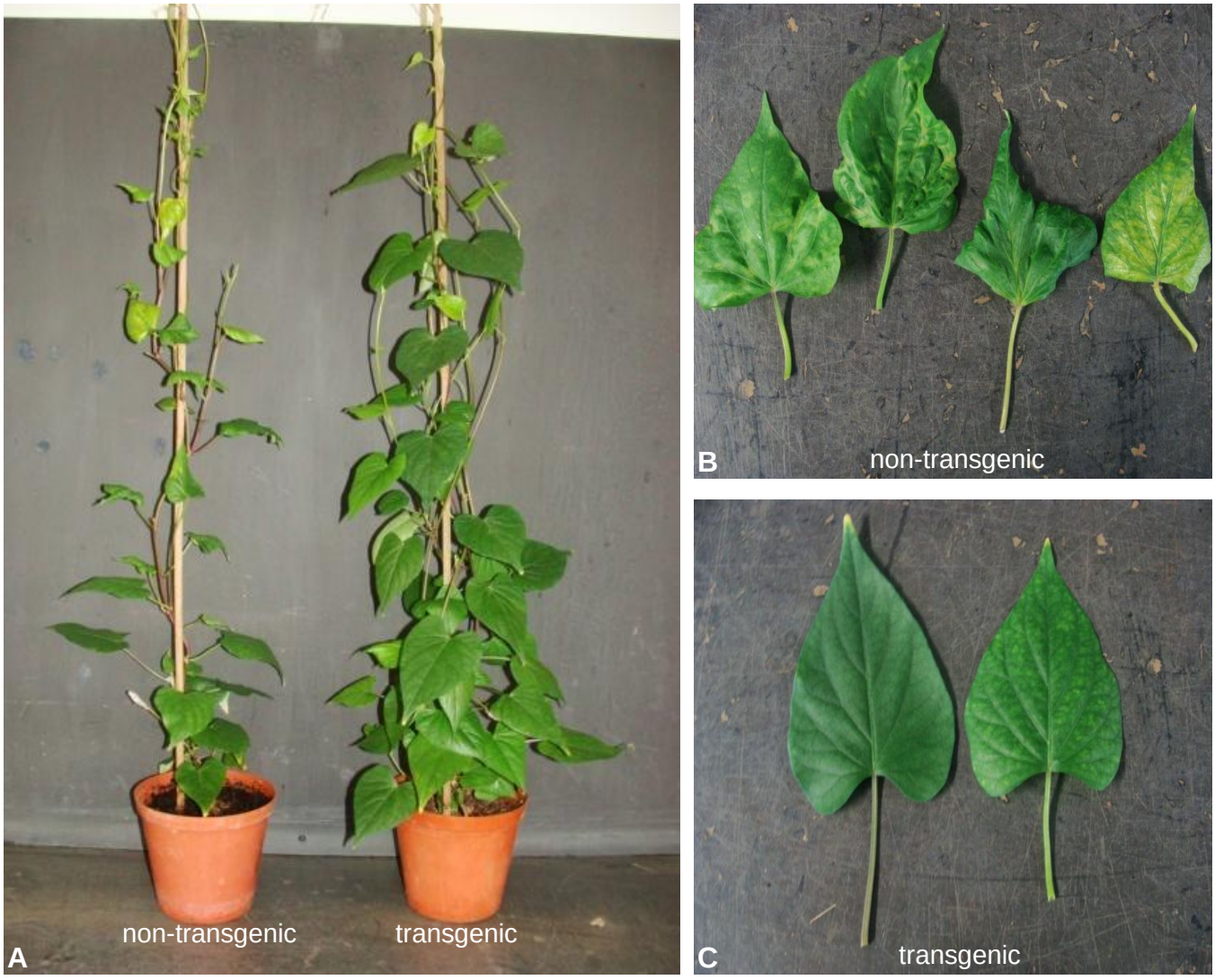


**Figure 4.10** Greenhouse evaluation of transgenic sweet potato (*Ipomoea batatas* Lam.) for resistance against Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato virus G (SPVG) and Sweet potato mild mottle virus (SPMMV). (A) Comparison of virus-challenged non-transgenic and transgenic plants 12 weeks post inoculation. (B) Symptom reactions on leaves of challenged non-transgenic control plants showing severe mosaic, strap, vein clearing, crinkling and leaf curl. (C) Asymptomatic upper leaf (left) and symptomatic lower leaf showing mild mottle (right) of challenged transgenic plants.


**Figure 4.11** Greenhouse evaluation of transgenic sweet potato (*Ipomoea batatas* Lam.) for resistance against Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato virus G (SPVG) and Sweet potato mild mottle virus (SPMMV). At 12 weeks post inoculation, transgenic plants T7 and T17 displayed a milder level of disease than transgenic plants T1, T2, T3, and T4.

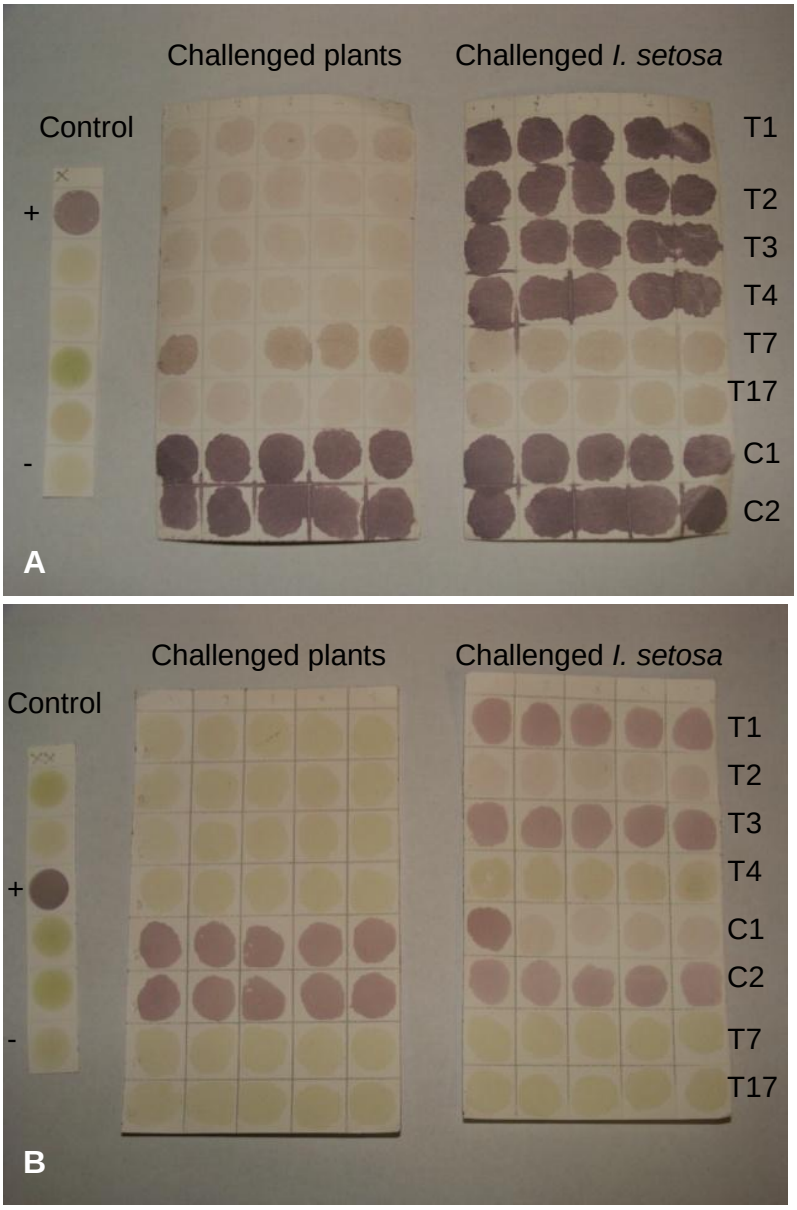


**Figure 4.12** Nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) detection of (A) Sweet potato feathery mottle virus (SPFMV) and (B) Sweet potato virus G (SPVG) in challenged transgenic and non-transgenic sweet potato (*Ipomoea batatas* Lam.) plants and their corresponding graft-inoculated *Ipomoea setosa* Ker. plants. Transgenic plants: T1, T2, T3, T4, T7 and T17. Non-transgenic control plants: C1 and C2. NCM control strips pre-spotted with sap from virus-positive (positive [+] control) and healthy plants (negative [-] control) were included.

## 4.5 DISCUSSION

Transgenic sweet potato plants of the cultivar Blesbok showing resistance to multiple viruses were developed in this study. The approach used in this study extends from studies by Jan *et al*. (2000) who demonstrated that transgenic plants with resistance to multiple viruses can be obtained by transforming plants with a transgene construct consisting of fused gene segments of different viruses linked to a DNA silencer, which could also be a viral gene. In this study, a single segmented transgene construct containing four partial CP encoding gene segments of SPFMV, SPCSV, SPVG and SPMMV fused to a silencer DNA, the middle half of the nucleocapsid (N) gene of TSWV was used to induce PTGS in transgenic sweet potato. Results indicated that resistance to SPFMV, SPCSV, SPVG and SPMMV manifested as alleviated and delayed symptom severity with possible reduced virus titers. This is the first report of transgenic sweet potato showing multiple resistance to at least two viruses and the SPVD complex.

A simple, efficient and reproducible method for plant regeneration of sweet potato was established in this study. This protocol was designed for the popular commercial variety - Blesbok, and is the first regeneration procedure based on a commercial South African sweet potato cultivar. Studies have shown sweet potato regeneration to be highly genotype-dependent because of the variation among cultivar responses to *in vitro* treatments (Dessai *et al*., 1995; González *et al*., 2008). Due to these noted genotype-dependent responses, a customized regeneration protocol was designed specifically for the cv. Blesbok using hormone concentration and explant type as parameters for optimization. Results of this study indicate that an acceptable percentage of shoot regeneration was obtained when apical shoot tips are cultured on medium containing the combined presence of 0.01mg/L NAA and 1mg/L BAP. Data on explant type and hormone concentrations needed for optimal shoot regeneration has allowed for the establishment of an efficient and reproducible regeneration system that will provide the initial framework in the development of a transgenic sweet potato cultivar.

Fully regenerated putative transgenic plantlets took four months to develop from the beginning of the transformation procedure. This time period was significantly longer in comparison to the two months observed in the previous study on the regeneration of untransformed sweet potato plants. The antibiotic carbenicillin, used for inhibition of *Agrobacterium* growth might have had a negative effect on plant regeneration. Similar instances of slow plant regeneration due to carbenicillin selection have also been reported (Nyaboga *et al*., 2008; Yang *et al*., 2011). The study of Yang *et al*. (2011) also reported varied sensitivity to carbenicillin by different sweet potato cultivars and suggested that the sensitivity of sweet potato to carbenicillin might be cultivar dependent. The authors go on to recommend that when plant regeneration is dramatically hindered by cultivar sensitivity to carbenicillin, alternative antibiotics such as cefotaxime may be used. Shortening the plant regeneration process after co-cultivation is a key step in the optimization of the transformation procedure used in this study. Therefore, future studies on the improvement of the transformation of sweet potato cv. Blesbok should investigate the use of cefotaxime as an alternate bactericide.

The sweet potato transformation protocol reported in this study resulted in a low efficiency. The use of kanamycin in the selection of putative transformants resulted in the regeneration of 24 plants out of the total explants infected with the recombinant *Agrobacterium*. However, subsequent screening of putative regenerated transformants using PCR (Fig. 4.8) and Southern blot analysis (Fig. 4.9) confirmed the positive transformation of 6 out of the 24 regenerated plants. The low efficiency of kanamycin in the selection of positive transformants led to the low final transformation efficiency reported in this study. Low numbers of transgenic events of sweet potato have also been reported in many previous studies (Newell *et al*., 1995; Gama *et al*., 1996; Morán *et al*., 1998; Cipriani *et al*., 2001; Otani *et al*., 2003; Song *et al*., 2004; Kreuze *et al*., 2008). In addition, studies have also reported the escape of untransformed plantlets from selection using kanamycin (Luo *et al*., 2006; Xing *et al*., 2008). The presence of low numbers of transformed cells in the growing plant tissues might have allowed the regeneration of untransformed plantlets in the presence of kanamycin. During selection, the strong regenerative capacity of these tissues was able to compete with regeneration

of rare transformed cells. Due to the lower concentration of kanamycin used for the root induction stage, untransformed cells were able to overcome low numbers of transformed cells resulting in the regeneration of an untransformed plant in the presence of kanamycin. The reduced kanamycin concentration in the RIM and its omission in the hormone-free BM was the result of a calculated strategy to allow for the regeneration of transformed plantlets in a shorter time period. However, this decision had a negative impact of the efficiency of selection. To improve selection, further studies will use a higher concentration kanamycin at the sacrifice of a prolonged regenerative period.

Screening of putative transformants by PCR (Fig. 4.8) and Southern blot (Fig. 4.9) analysis proved to be a critical aspect in the accurate identification of positively transformed plantlets. Results of the analyses indicated that the transgene construct consisting of the partial CP segments of SPFMV, SPCSV, SPVG and SPMMV were integrated into the sweet potato genome. Southern blot analysis of six transgenic plants showed that there was two insertion loci with the same pattern of hybridization. This would indicate that all six transgenic plants are not the result of independent transformation events but are more likely to be clones of one transgenic event with each clone having two copies of the cassette. Hybridization signals in positive control reactions and absence of signal in negative control reactions confirm stable integration and authenicity of the transgene.

Transgenic sweet potato plants were evaluated for virus resistance by graft-inoculation with SPFMV, SPCSV, SPVG and SPMMV. Sweet potato is a vegetatively propagated plant and herbaceous cuttings of the six transgenic sweet potato plants were used as progeny in the evaluation. Following inoculation, all transgenic plants were detectably infected with SPFMV and SPVG (Fig. 4.12). Therefore, none of the transgenic plants exhibited immunity to virus infection. However, resistance encompasses a wide variety of host-pathogen interactions. A resistant phenotype reduces the growth, replication or disease-producing activities of the pathogen. Disease symptoms are less severe on resistant hosts than on susceptible hosts (Pataky & Carson, 2004). The non-transgenic sweet potato plants were used as susceptible controls and their phenotype was unable

to restrict the replication and disease-producing properties of SPFMV and SPVG (Fig. 4.10A/B). Therefore, symptoms produced on control plants were severe and virion accumulation levels were high enough to enable detection by NCM-ELISA in both the non-transgenic plant and its corresponding graft-inoculated indicator plant. Transgenic plants displayed delayed and milder symptoms, if any, of chlorosis and mottle of lower leaves when compared to the untransformed control plants (Fig. 4.11). Delayed and attenuated symptom development is an important characteristic of transgenic virus resistance and could also be a good indication of reduced virus replication and titer. A tolerant and recovery phenotype was observed in transgenic plants and this is indicative of a milder form of plant infection in which the virus has still accumulated without causing significant damage to the plant. This phenotype is of commercial value as it reduces economic losses that result from severe symptom expression.

It was expected that all transgenic plants, being derived from the same transgenic event, would produce the same host reaction when challenged with virus infection. However, resistance reactions produced in transgenic plants T7 and T17 varied in degree and kind from those produced in plants T1, T2, T3 and T4 (Fig. 4.11). The latter plants proved more susceptible to virus infection by exhibiting a more severe disease reaction than transgenic plants T7 and T17. In addition, weak positive reactions in the NCM-ELISA for T7 and T17 indicated a low level of virus accumulation in both the transgenic and indicator plants. This variation can be attributed to the presence of lower virus titer levels in scions used in the graft inoculation of transgenic plants. An initial lower concentration of virus levels in scions may result in a lower concentration of virus in infected transgenic plants which can manifest as slower and less severe disease development. However, the difference in host reaction and virus accumulation levels is significant and will justify the basis for the preferable selection of the T7 and T17 plants in further testing.

SPCSV and SPMMV were not detected by NCM-ELISA in graft-inoculated transgenic, non-transgenic plants and their corresponding graft-inoculated indicator plants. This would indicate that graft-inoculation was not successful in the transfer of these two

viruses into transgenic and non-transgenic plants. The dynamics of disease development through graft inoculation might have favoured the proliferation of SPFMV and SPVG over SPCSV and SPMMV. Repeat grafting procedures should focus on the inoculation of each virus singly rather than simultaneous inoculation of all four viruses. In this regard, insect inoculation using aphids (SPFMV and SPVG) and whiteflies (SPCSV and SPMMV) could provide a more efficient means of virus transmission.

In this study, data on virus titers in transgenic plants showing varying levels of virus resistance would have been a desirable factor in the evaluation of transgenic resistance. However, standard quantitative ELISA assays are not accurate for this purpose, due to large quantities of interfering substances such as phenols, polysaccharides and latex that are naturally present in sweet potato (Esbenshade & Moyer, 1992; Abad & Moyer, 1992; Miano *et al*., 2008). Real-time quantitative PCR may be used as an alternative means of determining concentration levels of viruses in challenged transgenic plants and this information could provide a better understanding of the virus titer levels associated with varying levels of resistance (Kokkinos & Clark, 2006; Miano *et al*., 2008).

Symptom development on transgenic plants could be due to the graft-inoculation method where the susceptible scion provided a continuous source of virus that may have overcame resistance. Graft inoculation is known to represent the most potent means of supplying a continuous and high dose of virus inoculum to a plant (Wroth & Jones, 1992; Njeru *et al*., 1995; Okada & Saito, 2008). Previous studies have shown that resistance can be overcome by graft-inoculations even if the sweet potato genotype shows high-field resistance to virus infection (Mwanga *et al*., 2002; Miano *et al*., 2008; Okada & Saito, 2008). SPFMV and SPVG are transmitted by aphids and SPCSV and SPMMV by whiteflies under natural conditions (Loebenstein *et al*., 2009). Therefore, to fully determine the efficiency of the transgene, further experimentation in the field is needed where aphid and whitely inoculation will provide a natural route to infection (Nyaboga *et al*., 2008). A higher level of resistance could be obtained under field

conditions due to the lower and variable dose of inoculum provided by vector transmission. Further field experiments are now being planned.

The approach used in this study was through a sense structure involving the linking of small gene segments of SPFMV, SPVG, SPCSV and SPMMV to a silencer DNA, the middle half of the N gene of TSWV. It is proposed that gene silencing involving PTGS is the mechanism of resistance. Northern blot analysis of the resistance plants for accumulation of transgene-derived siRNAs would have provided evidence for PTGS. Such an analysis was not carried out in this study and therefore, the proposed mechanism cannot be conclusively proven. However, previous studies reported the detection of siRNAs in transgenic plants that were engineered using the same strategy described in this study (Pang *et al*., 1997; Jan *et al*., 2000, Lin *et al*., 2011). This would indicate that mechanism for resistance in transgenic sweet potato plants was via PTGS, which is a sequence-specific RNA degradation system.

This study demonstrates that small segments of viral genes can confer resistance when fused with a silencer DNA. The usefulness of this strategy has been previously demonstrated to enhance the induction of gene silencing. Initially, Pang *et al.* (1997) showed that small gene segments (110-235bp) were ineffective when expressed alone but were effective when fused to a silencer DNA (e.g. green fluorescent protein (GFP)). This study showed that transgenic plants with a transgene of GFP fused to small segments of the N gene of TSWV were resistant to TSWV through the mechanism of PTGS. Later, Jan *et al*. (2000) proposed that multiple resistance was also likely to be obtained if a viral gene was used in place of the GFP. This study demonstrated that transgenic plants containing a transgene consisting of the Turnip mosaic virus (TuMV) CP gene fused to a short TSWV N gene segment were resistant to both viruses via the PTGS mechanism. Similarily, Lin *et al*. (2011) showed that multiple resistance toTSWV and Tomato leaf curl Taiwan virus (ToLCTWV) was obtained in tobacco and tomato plants transformed with a single chimeric transgene consisting of the partial N gene of TSWV fused to the C2 gene segment of ToLCTWV.

There are many advantages for inducing the gene silencing state with a chimeric transgene consisting of silencer DNA fused with one or more small nontranslatable segments of a viral genome(s) (Pang *et al*., 1997). First, Jan *et al.* (2000) and Pang *et al.* (1997) speculate that the silencer DNA can enhance the induction of gene silencing. Second, they propose that the linking of several segments of the viral genes could provide multiple virus resistance. Third, they conclude that the small non-translatable segments minimize the risks of recombination, synergism, complementation or transcapsidation which are concerns that have been raised when using full-length of viral genes.

Studies on the production of transgenic sweet potato with resistance to SPFMV showed high levels of protection in the greenhouse evaluations (Kreuze, 2002). Once these plants were subjected to field conditions, the high levels of resistance to SPFMV broke down following infection with SPCSV and the plants succumb to severe SPVD (Karyeija *et al*., 2000; Kreuze *et al*., 2008). It thus can be seen that infections by multiple viruses is a severe impendent to the comprehensive control of virus disease in sweet potato (Loebenstein *et al*., 2009). The genetic engineering strategies employed in this study show a novel approach of addressing the problem of the multiplicity and synergism of sweet potato viruses. Following proper field testing, this approach could be used as the framework of controlling multiple virus infection other crops.

## 4.6 REFERENCES

**Abad, J. A. & Moyer, J. W. (1992).** Detection and distribution of sweetpotato feathery mottle virus in sweetpotato by in vitro-transcribed RNA probes (riboprobes), membrane immunobinding assay, and direct blotting. *Phytopathology* **82,** 300-305.

**An, G., Ebert, P. R., Mitra, A. & Ha, S. B. (1988).** Binary vectors. *Plant Molecular Biology Manual* **A3,** 1-19.

**Clark, C. A, Valverde, R. A., Fuentes, S., Salazar, L. F. & Moyer, J. W. (2002).** Research for improved management of sweet potato pests and diseases: cultivar decline. *Acta Horticulturae* **583,** 103-113.

**Cipriani, G., Fuentes, S., Bello, V., Salazar, L. F., Ghislain, M. & Zhang, D. P. (2001).** Transgene expression of rice cysteine proteinase inhibitors for the development of resistance against sweetpotato feathery mottle virus. In *CIP Program Report 1999-2000*. pp. 267-271. Edited by Fergusson, P., Parrott, S., Sheridan, K., Smith, B. & Stares, J. Lima: International Potato Center.

**Collinge, D. B., Jorgensen, H. J. L., Lund, O. S. & Lyngkjaer, M. J. (2010).** Engineering pathogen resistance in crop plants: current trends and future prospects. *Annual Review of Phytopathology* **48,** 269-291.

**Dasgupta, I., Malathi, V. G. & Mukherjee, S. K. (2003).** Genetic engineering for virus resistance. *Current Science* **84**, 341-354.

**Dessai, A. P., Gosukonda, R. M., Blay, E., Dumenyo, C. K., Medina-Bolivar, F. & Prakash, C. S. (1995).** Plant regeneration of sweetpotato (*Ipomoea batatas* L.) from leaf explants in vitro using a two-stage protocol. *Scientia Horticulturae* **62,** 217-224.

**Domola, M. J., Thompson, G. J., Aveling, T. A. S., Laurie, S. M., Strydom, H. & Van den Berg, A. A. (2008).** Sweet potato viruses in South Africa and the effect of viral infection on storage root yield. *African Plant Protection* **14,** 15-23.

**Esbenshade, P. R. & Moyer, J. W. (1982).** Indexing system for *Sweet potato feathery mottle virus* in sweet potato using enzyme-linked immunosorbent assay. *Plant Disease* **66,** 911-913.

**Fuchs, M. & Gonsalves, D. (2007).** Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. *Annual Review of Phytopathology* **45,** 173-202.

**Gama, M. I. C. S., Leite, R. P., Cordeiro, A. R. & Cantliffe, D. J. (1996).** Transgenic sweet potato plants obtained by *Agrobacterium tumefaciens*- mediated transformation. *Plant Cell Tissue Organ Culture* **46,** 237-244.

**Gibson, R. W., Kaitisha, G. C., Randrianaivoriavony, J. M. & Vetten, H. T. (1998).** Identification of the East African strain of *Sweet potato chlorotic stunt virus*

(SPCSV) as a major component of sweet potato virus disease (SPVD) in southern Africa. *Plant Disease* **82,** 1063.

**Gibson, R. W., Aritua, V., Byamukama, E., Mpembe, I. & Kayongo, J. (2004).** Control strategies for sweet potato virus disease in Africa. *Virus Research* **100,** 115-122.

**González, R. G., Sánchez, D. S., Guerra, Z. Z., Campos, J. M., Quesda, A. L., Valvida, R. M., Arencibia, A. D., Bravo, K. Q. & Caligari, P. D. S. (2008).** Efficient regeneration and *Agrobacterium tumefaciens* mediated transformation of recalcitrant sweet potato (*Ipomoea batatas* L.) cultivars. *Asia Pacific Journal of Molecular Biology and Biotechnology* **16,** 25-33.

**Jan, F-J. (1998).** Roles of nontarget DNA and viral gene length in influencing multi-virus resistance through homology dependent gene silencing. USA: Cornell University (Doctoral thesis).

**Jan, F-J., Fagoaga, C., Pang, S-Z. & Gonsalves, D. (2000).** A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *Journal of General Virology* **81,** 2103-2109.

**Karuri, H. W., Ateka, E. M., Amata, R., Nyende, A. B. & Muigai, A. W. T. (2009).** Characterization of Kenyan sweet potato genotypes for SPVD resistance and high dry matter content using simple sequence repeat markers. *African Journal of Biotechnology* **8,** 2169-2175.

**Karyeija, R. F., Kreuze, J. F., Gibson, R. W. & Valkonen, J. P. T. (2000).** Two serotypes of *Sweetpotato feathery mottle virus* in Uganda and their interaction with resistant sweetpotato cultivars. *Phytopathology* **90,** 1250-1255.

**Kokkinos, C. D. & Clark, C. A. (2006).** Real-time PCR assays for detection and quantification of sweetpotato viruses. *Plant Disease* **90,** 783-788.

**Kokkinos, C. D., Clark, C. A., McGregor C. E. & LaBonte, D. R. (2006).** The effect of sweet potato virus disease and its viral components on gene expression levels in sweetpotato. *Journal of the American Society of Horticultural Science* **131,** 657-666.

**Kreuze, J.** (**2002).** *Molecular Studies on the Sweet Potato Virus Disease and its Two Causal Agents*. Uppsala: Swedish University of Agricultural Sciences (Doctoral thesis), pp. 24-33.

**Kreuze, J. F., Salmolski, K. I., Lazaro, M. U., Chuquiyuri, W. J. C., Morgan, G. L., Mejla, P. G. C., Ghislain, M. & Valkonen, J. P. T. (2008).** RNA silencing-mediated resistance to a *Crinivirus* (*Closteroviridae*) in cultivated sweet potato (*Ipomoea batatas* L.) and development of sweetpotato virus disease following co- infection with a potyvirus. *Molecular Plant Pathology* **9,** 589-598.

**Lin, S-S., Henriques, R., Wu, H-W., Nui, Q-W. & Chua, N-H. (2007).** Strategies and mechanisms of plant virus resistance. *Plant Biotechnology Reports* **1,** 125-134.

**Lin, C-Y., Ku, H-M., Tsai, W-S., Green, S. K. & Jan, F-J. (2011).** Resistance to a DNA and a RNA virus in transgenic plants by using a single chimeric transgene construct. *Transgenic Resistance* **20,** 261-270.

**Lindbo, J. A. & Dougherty, W. G. (2005).** Plant pathology and RNAi: A brief history. *Annual Review of Phytopathology* **43,** 191-204.

**Loebenstein, G., Thottappilly, G., Fuentes, S. & Cohen, J. (2009).** Viruses and phytoplasma diseases. In *The Sweet Potato,* pp. 114. Edited by Loebenstein, G. & Thottappilly, G. The Netherlands: Springer publications.

**Lomonossoff, G. P. (1995).** Pathogen-derived resistance to plant viruses. *Annual Review of Phytopathology* **33,** 323-343.

**Luo, H. R., Santa Maria, M., Benavides, J., Zhang, D. P., Zhang, Y. Z. & Ghislain, M. (2006).** Rapid genetic transformation of sweetpotato [*Ipomoea batatas* (L.) Lam] via organogenesis. *African Journal of Biotechnology* **5,** 1851-1857.

**Miano, D. W., LaBonte, D. R. & Clark, C. A. (2008).** Identification of molecular markers associated with sweet potato resistance to sweet potato virus disease in Kenya. *Eyphytica* **160,** 15-24.

**Morán, R., García, R., López, A., Zaldúa, Z., Mena, J., García, M., Armas, R., Somonte, D., Rodríguez, J., Gómez, M. & Pimentel, E. (1998).** Transgenic sweet potato plants carrying the delta-endotoxin gene from *Bacillus thuringiensis* var. *tenebrionis. Plant Science* **139,** 175-184.

**Mukasa, S. B., Rubaihayo, P. R. & Valkonen, J. P. T. (2006).** Interactions between a Crinivirus, an ipomovirus and a potyvirus in coinfected sweet potato plants. *Plant Pathology* **55,** 458-467.

**Murashige, T. & Skoog, F. (1962).** A revised medium for the rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarium* **15,** 473-497.

**Mwanga, R. O. M., Yencho, G. C. & Moyer, J. W. (2002).** Dialled analysis of sweetpotatoes for resistance to sweetpotato virus disease. *Euphytica* **128,** 237-249.

**Naylor, R. L., Falcon, W. P., Goodman, R. M., Jahn, M. M., Sengooba, T., Telera, H. & Nelson, R. J. (2004).** Biotechnology in the developing world: a case for increased investments in orphan crops. *Food Policy* **29,** 15-44.

**Newell, C. A., Lowe, J. M., Merryweather, A., Rooke, L. M. & Hamilton, W. D. O. (1995).** Transformation of sweet potato (*Ipomoea batatas* Lam.) with *Agrobacterium tumefaciens* and regeneration of plants expressing cowpea trypsin inhibitor and snowdrop lectin. *Plant Science* **107,** 215-227.

**Njeru, R., Jones, R. A. C., Sivasithamparam, K. & Jones, M. G. K. (1995).** Resistance to subterranean clover mottle virus in subterranean clover results from restricted cell-to-cell movement. *Australian Agricultural Research* **46,** 633-643.

**Nyaboga, E. N., Ateka, E. M., Gichuki, S. T. & Bulimo, W. D. (2008).** Reaction of transgenic sweet potato (*Ipomoea batatas* L.) lines to virus challenge in the glasshouse. *Journal of Applied Biosciences* ***9,*** 362-371.

**Okada, Y. & Saito, A. (2008).** Evaluation of resistance to complex infection of SPFMVs in transgenic sweet potato. *Breeding Science* **58,** 243-250.

**Okada, Y., Nishiguchi, M., Saito, A., Kimura, T., Mori, M., Hanada, K., Sakai, J., Matsuda, Y. & Murata, T. (2002).** Inheritance and stability of the virus-resistant gene in the progeny of transgenic sweet potato. *Plant Breeding* **121,** 249-253.

**Otani, M., Shimada, T., Kimura, T. & Saito, A. (1998).** Transgenic plant production from embryogenic callus of sweet potato [*Ipomoea batatas* (L.) Lam] using *Agrobacterium tumefaciens*. *Plant Biotechnology* **15,** 11-16.

**Otani, M., Wakita, Y. & Shimada, T. (2003).** Production of herbicide-resistance sweetpotato (*Ipomoea batatas* (L.) Lam) plants by *Agrobacterium tumefaciens*-mediated transformation. *Breeding Science* **53,** 145-148.

**Pang, S-Z., Jan, F-J. & Gonsalves, D. (1997).** Nontarget DNA sequences reduce the transgene length necessary for RNA-mediated tospovirus resistance in transgenic plants. *Proceedings fo the National Academy of Sciences, USA* **94,** 8261-8266.

**Pang, S-Z., Jan, F-J., Tricoli, D. M., Russel, P. F., Carney, K. M., Hu, J. S., Fuchs, M., Quemada, H. D. & Gonsalves, D. (2000).** Resistance to squash mosaic comovirus in transgenic squash plants expressing its coat protein genes. *Molecular Breeding* **6,** 87-93.

**Pataky, J. K. & Carson, M. L. (2004).** Host Resistance. In *Plant Pathology: Concepts and Laboratory Exercises,* pp. 295-233. Edited by Trigiano R.N., Windham M.T. & Windham A.S. New York: CRC Press.

**Sanford, J. C. & Johnston, S. A. (1985).** The concept of pathogen-derived resistance-deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology* **113,** 395-405.

**Shepherd, D. N., Martin, D. P. & Thomson, J. A. (2009).** Transgenic strategies for developing crops resistant to geminiviruses. *Plant Science* **176,** 1-11.

**Song, G-Q., Honda, H. & Yamaguchi, K-I. (2004).** Efficient Agrobacterium tumefaciens- mediated transformation of sweet potato [*Ipomoea batatas* (L.) Lam.] from stem explants using a two-step kanamycin- hygromycin selection method. *In Vitro Cellular and Developmental Biology* **40,** 359-365.

**Tenllado, F., Llave, C. & Díaz-Ruíz, J. R. (2004).** RNA interference as a new biotechnology tool for the control of virus diseases in plants. *Virus Research* **102,** 85-96.

**Untiveros, M., Fuentes, S. & Salazar, L. F. (2007).** Synergistic interactions between *Sweet potato chlorotic stunt virus* (Crinivirus) with Carla-, Cucumo-, Ipomo- and Potyviruses infecting sweet potato. *Plant Disease* **91,** 669-676.

**Usugi, T., Onuki, M. & Hayashi, T. (1990).** Detection of some viruses by protein A sandwich ELISA (PAS-ELISA). *Kyushu Agricultural Research* **53,** 106.

**Wroth, J. M. & Jones, R. A. C. (1992).** Subterranean clover mottle sobomovirus: its host range, resistance in subterranean clover and transmission through seed and by grazing by animals. *Annals of Applied Biology* **121,** 329-343.

**Xing, Y-J., Ji, Q., Yang, Q., Luo, Y-M., Li, Q. and Wang, X. (2008).** Studies on *Agrobacterium*-mediated genetic transformation of embryogenic suspension cultures of sweet potato. *African Journal of Biotechnology* **7,** 534-540.

**Yang, J., Bi, H-P., Fan, W-J., Zhang, M., Wang, H-X. & Zhang, P. (2011).** Efficient embryogenic suspension culturing and rapid transformation of a range of elite genotypes of sweet potato (*Ipomoea batatas* [L.] Lam.). *Plant Science* doi:10.1016/j.plantsci.2011.01.005.

**Yi, G., Yang, K. & Kim, K-M. (2006).** Sweetpotato transformation mediated by *Agrobacterium tumefaciens*. *Korean Journal of Breeding* **38,** 13-18.

# CHAPTER FIVE
# THESIS OVERVIEW

The importance of sweet potato (*Ipomoea batatas* Lam.) as a staple food in Africa and its growing importance in South Africa (SA) despite its global status as a low economic valued 'orphan' crop, attests to the vital role that it has historically played in meeting the food and income needs of the world's poorest and fastest-growing populations (Fugile, 2007). Its broad adaptability to marginal environments, major contribution to household food security and flexibility in mixed farming systems has affirmed the position of the sweet potato as a fixed staple crop in the livelihoods of resource-poor farmers in SA.

In recent years, a progressive degeneration in sweet potato yield and quality has been reported by farmers across SA (Laurie *et al*., 2002). This phenomenon, commonly referred to as 'cultivar decline' has been attributed to the accumulation of viral pathogens in planting material resulting in a decline in yield and/or quality of the crop over time (Clark *et al*., 2002). The incidence of cultivar decline due to viral disease has been reported in major sweet potato growing provinces in SA including KwaZulu-Natal (KZN). The continuous decline in tuber yield and quality has serious implications on the household security of resource-poor farmers. Conventional breeding strategies have had limited success in the management of the multitude of viruses that infect sweet potato. Therefore, the development of a transgenic sweet potato cultivar with broad virus resistance was undertaken in this study to address the problem of multiple virus infections which are common wherever the crop is grown.

The findings presented in this study are based on the identification and distribution of viruses infecting sweet potato in KZN, molecular resolution of the genetic variability of the three major viruses, development of an efficient plant regeneration protocol for cv. Blesbok and the development of transgenic sweet potato with broad virus resistance to Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato virus G (SPVG), and Sweet potato mild mottle virus (SPMMV). Based on results, it can be speculated that:

- A total of seven viruses out of the 10 viruses tested for were identified in KZN. The seven viruses are: SPFMV, SPMMV, SPCSV, SPVG, Sweet potato chlorotic fleck virus (SPCFV), Sweet potato mild speckling virus (SPMSV), and Sweet potato caulimo-like virus (SPCaLV).
- SPFMV, SPVG, SPCSV, and SPMMV were the most prevalent viruses detected.
- Mixed infections of the O (ordinary) and C (common) strains of SPFMV were present in sweet potato crops from KZN.
- The SPCSV isolate from KZN belongs to the West African (WA) strain group of SPCSV and showed close relatedness to an isolate from Argentina.
- The SPFMV, SPVG and SPCSV strains showed close lineage with isolates from South America, East Asia and Africa.
- Apical shoot explants cultured on Murashige and Skoog (MS) media supplemented with 0.01mg/L naphthalene acetic acid (NAA) and 1mg/L 6-benzylaminopurine (BAP) gave the highest percentages of *de novo* organogenic shoot regeneration for the cv. Blesbok.
- Transgenic sweet potato transformed with a sense orientated chimeric transgene consisting of (CP) gene segments of SPFMV, SPCSV, SPVG and SPMMV fused to a silencer DNA, the middle half of the nucleocapsid (N) gene of Tomato spotted wilt virus (TSWV) displayed resistance against SPFMV and SPVG.

## 5.1 Viruses infecting sweet potato in KZN

Durable transgenic resistance to viral disease in any crop requires the determination of the identification, distribution and importance of all viruses infecting that crop in a specific area . Therefore, a field survey for viruses infecting sweet potato in KZN was the first essential step taken in the development of transgenic sweet potato with broad virus resistance. The identification of the four most prevalent viruses infecting sweet

potato in KZN using nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) and electron microscopy (EM) made it possible to develop transgenic sweet potato with resistance to SPFMV, SPVG, SPCSV, and SPMMV.

Serological assessment using NCM-ELISA was the foremost test used in the identification of viruses in sweet potato. However, the current commercial NCM-ELISA kit provides antiserum for only 10 out of the almost 20 viruses known to infect sweet potato. The lack of sweet potato antisera in present day serological testing could be attributed to the shortage of characterization data of newly identified and constantly emerging sweet potato-infecting viruses. Although NCM-ELISA testing covered the major viruses such SPFMV and SPCSV, the lack of suitable antisera to all known viruses in commercial tests might have resulted in the possible omission of other important viruses in identification procedures. Based on previous reports on the viruses known to infecting sweet potato in KZN (Ateka *et al*., 2007; Domola *et al*., 2008), Sweet potato virus 2 (SPV2) is a virus that could possibly have been overlooked in the preliminary serological testing. SPV2, a tentative new member of the genus *Potyvirus*, has been previously detected in KZN (Souto *et al*., 2003; Ateka *et al*., 2004). The presence of SPV2 on sweet potato production in KZN is significant due to reports of increased symptom severity when it interacts synergistically with SPCSV (Kokkinos & Clark, 2006; Tairo *et al*., 2006). This suggests that SPV2 might be economically important in areas of KZN where SPCSV occurs (Loebenstein *et al*., 2009). The threat posed by the possible existence of synergistic SPV2 in areas surveyed in this study warrants the use of alternative methods in the identification of SPV2. Further studies should include testing for SPV2 using molecular methods such as quantitive real-time PCR and next generation sequencing to circumvent the deficiency apparent in serological assays.

In a previous survey of sweet potato infecting viruses in SA, Domola *et al*. (2008) identified the presence of SPFMV, SPVG and SPV2 at low incidences in KZN. This present survey reports the identification of an additional five viruses in KZN including the novel emergence of Sweet potato caulimo-like virus (SPCaLV) in SA. This alarming

upsurge of viral incidence, diversification and the emergence of first-time encountered viruses in KZN justifies the need for continuous updated information on virus identity and distribution. Such information can be obtained by conducting field surveys on an annual basis in previously surveyed virus endemic locations in KZN. A continuous updated record of viral prevalence and distribution in KZN will be crucial in the understanding of the disease dynamics involved in viral disease development.

## 5.2 Genetic variability of viruses infecting sweet potato in KZN

Viruses infecting vegetatively propagated plants are historically known to exist as complex populations of highly adaptable isolates with a predisposition for increased genetic diversification in nature (Jerger *et al*., 2006). Given these attributes, it was essential to determine the genetic variability and strain distribution of the major viruses infecting sweet potato in KZN. The knowledge of the genetic variability of SPFMV, SPVG and SPCSV isolates in KZN contributes to the total body of research available for these viruses.

A few SPFMV (Rännäli *et al*., 2009) and SPVG (Rännäli *et al*., 2008) sequences from SA have been reported and were incorporated into this study. However, there is no sequence data on SPCSV isolates from SA. This study presents the first nt sequence and phylogenetic analysis of SPCSV from SA. Therefore, inferences on the genetic variability of SPCSV in SA could not be determined and the efficacy of the resistance conferred by the transgenic sweet potato against other SPCSV isolates in SA is questionable. More isolates from the rest of SA need to be identified and characterized. The nucleotide sequence data will provide the foundation to elucidate any phylogenetic relationships between isolates.

From a disease management standpoint, it is critical to frequently and proactively monitor any changes in the genetic variation of virus populations in KZN. This will be important to maximize the effectiveness of the engineered resistance over time. In

addition, developing new resistant transgenic plants is practical so solutions can be obtained in a timely manner, should a divergent virus population be detected.

### 5.3 Transgenic sweet potato

The progression of preliminary research conducted in this study culminated in the development of transgenic sweet potato with resistance to SPFMV and SPVG. A crucial step in this progression was the development of an efficient and reproducible regeneration protocol of sweet potato cv. Blesbok. However, when this regeneration protocol was used as a method for *Agrobacterium*-mediated transformation of explants, a low selection and transformation efficiency was observed. The use of cefotaxime as an alternate bactericide and increased concentrations of kanamycin for selection were considered as optimization parameters for the improvement of the transformation efficiencies of sweet potato cv. Blesbok. Further studies should evaluate the effect of different kanamycin and cefotaxime concentrations on the transformation efficiency of the cv. Blesbok. However, studies have shown sweet potato transformation to be highly genotype-dependent because of the variation among cultivar responses to *in vitro* treatments (Dessai *et al*., 1995; González *et al*., 2008). If these noted genotype dependent responses continue to hinder the rate of transformation, alternate cultivars should be considered for use in future genetic transformation.

Resistance to specific viruses has been achieved in many of the first genetically modified (GM) cultivars using the simple expression of coding sequences in the sense orientation (Tricoli *et al*., 1995; Jan *et al*., 2000; Lawson *et al*., 2001; Scorza *et al*., 2001). A shortcoming of this first generation approach is that strong resistance is displayed by only a small number of transformants, presumably due to a major dependency on insertional context. Conversely, once identified by extensive screening, the traits of transgenic resistance derived from this type of construct design appear to be durable and stably inherited in new cultivars (Collinge *et al*., 2010). This is exemplified by the triple virus resistance in the transgenic squash cultivar CZW-3 (Tricoli *et al*., 1995) which, after 14 years, is still on the market.

The new technology of transgene design has been refined to induce direct repeat (DR) or inverted repeat (IR) hairpin RNA. This hpRNA activates the plant's silencing machinery, which then processes it into siRNAs that elicit degradation of homologous RNAs (Smith *et al*., 2000; Gaba *et al*., 2010; Frizzie & Huang, 2010). Although, the hpRNA technology has been shown to provide efficient and reproducible resistance, no cultivars harnessing this technology have been released onto the market (Collinge *et al*., 2010).

This study demonstrates that small segments of viral genes can confer resistance when fused with a silencer DNA. The usefulness of this strategy has been previously demonstrated to enhance the induction of gene silencing (Jan *et al*., 2000; Lin *et al*., 2011). There are many advantages for inducing the gene silencing state with a chimeric transgene consisting of silencer DNA fused with one or more small nontranslatable segments of a viral genome(s) (Pang *et al*., 1997). The use of the 'silencer DNA' in our transgene design may circumvent problems experienced in producing sustainably resistant transgenic plants using other transgene design strategies.

In this study, grafting was used as the method of inoculation in the evaluation of transgenic sweet potato plants. The method proved to be an efficient and reliable means of transmitting virus infection. However, inoculation of large numbers of sweet potato genotypes requires large quantities of inoculum, greenhouse space, and pest control. In addition, if virus-infected scions from indicator plants are used as inoculum, the task becomes labor intensive and requires more time to allow for the infection of inoculated indicator plants. Therefore, more rapid, efficient and less labor intensive ways of inoculating sweet potato with viruses is needed to optimize the efficiency and accuracy of further transgenic plant evaluations.

Symptom expression and serological virus detection were used as the criteria for defining resistant genotypes in the greenhouse evaluation of transgenic sweet potato plants. However, these criteria may not be the most suitable in providing an accurate evaluation of transgenic resistance. Virus disease of sweet potato in KZN is a severe

impendent to tuber yield and quality. In addition, reduced virus accumulation in challenged transgenic plants is a good indication of resistance to virus infection. Therefore, yield and detectable virus titer are needed to provide a more accurate assessment of transgenic resistance. The addition of yield and virus titer as criterion for resistance evaluation can be incorporated into field assessment studies where aphid and whitely inoculation will provide a natural route to infection. In fact, a higher level of resistance could be obtained under field conditions due to the combined effect of a lower and variable dose of inoculum provided by vector transmission and use of refined and applicable criteria for resistance evaluation.

The effectiveness of the resistance displayed by transgenic sweet potato plants developed in this study against multiple virus infection in the field is unknown and remains to be tested. What is known and evidently clear is that the research presented in this study shows a novel approach of addressing the multiplicity of viruses that infecting sweet potato in KZN and the potential impact of such a virus-resistant transgenic sweet potato cultivar is immeasurable.

Transgenic virus-resistant sweet potato in SA has the potential to mitigate some of the most difficult problems facing resource-poor farmers and consumers. The likely effects of transgenic sweet potato should resonate from the level of individual farms. Enhanced crop productivity through reduced losses to virus disease and the eventual increase in tuber yield will help contribute to the reduction of the real cost of food while preserving or increasing the income of the farmer (Zeigler, 2001). Growing cash revenues as well as the greater availability of sweet potato for subsistence consumption will contribute significantly to the improved food security for rural households.

### 5.4 The way forward

In the politically and emotionally charged atmosphere surrounding transgenic crop technology, it will be essential that the virus-resistant sweet potato developed in this study be subjected to extensive screening under field conditions. If the resistance is

validated after field assessments, the research could be used as a model in controlling multiple virus infection of other crops.

The first resistant horticultural crops resulting from genetic engineering have made their mark as providing a novel and effective means of controlling virus disease. For example, the creation and deployment of Papaya ringspot virus (PRSV)-resistant papaya has provided a safe and effective way of saving an entire fruit industry in Hawaii. The same could be true for transgenic sweet potato with broad virus resistance. It is hoped that the virus-resistant transgenic sweet potato developed in this study will fulfill its intended purpose of mitigating the devastating effects of viral disease faced by SA farmers and, in doing so, mark its own place in history as an application of pathogen derived resistance (PDR) that demonstrates the durability of genetic engineering in reducing the impact of virus disease on agriculture.

## 5.5 References


**Ateka, E. M., Barg, E., Njeru, R. W., Lesemann, D. E. & Vetten, H. J. (2004).** Further characterization of '*Sweet potato virus 2*': a distinct species of the genus Potyvirus. *Archives of Virology* **149,** 225-239.

**Ateka, E. M., Barg, E., Njeru, R. W., Thompson, G. & Vetten, H. J. (2007).** Biological and molecular variability among geographically diverse isolates of *Sweet potato virus 2*. *Archives of Virology* **152,** 479-488.

**Clark, C. A, Valverde, R. A., Fuentes, S., Salazar, L. F. & Moyer, J. W. (2002).** Research for improved management of sweet potato pests and diseases: cultivar decline. *Acta Horticulturae* **583,** 103-113.

**Collinge, D. B., Jorgensen, H. J. L., Lund, O. S. & Lyngkjaer, M. J. (2010).** Engineering pathogen resistance in crop plants: current trends and future prospects. *Annual Review of Phytopathology* **48,** 269-291.

**Dessai, A. P., Gosukonda, R. M., Blay, E., Dumenyo, C. K., Medina-Bolivar, F., Prakash, C. S. (1995).** Plant regeneration of sweetpotato (*Ipomoea batatas* L.)

from leaf explants in vitro using a two-stage protocol. *Scientia Horticulturae* **62,** 217-224.

**Domola, M. J., Thompson, G. J., Aveling, T. A. S., Laurie, S. M., Strydom, H. & Van den Berg, A. A. (2008).** Sweet potato viruses in South Africa and the effect of viral infection on storage root yield. *African Plant Protection* **14,** 15-23.

**Frizzie, A. & Huang, S. (2010).** Tapping RNA silencing pathways for plant biotechnology. *Plant Biotechnology Journal* **8,** 655-677.

**Fugile, K. O. (2007).** Priorities for sweetpotato research in developing countries: results of a survey. *HortScience* **42,** 1200-1206.

**Gaba, V., Rosner, A., Maslenin, L., Leibman, D., Singer, S., Kukurt, E., Shiboleth, Y. M. & Gal-On, A. (2010).** Hairpin-based virus resistance depends on the sequence similarity between challenge virus and discrete, highly accumulating siRNA species. *European Journal of Plant Pathology* **128,** 153-164.

**González, R. G., Sánchez, D. S., Guerra, Z. Z., Campos, J. M., Quesda, A. L., Valvida, R. M., Arencibia, A. D., Bravo, K. Q. & Caligari, P. D. S. (2008).** Efficient regeneration and *Agrobacterium tumefaciens* mediated transformation of recalcitrant sweet potato (*Ipomoea batatas* L.) cultivars. *Asia Pacific Journal of Molecular Biology and Biotechnology* **16,** 25-33.

**Jan, F-J., Fagoaga, C., Pang, S-Z. & Gonsalves, D. (2000).** A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *Journal of General Virology* **81,** 2103-2109.

**Jerger, M. J., Seal, S. E. & Van den Bosch, F. (2006).** Evolutionary epidemiology of plant virus disease. *Advances in Virus Research* **67,** 163-203.

**Kokkinos, C. D. & Clark, C. A. (2006).** Interactions among *Sweetpotato chlorotic stunt virus* and different potyviruses and potyvirus strains infecting sweetpotato in the United States. *Plant Disease* **90,** 1347-1352.

**Laurie, L. M. (2002).** Overview of breeding and evaluation of orange-fleshed sweet potato in South Africa. http://www.cipotato.org/Vitaa?Proceedings/VITAA-paper-SLAURIE-FINAL-11Feb2002.pdf

**Lawson, E. C., Weiss, J. D., Thomas, P. E. & Kaniewski, W. K. (2001).** NewLeaf Plus (R) Russet Burbank potatoes: replicase-mediated resistance to *potato leafroll virus*. *Molecular Breeding* **7,** 1-12.

**Lin, C-Y., Ku, H-M., Tsai, W-S., Green, S. K. & Jan, F-J. (2011).** Resistance to a DNA and a RNA virus in transgenic plants by using a single chimeric transgene construct. *Transgenic Resistance* **20,** 261-270.

**Loebenstein, G., Thottappilly, G., Fuentes, S. & Cohen, J. (2009).** Viruses and phytoplasma diseases. In *The Sweet Potato,* pp. 114. Edited by Loebenstein, G. & Thottappilly, G. The Netherlands: Springer publications.

**Pang, S-Z., Jan, F-J. & Gonsalves, D. (1997).** Nontarget DNA sequences reduce the transgene length necessary for RNA-mediated tospovirus resistance in transgenic plants. *Proceedings fo the National Academy of Sciences, USA* **94,** 8261-8266.

**Ränäli, M., Czekaj, V., Jones, R. A. C., Fletcher, J. D., Davis, R. I., Mu, L., Dwyer, G. I., Coutts, B. A. & Valkonen, J. P. T. (2008).** Molecular genetic characterization of *Sweet potato virus G* (SPVG) isolates from areas of the Pacific Ocean and southern Africa. *Plant Disease* **92,** 1313-1320.

**Ränäli, M., Czekaj, V., Jones, R. A. C., Fletcher, J. D., Davis, R. I., Mu, L. & Valkonen, J. P. T. (2009).** Molecular genetic characterization of *Sweet potato feathery mottle virus* (SPFMV) isolates from Easter Island, French Polynesia, New Zealand, Southern Africa. *Plant Disease* **93,** 933-939.

**Scorza, R., Callahan, A., Levy, L., Damsteegt, V., Webb, K. & Ravelonandro, M. (2001).** Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene. *Transgenic Research* **10,** 201-209.

**Shepherd, D. N., Martin, D. P. & Thomson, J. A. (2009).** Transgenic strategies for developing crops resistant to geminiviruses. *Plant Science* **176,** 1-11.

**Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. (2000).** Gene expression: total silencing by intron-spliced hairpin RNAs. *Nature* **407,** 319-320.

**Souto, E. R., Sim, J., Chen, J., Valverde, R. A. & Clark, C. A. (2003).** Properties of strains of *Sweet potato feathery mottle virus* and two newly recognized Potyviruses infecting sweetpotato in the United States. *Plant Disease* **87,** 1226-1232.

**Tairo, F., Mukasa, S. R., Jones, R. A. C. Kullaya, A., Rubaihayo, P. R. & Valkonen, J. P. T. (2005).** Unraveling the genetic diversity of the three main viruses involved in sweet potato virus disease (SPVD), and its practical implications. *Molecular Plant Pathology* **6,** 199-211.

**Tairo, F., Jones, R. A. C. & Valkonen, J. P. T. (2006).** Potyvirus complexes in sweetpotato: Occurrence in Australia, serological and molecular resolution, and analysis of the *Sweet potato virus 2* (SPV2) component. *Plant Disease* **90,** 1120-1128.

**Tricoli, D. M., Carney, K. J., Russell, P. F., McMaster, J. R., Groff, D. W., Hadden, K. C., Himmel, P. T., Hubbard, J. P., Boeshore, M. L. & Quemada, H. D. (1995).** Field evaluation of transgenic squash containing single or multiple virus coat protein constructs for resistance to *Cucumber mosaic virus*, *Watermelon mosaic virus 2*, and *Zucchini yellow mosaic virus*. *Biotechnology* **13,** 1458-1465.

**Ziegler, R. S. (2001).** Agricultural biotechnology, reducing poverty in developing countries. *Plant Disease* **85,** 568-579.

# CONFERENCES TO DATE

**Sivparsad, B.J., and Gubba, A.** The development of transgenic sweet potato (*Ipomoea batatas* L.) with broad virus resistance in South Africa. *University of KwaZulu-Natal Faculty of Science & Agriculture Postgrad Research day*. University of KwaZulu-Natal, Pietermaritzburg, South Africa, 4th November 2008.

**Sivparsad, B.J., and Gubba, A.** The development of transgenic sweet potato (*Ipomoea batatas* L.) with broad virus resistance in South Africa. *University of KwaZulu-Natal School of Biochemistry, Genetics, Microbiology and Plant Pathology Postgrad Research day*. University of KwaZulu-Natal, Pietermaritzburg, South Africa, 6th November 2008.

**Sivparsad, B.J., and Gubba, A.** The development of transgenic sweet potato (*Ipomoea batatas* L.) with broad virus resistance in South Africa. *46th Congress of the Southern African Society for Plant Pathology (SASPP)*. Gordon's Bay, South Africa, 25-28th January 2009.

**Sivparsad, B.J., and Gubba, A.** Phylogenetic analysis of the most prevalent viruses infecting sweet potato (*Ipomoea batatas* L.) in KwaZulu-Natal. *47th Congress of the Southern African Society for Plant Pathology (SASPP)*. Kruger National Park, South Africa, 23-26th January 2011.

# PUBLICATIONS TO DATE

**Sivparsad, B.J. & Gubba, A. (2013).** Identification and distribution of viruses infecting sweet potato (*Ipomoea batatas* L.) in KwaZulu-Natal. *South African Journal of Plant and Soil* (accepted for review in January 2013).

**Sivparsad, B.J. & Gubba, A. (2013).** Development of transgenic sweet potato (*Ipomoea batatas* L.) cv. Blesbok with broad virus resistance in South Africa (SA). *Transgenic Research* (accepted for review in January 2013).

**Sivparsad, B.J. & Gubba, A. (2012).** Development of efficient plant regeneration protocol for sweet potato (*Ipomoea batatas* L.) cv. Blesbok. *African Journal of Biotechnology* **11,** 14982-14987.

**Sivparsad, B.J. & Gubba, A. (2012).** Molecular resolution of the genetic variability of major viruses infecting sweet potato (*Ipomoea batatas* L.) in the province of KwaZulu-Natal in the Republic of South Africa. *Crop Protection* **41,** 49-56.