

TABLE OF CONTENTS	PAGE
PREFACE	v
ABSTRACT	vii
LIST OF TABLES	xi
LIST OF FIGURES	xii
ABBREVIATIONS	xiii
DEDICATION	xvii
CHAPTER 1	1
GENERAL INTRODUCTION	
1.1 Cassava (<i>Manihot esculenta</i> Crantz)	2
1.2 Geminiviruses	7
1.3 The transmission of cassava mosaic virus by the whitefly <i>Bemisia tabaci</i>	17
1.4 Genetic engineering	19
1.4.1 Cassava <i>in vitro</i> regeneration	20
1.4.2 Cassava genetic transformation	25
1.4.2.a <i>Agrobacterium</i> -mediated gene transfer	25
1.4.2.b Particle bombardment	27
1.4.3 Mechanisms of genetic engineering for virus resistance	32
1.4.3.a Coat protein-mediated resistance	33
1.4.3.b Replicase-mediated resistance	34
1.4.3.c RNA-mediated resistance	35
1.5 Objectives and thesis plan	38
1.6 References	40

CHAPTER 2	62
ATTEMPTS TO INDUCE DIRECT ORGANOGENESIS FROM TUBER TISSUE OF CASSAVA (<i>MANIHOT ESCULENTA</i> CRANTZ)	
2.1 INTRODUCTION	63
2.2 MATERIALS AND METHODS	65
2.2.1 Preparation of roots and tuber tissues	65
2.2.2 Plant growth media	66
2.2.3 Microscopy	67
2.3 RESULTS AND DISCUSSION	67
2.3.1 Effects of cytokinin in combination with auxin on callus induction	67
2.3.2 Effect of auxins and cytokinins on tuber organogenesis response	73
2.3.3 Effect of root explants on organogenesis response	75
2.4 REFERENCES	77
CHAPTER 3	82
BIOLISTIC INOCULATION OF CASSAVA (<i>MANIHOT</i> <i>ESCULENTA</i> CRANTZ) WITH SOUTH AFRICAN CASSAVA <i>MOSAIC VIRUS</i>	
3.1 INTRODUCTION	83
3.2 MATERIALS AND METHODS	84
3.2.1 Plasmid constructs	84
3.2.2 Preparation of gold particles	84
3.2.3 Bombardment of SACMV dimers into tobacco and cassava plants	85
3.3 RESULTS AND DISCUSSION	86
3.4 ACKNOWLEDGEMENTS	90
3.5 REFERENCES	91

CHAPTER 4	94
SCREENING OF FOUR SELECTED SOUTH AFRICAN CASSAVA (<i>MANIHOT ESCULENTA</i> CRANTZ) CULTIVARS FOR PRODUCTION OF EMBRYOGENIC TISSUES	
4.1 INTRODUCTION	95
4.2 MATERIALS AND METHODS	97
4.2.1 Culture conditions	97
4.2.2 Plant material	97
4.2.3 Induction of embryogenic tissues	98
4.2.4 Regeneration of <i>in vitro</i> plantlets	98
4.3 RESULTS AND DISCUSSION	99
4.4 ACKNOWLEDGEMENTS	108
4.5 REFERENCES	109
CHAPTER 5	112
OPTIMISATION OF SACMV N-REP TRANSFORMATION EFFICACY USING TOBACCO LEAF DISKS AND CASSAVA FEC	
5.1 INTRODUCTION	113
5.2 MATERIALS AND METHODS	116
5.2.1 Plant material	116
5.2.2 Preparation of the first 600bp N-Rep construct	116
5.2.3 Preparation of the second 621bp N-Rep construct	117
5.2.4 Transformation into <i>A.tumefaciens</i>	118
5.2.5 Transformation of <i>N.tabacum</i> leaf disks	118
5.2.6 Hardening-off	119
5.2.7 Transformation of cassava FEC tissues	120
5.2.8 Molecular analysis of the second 600bp N-Rep construct	121
5.3 RESULTS AND DISCUSSION	121

5.3.1 Cloning of the first 600bp N-Rep construct	121
5.3.2 Expression of the GUS gene in 600bp N-Rep transgenic tobacco plants and pCAMBIA2301-anti-N-Rep (621bp) transformed TMS60444 FEC tissues	122
5.3.3 Molecular characterization of control and anti-N-Rep transgenic tobacco plants	125
5.3.4 Transient viral replication assay	129
5.4 ACKNOWLEDGEMENTS	133
5.5 REFERENCES	134
CHAPTER 6	141
GENERAL CONCLUSIONS	
REFERENCES	146

PREFACE

Wow, it's finally time for me to graduate!

Sincere thanks are expressed to my supervisor Prof MEC Rey. Your regular and prompt feedback and guidance is much appreciated. Your support for my endeavours and believing in me made who I am today. I am also indebted to my adopted co-supervisor Dr VM Gray who made significant contributions particularly during the early phase of my work. I also gratefully acknowledge Prof D Mycock's assistance with the design of the experiments particularly in Chapter 2 of the thesis. Dr D Kieck who was my first main supervisor in the initial stages of my work, although he was unable to see it to the end. His guidance gave me sense to design experiments and interpretation of results.

It brings me profound pleasure to express my heartfelt gratitude to Dr C Fauquet for hosting me at the International Laboratory for Tropical Agriculture (ILTAB), Dr NJ Taylor for supervising the cassava biotechnology work, Dr F Ogbe and Dr J Pita for SACMV challenge work. Other ILTAB members I can't afford to leave out are Denise Peterson, Dr J Yadav, Dr V Ramachandran, Dr A Akano for good company, Dr B Fofana, Dr N Kokora for experiments we did together. Thanks to the President of The Donald Danforth Plant Science Center, Dr RN Beachy and the rest of the team I can't mention all here.

It would be unpardonable of me not to mention Bernadette Delaney and Patricia Cosgrove for their assistance in sorting out my visa, my wife and daughter's visas for our stay in St. Louis, Missouri.

A special thanks to Mrs Margaret Grant for ordering my chemicals at the University of the Witwatersrand and all staff members of the School of Molecular and Cell Biology for their moral support.

Finally, I owe a lot to my parents and family for their moral support without which it would have been difficult to pursue my PhD. My profound thanks to my wife, Melica who has spent most of her time looking after Andani and Mulisa and therefore taking over our household which have sustained me throughout my PhD studies.

With great sadness, I am also indebted to my late uncle Don and his late wife Sophie Makwarela for their never dying moral support and friendship.

This study was undertaken with the financial support of National Research Foundation (NRF), Cassava Starch Manufacturing Company (CSM), Oppenheimer memorial trust and UNESCO which is greatly appreciated.

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is a vegetatively propagated root crop used as a staple throughout the tropics and subtropics. It is the fourth most important and cheapest staple food crop after rice, wheat and maize in developing countries, providing food for over 600 million people. However, its production is severely limited by a wide variety of viral and bacterial diseases, especially Cassava Mosaic Disease (CMD) which is caused by several geminivirus species including, *South African cassava mosaic virus* (SACMV), *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *Indian cassava mosaic virus* (ICMV) and the *Ugandan recombinant virus* (UgV). In South Africa (SA), there has recently been an enormous upsurge of interest in cassava for industrial applications such as the manufacture of starch, animal feeds, and in its potential as a food security crop for marginalised farmers. However, due to serious losses in cassava yields by begomoviruses, such as SACMV, there is an urgent need for the development of appropriate systems that allows for transformation and regeneration of virus-resistant transgenic cassava cultivars suitable for diverse needs and growth requirements in different geographical areas in southern Africa.

The potential application of cassava tuber disks as an alternative system to leaf tissue for transformation and regeneration was investigated. Furthermore, the antibiotic, carbenicillin, was tested as a possible shoot inducing factor. Disks from freshly-harvested cassava tubers were cultured on 25 different sets of MS supplemented with zeatin (0.01-5 mg l⁻¹) and indole-3-acetic acid (0.01-5 mg l⁻¹). Carbenicillin at 500 µg l⁻¹ was included in each treatment as a potential

organogenesis inducing factor. The results observed after 21 days in culture indicated that non-embryogenic friable callus formed readily on MS medium supplemented with MS vitamins, 30 g l⁻¹ sucrose, 0.01 mg l⁻¹ indole-3-acetic acid (IAA), 0.01 mg l⁻¹ zeatin (ZEA), 500 µg ml⁻¹ carbenicillin and 0.8% agar, pH 5.8. Shoots or somatic embryos were never formed and only adventitious roots developed at a frequency of 60% on shoot induction medium supplemented with 2 µM copper sulphate (CuSO₄), 1 mg l⁻¹ 6-benzylaminopurine (BAP) and 0.5 mg l⁻¹ indole-3-butyric acid (IBA).

The current study also investigated infection of cassava and tobacco by the SA begomovirus species SACMV, dimer A and B using the particle inflow gun. Full-length head-to-tail dimers of DNA-A and DNA-B of SACMV were constructed by digestion with *Sa*I or *Eco*RI, respectively. The DNA-coated particles were used to shoot 3-week-old cassava plantlets (cv. TMS60444) at a pressure of 1500 psi using the Bio-Rad biolistic device. Thirty-day-old *N. benthamiana* seedlings were also inoculated in the same manner. In both cases young tender uppermost leaves were targeted (five plants inoculated and another 5 as control). Disease symptoms were recorded daily on the first emerging leaves. Cassava plantlets and tobacco seedlings showed infection by visibility of symptoms. On the other hand, control plantlets that were not inoculated were symptomless. Symptoms appeared 7 dpi in tobacco whereas mosaic symptoms became visible 14 dpi in cassava.

The pre-requisite for any cassava transformation program that proposes to develop improved plants is the availability of a reliable regeneration system. Presently many laboratories that prioritize cassava research are able to reliably

regenerate plants from a limited range of cultivars. Unfortunately, some cultivars are still recalcitrant to induce useful levels of embryogenesis from their tissues. In this study, the production of organized embryogenic structures (OES) from five cultivated southern African regionally important cvs. T200, T400, P4/4, P4/10 and TMS303337 was investigated. A west African cv. TMS60444 was used as a control as it has been adopted as a good model cultivar. By utilizing improved procedures developed at ILTAB for producing embryogenic tissues from various other African cassava cultivars, OES were produced from leaf lobe explants of all the above locally-grown cassava cultivars. South African cvs. T200 and T400 performed well, producing OES at a frequency and quality approaching that of the model cv. TMS60444. Both were shown to be significantly superior for the production of embryogenic structures to the other two SA cvs. P4/4, P4/10 and a Zimbabwean cv. TMS303337.

Genetically-engineered expression of viral gene sequences has been proposed as an efficient system to confer protection against virus diseases by eliciting protection mechanisms in the plant. Our collaboration with ILTAB aimed at transferring cassava transformation techniques to the University of the Witwatersrand by adapting ILTAB cassava transformation and regeneration system into local cassava landraces. We isolated N-terminus truncated replicase (N-Rep) by PCR and transformed both tobacco leaf disks and cassava FEC tissues using these two constructs. Anti-sense N-Rep gene in the pCAMBIA2301 vector was then used to transform both S.A. cassava cv. T200 FEC tissues by microparticle bombardment and tobacco leaf disks by *Agrobacterium* co-cultivation. Hundred and thirty eight tobacco plants transformed with the plasmid

pCAMBIA2301 and eighty four tobacco plants transformed with pCAMBIA2301-anti-N-Rep under the transcriptional control of 35S promoter were obtained from twenty five and thirty three independent leaf disk explants, respectively. The phenotype of these plants was observed as normal. Transformants were further analysed by PCR for the presence of the truncated N-Rep gene. The results of southern blot hybridization analysis of nine transgenic tobacco lines confirmed stable integration of the introduced DNA.

LIST OF TABLES	PAGE
Table 1.1 Cassava production trends in selected regions	7
Table 1.2 Annual cassava production in major African cassava-producing countries	7
Table 1.3 Typical geminiviruses consist of two quasi-isometric subunits with a characteristic 'waist' constriction and pointed ends	12
Table 1.4 Cassava mosaic geminivirus species and strains	13
Table 1.5 Surveys of the incidence of cassava mosaic disease (CMD) in 18 African countries	18
Table 1.6 A summary of methods used in genetic engineering cassava programs.	31
Table 2.1 Effect of combining IAA and ZEA on callus induction from tuber explants of <i>Manihot esculenta</i> Crantz cv. T200 after 4 weeks of culture under light and dark incubations	68
Table 2.2 SIM treatments used for the induction of shoot regeneration from <i>Manihot esculenta</i> Crantz cv T200 tuber disks	69
Table 3.1 Primers used to amplify AC1 N-Rep from both cassava and tobacco infected plants	87
Table 4.1 Amount of organised embryogenic structures produced by <i>in vitro</i> leaf lobe explants on induction medium	101

LIST OF FIGURES	PAGE
Figure 1.1 Cassava small-scale farming for subsistence by Mr. Flawana Masingi of Buyisonto, Bushbuckridge, in Mpumalanga Province	5
Figure 1.2 Mrs. Daina Sekulane's subsistence cassava field approximately 600m north of Mr. Masingi's field	6
Figure 1.3 Typical geminiviruses consist of two quasi-isometric subunits with a characteristic 'waist' constriction and pointed ends	8
Figure 1.4 Schematic representation of different regeneration steps in cassava	23
Figure 2.1 Callus and root induction in <i>Manihot esculenta</i> Crantz cv. T200	70
Figure 3.1 Infection with SACMV	88
Figure 3.2 Symptom development after particle bombardment with SACMV	89
Figure 4.1 The effect of induction medium on the production of organised embryogenic structures from Southern African cassava cultivars	100
Figure 4.2 Organised embryogenic structures produced by immature leaf lobes after 28 days in MS2 medium supplemented with 50µM picloram	102
Figure 4.3 Stages involved in OES production	103
Figure 4.4 Influence of various NAA concentrations and different light regimes on the production of embryogenic structures in three cassava cultivars	104
Figure 5.1 PCR analysis of <i>Agrobacterium</i> C58C1- pCAMBIA2301-pMON999 and C58C1- pCAMBIA2301-anti-N-Rep using N-Rep primers	123
Figure 5.2 Diagrams represent constructs containing fusions between the GUS coding sequence and the AC1 sequence in the sense orientation (pBI121-N-Rep) or in the anti orientation (pBI121-anti-N-Rep)	123
Figure 5.3 Schematic representation of the T-DNA of pCAMBIA2301-anti-N-Rep containing the <i>NptII</i> , anti N-Rep and <i>uidA</i> genes	124
Figure 5.4 Histochemical GUS assay of FEC tissues	127
Figure 5.5 Transformation of TMS60444 FEC tissues with <i>Agrobacterium</i> C58C1- pCAMBIA2301-anti-NRep	128
Figure 5.6 Transformation of <i>N.tabacum</i> leaf disks with <i>Agrobacterium</i> C58C1- pCAMBIA2301-anti-NRep	129
Figure 5.7 Southern blot analysis of genomic DNA of 2 selected transgenic C58C1-pCAMBIA2301-pMON999 and 9 selected C58C1- pCAMBIA2301-anti-NRep tobacco plants	130

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
aa	Amino acid
AC1	Replicase gene
ACMV	African cassava mosaic virus
ACMV-CM	African cassava mosaic virus – Cameroon
ACMV-IC	African cassava mosaic virus – Ivory Coast
ACMV-I	African cassava mosaic virus – India
ACMV-KE	African cassava mosaic virus – Kenya
ACMV-NG	African cassava mosaic virus – Nigeria
ACMV-UG-Mld	African cassava mosaic virus – Uganda (mild)
ACMV-UG-Svr	African cassava mosaic virus – Uganda (Severe)
ARC	Agricultural Research Council
BA or BAP	6-Benzylaminopurine
bp	base pair
°C	Degrees Celcius
CaMV	Cauliflower mosaic virus
CIAT	International Center for Tropical Agriculture, Columbia
cm	centimeter
CMD	Cassava mosaic disease
CP	Coat protein
CR	Core region
cv	cultivar
DI	DeFEctive interfering
DIG	Digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
Dpi	Days post inoculation
ds DNA	double-stranded DNA
EACMV	East African cassava mosaic virus
EACMV-CM	East African cassava mosaic virus – Cameroon

EACMV-KE	East African cassava mosaic virus – Kenya
EACMV-MW	East African cassava mosaic virus – Malawi
EACMV-TZ	East African cassava mosaic virus – Tanzania
EACMV-UG1	East African cassava mosaic virus – Uganda
EACMV-UG-Mld	East African cassava mosaic virus – Uganda (mild)
EACMV-UG-Svr	East African cassava mosaic virus – Uganda (Severe)
EDTA	ethylenediamine tetra-acetic acid
<i>et al.</i>	and others
FAO	Food and Agricultural Organisation of the United Nations
FEC	friable embryogenic callus
Fig./s.	figures
g	gram
GD	Gresshoff and Doy medium
GFP	green fluorescent protein
GM	genetically modified
GUS	β-glucuronidase
h	hour
<i>hpt</i> or <i>hph</i>	hygromycin phosphotransferase gene
IBA	Indole-3-butyric acid
ICMV	Indian cassava mosaic virus
ICTV	International Committee for the Taxonomy of Viruses
IITA	International Institute of Tropical Agriculture
ILTAB	International Laboratory for Tropical Agricultural Biotechnology
IPTG	isopropyl-β-D-galactopyranoside
IR	intergenic region
kan ^R	kanamycin resistance
kb	kilobase
kbp	kilobase pairs
km	kilometres
l	litres
LB	Left border

M	Molar
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
min	minute/s
MP	Movement protein
Mr	molar ratio
mRNA	messenger RNA
MS	Murashige and Skoog
MSV	Maize streak virus
MW	Molecular weight
NAA	α-naphthalene acetic acid
ng	nanograms
nm	nanometres
No.	number
NOS-pro	nopaline synthase gene promoter
NOS-ter	nopaline synthase gene terminator
nt	nucleotide
ORF/s	open reading frame/s
pBKS ⁺	pBluescript KS
PCR	polymerase chain reaction
PEG	polyethylene glycole
pers.comm.	personal communication
pg	picograms
PIG	particle inflow gun
pmoles	picomoles
psi	pounds per square inch
RB	right border

Rep	replicase
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SACMV	South African cassava mosaic virus
SD	Standard deviation
SH	Schenk and Hildebrandt medium
sdH ₂ O	sterile distilled water
sec	seconds
Sp ^R	spectinomycin resistance
ssDNA	single stranded DNA
T-DNA	transferred DNA
TE	Tris-EDTA
Ti-plasmid	tumour-inducing plasmid
TMV	Tobacco mosaic virus
TrAP	Transcriptional activator protein
Tris	Tris(hydroxymethyl)aminomethane
USA	United States of America
Vir	virulence
v/v	volume per volume
WTGs	Whitefly-transmitted geminiviruses

DEDICATION

To my parents, wife and two daughters