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Optimisation of regeneration systems for a range of Cassava (Manihot Esculenta Crantz) cultivars suitable for growth in South Africa and transformation with SACMV N-REP gene

Makwarela, Murunwa

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PREFACE

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

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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 mgl^{-1}) and indole-3-acetic acid (0.01-5 mgl^{-1}). Carbenicillin at 500 μgl^{-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 gl^{-1} sucrose, 0.01 mgl^{-1} indole-3-acetic acid (IAA), 0.01 mgl^{-1} zeatin (ZEA), 500 μgml^{-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 2μM copper sulphate ($CuSO_4$), 1 mgl^{-1} 6-benzylaminopurine (BAP) and 0.5 mg^{-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 *Sal*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.

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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
et al.	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
hpt or *hph*	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	millitre
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_2O	sterile distilled water
sec	seconds
Sp^R	spectinomycin resistance
ssDNA	simgle 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