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Evaluation of virus resistance and storage root yield in cassava germplasm

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Evaluation of virus resistance and storage root yield in cassava germplasm

by

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Dissertation

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Advisor: Prof. G. Bouwer

June 2022

Declaration

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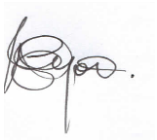
Signature: 

13/06/2022

Abstract

Cassava mosaic disease (CMD) remains the biggest challenge to cassava cultivation and deployment of CMD resistant landraces has been one of the key strategies to minimize the spread of CMD in open fields and avert losses. The aim of the project was to screen drought-tolerant South African cassava germplasm for responses to *South Africa cassava mosaic virus* (SACMV) challenge and evaluate previously transformed cassava lines, where model cultivar cv. 60444 was transformed with a hairpin DNA cassette transcribing a hairpin RNA (hp-RNA) targeting either plastidial *adenylate kinase* (*ADK*) or *uridine monophosphate synthase* (*UMPS*) under the control of a patatin root promoter in an attempt to increase starch production and storage root yield. Evaluation of infectivity in cassava germplasm indicated that Ukulinga 12 genotype was susceptible to SACMV, showing severe CMD symptoms and high viral load. Ukulinga 2 exhibited high levels of resistance to SACMV, and the CMD symptoms emerged very late post 32 days post inoculation (dpi) and remained mild, with low viral load. Ukulinga 8 germplasm did not exhibit any symptoms and viral load was minimal for the duration of the study. There was a weak Pearson's correlation (r=0.37) between mean symptom severity index and viral load. Two trials were conducted in different growth environments to evaluate increased starch content and storage root yield. The majority of control and transformed plants from trial 1 did not produce storage roots, and a few plants produced small (below 2g) storage roots whilst trial 2 plants produced a high number of average to large ($2g \leq x \leq 5g$) storage roots. There was statistically significantly ($p<0.05$) increase in percentage dry weight (29.4 ± 1.0%) of storage roots from UMP 26 line in comparison to untransformed cv. 60444 (22.8 ± 1.6%). Both ADK 5 and UMP 26 lines produced a significant increase ($p<0.05$) in total percentage starch content of storage roots of (32.3 ± 0.6g/100g and 40.2 ± 1.3g/100g, respectively), while cv. 60444 control had 27.9 ± 1.3g/100g. UMP 28 line had the least productivity, producing the least number of storage roots per plant, the lowest starch content and least percentage dry weight of storage roots. Principal component 1 (PC1) had standardized variation of 98.4% and 93.3% in trial 1 and 2 respectively and plant height was the biggest contributor to the separation of genotypes in both trials. ADK 5 line produced the highest total yield (53.9g) in all of trial 2 tested genotypes, in comparison to the high-yielding T200 landrace and model cv. 60444 (46.3g and 18.3g respectively). The correlation between total starch and percentage dry weight of the storage roots from trial 1 was ($r=0.63$, $p=8.4 \times 10^{-6}$) compared to ($r=0.3$, $p=0.042$) for trial 2. The expression

of *ADP-glucose pyrophosphorylase* (*AGPase*) was found to be significantly upregulated in all transgenic lines tested in both trials compared to non-transgenic and wild-type controls. *ATP/ADP antiporter* (*AATP1*) expression was significantly downregulated in all but one (ADK 1) of the transgenic lines from trial 1 and significantly downregulated in all the tested transgenic lines in trial 2. The identification of local CMD resistant genotypes and high-yielding transgenic lines in this study is another step towards delivering locally-adapted, yield-improved and CMD resilient cassava genotypes that will offer greater opportunities and ease of adoption for full-scale commercial utilization in response to existing and future demands in the starch and biofuel energy industrial sectors.

Dedication

To my most beloved

grandfather

Miwuwe Kasembe

Research Outputs

E.E Nyoni and M.E.C Rey: (Poster Presentation) Response of local cassava germplasm to infection by *South African cassava mosaic virus*. South African Society of Microbiology Virtual Conference, 2021

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Table of contents

Declaration i

Abstract ii

Dedication iv

Research Outputs v

Acknowledgements vi

Table of contents vii

List of figures xi

List of tables xv

List of supplementary data xvi

List of abbreviations xvii

Chapter 1: Literature Review 1

1.1 Cassava, the crop 1

1.1.1 Origin and distribution of cassava 1

1.1.2 Importance and utilization of cassava 1

1.1.3 Cassava production 3

1.1.4 Constraints to cassava productivity 4

1.2 Cassava mosaic disease 5

1.2.1 Classification of cassava mosaic begomoviruses 5

1.2.2 Transmission and host range 6

1.3 Genetic Engineering 8

1.3.1 Genetic engineering of cassava 8

1.3.2 Genetic engineering of cassava for desirable agronomic traits 9

1.4 Cassava starch biosynthesis 10
1.4.1 Cassava starch biosynthetic genes of interest in this study 13
1.5 Structure and composition of cassava starch..... 13
1.6 Cassava starch and yield improvement 14
1.6.1 Strategies to increase source metabolism 15
1.6.2 Strategies to increase sink metabolism..... 15
1.6.3 Strategies to simultaneously increase source and sink metabolism 18
1.7 Storage root formation 19
1.8 Background and generation of transgenic cassava starch lines..... 21
Chapter 2: Rationale of study 24
2.1. Aim of the study..... 26
2.2. Objectives..... 26
Chapter 3: Materials and methods 28
3.1 Objective 1: Evaluation of drought-tolerant Ukulinga cassava germplasm for cassava mosaic disease (CMD) resistance or susceptibility..... 28
3.1.1 Micropropagation and acclimatization of Ukulinga germplasm 29
3.1.2 *Agrobacterium* inoculation of plants 29
3.1.3 Cassava mosaic disease symptom development and assessment 30
3.1.4 Total nucleic acid extraction..... 30
3.1.5 Determination of viral load by real-time quantitative PCR..... 31
3.1.6 Experimental design 31
3.2 Objective 2: Evaluation and comparison of storage root yield of three cassava transgenic cassava lines 32
3.2.1 Characterization of transgenic lines 33
3.2.2 Micropropagation and acclimatization of ADK and UMP transgenic plants..... 33
3.2.3 Cassava storage root weight and amylose/ amylopectin content 33

3.2.4 Starch determination and microscopy 34
3.2.5 RNA extraction and cDNA synthesis 34
3.2.6 Real-time quantitative PCR 35
3.2.7 Experimental design 35
Chapter 4: Results 37
4.1 Evaluation of drought-tolerant Ukulinga cassava germplasm for response to SACMV 37
4.1.1 Symptom severity evaluation 37
4.1.2 Cassava plant height evaluation 41
4.1.3 Cassava plant viral load 42
4.2 Evaluation of transgenic cassava starch lines for storage root yield 45
4.2.1 Molecular screening of transgenic cassava starch lines 45
4.2.2 Cassava plant height 46
4.2.3 Storage roots production and characteristics 47
4.2.4 Storage root yield 51
4.2.5 Gene expression study 58
4.2.6 Pearson's correlation 62
4.2.7 Principal component analysis (PCA) 63
Chapter 5: Discussion 66
5.1 CMD resistance 66
5.2 Pot trials on storage root yield 70
5.2.1 Plant Growth Conditions 70
5.2.2 Gene expression of *AATPI* and *AGPase* associated with starch biosynthesis pathway efficiency 71
5.2.3 Starch content in UMP and ADK lines 73
5.2.4 Morphological analyses of starch granules 74

Chapter 6: Conclusion..... 76

References..... 78

Supplementary data..... 96

List of figures

Figure 1.1: Cassava starch derivatives and their uses (Sanginga, 2015) 3

Figure 1.2: Cassava yield per unit area across the world (FAO, 2016) 4

Figure 1.3: Genome organization of bipartite geminivirus. The ORFs (AV1, AC1, AC2, AC3, AV1, BC1, and BV1) are colour-coded according to the function of their protein products (rep, replication-associated protein; ren, replication enhancer protein; trap, transcriptional activator protein; cp, capsid protein; mp, movement protein; nsp, nuclear shuttle protein). LIR, long intergenic region; SIR, short intergenic region; CR, common region. The hairpin which includes the origin of replication is indicated in the LIR (Zerbini et al., 2017). 6

Figure 1.4: Cassava plants agro-inoculated with *South African cassava mosaic virus* showing leaf curling and mosaic developmental defects associated with CMD at 32 days post infection (dpi). 7

Figure 1.5: Starch synthesis pathway in vascular plants (Lloyd and Kossmann, 2019)..... 13

Figure 1.6: *De novo* biosynthetic pathway of pyrimidine nucleotides in plants. Enzymes shown are: (1) *carbamoyl phosphate synthase* (*CPS*), (2) *aspartate transcarbamoylase*, (3) *dihydroorotase*, (4) *dihydroorotate dehydrogenase*, (5)-(6) *UMP synthase* (*orotate phosphoribosyltransferas*e plus *orotidylate decarboxylase*) (Stasolla et al., 2003). 16

Figure 1.7: Salvage reactions of uracil pyrimidine and nucleosides in plants. Enzymes shown are: (1) *uracil phosphoribosyltransferase (UPRT)*, (2) *uridine kinase (U*K), (3) non-specific *nucleoside phosphotransferase*, (7) *uridine phosphorylase*, (8) *uridine nucleosidase* (Stasolla et al., 2003). 18

Figure 1.8: Cross-section of phloem and xylem cells in fibrous root and developing storage roots. Ex-exodermis; P-phloem; PC-procambium; Per-periderm; VC-vascular cambium; X-xylem. Arrowheads denote vascular rays (Mehdi et al., 2019; Sonnewald et al., 2020). 20

Figure 1.9: Model of cassava assimilates transport and potential targets for biotechnological yield improvement. H^+- proton; Suc - sucrose; Frc - fructose; *SUS* - *sucrose synthase*; UDP-Glc - UDP-glucose (Mehdi et al., 2019) 21

Figure 1.10: Schematic representation of the hairpins. (A) *Adenylate kinase (ADK)* (831bp) hairpin, and (B) *Uridine monophosphate synthase (UMPS)* (1087bp) hairpin. The forward and reverse arms of the hairpins are separated by an 81bp intron. Each hairpin is flanked by the

patatin root promoter (B33) and an *octopine synthase* transcription terminator (OCS). The hairpins were each separately inserted into the vector pCB33 at the MCS site (Walsh, 2019).... 22

Figure 2.1: South Africa starch industry (a) Top importers; (b) trade balance sheet (Phaleng, 2017) 26

Figure 3.1: Workflow diagram for the evaluation of Ukulinga cassava germplasm for response against cassava mosaic geminiviruses 28
Figure 3.2: The modified symptom severity index to determine symptom severity score of plants infected with *South African cassava mosaic virus.* On the scale 0: Healthy (no symptoms), 1: Light mosaic and curling, 2: Medium mosaic curling and 3: Severe mosaic curling with reduction in leaf size 30
Figure 3.3: Workflow diagram for screening and evaluation of transgenic cassava lines for storage root yield..... 32

Figure 4.1: Cassava plant genotypes agro-infected with infectious clones of South African cassava mosaic virus showing cassava mosaic disease (CMD) symptom severity at 32 dpi. 38
Figure 4.2: The mean symptom severity scores in cv. 60444, TME3, Ukulinga 2, Ukulinga 8 and Ukulinga 12 germplasm. Values indicate means of biological replicates with 6 plants per treatment. Bars indicate mean standard error and letters indicate the results of Tukey HSD test comparing values among the timepoints in the same genotypes ($p<0.05$). 39
Figure 4.3: Cassava plant genotypes agro-infected with South African cassava mosaic virus showing CMD symptom severity at 65 dpi. 40
Figure 4.4: The mean change in height (plant growth) in healthy and mock inoculated plants compared to SACMV-infected cassava plants for each cassava genotype. Values indicate means of three trials with 6 plants per treatment. Bars indicate mean standard error and letters indicate the results of Tukey HSD test comparing values among treatments in the same genotype ($p<0.05$)..... 42
Figure 4.5: The viral load relative to reference gene *UBQ10* in model cultivar cv. 60444, Ukulinga 2, Ukulinga 8 and Ukulinga 12 and TME3 germplasm infected with South African cassava mosaic virus at 14 dpi, 32 dpi and 65 dpi. Error bars indicate mean standard error and

letters indicate the results of Tukey HSD test comparing values among genotypes in the same timepoint ($p<0.05$). 43
Figure 4.6: qPCR amplification products viral coat protein (AV1) loaded on a 1% (w/v) agarose gel of cassava genotypes at three time points; 14 dpi, 32 dpi and 65 dpi. M is the GeneRuler 1kb Plus DNA Ladder molecular weight marker, NTC is the non-template control and AGL is the negative control (mock inoculated). 44
Figure 4.7: Correlation among the relative viral load, mean CMD symptomatic score and time points post SACMV infection. 45
Figure 4.8: PCR detection of *Gus* (191 bp) and *Hyg* (498 bp) in genomic DNA extracted from transgenic cassava starch lines (ADK 1, ADK 5, UMP 17, UMP 26 and UMP 28). The GeneRuler 1 kb Plus molecular weight marker (M) (Thermo Fisher Scientific) was loaded on the first and eleventh lane and NTC is the non-template control containing deionised water. (+) GUS and (+) HYG are the positive controls containing the pCB33. 46
Figure 4.9: The average plant height of cassava genotypes over 6 months. Error bars indicate mean standard error and letters indicate the results of Tukey HSD test comparing values among genotypes in the same month ($p<0.05$). 47
Figure 4.10: Morphology and size of starch granules in homogenized storage root tissue of various cassava genotypes stained with 50% (v/v) Lugol's solution 50
Figure 4.11: Storage root morphology and harvest yield from greenhouse facility-grown transgenic cassava lines, cv. 60444 and T200 landrace in trial 2. 51
Figure 4.12: The total cassava storage root yield (a) from the controlled conditions growth chamber facility and (b) from the greenhouse facility. Each bar represents the total overall weight of all the pooled storage roots produced by each genotype. 53
Figure 4.13: Transverse (a) and longitudinal (b) sections of storage root disks from different cassava genotypes stained with 50% (v/v) Lugol's solution to visualize presence of starch 55
Figure 4.14: Thin hand-cut cross section of storage roots fresh tissue stained with 50% (v/v) Lugol's solution to visualize starch granule density in various cassava genotypes. 56
Figure 4.15: Cross section of secondary storage roots showing phloem and xylem cells in cassava storage root of various genotypes stained with 0.1% (w/v) toluidine. X - xylem, p - phloem, pc - procambium, arrowhead denotes phloem sieve plates (PSPs). 57

Figure 4.16: Relative gene expression of starch biosynthesis-associated genes; *ATP/ADP antiporter* (*AATP1*), *adenine diphosphate glucose pyrophosphorylase* (*AGPase*) and pyrimidine biosynthesis-linked gene *carbamoyl phosphate synthas*e (*CPS*) of mature cassava storage roots in trial 1. 60
Figure 4.17: Relative gene expression of starch biosynthesis-associated genes; *ATP/ADP antiporter* (*AATP1*), *adenine diphosphate glucose pyrophosphorylase* (*AGPase*) and pyrimidine biosynthesis-linked gene *carbamoyl phosphate synthas*e (*CPS*) of mature cassava storage roots in trial 2. 61
Figure 4.18: Scatter plot with trend line displaying the correlation (Pearson's) between total starch and percentage dry weight of storage roots from trial 2 62
Figure 4. 19: Scatter plot with trend line displaying the significant Pearson's correlation between total starch and percentage dry weight of storage roots from trial 1 63
Figure 4. 20: Principal component analysis (PCA) of storage root traits and plant height in trial 2 and respective loading points including the variables that contributed the most in the separation in PC1 and PC2. 64
Figure 4. 21: Principal component analysis (PCA) of storage root traits and plant height measured in trial 1, and respective loading points including the variables that contributed the most in the separation in PC1 and PC2. 65

List of tables

Table 1.1: Nine cassava mosaic geminiviruses species described to date (Fondong, 2017) 5

Table 3.1: Primers used for relative expression of starch biosynthesis-associated genes 36

Table 4.1: Characteristics of storage roots produced from the transgenic cassava starch lines grown in the greenhouse facility..... 49

List of supplementary data

Figure *S1:* The percentage of cassava plants from each genotype that developed systemic CMD symptoms after infection with SACMV. 96

Figure *S2*: Morphology of apex cassava leaves from different cassava genotypes, and their corresponding petioles lengths, before inoculating with the infectious SACMV clones. 97

Figure *S3*: Mid-stem cuttings from different cassava genotypes showing internodal length at 65 dpi. 98

Figure *S4*: Boxplot of the distribution of petiole and internodal lengths in various cassava germplasm and in the model cultivar cv. 60444. The bottom whisker denotes the lower extreme, the top whisker denotes the upper extreme. The bottom and top of the box denote the lower quartile and upper quartile respectively, and the middle of the box is the median..... 98

Figure *S5*: Canopy and leaf loss (senescence) from different cassava transgenic lines and landrace grown in the greenhouse facility at 4 MAP; h is the height from the ground to the lowest node-bearing leaf. 99

Figure *S6*: Storage root morphology and yield harvest from controlled conditions growth chamber facility-grown transgenic cassava lines, cv. 60444 and T200 landrace in trial 1..... 100

List of abbreviations

AATP1:	*ATP/ADP antiporter*
AC1:	Replication-associated protein
AC2:	Transcriptional activator protein
AC3:	Replication enhancer protein
AC4:	Putative viral suppressor protein
ACMV:	African cassava mosaic virus
ADK:	*Adenylate kinase*
ADP:	Adenosine diphosphate
ADP-glucose:	Adenosine diphosphate-glucose
AGO:	Argonaut protein
AGPase:	*ADP-glucose pyrophosphorylase*
AMP:	Adenosine monophosphate
ANOVA:	Analysis of variance
ATP:	Adenosine triphosphate
AV1:	Coat Protein
AV2:	Pathogenicity determinant
B33:	Patatin class I promoter
BC1:	Movement protein
BV1:	Nuclear shuttle protein
CaMV:	Cauliflower mosaic virus

Cas9:	CRISPR-associated 9 protein
cDNA:	Complementary DNA
CMD:	Cassava mosaic disease
CMG:	Cassava mosaic geminivirus
CPS:	*Carbamoyl phosphate synthase*
CRISPR:	Clustered regularly interspaced short palindromic repeats
cv.:	Cultivar
DCL:	DICER-like proteins
dpi:	Days post inoculation
DNA:	Deoxyribonucleic acid
DRB:	dsRNA binding proteins
dsDNA:	Double-stranded DNA
dsRNA:	Double-stranded RNA
EACMV:	East African cassava mosaic virus
g:	Gram
g	Gravity
GOPOD:	Glucose oxidase plus peroxidase
GBSS:	*Granule bound starch synthase*
h:	Hour
hpRNA:	Hairpin RNA
IR:	Intergenic region

LAI:	Leaf area index
MAP:	Months after planting
mg:	Milligram
miRNA:	MicroRNA
min:	Minute
mM:	Millimolar
MS20:	Murashige and Skoog with 20g/L sucrose
ng:	Nanogram
nt:	Nucleotide
OD_{600}	Optical density (600 nanometres)
ORF:	Open reading frame
PCR:	Polymerase chain reaction
PTGS:	Post-transcriptional gene silencing
qPCR:	Quantitative PCR
QTL:	Quantitative trait locus
ren:	Replication enhancer protein
RISC:	RNA-induced silencing complex
RDR:	RNA-dependent RNA polymerases
Rep:	Replication associated protein
RNA:	Ribonucleic acid
RNAi:	RNA interference

SACMV:	South African cassava mosaic virus
siRNA:	Small interfering RNA
SNP:	Single nucleotide polymorphism
SPS:	Sucrose phosphate synthase
SSR:	Simple sequence repeats
SSS:	*Soluble starch synthase*
TGS:	Transcriptional gene silencing
TME:	Tropical *Manihot esculenta*
TMS:	Tropical Manioc Selection
TNA	Total nucleic acid
UBQ10:	*Ubiquitin 10*
UDP-glucose:	Uridine diphosphate glucose
UK:	*Uridine kinase*
UMPS:	*Uridine monophosphate synthase*
UPRT:	*Uracil phosphoribosyltransferase*
vsRNA:	Viral small RNA
v/v:	Volume for volume
v/w:	Volume for weight
µg:	Microgram
µM:	Micromolar
µl:	Microlitre

Chapter 1: Literature Review

1.1 Cassava, the crop

1.1.1 Origin and distribution of cassava

Cassava (*Manihot esculenta* Crantz) is a woody shrub of the *Euphorbiaceae* family and is a staple diet to a billion people in over a hundred countries (Chetty et al., 2013; El-Sharkawy, 1993). It is native to South America (Nassar, 2007) and was introduced to Africa by the Portuguese traders around the 16th century, subsequently reaching East Africa in late 18th century (Legg and Thresh, 2000). Cassava has been extensively and almost exclusively grown by smallholder farmers for its starch-rich storage roots in isolated areas where soils are infertile and rainfall patterns are erratic (Thro et al., 1998). Cassava was introduced into South Africa by the Tsonga tribesmen, but it is not known exactly when this occurred. It is grown marginally as a secondary crop alongside the usual maize staple crop as a means of supplementing and fortifying food security, mainly in isolated provinces of northern Kwazulu Natal, Limpopo and Mpumalanga (Phaleng, 2017).

1.1.2 Importance and utilization of cassava

Cassava provides the highest dietary energy per unit area than any other staple crop. It is ranked the fourth most vital staple food crop after rice, wheat and maize in developing countries (Taylor et al., 2004). Its starchy roots contribute over a quarter of the daily calorie intake to some rural less resourced communities of sub-Saharan Africa (FAO, 2021). However, cassava utilization varies geographically, for instance in far-east Asia it is chiefly cultivated for biofuel provision (Maziya-Dixon et al., 2007). Favourable characteristics of cassava encompass affordable readily available planting material to low-income subsistence farmers, flexible harvest time, significant yield harvest under low rainfall and marginal soil fertility. Also, it has the ability to withstand acidic soils and its symbiotic relationship with soil fungi assists in the uptake of phosphorus and micronutrients (Sonnewald et al., 2020). Climatic change as a consequence of global warming is forecast to have a negative impact on a vast majority of cultivated staple crops (Jarvis et al., 2012). As a C_3 crop, increased CO_2 will benefit cassava productivity more than C_4 crops such as maize (*Zea mays*), millet (*Pennisetum glaucum*) and sorghum (*Sorghum color*) (Rosenthal et al.,

2012; Roudier et al., 2011). This further exemplifies the critical role cassava will assume in the future to guarantee food security, more so in the sub-Saharan Africa region which has been projected to have more than a 2-fold increase in population by 2050 (United Nations, 2019).

The status of cassava cultivation today is changing from subsistence farming to an industrialized agro-system designed to process cassava into a diverse spectrum of products (Baguma et al., 2017). In the South African context, there has been a lag, but more recently private-government partnership engagements have been established to fully harness the untapped potential in commercialising the crop. The Industrial Development Corporation (IDC) is currently developing a strategy to commercial cassava cultivation for a range of industrial products (Industrial Development Corporation, 2017). Local commercial entities Enterprise Foods (Pty) Ltd and Lucky Star (Pty) Ltd both incorporate cassava starch as a binder in sausages and a thickener in sauces of tinned fish products respectively. The sausages are famously known to possess a shelf life of six times longer than those that have maize and wheat starch as a binding (Mudombi, 2010). Furthermore, the global conglomerate AB InBev (formerly SABMiller) piloted the brewing of cassava lager beer in 2011 (https://www.dailymaverick.co.za/article/2011-11-02-sabmiller-launches-entry-level-cassava-beer/). In addition to being a major source of dietary carbohydrates in Africa, cassava starch also has an excellent potential as livestock feed, in textiles, plywood and paper industry, brewing, chemical, biofuels and pharmaceutical industries (**Figure 1.1**) (Breuninger et al., 2009; Tonukari, 2004). The different applications of cassava starch rely on its granule structure and crystalline nature which exert influences on the physicochemical properties (Nuwamanya et al., 2010).

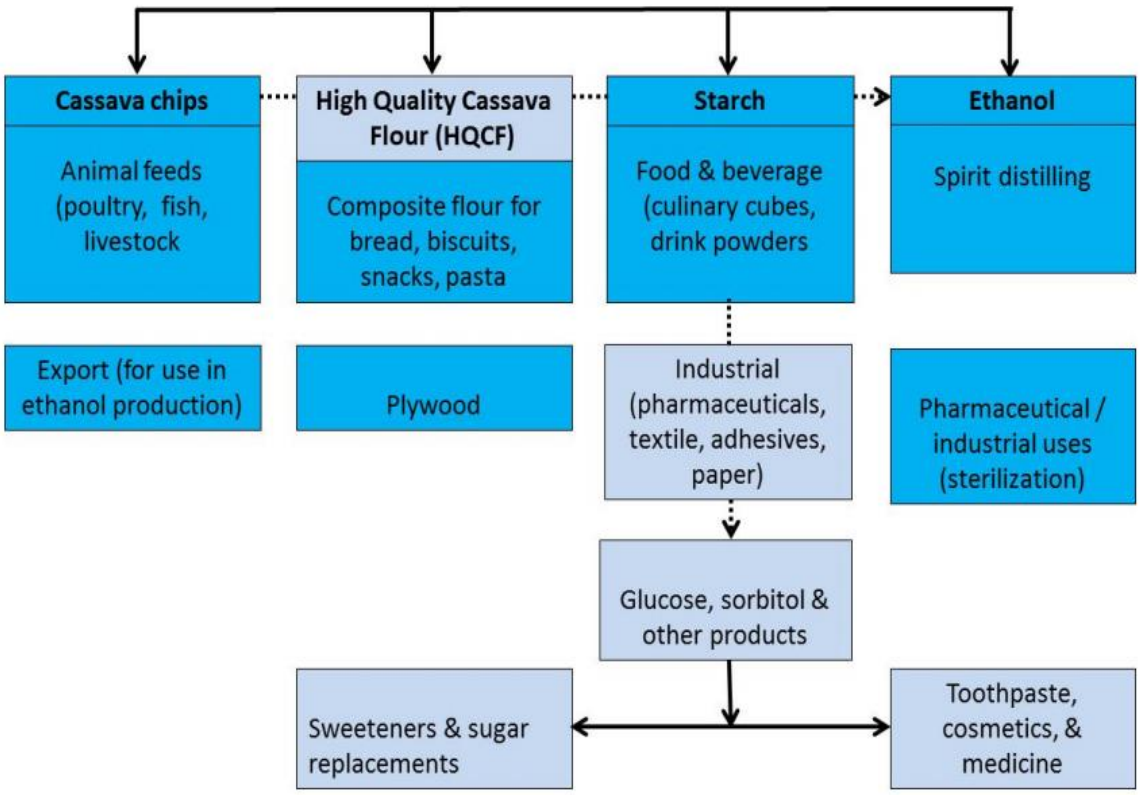


Figure 1.1: Cassava starch derivatives and their uses (Sanginga, 2015)

1.1.3 Cassava production

Cassava production requires minimal inputs, has high return on investment giving significant harvests where other staple crops demonstrate low yields (FAO, 2016). The sub-Saharan Africa region cultivates just over 75% of the total world cassava area, resulting in 60% total yield of the world (FAO, 2019). Furthermore, in this region yields have been on a declining trend, exacerbated by poor agronomic practices, dwindling arable land and biotic factors. The average yields of African smallholder farmers are below the world standard and 2.5 times below the yields achieved in far east-Asia (**Figure 1.2**) (FAO, 2016).

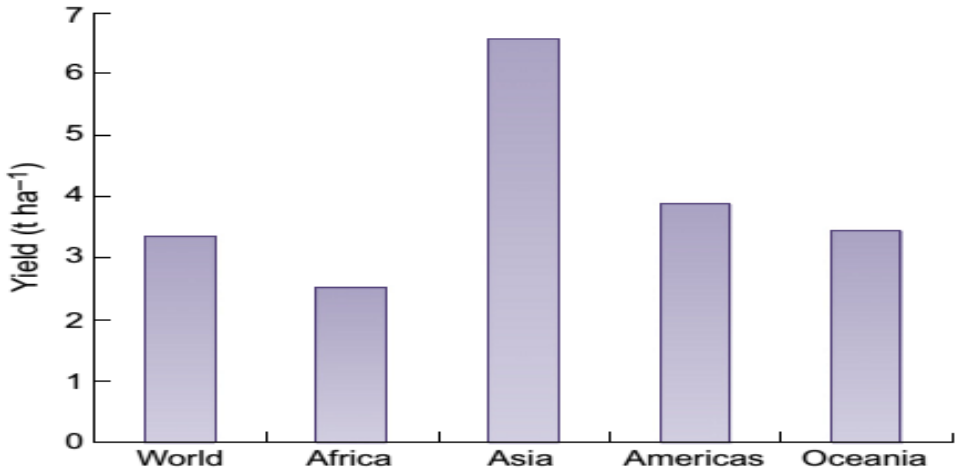


Figure 1.2: Cassava yield per unit area across the world (FAO, 2016)

1.1.4 Constraints to cassava productivity

Cassava mosaic disease (CMD) is amongst the critical diseases limiting cassava productivity in Africa (Fondong, 2017; Houngue et al., 2019; Rey and Vanderschuren, 2017), and can be traced to all cassava growing regions other than in South America (Patil and Fauquet, 2009; Rey and Vanderschuren, 2017). CMD contributes to loss of billions of dollars in farmers' revenue, (Scholthof et al., 2011), where in excess of 82% production loss has been cited in some recent case studies (Graziosi et al., 2016). The incidence and severity of CMD varies between countries, regions, cultivar and virus type (Houngue et al., 2019; Rey and Vanderschuren, 2017; Sseruwagi et al., 2004). Despite the potential socio-economic importance of cassava, there has been little impetus from public-private-partnerships to invest in research and development of cassava productivity enhancement in comparison to commodity crops such as rice, wheat, maize, and potatoes (Ceballos et al., 2004). New biotechnologies will be needed to address cassava production constraints that hinder commercial viability.

Table 1.1: Nine cassava mosaic geminiviruses species described to date (Fondong, 2017)

CMG	Occurrence
African cassava mosaic virus (ACMV)	African continent
Cassava mosaic Madagascar virus (CMMGV)	Madagascar
East African cassava mosaic Kenya virus (EACMKV)	Eastern and Africa
East African cassava mosaic Malawi virus (EACMMV)	Eastern and Southern Africa
East African cassava mosaic virus (EACMV)	Eastern Africa
East African cassava mosaic Zanzibar virus (EACMZV)	Eastern Africa
Indian cassava mosaic virus (ICMV)	Indian Subcontinent
South African cassava mosaic virus (SACMV)	Southern Africa
Sri Lankan cassava mosaic virus (SLCMV)	Indian Subcontinent

1.2 Cassava mosaic disease

1.2.1 Classification of cassava mosaic begomoviruses

Cassava mosaic disease is caused by the cassava mosaic geminiviruses (CMGs) of the family *Geminiviridae* and genus *Begomovirus* (Patil and Fauquet, 2009) which are regarded as amongst the most damaging vector-borne plant pathogens (Houngue et al., 2019; Kuon et al., 2019). The *Begomovirus* genus comprises of 388 species and is the largest of the nine described genera (Rojas et al., 2018; Zerbini et al., 2017). The genomes of CMGs are bipartite, with two single stranded circular DNAs (A and B) encapsulated in twinned icosahedral particles. DNA-A encodes proteins necessary for viral replication, transcription, virus assembly and pathogenicity and DNA-B component has 2 open reading frames (ORFs) necessary for intracellular movement and intercellular transport (**Figure 1.3**) (Castillo et al., 2004; Chellappan et al., 2005; Gutierrez, 1999; Zerbini et al., 2017). To date, seven of the nine reported species of CMGs are endemic in sub-Saharan Africa and two are found on the Indian sub-continent (**Table 1**) (Rey and Vanderschuren, 2017; Patil and Fauquet, 2009, 2015). CMD was identified as early as 1894 in Tanzania (Lin et al., 2019) and of interest to this particular study, the first molecular characterization of South African cassava mosaic virus (SACMV) was reported in 1998 (Berrie et al., 1998).

Advancement in molecular and next generation sequencing tools allows continual identification of a wide spectrum of strains and variants of CMGs possessing significant sequence and biological differences in the endemic regions of sub-Saharan African and in the Indian continent (Brown et al., 2015; Legg et al., 2014).

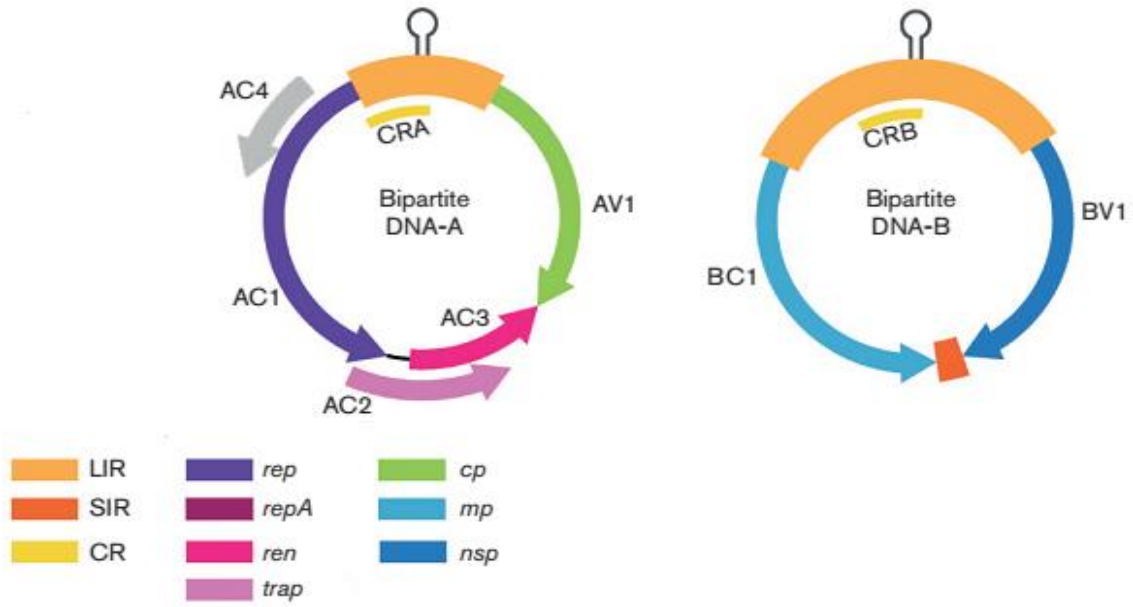


Figure 1.3: Genome organization of bipartite geminivirus. The ORFs (AV1, AC1, AC2, AC3, AV1, BC1, and BV1) are colour-coded according to the function of their protein products (rep, replication-associated protein; ren, replication enhancer protein; trap, transcriptional activator protein; cp, capsid protein; mp, movement protein; nsp, nuclear shuttle protein). LIR, long intergenic region; SIR, short intergenic region; CR, common region. The hairpin which includes the origin of replication is indicated in the LIR (Zerbini et al., 2017).

1.2.2 Transmission and host range

CMGs are transmitted by whitefly (*Bemisia tabaci* Gennadius) and through propagation of infected stem cuttings when preparing for next cropping season (Legg and Fauquet, 2004). In the open field, geminivirus species can induce CMD in cassava plants either in single or mixed infections resulting in severe disease (Berry and Rey, 2001). When infections are coupled with recombination, they may lead to the emergence of novel virus species and strains (Hanley-

Bowdoin et al., 2013). Cassava mosaic disease symptoms present as irregular yellow-green chlorotic mosaic patterns of leaves, curly leaves, stunted growth and reduced storage root yield due to compromised photosynthetic capacity (**Figure 1.4**) (Narayanan et al., 2021). In highly vulnerable cultivars, complete devastation of the above ground biomass and storage root losses may occur (Masinde et al., 2016). The deployment of resistant varieties has been one of the most effective strategies to combating the negative effects of CMD in Africa (Akano et al., 2002).



Figure 1.4: Cassava plants agro-inoculated with *South African cassava mosaic virus* showing leaf curling and mosaic developmental defects associated with CMD at 32 days post infection (dpi).

There are three sources of cassava plant host resistance and tolerance mechanisms observed to protect against CMD (Beyene et al., 2015). The first mechanism was introgressed from *Manihot glaziovii* Muell. Arg. (ceara rubber) was designated CMD1 and is polygenic recessive loci (Hahn et al., 1980). The CMD2 form of resistance is monogenic dominant and is endemic in West African cassava landraces mostly the Tropical *Manihot esculenta* (TME) series (Akano et al., 2002; Okogbenin et al., 2013). Of interest to this study, the TME3 genotype is known to habour the tolerance-recovery phenotype to a range of CMG species particularly SACMV (Allie et al., 2014; Louis and Rey, 2015). CMD2 resistance is employed in breeding programs as it is

easily inheritable and imparts tolerance to several CMG species (Rabbi et al., 2014). Plants harbouring CMD1 or CMD2 resistance initially become susceptible exhibiting classical CMD symptoms, but subsequently are able to show recovery and fend off the viral attack entirely resulting in asymptomatic newly formed leaves (Okogbenin et al., 2013). The third resistance mechanism, CMD3 is a quantitative trait locus (QTL) providing greater resistance to CMD with minor or no disease symptoms observable on the plants (Okogbenin et al., 2012). The particular molecular mechanisms governing the three disease resistances remain a grey area.

1.3 Genetic Engineering

1.3.1 Genetic engineering of cassava

Cassava is an allotetraploid demonstrating high heterozygosity which, alongside low fertility and unsynchronised flowering results in cumbersome and laborious traditional breeding techniques (Ceballos et al., 2004; Lentz et al., 2018). The high heterozygous nature of cassava crop makes it a challenging prospect to choose parents with desirable traits for breeding purposes. As an alternative, genetic engineering provides a potential for cassava crop trait enhancement (Liu et al., 2011). Developing resistant varieties through genetic engineering is amongst the most robust, cost effective and long term solution of mitigating and controlling disease outbreaks and suppressing viral proliferation in the farming ecosystem (Rabbi et al., 2014). Transgenic interventions are favoured as they evade the trait separation that is encountered in conventional breeding. Cassava transformation is extremely difficult, but the technology of *Agrobacterium*-mediated methods have over time made great progress (Bull et al., 2009). More recently, the technology has been extended from the model cultivar cv.60444 to include recalcitrant farmer-preferred landraces (Elegba et al., 2021; Lentz et al., 2018). It has opened new avenues where desirable traits such as viral resistance, mineral biofortification in the roots and leaves, waxy cassava starch, polyploidy induction, and glyphosate-tolerant cassava have each been explored and integrated into the cassava plants (Walsh et al., 2019; Chavarriaga-Aguirre et al., 2016; Chen et al., 2021; Narayanan et al., 2020).

1.3.2 Genetic engineering of cassava for desirable agronomic traits

Plants have efficient innate antiviral defense mechanisms, based on the RNA silencing also referred to as gene silencing or RNA interference (Yang and Li, 2018). This defense mechanism is a conserved in plants and shields the host genome from compromise by invading viruses and transmissible genetic elements (Hannon, 2002; Pooggin, 2017). Post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) processes trigger effective defence mechanisms against invading genetic elements such as viruses (Pooggin, 2017; Waterhouse et al., 2001). Key components of silencing pathways include dicer-like (DCL) ribonucleases and associated dsRNA binding (DRB) proteins, RNA-dependent RNA polymerases (RDR), and argonaute (AGO) proteins (Yang and Li, 2018).

The PTGS mechanism detects double-stranded RNA (dsRNA) of the invading foreign nucleic acids and degrades them into oligonucleotides of between 21-24 in length known as small interfering RNAs (siRNAs). The generated siRNAs then act as guides for specific protein-siRNA complexes to target and cause destruction to foreign evading homologous genetic elements (Pooggin, 2017; Waterhouse et al., 2001). This highlights the critical function of dsRNA is a potent precursor of gene silencing in sequence-specific RNA degradation.

PTGS system in plants can be further exploited to engineer other desirable traits of agronomic value into crops (Angaji et al., 2010), encompassing enhancing crop productivity by targeting those genes associated with biomass and yield, prolonging produce shelf life, nutritional biofortification and tolerance to abiotic stresses like salinity and cold (Kamthan et al., 2015).

Antisense constructs

RNA silencing is commenced by dicer-like (DCL) protein, a RNaseIII-type enzyme, which catalyses long dsRNA trigger to produce siRNA (Yang and Li, 2018). The transgenes are constructed and structured in a manner to lead in production of self-complementary either sense or antisense oligonucleotides which lead to induction of PTGS of different efficacies (Thakur, 2003). Challenges have been encountered using antisense technology in vivo due to problems of accessing of target sites on mRNA and delivering transcripts (Caplen et al., 2001).

Antisense technology was employed by Regierer et al., 2002 to inhibit expression of plastidial *adenylate kinase* (*ADK*) in potato plants by inserting a cassette of plastidial *ADK* in an antisense

orientation between the constitutive promoter cauliflower mosaic virus (CaMV) 35S and ocs terminator. The screening of the transgenic lines revealed a suppressed expression of *ADK* substantial in transcript level in both leaves and tubers. More importantly, it led to significant gains in tuber yield productivity and starch content (Regierer et al., 2002).

Hairpin RNA constructs

Hairpin RNA based-constructs are potent silencing constructs that harbour both a sense and antisense oligonucleotides, creating RNA that fold back into hairpin (hp) form (Pooggin, 2017). The expression of inverted repeat transgenes result in increased hp-RNA fostering multiple DCLs activity, which leads to a high number of siRNAs to target incoming viral mRNA or host genomic RNA (Smith et al., 2000). Numerous reports are have empirically exhibited that cassettes engineered to express dsRNA, in the form of hp-RNA trigger a high efficacy of inducing PTGS of viral genes, transgenes and endogenous genes in plants (Pooggin, 2017; Yang and Li, 2018). The stability of the hp-RNA-induced gene silencing over many generations is an added advantage in reliable application of gene silencing in plants (Pooggin, 2017). Transgene cassettes encoding hp-RNA have been efficiently employed in conferring geminivirus resistance in cassava plants (Walsh et al., 2019). Furthermore, hp-RNA constructs can be used as a tool for gene discovery and functional analysis (Pooggin, 2017; Wesley et al., 2001).

1.4 Cassava starch biosynthesis

Starch biosynthesis is a highly conserved pathway in most photosynthetic plants (Martin and Smith, 1995), the major variation with plant species occurs on the process of starch accumulation (Hannah and James, 2008). Starch is the major carbohydrate reserve in plants and is found both in autotrophic and heterotrophic tissues. Starch biosynthesis is largely localised in the plastids and it is widely accepted that it is synthesized from sucrose (**Figure 1.5**) (Regierer et al., 2002). Forty five genes with a role in starch biosynthesis in cassava, including ADPG *adenine diphosphate glucose pyrophosphorylase* (*AGPase*), *granule bound starch synthase* (*GBSS*), *starch synthase* (*SS*), *starch branching enzyme* (*SBE*), *de-branching enzyme* (*DBE*) and *glucan, water dikinase* (*GWD*), have been identified and their functions characterized (Tappiban et al., 2019). To date, the identified 110 QTL for starch content and pasting traits will play a crucial role in improving starch quality by genetic engineering and molecular marker assisted selection

(Tappiban et al., 2019). Within the cassava storage roots, when sucrose symplastically reaches the cytosol of parenchyma tissues through the plasmodesmata it is catalysed to uridine diphosphate-glucose (UDP-glucose) and fructose by *sucrose synthase* (Li et al., 2016). ADP-glucose is the precursor to starch biosynthesis (Huang et al., 2020). Fructose is then phosphorylated by *fructokinase* to fructose 6-phosphate and UDP-glucose is synthesized to glucose 1-phosphate by *UDP-glucose pyrophosphate* (*UGPase*). In the cytosol, hexose phosphates fructose 6-phosphates, glucose 1-phosphate and glucose 6-phosphate are interchangeably converted through cytosolic *phosphoglucose isomerase* (c*PGI*) and cytosolic *phosphoglucomutase* (c*PGM*) (Geigenberger et al., 2004). The *glucose 6-phosphate/phosphate translocator* (GPT) facilitates the entry of glucose 6-phosphate into the amyloplast (Kammerer et al., 1998) and is subsequently reversibly isomerized into glucose 1-phosphate by plastidial *phosphoglucomutas*e (p*PGM*) (Geigenberger et al., 2004).

The conversion of intraplastidial glucose 1-phosphate to adenine diphosphate glucose (ADP glucose) releasing pyrophosphate as a by-product represents both the key initial committed step and a rate limiting reaction in starch biosynthesis pathway. This particular energy requiring reaction is driven by *adenine diphosphate glucose pyrophosphorylase* (*AGPase*) (Preiss, 1988), and adenosine triphosphate (ATP) is imported to the amyloplasts across the plastidial membrane cytosolic region using the *ATP/ADP antiporter* (*AATP1*) (Tjaden et al., 1998). These two previously mentioned key proteins (*AATP1* and *AGPase*) were amongst the subject of particular interest for this study to determine gene expression and extent of their roles towards overall storage root bulking. ADP glucose is the main substrate for synthases and is processed into starch. *Granule bound starch synthase* (*GBSS*) synthesizes ADP glucose into amylose whilst both *branching enzymes* (*BE*) and *soluble starch synthases* (*SSS*) are specifically responsible for amylopectin production.

Efforts to increase starch yield have mainly targeted three key areas of starch biosynthesis namely *AGPase* activity, starch synthases overexpression and the elevated provision of ATP via the *ATP/ADP antiporter* (*AATP1*) system to the plastid (Smith, 2008). Empirical evidence demonstrates that there is a direct relationship between a rise in the expression of *AGPase* in storage tissues and the accumulation of starch content (Sonnewald and Kossmann, 2013; Tuncel and Okita, 2013; Zeeman et al., 2010). Some of the strategies utilized to influence the rate

limiting AGPase step in storage organs has been to increase supply of its substrates, ATP and/or glucose 1-phosphate. In dicotyledonous plants, the *AGPase* activity within plastids is limited by energy supply (Geigenberger et al., 2001), suggesting that increasing ATP supply would elevate the starch contents in sink tissues. Geigenberger et al., (2001) was able to increase this limited ATP supply in potatoes through overexpression of a plastidial *adenylate antiporter* (*AATP1*) which counter exchanges ATP for ADP. Interestingly, a separate study described that overexpression of either *AATP1* or plastidial *glucose 6-phosphate translocator* (*GPT*) alone in potatoes did not yield significant increase in starch and tubers. However, upon stacked overexpression of both *AATP1* and a plastidial *GPT* gave rise to substantial increase in starch and tuber yield (Zhang et al., 2008), highlighting that starch biosynthesis was co-limited by the import of energy supply and the carbon source allocation. This could also suggest that the observed differences between the separate studies could be due to different environmental conditions.

Furthermore, research has shown that the supply of adenylates to the plastid is of fundamental requirement to starch formation in storage tissues like the potato tubers (Loef, 2001). Plastidial *adenylate kinase (ADK)* is a key enzyme with a function in equilibrating the amounts of adenylates within the amyloplast via the reversible catalysing ATP and adenine monophosphate (AMP) into ADP (Noda, 1973). Antisense downregulation of the plastidial *ADK* produced significant increases in both starch accumulation and tuber yield weight of 60% and 84% respectively in transgenic potatoes (Regierer et al., 2002). This was later empirically proved to be as a result of increased respiratory oxygen consumption and uptake of carbon fluxes into starch translating into post translational redox-activation of *AGPase* (Oliver et al., 2008).

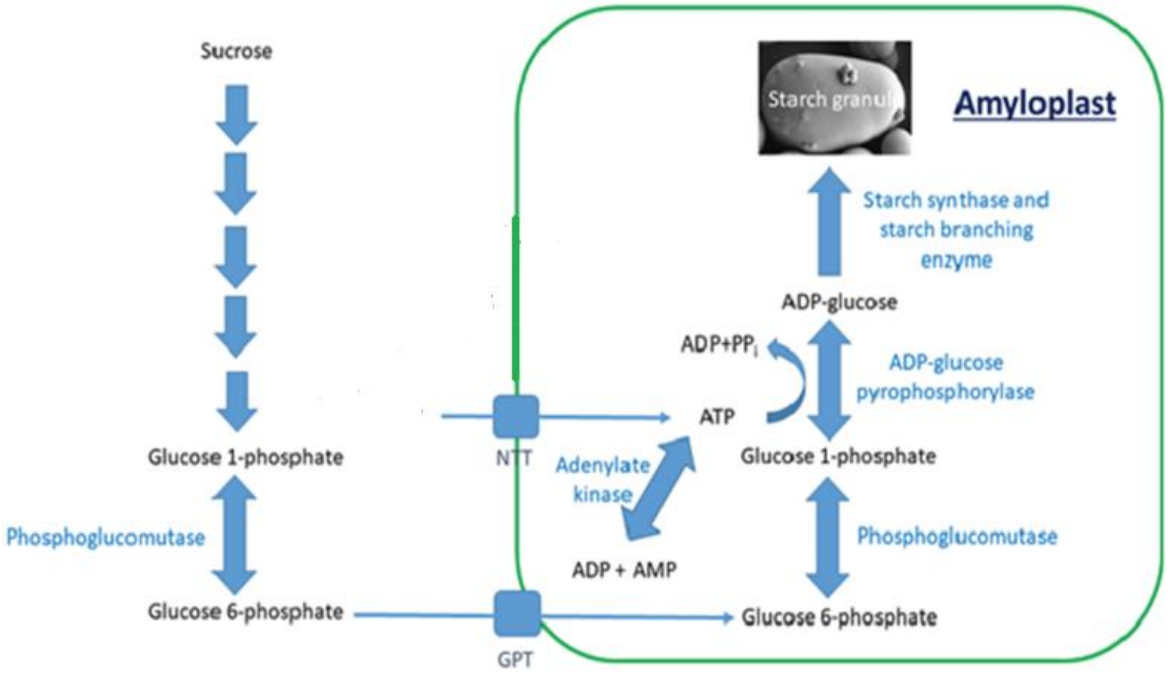


Figure 1.5: Starch synthesis pathway in vascular plants (Lloyd and Kossmann, 2019)

1.4.1 Cassava starch biosynthetic genes of interest in this study

Glucose-1- phosphate adenylyltransferase (*AGPase*), which is a key rate-limiting enzyme in the cassava starch biosynthetic pathway, that is encoded by a multi-gene family. The *MeAGPase* genes are located on seven chromosomes (Chr1, Chr3, Chr11, Chr12, Chr13, Chr15 and Chr18) (https://phytozome-next.jgi.doe.gov/). Numerous distinct *AGPase* isozymes have been classified in plant species such as rice (*Oryza sativa*) (Hirose et al., 2006). This particular study focused on the gene situated on chromosome 15. Another gene of interest, *carbamoyl phosphate synthase* (*CPS*) which catalyzes the first committed step in *de novo* pyrimidine synthesis is found distributed on 2 chromosomes (Chr4 and Chr10). Due to limited resources, the study focused on the gene located at chromosome 4. *ATP-ADP transporter (AATP1)* has a single location on chromosome 10 (https://phytozome-next.jgi.doe.gov/).

1.5 Structure and composition of cassava starch

Cassava starch granules are circular with a flat side exhibiting a conical pit, ranging from 5-40µm in size which grow in relation to the age of the crop for up to 6 months (Moorthy and

Ramanujam, 1986), and are packaged in specialized plastids known as amyloplasts in both the source and sink tissues (Slattery et al., 2000). Starch comprises two major polymers, amylose which has mostly linear α-1,4 glucose residue linkages and low degree of α-1,6 branching and amylopectin is essentially build from α-D glucopyranose residues by α-1,4 and α-1,6 bonds (Kawabata et al., 1984; Moorthy and Ramanujam, 1986). Amylose varies between 13.6% and 23.8% of total cassava starch contributing distinct characteristics vital for starch quality, whilst amylopectin impacts viscosity and high elasticity to thickeners and pastes in both food and non-food sectors (Kawabata et al., 1984; Munyikwa et al., 1997).

Plant starch is amenable to modification by biochemical and genetic engineering interventions to adjust its physico-chemical properties according to its intended end-use (Salehuzzaman et al., 1993).

1.6 Cassava starch and yield improvement

Cassava starch and yield improvement strategies have been inadequate with comparatively low capital investment channeled into the research (El-Sharkawy, 2004). Efforts have largely been centered on drought tolerance, lowering cyanogenic content, fortifying micronutrients in storage roots and conferring resistance to viral diseases such as cassava mosaic disease (De Souza et al., 2017; El-Sharkawy, 2004). *AGPase* is a key enzyme of starch biosynthesis and is regulated by allosteric and redox effectors (Kötting et al., 2010; Ligaba-Osena et al., 2018) Transgenic cassava harbouring modified bacterial *glgC* transgene to trigger maximum activity of *AGPase* and overcome negative feedback of fructose-1,6-biphosphate had 70% higher *AGPase* activity and more than 2 times in biomass accumulation in comparison to untransformed plants (Ihemere et al., 2006; Ligaba-Osena et al., 2018). When the cassava *granule-bound starch synthase 1* (*GBSS1*) gene was inserted in an antisense orientation in wild-type potatoes it yielded novel amylose-free starch also termed as waxy starch (Raemakers et al., 2005). Waxy starch has anti-retrograding effects and is widely applied in food science to lessen the aging of baked and confectionery products (Šárka and Dvořáček, 2017) Previous molecular and physiological studies carried out in some plant species have revealed a number of factors that impact source-to-sink relationship and the optimum assimilate distribution between source organs such as leaves and sink organs like roots or tubers as an influencer of yield determinant (Sonnewald et al., 2020). The potential of cassava yield to be increased relies on improving the limiting

interception efficiency (ε_i), and conversion efficiency (ε_c). These relate to canopy size, architecture and genetic engineering to improve the rate photosynthesis (source) jointly with sink capacity (De Souza et al., 2017). In cassava, specifically the past strategies to promote storage root development and starch accumulation have yielded significant outcomes, hence it is vital to elucidate the biosynthetic mechanisms of the source and sink organs (Sonnewald et al., 2020).

1.6.1 Strategies to increase source metabolism

There is sufficient theoretical evidence to suggest that there is direct proportion between crop yield increasing with increases in the efficiency of photosynthesis, even without the changes in assimilate storing and sink growth (Long et al., 2015). Broad strategies to increase source capacity have centered on either the efficiency of the light reactions, enhancing carboxylation, rate of sucrose biosynthesis or phloem loading (Sonnewald and Fernie, 2018). Two separate studies have shown that metabolic engineering of enzymes within the tricarboxylic acid cycle by antisense inhibition of *succinate dehydrogenase* and reducing *malate dehydrogenase* activity both led to improved photosynthetic performance, plant growth and yield (Araújo et al., 2011; Nunes-Nesi et al., 2005). Accelerated photosynthesis will consequently result in an accumulation in carbohydrate pools, however if not utilized in the sink tissues they can trigger negative feedback on the rate of photosynthesis (Sonnewald and Fernie, 2018).

1.6.2 Strategies to increase sink metabolism

Most attempts to increase sink capacity have examined potato and tomato using constructs driven by class 1 B33 patatin promoters (Rocha-Sosa et al., 1989; Zhang et al., 2008). These promoters are favoured as they have high activity in storage roots or tubers, and thus lessens the unintended effects on the above-ground parts of the plants (Rocha-Sosa et al., 1989). Sink strength in potatoes was improved by bolstering both levels uridine nucleotide and adenylate pool levels through the silencing of *uridine monophosphate synthase* (*UMPS*) and plastidial *adenylate kinase* (*ADK*) respectively (Geigenberger et al., 2005a; Regierer et al., 2002). *UMPS* has a rate-determining role in the pyrimidine synthesis, carbon pools are also known to be indirectly regulated by nucleotide cofactors (Traut and Jones, 1996), whilst plastidial *ADK* is a crucial enzyme with a function in equilibrating the amounts of adenylates within the amyloplast via the reversible catalysis of ATP and adenine monophosphate (AMP) into ADP (Noda, 1973).

The pyrimidine pathway

The conserved *de novo* pyrimidine biosynthesis otherwise termed the orotate pathway chiefly consists of 6 reactions ultimately leading in the formation of uridine monophosphate (UMP) from *carbamoyl phosphate* (*CPS*) (**Figure 1.6**) (Stasolla et al., 2003). Pyrimidines have multitude of roles in critical biochemical processes as precursors of nucleic acids formation and the biosynthesis of conjugated macromolecules associated with energy metabolism, sucrose and cellulose biosynthesis (Ohler et al., 2019; Stasolla et al., 2003). Notably, in plants the first reaction represents the committed step of the pathway and is catalyzed by *carbamoyl phosphate synthase* (*CPS*); whilst the last two vital regulatory stages of processing orotate to UMP are driven by a bi-functional polypeptide *uridine monophosphate synthase* (*UMPS*), which exhibits two catalytic activities of *orotate phosphoribosyltransferase* and *orotidylate decarboxylase* (Stasolla et al., 2003; Zrenner et al., 2006). All the orotate pathway reactions are all localized within plastids except the dehydrogenation step which is sited in the inner membrane of the mitochondria (Miersch et al., 1986; Zrenner et al., 2006).

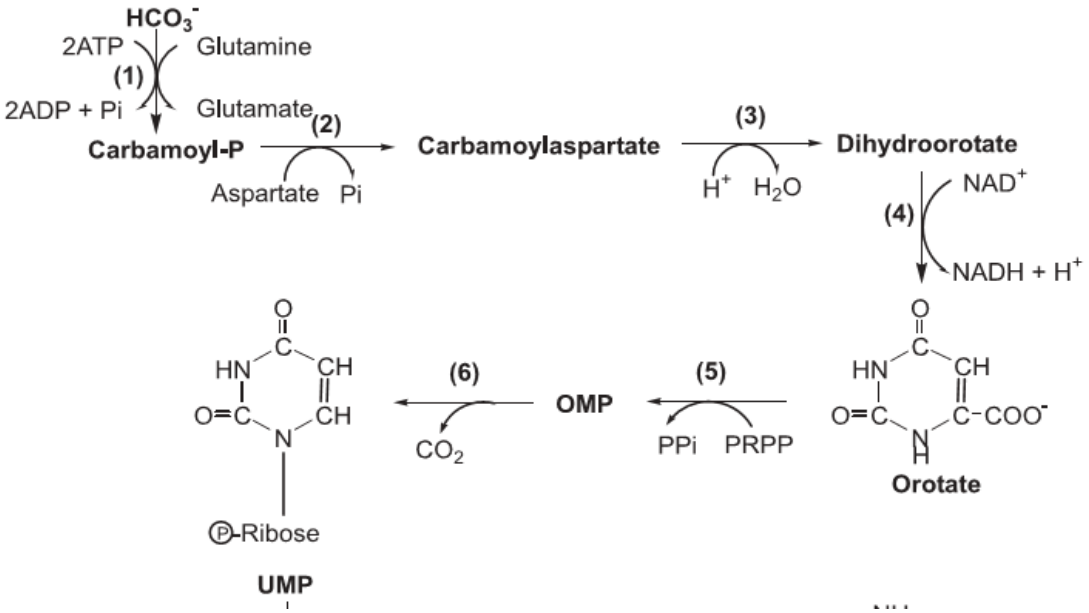


Figure 1.6: *De novo* biosynthetic pathway of pyrimidine nucleotides in plants. Enzymes shown are: (1) *carbamoyl phosphate synthase* (*CPS*), (2) *aspartate transcarbamoylase*, (3) *dihydroorotase*, (4) *dihydroorotate dehydrogenase*, (5)-(6) *UMP synthase* (*orotate phosphoribosyltransferas*e plus *orotidylate decarboxylase*) (Stasolla et al., 2003).

The pyrimidine salvage pathway

Some of the degraded nucleic acids and preformed nucleotides products; the deoxyribonucleosides and ribonucleosides can be repurposed by the salvage pathways and used for the synthesis of pyrimidine nucleotides in plants (Wagner and Backer, 1992). The *de novo* orotate pathway has high metabolic requirement, hence the salvage pathway plays a crucial role in supplying nucleobases to cells which are not able to meet demand at a much lower metabolic expense (Neuhard and Nygaard, 1987; Zrenner et al., 2006). Uracil and uridine are salvaged by the enzymatic activity of *uracil phosphoribosyltransferase (UPRT)* and *uridine kinase (UK)* respectively, to yield UMP (**Figure 1.7)** (Geigenberger et al., 2005b; Stasolla et al., 2003). There is yet to be documented evidence for salvage activity for cytosine pyrimidine in higher plants, however cytosine is first synthesized to uracil by *cytosine deaminase* (Katahira and Ashihara, 2002; Stasolla et al., 2003).

Potato plants were genetically engineered to express an antisense construct targeting *UMPS*, under the influence of a B33 patatin root-specific promoter. The downregulation of *UMPS* triggered the uridine salvaging pathway leading to upregulation of *CPS*, including salvage pathways associated enzymes *UPRT* and *UK*. The effect resulted in elevated uridine nucleotides in tubers and increased degradation of sucrose to starch and cell wall synthesis, and consequently the increased partitioning of starch contents and cell wall in the sink tissues overall attaining a 25% higher yield to wild type potato plants (Geigenberger et al., 2005b).

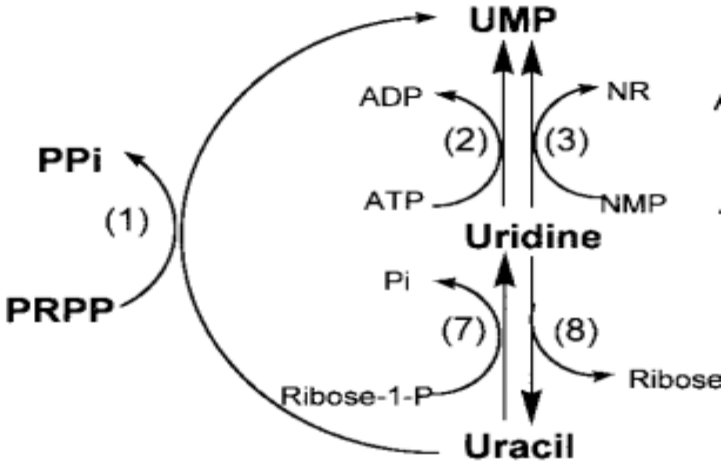


Figure 1.7: Salvage reactions of uracil pyrimidine and nucleosides in plants. Enzymes shown are: (1) *uracil phosphoribosyltransferase (UPRT),* (2) *uridine kinase (U*K), (3) non-specific *nucleoside phosphotransferase*, (7) *uridine phosphorylase*, (8) *uridine nucleosidase* (Stasolla et al., 2003).

1.6.3 Strategies to simultaneously increase source and sink metabolism

There exists conceptual evidence that plant carbon partitioning capabilities are co-limited by source and sink metabolic processes (Sonnewald and Fernie, 2018). Simultaneous engineering of multiple metabolic processes of source origin such as increased photosynthesis and metabolite transport coupled with those of sink region like sucrose degradation and starch biosynthesis will likely result in much increase in starch content and cassava storage root (Sonnewald et al., 2020).

The Cassava Sink-Source (CASS) research consortium is invested in designing various combinations of multigene stacking constructs for transformation into cassava based on the framework of genes associated with source metabolism, translocation and sucrose-to-starch pathways for example *Escherichia coli glycolate dehydrogenase (EcGlyDH),* the *Arabidopsis tonoplast sugar transporter (AtTST),* the *Pisum sativum glucose-6-phosphate/phosphate translocator 2 (PsGPT*2) and the *Arabidopsis nucleotide translocator 1 (AtNTT1*) (Sonnewald et al., 2020). *EcGlyDH* has a role in synthesizing glycerate and CO_2 by-product from 2-phosphoglycolate increasing both the photorespiratory efficiency and harvest (South et al., 2019). *AtTST* increases the rate of sugar transport from the leaf and changes cellular sugar

signalling evading metabolic feedback regulation and elevating the photosynthetic rate (Wingenter et al., 2010). *PsGPT2* and *AtNTT1* are carriers of the starch biosynthesis precursors; glucose 6-phosphate and ATP into the amyloplast and improve starch partitioning in potato tubers (Zhang et al., 2008).

1.7 Storage root formation

Cassava storage roots are technically not tubers as they do not harbour reproductive capability (Teerawanichpan et al., 2008). Thickening of cassava roots occurs when excess photoassimilates are transported to the roots after the plant has met its requirements for vegetative growth (Olasanmi et al., 2017). Photoassimilate partitioning is mediated by symplasmic phloem loading in which sucrose enters the radial movement to xylem parenchyma tissue resulting in storage root growth (**Figure 1.8** and **Figure 1.9**). Symplastic phloem unloading is thus, the major route where sucrose moves via plasmodesmata between phloem companion cells and parenchyma cells into the sink region (**Figure 1.9**) (Patrick, 1997). Post-phloem transport can either take place symplasmically and/or by the energy-requiring apoplasmic pathway hence it is vital to elucidate the cassava transport and photoassimilate storage in roots as avenues for biotechnological enhancement of cassava harvest (Mehdi et al., 2019). Root bulking resumes after 8 weeks of plant growth and thereafter root differentiation occurs in rapid succession until the completion of the initial phase of bulking at 9 months after planting (MAP). The plant then enters the recovery phase, followed by the second stage of root bulking at 15 MAP until 19 MAP (Olasanmi et al., 2017). Early bulking cassava landraces have been cultivated for years by smallholder subsistence farmers and harvested for consumption as from as early as 6 MAP (Jones, 1959).

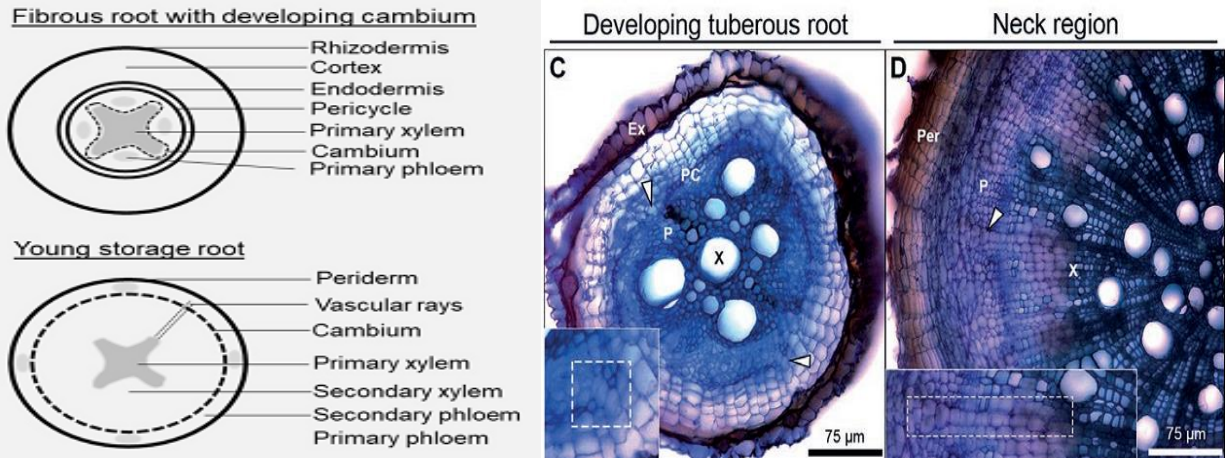


Figure 1.8: Cross-section of phloem and xylem cells in fibrous root and developing storage roots. Ex-exodermis; P-phloem; PC-procambium; Per-periderm; VC-vascular cambium; X-xylem. Arrowheads denote vascular rays (Mehdi et al., 2019; Sonnewald et al., 2020).

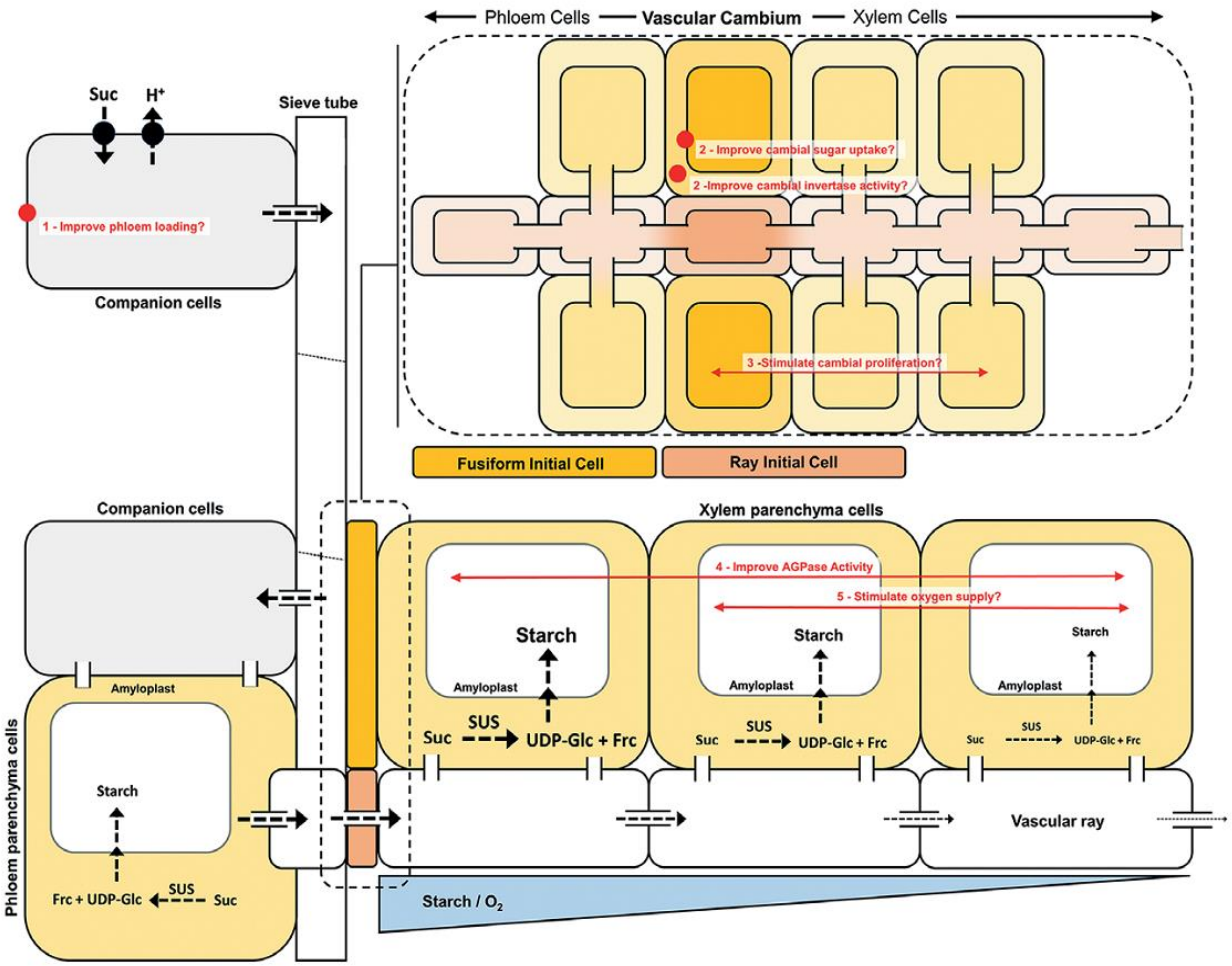


Figure 1.9: Model of cassava assimilates transport and potential targets for biotechnological yield improvement. H^+- proton; Suc - sucrose; Frc - fructose; *SUS* - *sucrose synthase*; UDP-Glc - UDP-glucose (Mehdi et al., 2019)

1.8 Background and generation of transgenic cassava starch lines

In order to attempt to increase starch and storage root yield, model cassava cultivar cv. 60444 was transformed with either the hairpin RNA (hp-RNA) cassette targeting *ADK* (referred to as ADK lines) or *UMP synthase* (UMP lines) under the control of a B33 patatin root promoter generated at the Plant Biotechnology Laboratory of the University of the Witwatersrand. The project further explores gene expression of the key starch biosynthesis enzymes *AATP1* and *AGPase* and their contributory roles in ADK lines towards storage root bulking; and gene expression of regulatory enzyme of the orotate pathway, *CPS* specifically for UMP cassava lines.

The transformation protocol used a plant transformation binary vector pCB33 which harbours both the *GUSPLUS* reporter gene for the visualization of transformation success and the *hygR* which confers resistance to hygromycin (**Figure 1.10**) (Bull et al., 2009; Walsh et al., 2019). The synthesised *ADK* (831bp) and *UMP synthase* (1087bp) hp-DNA were separately inserted into pCB33 through sticky end cloning. Model cassava cv.60444 friable embryogenic callus (FECs) were generated following Bull et al., 2009 protocol and utilized in *Agrobacterium*-mediated transformation. Resulting transgenic plantlets were then characterized by PCR to confirm integration of the genes of interest (Bull et al., 2009). Rooting tests were subsequently conducted to reliably screen transgenic plantlets from escaped non-transgenic plantlets that evaded the antibiotic selection phase. The characterized and confirmed transgenic ADK and UMP lines which showed great potential in the prior preliminary study were chosen and used in this particular study to evaluate starch content and storage root production.

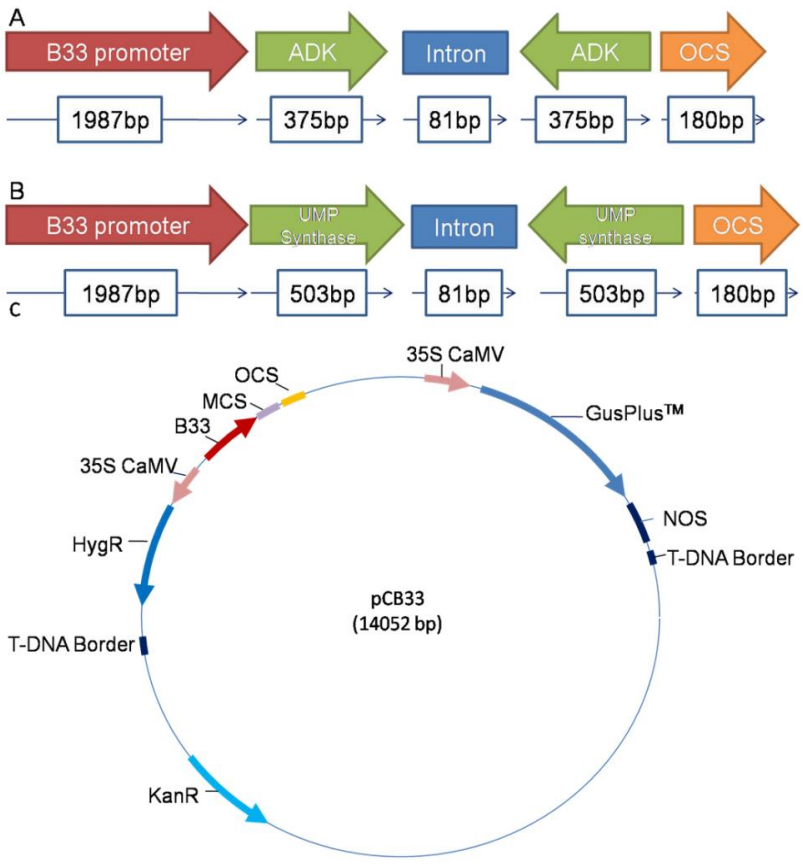


Figure 1.10: Schematic representation of the hairpins. (A) *Adenylate kinase (ADK)* (831bp) hairpin, and (B) *Uridine monophosphate synthase (UMPS)* (1087bp) hairpin. The

forward and reverse arms of the hairpins are separated by an 81bp intron. Each hairpin is flanked by the patatin root promoter (B33) and an *octopine synthase* transcription terminator (OCS). The hairpins were each separately inserted into the vector pCB33 at the MCS site (Walsh, 2019).

Chapter 2: Rationale of study

The mean cassava yield across Africa has remained largely unchanged with no significant increase in the past 60 years. Augmented efforts to improve cassava yield will be vital to meet food security demand for the projected 2-fold increase in population within the sub-Saharan Africa region by the year 2050 (United Nations, 2019). This would inadvertently reduce arable land available for agricultural purpose as more space is allocated or reserved for human settlement activities. As cassava gives a starch production per unit area much greater than that of maize and rice, it is an excellent crop for human sustenance and animal feed (Tonukari, 2004). There exist the need to exploit the technological advancements to elevate agricultural productivity via, (i) increased yield output per unit hectare of crops, (ii) tolerance to drought as a result of global warming and (iii) disease resistance so as to strengthen food security under changing climatic conditions (United Nations, 2019).

Cassava starch demonstrates an array of desirable functional properties such as odourlessness, superior paste clarity, high freeze-thaw characteristics, low concentration of proteins, phosphorus and crude fat content, making it an attractive choice to ease downstream processing in wide industrial applications (Balagopalan, 2002). The lower production costs should translate to the end user of products as affordable goods.

Given the versatile end-uses of cassava starch, it could be harnessed both as a food security and income generating crop to drive economic growth in South Africa. Domestic starch production is unable to meet local demand and the country imports R660 million of dextrins, and other modified starches. Imported cassava starch contributes over 19% of the stake which contributes to a negative trade balance (IDC, 2017) (**Figure 2.1b**). Research initiatives and outcomes focusing on producing a rugged, disease-resistant and high-yielding cassava will ease commercial uptake of a profitable crop. This will unlock new untapped local industries throughout the value chain, creating massive ancillary opportunities, alleviating the unemployment rate and bolstering gross domestic product (GDP) (Sanginga, 2015). Unlike other imported cereal crops, cassava starch is not prone to world market price volatility, hence African governments unable to meet their food demands could leverage that to reduce imports.

Environmentally-friendly green energy is rapidly being adopted globally in favour of fossil fuels by both governments and corporates, as part of their actions and commitments towards the United Nations' Paris Agreement treaty to mitigate greenhouse gases emissions and climate change strategies. Cassava-derived bioethanol has huge potential for South Africa in the manufacture of biodegradable packaging and disposable bags. This could cascade down to positively impact marginalized subsistence smallholder farmers who are the current primary cassava growers to participate in mainstream economy in this potentially multi-billion rand industry. Cassava drought-tolerant trait could make it a critical part of climate change adaptation strategies.

The inherent drought-tolerant South African local cassava Ukulinga germplasm which are CMD-resistant can be utilized in genetic engineering and breeding programmes to generate robust high-yielding, disease resistant plant that can be a potential cash crop resulting in easier funding by investors for a long sustainable and profitable business, as in tropical Asia where cassava is the chief raw material in starch industries and bioethanol production. This starch is eventually imported to emerging markets like South Africa, with Vietnam and India amongst the current top importers for the local market (**Figure 2.1a**) (Phaleng, 2017). Exploitation of High Quality Cassava Flour (HQCF) in food and other industrial applications will result in improved livelihoods for the smallholder farmers as they would be able to be key players in the mainstream economy and contribute towards the GDP index and import bills for the country.

The study seeks to make an effort towards aligning and contributing to the United Nations (UN) sustainable development goals to transform the world namely; no poverty, zero hunger, clean energy, industrial innovation, sustainable communities and action towards climate change.

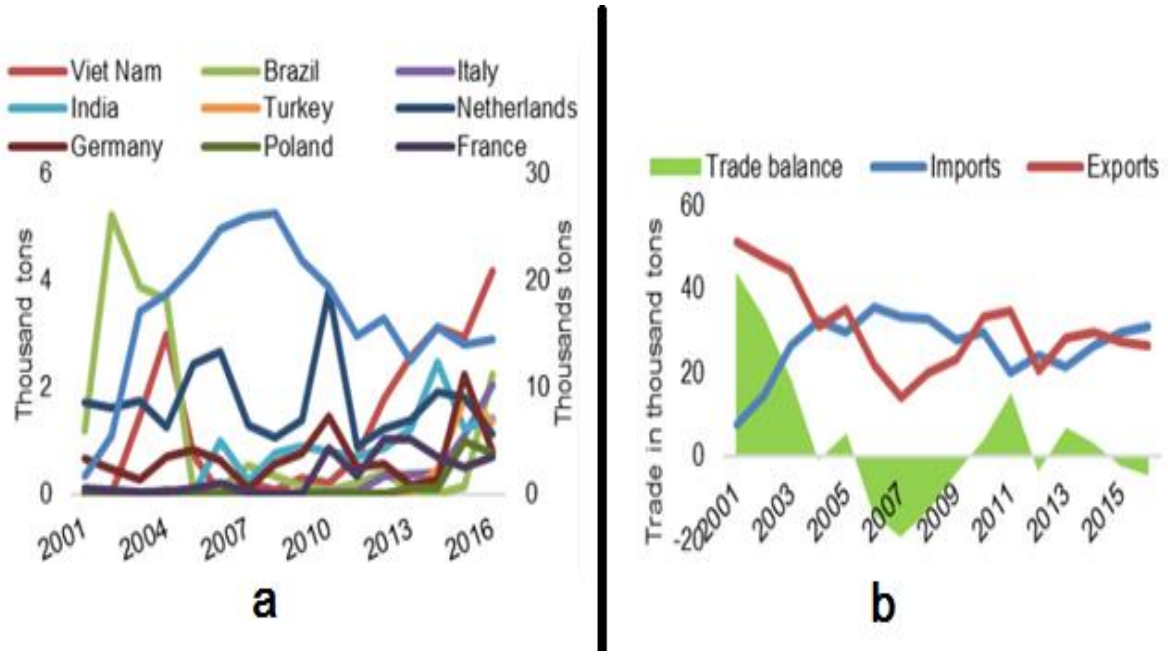


Figure 2.1: South Africa starch industry (a) Top importers; (b) trade balance sheet (Phaleng, 2017)

2.1. Aim of the study

The aim of the study was to screen local drought-tolerant South African cassava germplasm for responses to *South Africa cassava mosaic virus* (SACMV) challenge and evaluate previously transformed cassava lines, where model cv. 60444 was transformed with a hairpin DNA cassette transcribing a hp-RNA targeting either plastidial *adenylate kinase* (*ADK*) or *uridine monophosphate synthase* (*UMPS*) under the control of a B33 patatin root promoter in an attempt to increase starch production and storage root yield. The identified viral resistant local germplasm and high yielding cassava lines could eventually be utilized in breeding programs and transgenic interventions for strengthening food security and viable commercial exploitation in South African starch industry.

2.2. Objectives

In order to address the aim, the following objectives are proposed:

Objective 1: Evaluating and screening of drought-tolerant Ukulinga cassava germplasm for cassava mosaic disease (CMD) resistance or susceptibility

- Challenge Ukulinga cassava germplasm (Ukulinga 2, Ukulinga 8 and Ukulinga 12) with infectious clones of SACMV in three separate greenhouse trials.
- Characterize SACMV-challenged Ukulinga germplasm for virus infection response at 3 time points (14 dpi, 32 dpi and 65 dpi). CMD-tolerant TME3 and CMD-susceptible cv.60444 were used as comparative controls. Symptom severity index (SSI) was used to evaluate disease symptoms.
- Quantify relative viral load and determine correlation with plant tolerance or susceptibility.

Objective 2: Evaluation and comparison of storage root yield of five cassava transgenic cassava lines in greenhouse trials

- Screen 2 ADK transgenic lines (ADK 1 and ADK 5) and 3 UMP lines (UMP 17, UMP 26 and UMP 28) for presence of *hygR* antibiotic resistance and *GUSPLUS* reporter gene.
- Conduct two trials (1 controlled conditions growth chamber-based and another greenhouse-based) for transgenic cassava *UMPS* and *ADK* lines and measure growth parameters such plant height and storage root weight.
- Compare relative expression of starch-biosynthesis associated genes such as *adenylate transporter (AATP1)* and *ADP glucose pyrophosphorylase* (*AGPas*e) for both ADK and UMP transgenic lines; *carbamoyl phosphate synthase* (*CPS*) for UMP transgenic lines only. The untransformed cassava model cv. 60444 and the high-yielding local landrace T200 were used as comparative controls.
- Quantify total starch content and amylose/amylopectin ratios in harvested storage roots.

Chapter 3: Materials and methods

3.1 Objective 1: **Evaluation of drought-tolerant Ukulinga cassava germplasm for cassava mosaic disease (CMD) resistance or susceptibility**

The outline of the workflow is viewed in **Figure 3.1**.

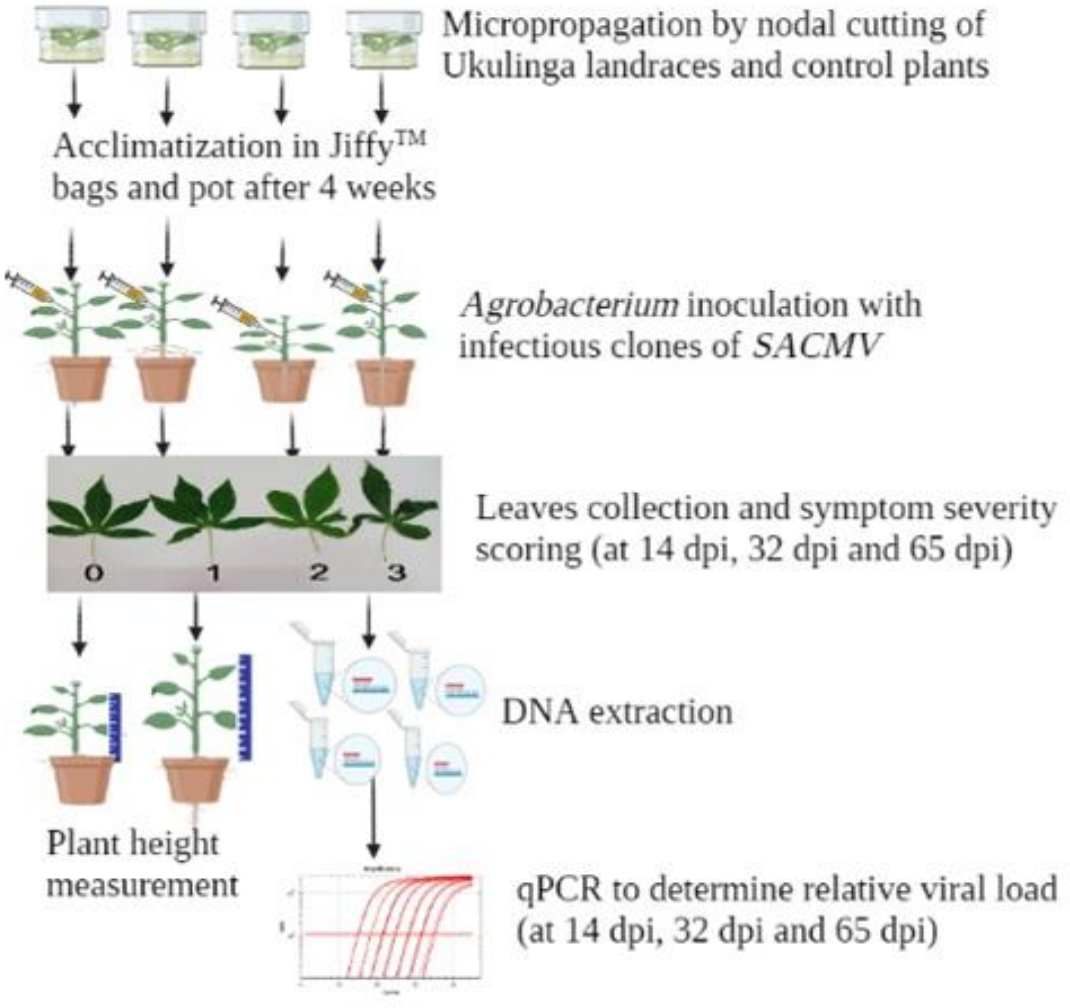


Figure 3.1: Workflow diagram for the evaluation of Ukulinga cassava germplasm for response against cassava mosaic geminiviruses

3.1.1 Micropropagation and acclimatization of Ukulinga germplasm

The three selected Ukulinga germplasm (Ukulinga 2, Ukulinga 8 and Ukulinga 12) were propagated by nodal cutting in tissue culture on Murashige and Skoog (MS20) medium containing 4.4 g/L MS salts, 20 g/L sucrose and 6.8 g/L plant agar, pH 5.8. For the experimental controls, the model cultivar cv. 60444 which is susceptible to CMD and the CMD2 loci-bearing TME3 (CMD-tolerant) were also propagated. The cassava plantlets were grown in growth cabinets with controlled parameters set at 28 °C/25 °C under 16h light/8h dark photoperiod. When sufficient roots had emerged at four weeks, an average of 30 plantlets per genotype and corresponding controls were each transferred to Jiffy bags (Jiffy International). The plantlets were then placed in deep plastic trays and covered with translucent cling wrap to help acclimatize the plantlets. The trays were placed in a growth facility with pre-set conditions of 28 °C/25 °C under 16h light/8h dark photoperiod respectively and approximately 60 % humidity. After a week a couple of medium-sized slits were made on the cling wrap using a scalpel, thereafter, it was repeated each subsequent day for 7 days and eventually removed to assist in acclimatizing the growing plantlets. The cassava plantlets were then potted in a soil: vermiculite mixture ratio of 3:1 so they could further harden off for 2 more weeks.

3.1.2 *Agrobacterium* inoculation of plants

Subsequent to the acclimatization and hardening off phase, the growing plantlets were infected with full length head-to-tail dimers of SACMV DNA-A and DNA-B immobilized in *Agrobacterium tumefaciens* strain AGL1 (OD_{600} of ±1.8) (Berrie et al., 2001). Initially, cassava plantlets were pricked numerous times along the stem and each individual plant was *Agrobacterium*-inoculated with 120 µl of the culture at different points on the stem beneath the apical leaves. The cassava plantlets were then placed in deep plastic trays and covered with translucent cling wrap and a couple of slits were then continuously made daily after a week, over 7 days until all the cling wrap was taken off. In summary, each biological replicate consisted of 6 SACMV-infected cassava plantlets for each of the cassava genotypes per treatment. Control treatments were both the healthy (uninfected) cassava plantlets and mock-inoculated plantlets with 120 µl of *A. tumefaciens* strain AGL1 only.

3.1.3 Cassava mosaic disease symptom development and assessment

Inoculated plants were assessed for symptoms development at 3 defined time points (14 days post inoculation (dpi), 32 dpi and 65 dpi. The corresponding plant heights were also measured for each of the plants. The symptom severity score index (SSI) system modified by Legg and Fauquet, (2004) was employed to assess for disease severity on two upper-most expanding leaves where; 0= no symptoms, 1= faint mosaic, 2= mosaic with mild curling, 3= severe mosaic, with severe curling (**Figure 3.2**). These upper-most expanding leaves were extracted and quick-frozen in liquid nitrogen then kept at -70°C for long term storage until required. Plant leaves were sampled as normally at 14 dpi but when no plant under study had shown symptom development by then, hence the SSI scoring was carried at 21 dpi. For each of the cassava genotypes, symptom severity was determined and the ratio of plants exhibiting CMD symptoms was expressed as a percentage of the plants that had been *Agrobacterium*-inoculated.

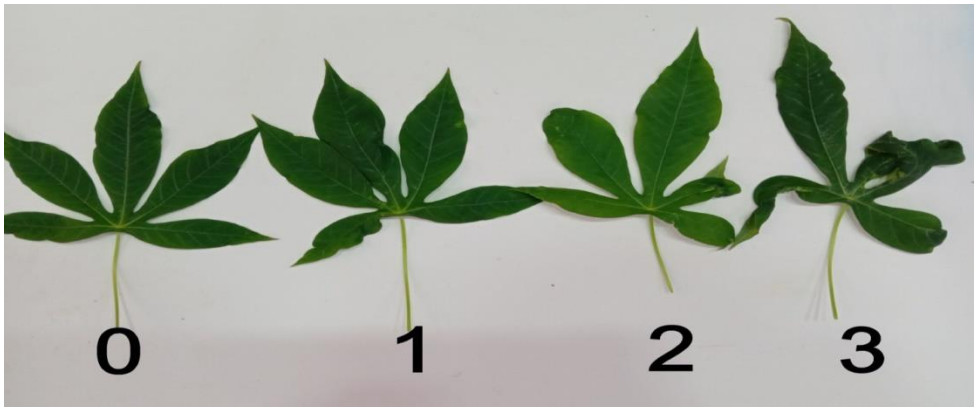


Figure 3.2: The modified symptom severity index to determine symptom severity score of plants infected with *South African cassava mosaic virus.* On the scale 0: Healthy (no symptoms), 1: Light mosaic and curling, 2: Medium mosaic curling and 3: Severe mosaic curling with reduction in leaf size

3.1.4 Total nucleic acid extraction

Total nucleic acid (TNA) was extracted from 50mg of harvested leaf samples using the CTAB method described by (Doyle and Doyle, 1987). The TNA pellet was re-suspended in RNAse A (20 µg/ml): TE (10 mM Tris pH 8.0 and 1 mM EDTA) buffer ratio of 1:100 and incubated at

37°C for 60 min. The quantity and purity of the extracted TNA were assessed using the Nanodrop ND-100 spectrophotometer (Nanodrop). The TNA samples were further standardized to a concentration of 20 ng/µl.

3.1.5 Determination of viral load by real-time quantitative PCR

The relative viral load of SACMV was determined by real-time quantitative PCR (RT-qPCR) at 14 dpi, 32 dpi and 65 dpi in relation to *Ubiquitin 10 (UBQ10)* as a reference gene (Allie et al., 2014) on the C1000 thermocycler (Biorad). *UBQ10* was amplified using the forward primers 5` TGCATCTCGTTCTCCGATTG 3` and reverse primers 5` GCGAAGATCAGTCGTTGTTGG 3`. Each reaction mixture was set up as 5 µl of Maxima SYBR green master mix, coat protein (AV1) forward primer (5` ACGTCCGTCGCAAGTACGAT 3`) and coat protein (AV1) reverse primer (5' ATTGTCATGTCGAATAGTACG 3`) to a final concentration of 0.3 µM for each primer, 1 µl of TNA sample and nuclease-free water to a total volume of 10 µl. The RT-qPCR amplification was carried out under the following conditions; initial denaturation and enzyme activation were carried out at 95°C for 10 min followed by 39 cycles of denaturation at 95°C for 15s, annealing at 60°C for 30s and elongation at 72°C for 30s.

After the conclusion of the run, the products of RT-qPCR were electrophoresed on 1% (w/v) agarose gel at 100V for 30 min and then viewed under UV on the Chemidoc MP Imaging System (Biorad). The relative band intensities were quantified relative to the cv. 60444 14 dpi samples using the Image Lab version 6.0 software (Biorad).

3.1.6 Experimental design

The experiment was set up as a randomized block design with 15 treatments (5 genotypes x 3 plant treatments of SACMV inoculated, mock-inoculated and healthy plants); each treatment comprised of at least 6 plants (n=6) and 3 trials were concluded. The two leaves below the apical leaves were collected per 2 plants and were pooled into 3 groups of three biological and 2 technical replicates per biological replicate.

Relative viral load was quantified using the CFX Maestro software version 1.1 (Biorad). ANOVA ($p<0.05$) was performed to establish a statistically significant difference between cassava germplasm and control plants (cv. 60444 and TME3), and Pearson's correlation test was

performed and analyzed using the R corrplot package to determine the correlation between the relative viral load and symptom severity using R program version 4.04 (R Core Team, 2014).

3.2 Objective 2: Evaluation and comparison of storage root yield of three cassava transgenic cassava lines

The outline of the workflow is viewed in **Figure 3.3**.

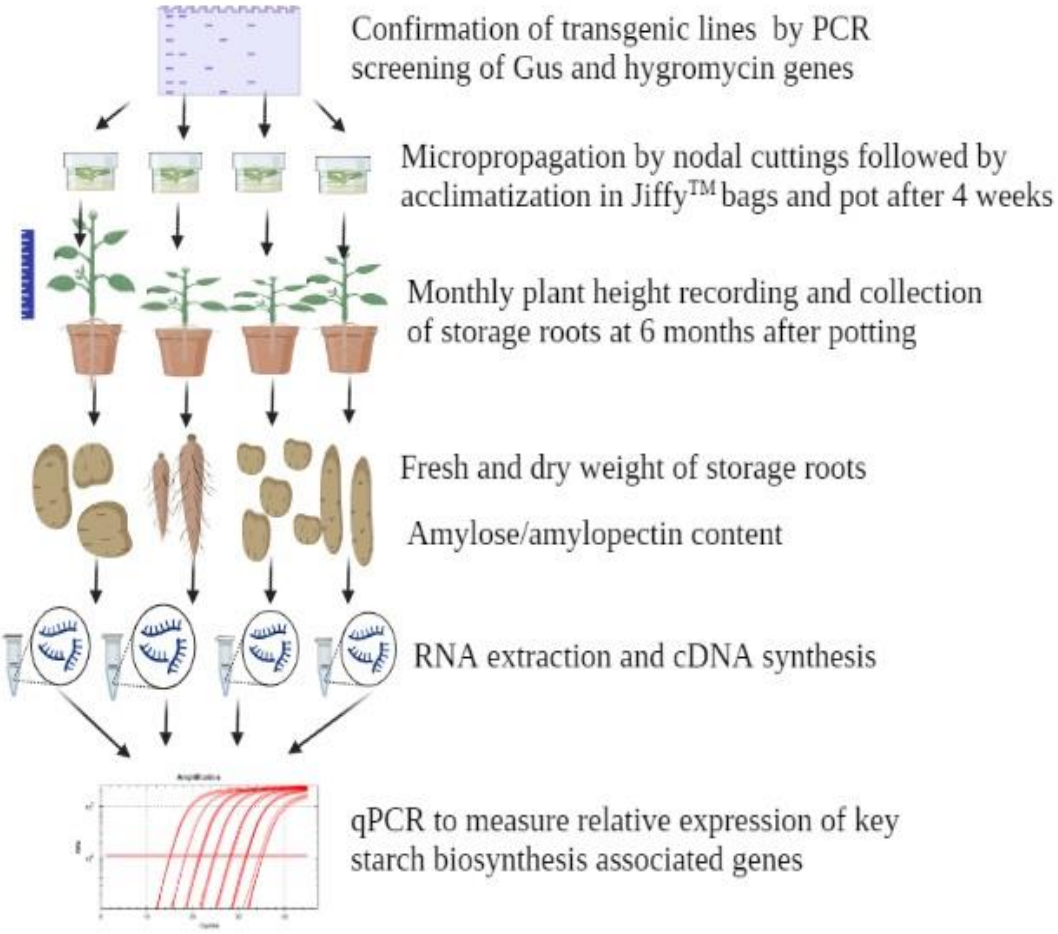


Figure 3.3: Workflow diagram for screening and evaluation of transgenic cassava lines for storage root yield

3.2.1 Characterization of transgenic lines

To confirm the successful integration of the plant transformation cassettes of model cassava cv.60444 with either pCB-ADK or pCB-UMP constructs were selected. The selected transgenic lines were screened for the presence of reporter gene, *Gus* and antibiotic resistance gene, *Hyg* by PCR and then visualized on 1% (w/v) agarose gel. The screening for *Gus* was carried out using forward primer (5` CAACATCCTCGACGATAGCA 3`) and reverse primer (5` GGTCACAACCGAGATCTCCT 3`) expected to amplify a 181 bp fragment and *Hyg* forward primer (5` TCTCGATGAGCTCATGCTTTGG 3`) and reverse primer (5` AGTACTTCTACACAGCCATGG 3`) to give a 485 bp product. Once the stable transformations of the lines were re-confirmed bulking up of transgenic lines and control plants was commenced.

3.2.2 Micropropagation and acclimatization of ADK and UMP transgenic plants

Micropropagation and subsequent acclimatization was undertaken on the following 5 transgenic lines ADK 1, ADK 5, UMP 17, UMP 26 and UMP 28 including the control plants cv. 60444 and T200 using the same procedure described in section **3.1.1**. However, the potting of cassava plantlets were carried out in 9×9×9.5 cm square pots with seedling mix: perlite ratio of 3:1 as previous projects within the laboratory proved perlite to be much superior for fostering root development in comparison to vermiculite. Plantlets were fertilized during potting using the Vita Veg 6.3.4 (Talborne Organics) organic fertilizer with a low ratio of phosphorus content to promote root development ahead of above-ground plant biomass and again at 4 months after potting (MAP). For trial 1, the plantlets were grown in a growth facility with environmentally pre-set conditions of 28 °C/25 °C under 16h light/8h dark photoperiod respectively, the light intensity average of 9000 lux, air flow and 60% humidity. However, for trial 2 the plantlets were grown in the greenhouse facility exposed to natural light from the beginning of October to the end of March the subsequent year where ambient daytime temperatures inside the greenhouse with average daytime of 32 °C and night time average temperature of 18 °C. Plant height measurements were each carried out monthly for 6 months.

3.2.3 Cassava storage root weight and amylose/ amylopectin content

Six MAP the storage roots of cassava plants were harvested, weighed using an electronic balance and then stored at -70 °C until further use. Storage roots were oven-dried at 50 °C for 48h and, the dry storage root weight was recorded. The Amylose/Amylopectin kit (Megazyme) was used

for the measurement of the amylose to amylopectin ratios of starch in storage roots following the manufacturer's instructions. A total of 2 batches were conducted for each test sample with the corresponding reference sample incorporated. Each of the test samples had a technical repeat, with an additional repeat with every fifth test sample to ensure batch reproducibility and accuracy. The principle of the assay is to initially get rid of the lipids and any soluble sugars present in the sample through precipitation with alcohol. Acetate/salt solution is then utilized in the dissolution of the precipitated sample followed by targeted precipitation of amylopectin by addition of concanavalin A and removal by centrifugation. Amylose and corresponding total starch are both enzymatically hydrolyzed to D-glucose by amylases and amylogucosidase and measured colourmetrically by spectrophotometer. The amount of amylose in the test sample is calculated as a ratio of precipitated concanavalin A sample to that of total starch sample.

3.2.4 Starch determination and microscopy

Six-month old storage root material was prepared for staining with either 50% (v/v) Lugol's solution or 0.1% (w/v) toluidine solution. The total starch content of the storage roots were estimated from the measurements obtained from the Amylose/Amylopectin kit assaying, using the Megazyme Total Starch calculator (Megazyme). Each sample comprised 3 technical replicates, with control sample included for validation. For the qualitative determination of starch, transversely hand-cut disks of fresh storage roots were immersed in 50% (v/v) Lugol's solution for 1 minute, followed by double rinsing in deionised water for 15s each time before the still images were captured. The visualization of starch distribution in the storage roots was done by staining thin, transversely hand-cut fresh storage root disks with 10% (v/v) Lugol's solution and fixing onto microscope slides for viewing. For the visualization of starch granules, storage root disks were oven dried at 50 °C for 48h. The dried storage root disks were then ground into fine powder using pestle and mortar. Cassava flour was then stained with Lugol's solution and fixed to the microscope slides. The microscope slides were viewed using the Carl Zeiss Axiocam 506 microscope (Zeiss) and starch granule sizes measured using the microscope's in-built software.

3.2.5 RNA extraction and cDNA synthesis

Mature secondary storage roots were ground in the presence of nitrogen into a fine powder using pestle and mortar; pooled into 2 groups. Approximately 0.1g of frozen powder was quickly

transferred into 600 µl pre-warmed extraction buffer [100 mM Tris–HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% (w/v), CTAB, 2% (w/v) PVP and 2% (v/v) *β*-mercaptoethanol] with 12 µl *β*-mercaptoethanol added just before use. The mixture was vortexed and incubated at 65 °C for 15 min with vigorous shaking every 5 min. Five hundred µl of chloroform was added, mixed well and then centrifuged at 12000*g* for 10 min at 4 °C. The viscous supernatant was transferred to a new tube and total RNA extracted using TRIzol reagent according to manufacturer's protocol (Zymo Research). RNA purity and quantity was measured on the Nanodrop ND-100 spectrophotometer (Nanodrop). The RNA integrity was assessed on a 1% (w/v) agarose gel run in 0.5X TBE. Genomic DNA was removed enzymatically using DNase I protocol from Thermoscientific. Complementary DNA (cDNA) was synthesized from 1µg of total RNA with oligo $(dT)_{18}$ primers, using the RevertAid First strand cDNA synthesis kit (Thermoscientific).

3.2.6 Real-time quantitative PCR

The relative expression of the *glucose-1-phosphate adenylyltransferase large sub unit* (*AGPase*), *ATP-ADP transporter* (*AATP1*) and *carbamoyl phosphate synthase large subunit* gene (*CPS*) in various cassava genotypes were determined by RT-qPCR, with *Ubiquitin 10* (*UBQ10*) as the reference gene. One µl of cDNA product was used as the template for RT-qPCR amplification of the above mentioned genes according to the Maxima SYBR green (Thermoscientific) procedure; with 3 technical replicates per sample. The primer sequences used are listed in **Table 3.1** below and the exact thermocycler reaction conditions stated in section **3.1.5** were applied. The non-transgenic cv.60444 and T200 were included as controls.

3.2.7 Experimental design

The experiment was set up as a randomized block design with 7 treatments comprising 12 plants each (n=12) and over 2 separate trials were completed. Relative gene expression study was analyzed on the CFX Maestro software version 1.1 (Biorad). Two-way analysis of variance (ANOVA) was performed to establish a statistically significant difference in gene expression amongst the cassava genotypes followed by Tukey's post hoc test for overall statistically significant difference in group means. Data analysis for plant height, percentage amylose content, starch content and fresh and dry storage root weight was completed using ggplot2 function from R program version 4.04 (R Core Team, 2014). The Pearson's correlation test was performed and analyzed using the R corrplot package to determine the correlation between the

storage root weight and total starch content of tubers. Principal component analysis (PCA) was separately carried out for the growth facility and greenhouse datasets using the prcomp function.

Table 3.1: Primers used for relative expression of starch biosynthesis-associated genes

Gene name / Gene identifier	**Primer sequence 5`- 3`**	**Product size (bp)**
Ubiquitin 10 (UBQ10) *Manes.17G035200*	TGCATCTCGTTCTCCGATTG (Forward) GCGAAGATCAGTCGTTGTTGG (Reverse)	148 bp
ATP-ADP transporter (AATP1) *Manes.10G004900*	CATGAGTGGTTCAACACTCAATCC (Forward) CCTACGTTCAGACGTTTCACCT (Reverse)	156 bp
Glucose-1- phosphate adenylyltransferase (AGPase) *Manes.15G025400*	GTGCAAAATCATGCCCAACGA (Forward) GAAGGTTGCGCGGCC (Reverse)	99 bp
Carbamoyl phosphate synthase large subunit (CPS) *Manes.04G025400*	GGCAACTGCAGAGGCAATAAAG (Forward) CAAGCTTGGCCAGAAATGCTATC (Reverse)	157 bp

Chapter 4: Results

4.1 Evaluation of drought-tolerant Ukulinga cassava germplasm for response to SACMV

4.1.1 Symptom severity evaluation

There were no CMD symptoms noted in all the genotypes at 14 dpi. The phenotypic disease symptoms of CMD first emerged in cv. 60444, Ukulinga 12 and TME3 genotypes as faint mosaic with minor curling at 18 dpi. At 28 dpi, all the cv. 60444, Ukulinga 12 and TME3 infected plants were exhibited CMD symptoms. Minor mosaic and leaf curling symptoms progressed to mild mosaic with mild curling at 32 dpi in newly developing apex leaves on the infected plants of cv. 60444, Ukulinga 12 and TME3 **(Figure 4.1)**. The known susceptible model cv. 60444 plants had slower symptom progression, where 62.5% of all the infected plants showed systemic CMD symptoms at 21 dpi in comparison to drought-tolerant Ukulinga 12 and CMD-tolerant TME3 which had 75% and 100% respectively **(Figure *S1*)**. The mean symptom severity index (SSI) at 21 dpi for the control genotypes were 1.25 ± 0.41 for cv. 60444, 1.57 ± 0.43 for TME3 and for the local germplasm Ukulinga 12 was 1.38 ± 0.42 and 0 ± 0.00 for both Ukulinga 2 and Ukulinga 8 **(Figure. 4.2)**. Ukulinga 2 and Ukulinga 8 infected accessions remained asymptomatic at 32 dpi, with the first emergence of faint mosaic and minor curling in Ukulinga 2 at 34 dpi **(Figure 4.1)**. Symptom severity index increased progressively, and at 35 dpi reached a mean of 2.25 ± 0.31 in cv. 60444, 2.61 ± 0.19 in TME3, 0.38 ± 0.26 in Ukulinga 2, 2.1 ± 0.3 in Ukulinga 12 and 0 ± 0.00 for asymptomatic Ukulinga 8. The number of infected Ukulinga 2 plants only reached 100% CMD-associated symptoms late at 49 dpi, presenting as minor mosaic and leaf curling.

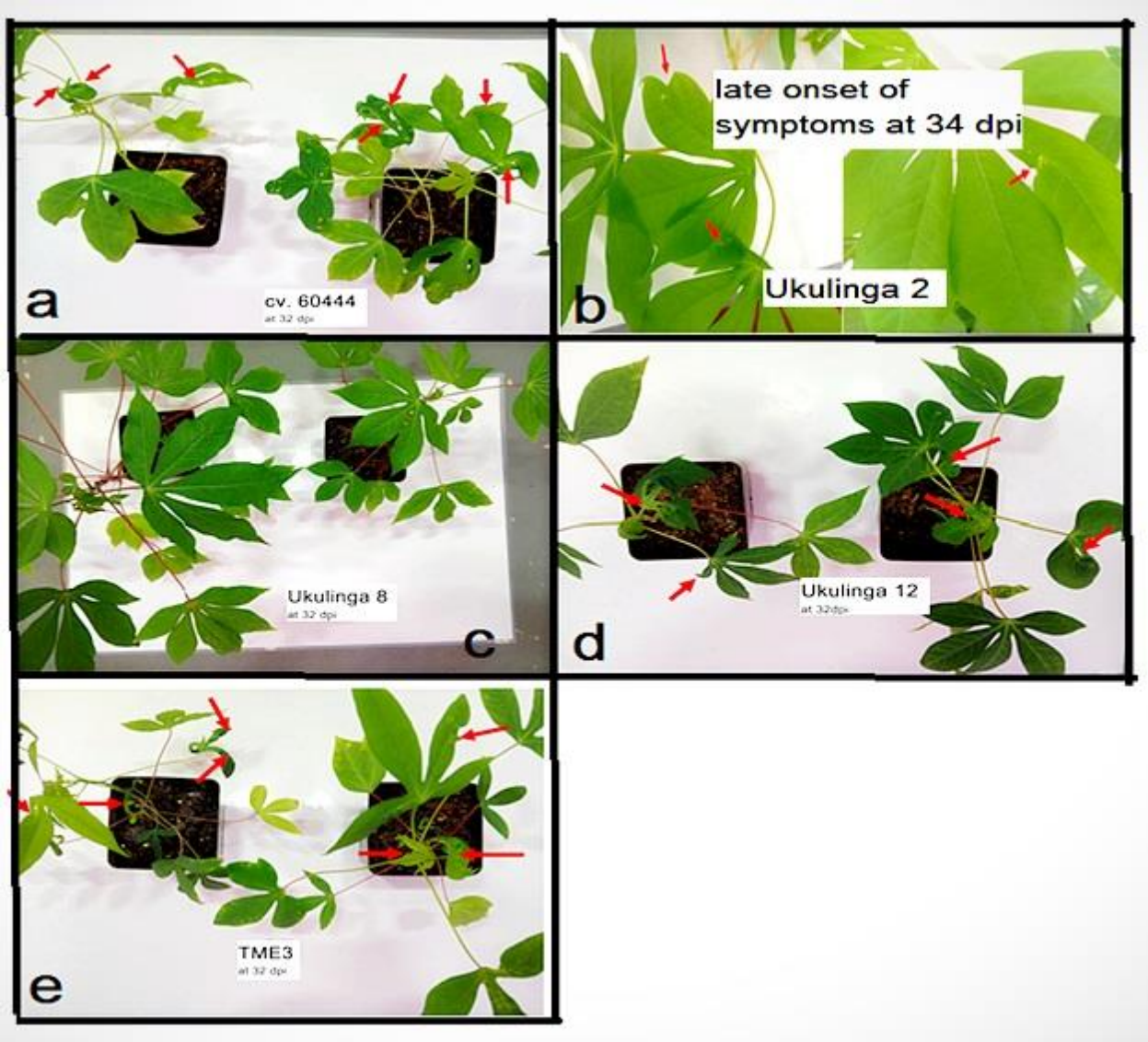


Figure 4.1: Cassava plant genotypes agro-infected with infectious clones of South African cassava mosaic virus showing cassava mosaic disease (CMD) symptom severity at 32 dpi.

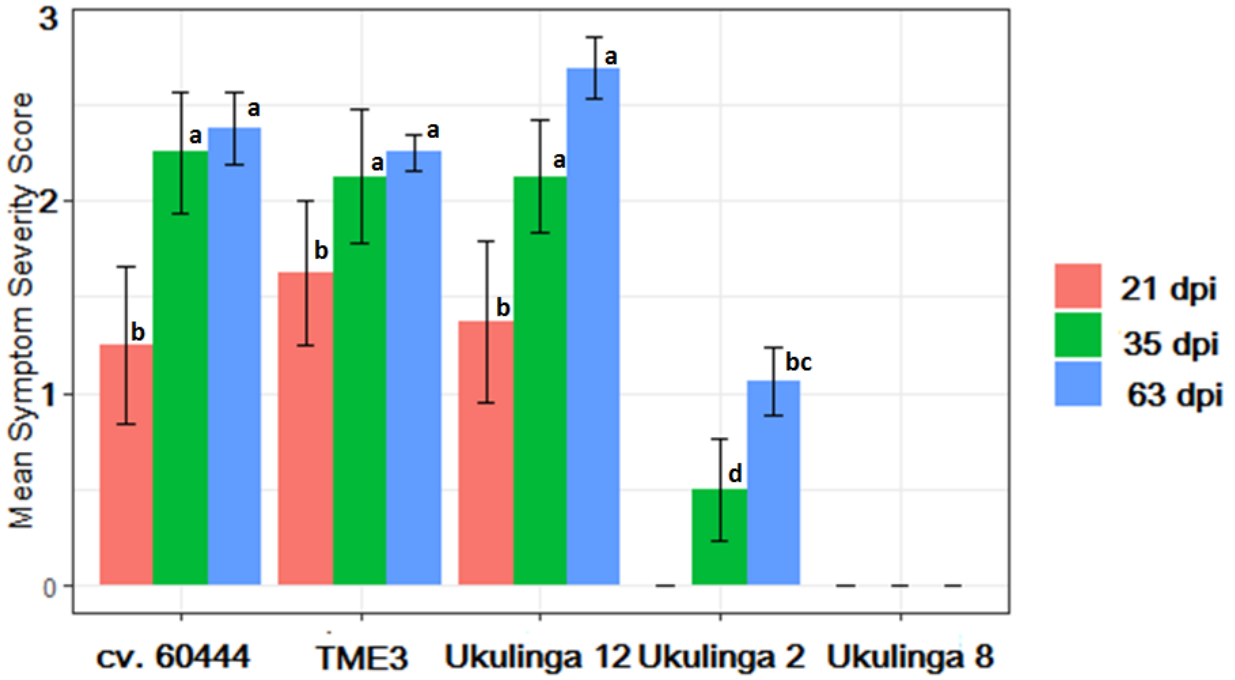


Figure 4.2: The mean symptom severity scores in cv. 60444, TME3, Ukulinga 2, Ukulinga 8 and Ukulinga 12 germplasm. Values indicate means of biological replicates with 6 plants per treatment. Bars indicate mean standard error and letters indicate the results of Tukey HSD test comparing values among the timepoints in the same genotypes ($p<0.05$).

When 63 dpi was reached, the disease symptoms were much more severe in cv. 60444, Ukulinga 12 and TME3 and remained mild for Ukulinga 2 **(Figure 4.3)**. The mean SSIs increased to peaks of 2.38 ± 0.18 in susceptible cv. 60444, 1.06 ± 0.18 in Ukulinga 2 and 2.69 ± 0.16 in Ukulinga 12, whilst a slight decline to 2.33 ± 0.18 was noted for TME3, known to show a degree of recovery **(Figure 4.2)**. Ukulinga 8 was the most CMD resistant without any observed systemic induction of symptoms by SACMV. The observed differences in SSIs between cv. 60444 and the two local germplasm Ukulinga 2 and Ukulinga 8 were subjected to Tukey's honest significant difference post hoc test which demonstrated statistically significant differences at ($p<0.05$) at all the 3 time points except at 21 dpi for Ukulinga 2. There was also significant differences ($p<0.05$) in average SSI between the CMD-tolerant control TME3 and the local germplasm Ukulinga 2 and Ukulinga 8 for the stated 3 time points.

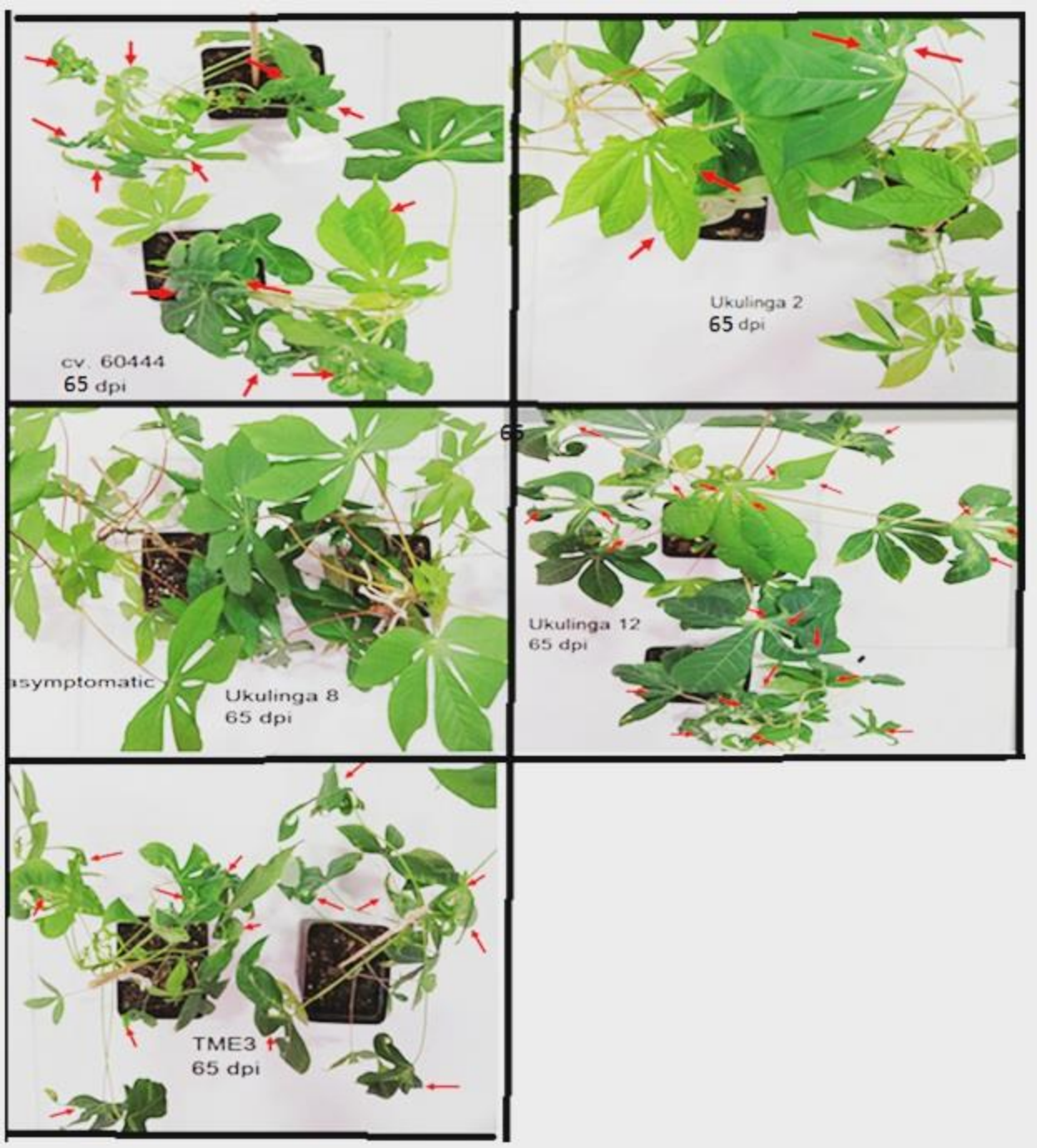


Figure 4.3: Cassava plant genotypes agro-infected with South African cassava mosaic virus showing CMD symptom severity at 65 dpi.

4.1.2 Cassava plant height evaluation

Ukulinga 2 and Ukulinga 8 cassava plants had higher increase in height with time compared with cv. 60444, Ukulinga 12 and TME3 in the duration of the study. Ukulinga 12 notably had the slowest change in height (**Figure 4.4**). One-way ANOVA demonstrated that there were significant statistical differences amongst the treatments. Tukey's honest significance difference (HSD) test was applied to identify the significant differences amongst the change in plant height of the genotypes. Both Ukulinga 2 and Ukulinga 8 change in plant height were significantly greater ($p<0.05$) than cv. 60444, whilst Ukulinga 12 was statistically significant slower ($p<0.05$) change in plant height. Furthermore, the petiole length and internodal length revealed that Ukulinga 2 and Ukulinga 8 had longer lengths in comparison to cv. 60444, Ukulinga 12 and TME3 (**Figure *S2***, **Figure *S3*** and **Figure *S4***).

In order to assess the extent of viral infection on plant growth, the plant height of SACMV-infected plants was compared to that of healthy and mock inoculated plants. The mean height difference among the healthy, the mock inoculated and SACMV-infected plants within the genotype was not significantly different ($p>0.05$) across all the treatments suggesting that plant growth rate was not affected negatively by the SACMV infection **(Figure 4.4)**. For example, the mean change of height within the healthy cv. 60444, mock inoculated cv. 60444 and SACMV-infected cv. 60444 was 160.71cm ± 11.35, 156.25cm ± 8.17 and 148.63cm ± 17.16, respectively.

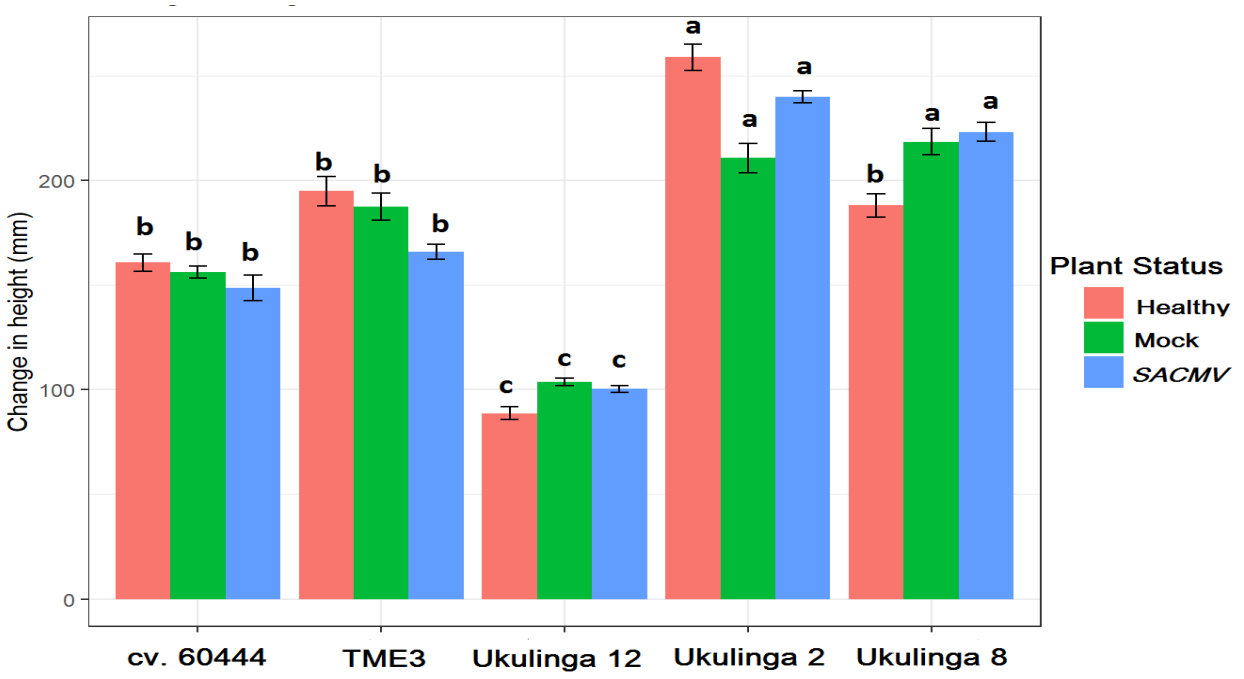


Figure 4.4: The mean change in height (plant growth) in healthy and mock inoculated plants compared to SACMV-infected cassava plants for each cassava genotype. Values indicate means of three trials with 6 plants per treatment. Bars indicate mean standard error and letters indicate the results of Tukey HSD test comparing values among treatments in the same genotype ($p<0.05$).

4.1.3 Cassava plant viral load

The relative viral load in infected cv. 60444, Ukulinga germplasm and TME3 were determined by real time quantitative PCR ($2^{-\Delta\Delta Cq}$) using the reference gene *UBQ10*. The relative viral load was calculated and compared for significant statistical differences ($p<0.05$) between all Ukulinga germplasm, TME3 and the cv. 60444 at three time points 14 dpi, 32 dpi and 65 dpi. The relative viral load of Ukulinga 12 germplasm was higher ($p<0.05$) compared to that of cv. 60444, whilst it was significantly lower for the Ukulinga 2, Ukulinga 8 and TME3 landraces at 14 dpi **(Figure 4.5)**. The relative viral load of Ukulinga 12 was significantly lower ($p<0.05$) than both of cv. 60444 and the known CMD-tolerant TME3 at 32 dpi, however Ukulinga 2 and Ukulinga 8 viral load remained significantly lower which corresponded with the asymptomatic plant leaf phenotypes observed at this stage. The susceptible cv. 60444 had 10800 fold ±396, TME3 had 7500 fold ±227, Ukulinga 12 was 1732 fold ±133 and Ukulinga 2 was 364 fold ±51 at 32 dpi in comparison to that of Ukulinga 8. At 65 dpi, cv. 60444 relative viral load accumulation had

increased significantly (54600 fold ± 4103) compared to that shown symptomless Ukulinga 8, whilst for Ukulinga 12 fold change was 7948 ± 669, for Ukulinga 2 382 ± 43, and for TME3 4496 ± 196 at $p<0.05$ **(Figure 4.5)**. A decline in virus load in the known recovery phenotype TME3 post 32 dpi was observed from 7500 fold ± 227 to 4496 fold ± 196 at 65 dpi, however persistent relatively mild CMD symptoms were observed. Overall, the noted low relative viral load of Ukulinga 2 and Ukulinga 8 at the 3 time points was synonymous with mild to no symptoms observed throughout the infection stages.

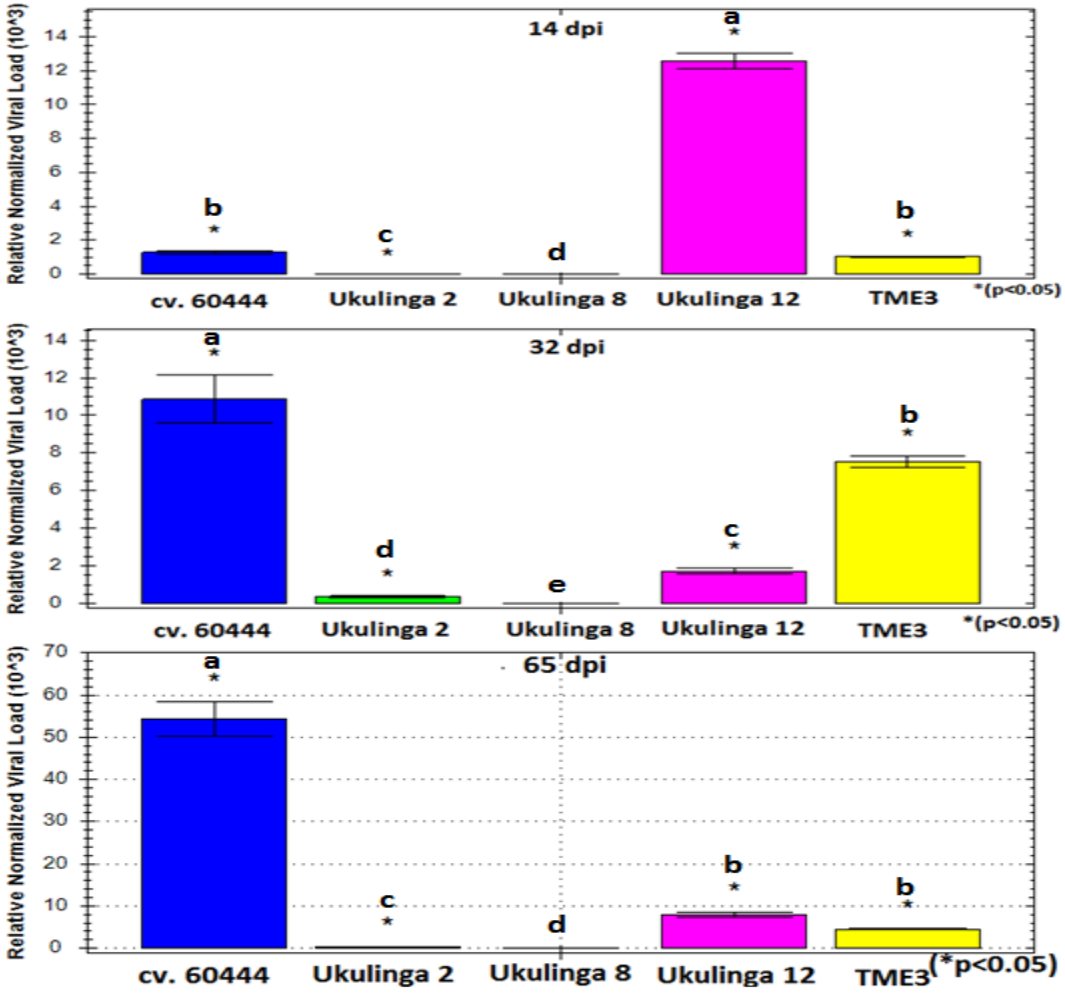


Figure 4.5: The viral load relative to reference gene *UBQ10* in model cultivar cv. 60444, Ukulinga 2, Ukulinga 8 and Ukulinga 12 and TME3 germplasm infected with South African cassava mosaic virus at 14 dpi, 32 dpi and 65 dpi. Error bars indicate mean standard error and

letters indicate the results of Tukey HSD test comparing values among genotypes in the same timepoint ($p<0.05$).

The virus load determinations were further substantiated by the relative band densities measured in various genotypes at the 14 dpi, 32 dpi and 65 dpi post real-time qPCR amplification of the section of the viral coat protein (150 bp). The model cv. 60444, Ukulinga 12 and TME3 demonstrated higher intensities of (1.00, 0.79, 1.21), (0.96, 0.97, 1.20) and (0.65, 1.34, 1.26) respectively at the 3 time points, relative to the resistant Ukulinga 2 and Ukulinga 8 germplasm which displayed lower band intensities of (0.50, 0.49, 0.40) and (0.12, 0.24, 0.43) (**Figure 4.6**).

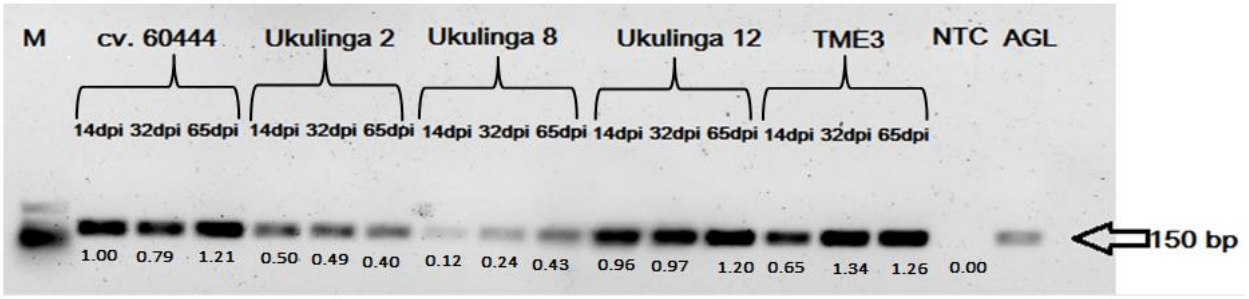


Figure 4.6: qPCR amplification products viral coat protein (AV1) loaded on a 1% (w/v) agarose gel of cassava genotypes at three time points; 14 dpi, 32 dpi and 65 dpi. M is the GeneRuler 1kb Plus DNA Ladder molecular weight marker, NTC is the non-template control and AGL is the negative control (mock inoculated).

There was no correlation between relative viral load and mean symptomatic score ($r = 0.37$), and there was also no significant correlation between the mean symptomatic score and time points (32 dpi and 65 dpi) ($r = 0.26$) (**Figure. 4.7**). There was a wide variation in relative viral accumulation patterns in the tested genotypes. For example, cv. 60444 had incremental viral titre accumulation at each subsequent time point, while Ukulinga 12 had an initial peak in relative viral titre at 14 dpi which declined at 32 dpi and then slightly increased at 65 dpi. Both TME3 and Ukulinga 2 relative viral load accumulation increased at 14 dpi to a peak at 32 dpi, and finally at the recovery stage (65 dpi), significant viral suppression were observed (**Figure. 4.5**).

There were no significant differences in the mean symptomatic scores of cv. 60444, Ukulinga 12 and TME3 at all the 3 time points despite the variation in relative viral accumulation trends (**Figure 4.5**). There was no correlation between the mean relative viral load and time points (r = 0.13), indicating variable viral responses amongst the tested genotypes.

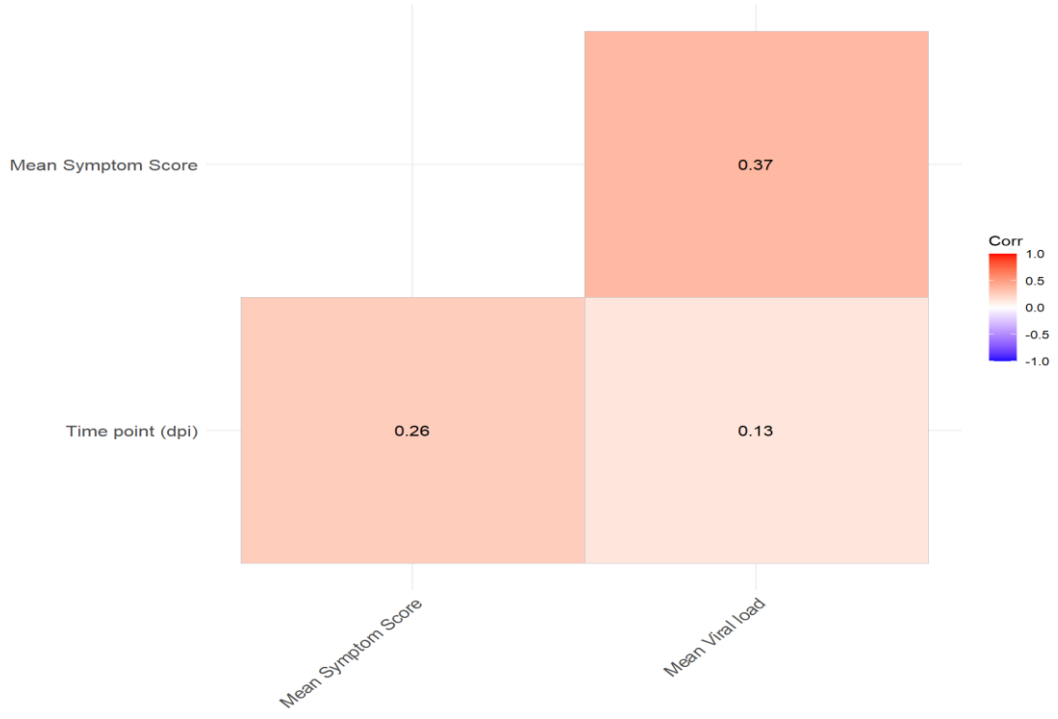


Figure 4.7: Correlation among the relative viral load, mean CMD symptomatic score and time points post SACMV infection.

4.2 Evaluation of transgenic cassava starch lines for storage root yield

4.2.1 Molecular screening of transgenic cassava starch lines

Confirmation of successful transformation of previously selected regenerated cassava lines for the hairpin cassettes harbouring either *adenine kinase* (*ADK*) or *uridiidine monophosphate synthase* (*UMPS*) genes was conducted by PCR. The presence of the reporter gene *Gus* (191bp) and antibiotic resistance selection gene *Hyg* (498 bp) were confirmed in the chosen transgenic starch lines, ADK 1, ADK 5, UMP 17, UMP 26 and UMP 28. The non-transgenic controls, cv. 60444 and T200 did not harbour the transgenes **(Figure 4.8)**. The transgenic line's capability to pass the rooting test (Bull et al., 2009) was further proof that they were successfully transformed.

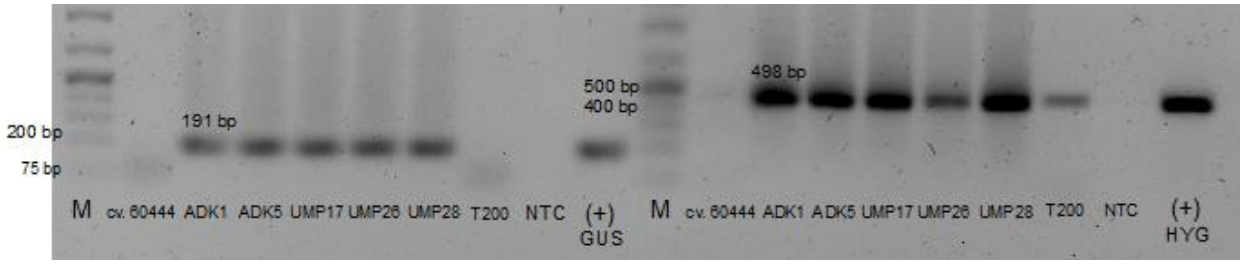


Figure 4.8: PCR detection of *Gus* (191 bp) and *Hyg* (498 bp) in genomic DNA extracted from transgenic cassava starch lines (ADK 1, ADK 5, UMP 17, UMP 26 and UMP 28). The GeneRuler 1 kb Plus molecular weight marker (M) (Thermo Fisher Scientific) was loaded on the first and eleventh lane and NTC is the non-template control containing deionised water. (+) GUS and (+) HYG are the positive controls containing the pCB33.

4.2.2 Cassava plant height

The cassava plants were measured every month for change in vertical height for a period of 6 MAP. There were no significant differences in the plant growth as measured by change of height between the two different growth environments of controlled growth conditions in the growth chamber facility and in the greenhouse facility. However, during trial 1, leaf shedding was minimal and the plant canopy (leaf area index) was very dense with visually higher leaf area index per plant in comparison to the greenhouse grown plants in trial 2. The denser canopy had the inadvertent effect of weighing heavily on the cassava stem and the stems had to be structurally supported using skewer woodsticks wedged into the soil running vertically upright. There were no structural support system needed for plants in the duration of trial 2. It was observed that in the greenhouse facility at approximately 4 months after planting, the cv. 60444 and the transgenic lines ADK 1, ADK 5, UMP 26 and UMP 28 were shedding more older leaves (senescence) and maintaining a very lean plant canopy in comparison to T200 and UMP 17 (**Figure *S5***). This effect is depicted by *h* which is the distance from ground to the point of the lowest leaf attachment on the stem.

The high-yielding T200 genotype had the slowest height change throughout the 6 month study period with significant difference in average plant height in month 2 (99.7mm), month 3 (175.8mm), month 4 (264.7mm) and month 6 (353.1mm) (**Figure 4.9**). The ADK 5 and the UMP 28 line had the highest change in height in the latter months of the study period, where for

month 4 both had signficant ($p<0.05$) higher average heights of 373.7mm and 402.8mm respectively. The Tukey's post hoc analysis proved significant ($p<0.05$) for month 5 as ADK 5 and UMP 28 achieved average heights of 421.8mm and 441.9mm respectively. At 6 MAP, only UMP 28 demonstrated a statistically ($p<0.05$) higher average plant height of 487.2mm compared to cv. 60444. Although ADK 5 and UMP 28 both had similar above-ground vertical change in height, the total storage root yield harvest of ADK 5 was more than 3 times achieved by UMP 28 specifically for the greenhouse-run trial.

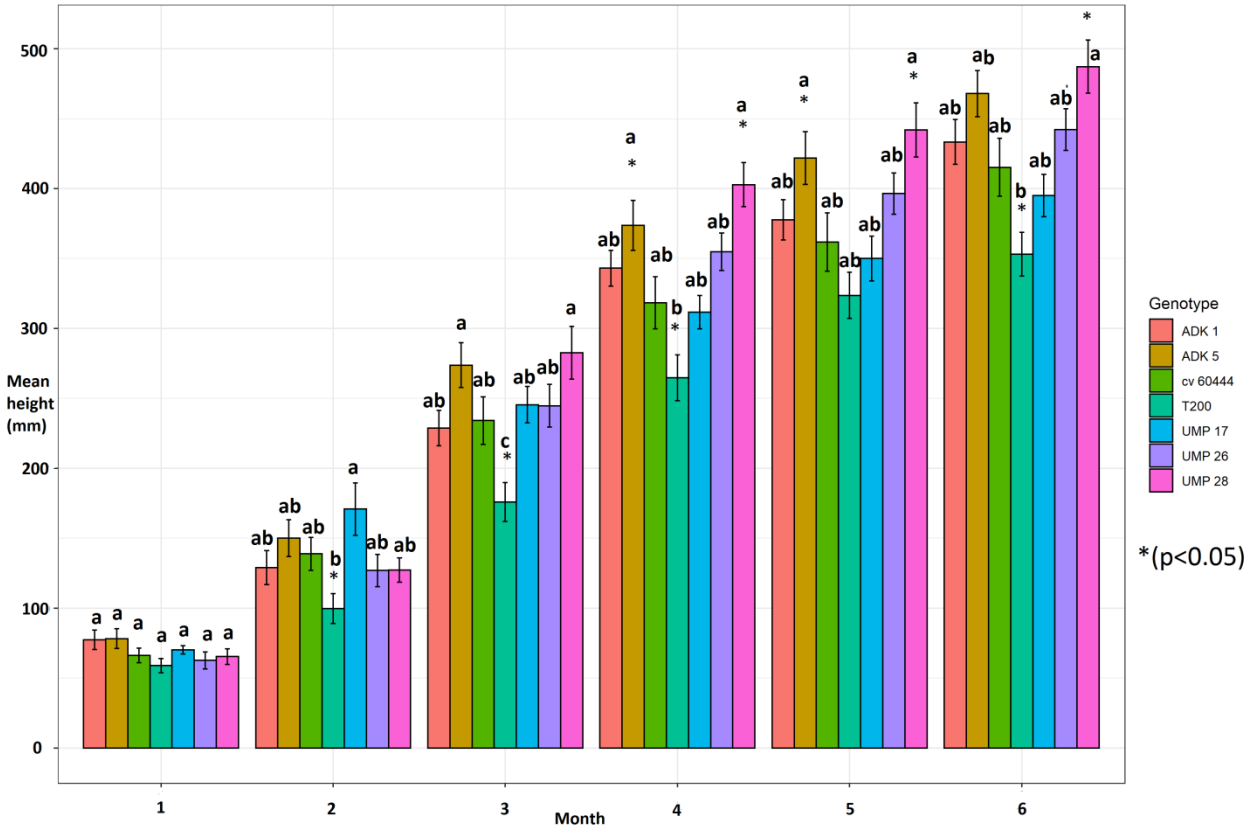


Figure 4.9: The average plant height of cassava genotypes over 6 months. Error bars indicate mean standard error and letters indicate the results of Tukey HSD test comparing values among genotypes in the same month ($p<0.05$).

4.2.3 Storage roots production and characteristics

Six months after planting, the transgenic cassava starch lines were harvested and assessed for production of storage roots. Characteristics of storage roots produced from the transgenic

cassava starch lines grown in the greenhouse facility are shown in **Table 4.1**. The one-way ANOVA test revealed that the total number of secondary storage roots produced by each plant of the T200 genotype was significant ($p<0.05$). Tukey's post hoc test was applied to identify the significant differences and showed that the T200 landrace produced significantly higher numbers of secondary storage roots per plant of 4.7 ± 0.7 than cv. 60444 (2.8 ± 0.6) while UMP 28 plants had significantly lower number (1.4 ± 0.2) of storage roots than the untransformed cv. 60444 (**Table 4.1**). The storage roots were classified using an adopted grading system such as small roots (<2g), medium roots (2-5g) and large roots (>5g) (Utsumi et al., 2020). Furthermore, the ADK 5 line had the highest significant ($p<0.05$) productivity per individual plant, contributing an average of 6.7 ± 1.1g. The UMP 28 line had the least number of roots produced per plant, and also had the lowest storage root yield productivity by weight per plant which was calculated to be a third of that obtained in the ADK 5 line and below the average of the control cv. 60444. UMP 17 and T200 individual plant storage root productivity of 5.2 ± 4.8g and 5.1 ± 1.3g, respectively, proved to be not statistically significant.

The roots harvested from the transgenic UMP 28 line had a significantly lower percentage dry weight of 16.8 ± 0.9 compared to cv. 60444, while UMP 26 storage roots had a significantly higher ($p<0.05$) percentage dry weight of 29.4 ± 1.0 compared to cv. 60444. The South African high-yielding T200 landrace was slightly lower than UMP 26 at 28.1 ± 2.4%. Notably, UMP 28 also had the lowest percentage amylose (11.9 ± 0.3%) of the total starch compared to the other genotypes that had percentage amylose contents in the range of 14.4% - 17.4%.

The transgenic ADK 1 line had a visual higher proportion of relatively small starch granules, UMP 26 and cv. 60444 had a greater proportion of below the average sized starch granules whilst ADK 5, UMP 17, UMP 28 and T200 storage roots had a greater proportion of above-average sized starch granules (**Figure 4.10**). The cv. 60444 had a characteristic mixture both oval and truncated shaped starch granules, while both ADK 5 and T200 storage roots presented largely truncated shaped granules. The remainder of the transgenic lines harboured spherical starch granules.

Table 4.1: Characteristics of storage roots produced from the transgenic cassava starch lines grown in the greenhouse facility

Genotypes	Σ Number of secondary storage roots/ plant	Average fresh storage roots yield/ plant (g)	% Dry weight	% Amylose (of total starch)	Range of starch granule sizes (µm)	Starch content (g/100g)
cv. 60444	2.8 ± 0.6^{ab}	3.1 ± 0.9^{ab}	22.8 ± 1.6^{ab}	17.4 ± 1.4^{a}	5.70 - 18.73	27.9 ± 1.3^{ab}
ADK 1	3.0 ± 0.6^{ab}	4.4 ± 0.8^{ab}	18.4 ± 2.7^{ab}	16.8 ± 0.3^{ab}	4.79 - 10.71	28.3 ± 1.4^{ab}
ADK 5	3.0 ± 0.4^{ab}	6.7 ± 1.1*^{a}	25.3 ± 1.5^{ab}	14.3 ± 0.8^{ab}	6.45 - 15.33	32.3 ± 0.6*^{a}
UMP 17	2.5 ± 1.5^{ab}	5.2 ± 4.8^{ab}	22.7 ± 2.9^{ab}	16.1 ± 1.7^{ab}	7.81 -14.39	12.8 ± 0.6*^{b}
UMP 26	2.8 ± 0.8^{ab}	4.7 ± 2.4^{ab}	29.4 ± 1.0*^{a}	15.7 ± 0.5^{ab}	6.62 -13.57	40.2 ± 1.3*^{a}
UMP 28	1.4 ± 0.2*^{b}	2.2 ± 0.6^{ab}	16.8 ± 0.9*^{b}	11.9 ± 0.3*^{b}	7.44 - 13.37	13.3 ± 1.0*^{b}
T200	4.7 ± 0.7*^{a}	5.1 ± 1.3^{ab}	28.1 ± 2.4^{ab}	16.0 ± 0.6^{ab}	8.62 - 16.43	21.6 ± 1.2*^{b}

* Asterisks indicate significant difference at $p<0.05$ and letters indicate the results of Tukey HSD test comparing values among the genotypes.

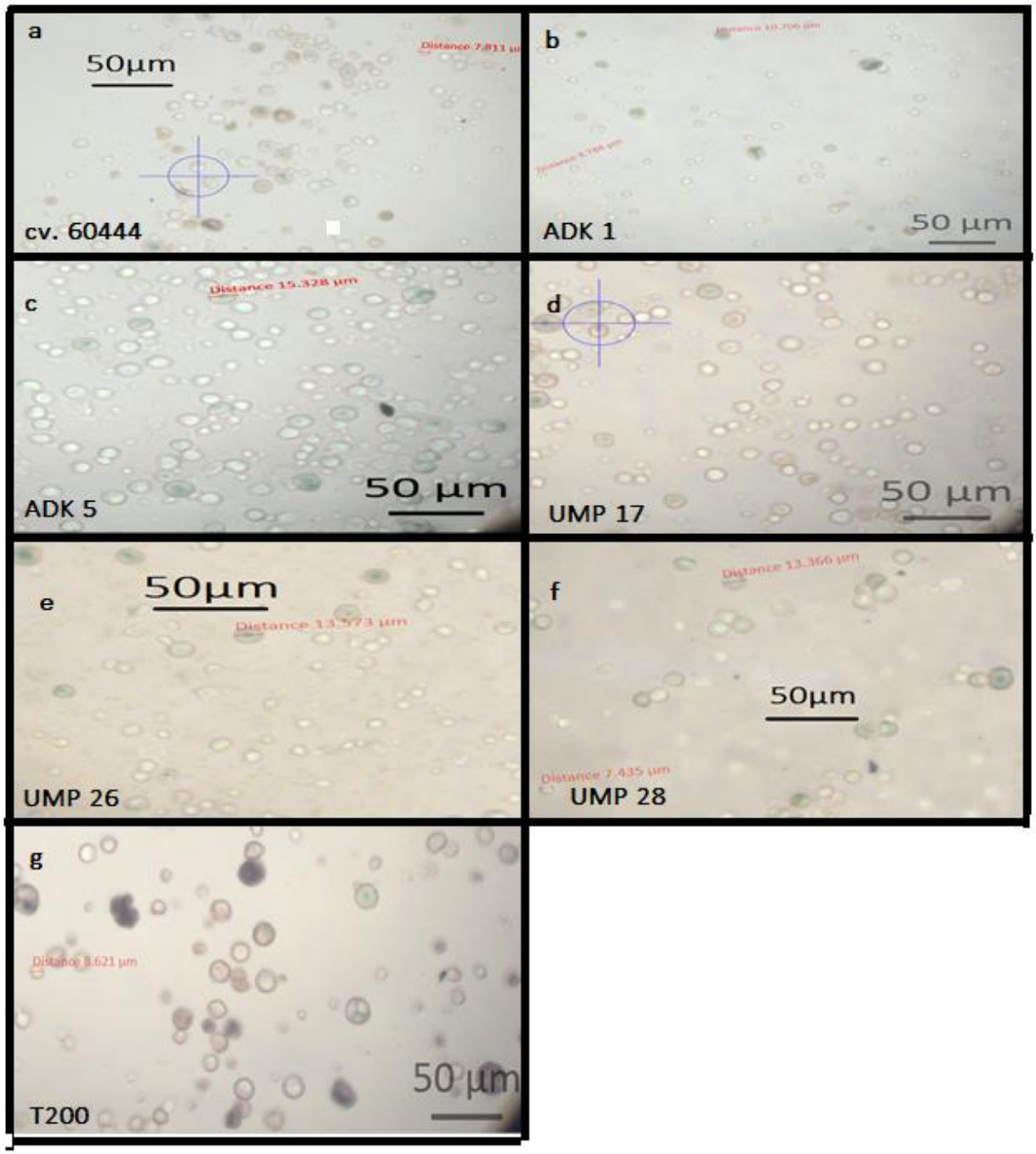


Figure 4.10: Morphology and size of starch granules in homogenized storage root tissue of various cassava genotypes stained with 50% (v/v) Lugol's solution

4.2.4 Storage root yield

The storage roots were harvested from cassava plants 6 MAP. The primary fibrous roots were stripped off and both secondary fibrous roots (>2mm) and storage roots were retained, washed, weighed and then stored at -70 °C until further use. The model cv. 60444, ADK 1, ADK 5 and UMP 28 produced storage roots with a dark brown periderm, whilst UMP 17 and UMP 26 had a much lighter brown periderm. T200 landrace presented a distinct off-white periderm colour (**Figure 4.11**).

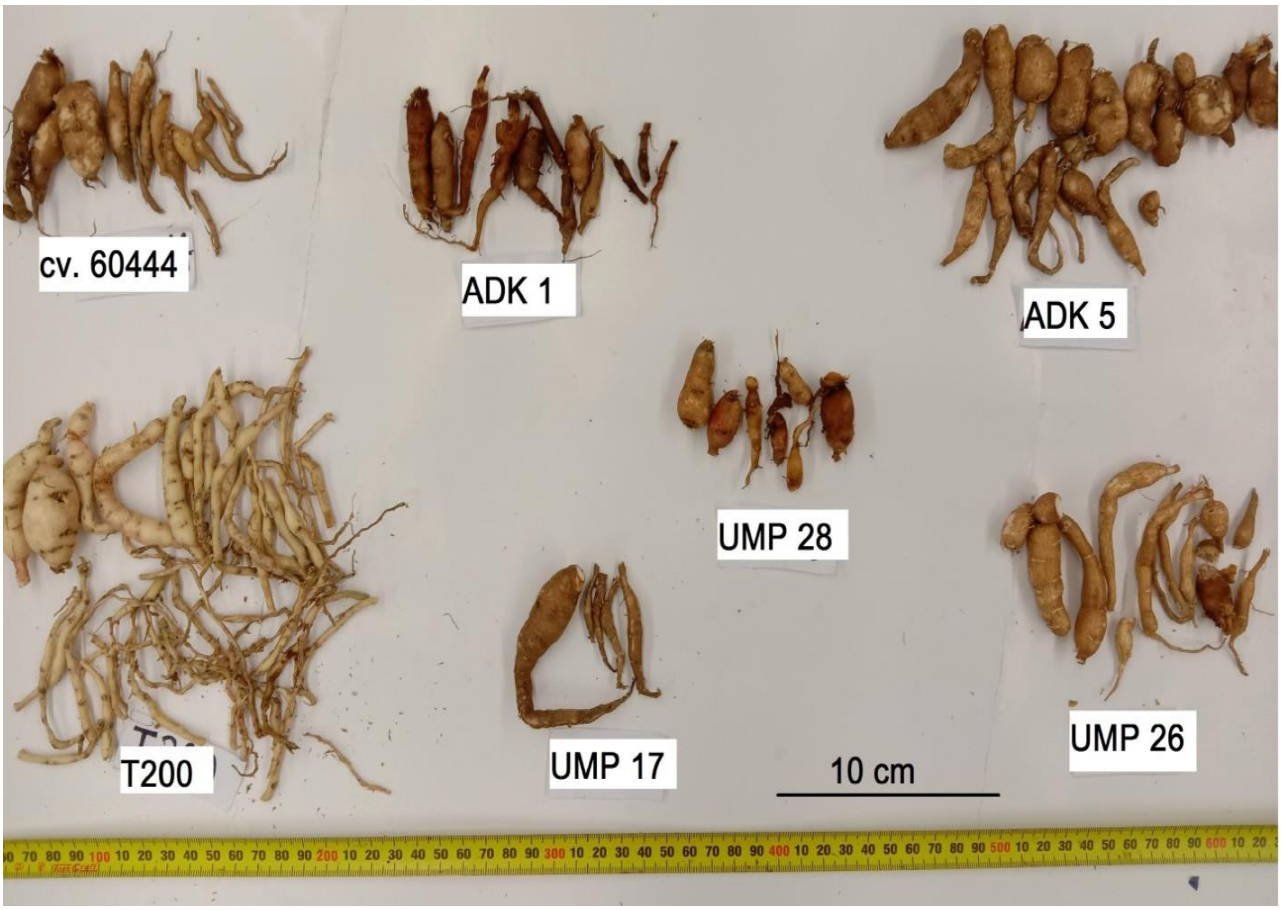


Figure 4.11: Storage root morphology and harvest yield from greenhouse facility-grown transgenic cassava lines, cv. 60444 and T200 landrace in trial 2.

Controlled conditions growth chamber facility (trial 1)

The ADK lines, ADK 1 and ADK 5 attained a low total yield harvest of 1.67g and 1.31g respectively (**Figure 4.12a**). The UMP lines all had a slightly higher total yield than the ADK

lines, however it was lower than the total yield of the control untransformed cv. 60444 which achieved a modest 6.84g (**Figure *S6***). Despite the high plant mortality rate in UMP 17, the total yield was 4.75g and UMP 26 achieved a yield of 5.50g. UMP 28 gave the lowest yield amongst the UMP lines of 3.53g. Overall, the controls cv. 60444 and T200 had much higher total yield in relation to the tested transgenic lines attaining 6.84g and 9.18g total yield, respectively.

Greenhouse facility (trial 2)

Twenty five percent of the cv. 60444 plants died and did not reach the full study term (6 months). The remaining plants yielded 18.3g of bulbous and elongated storage root matter (**Figure 4.11** and **Figure 4.12(b)**). The low harvest observed in ADK 1, UMP 17 and UMP 28 lines was to an extent due to transgenic plants which started to dry up 3 MAP before reaching full storage root development. The UMP 17 line had the highest incidence of plants drying up (67%) with a combined yield of 10.4g fresh weight, whilst ADK 1 and UMP 28 lines had 58% plant mortality and yields of 13.1g elongated storage roots and 11.1g bulbous storage roots, respectively. The UMP 26 line had 42% plant mortality, achieving 23.5g of mostly elongated-shaped roots. The ADK 5 lines and T200 landraces were largely not impacted by cassava plants premature dessication, with a comparatively low plant mortality of 8% and 0% respectively.

High mean number of storage roots produced per plant of each genotype and a high average storage root weight per individual plant both contributed to the overall high total yield output. For example, ADK 5 had significantly increased storage root weight per plant of 6.7 ± 1.1g and the average number storage root production per each plant was 3.0 ± 0.4 and T200 had a significantly high number of average storage root produced per plant of 4.7 ± 0.7 and a corresponding high average storage root weight per plant of 5.1 ± 1.3g (**Table 4.1**). Importantly, all ADK 5 plants attained the highest overall total yield harvest of mostly large (>5g), bulbous storage roots weighing 53.9g fresh weight in total, which was 195% higher than the untransformed cv. 60444 plants. The local landrace T200 had total storage root yield of 46.3g derived mainly from assorted big bulbous and elongated storage roots. This was 153% greater than cv. 60444 yield (**Figure 4.12(b)**). Also, the UMP 26 line attained an increased total yield (28%) in relation to the control cv. The comparison between plants grown in the growth chamber facility with controlled humidity, light and temperature, with those grown in the greenhouse

facility showed that cassava grown in the greenhouse produced significantly greater average number of storage roots with an overall higher average fresh root weight.

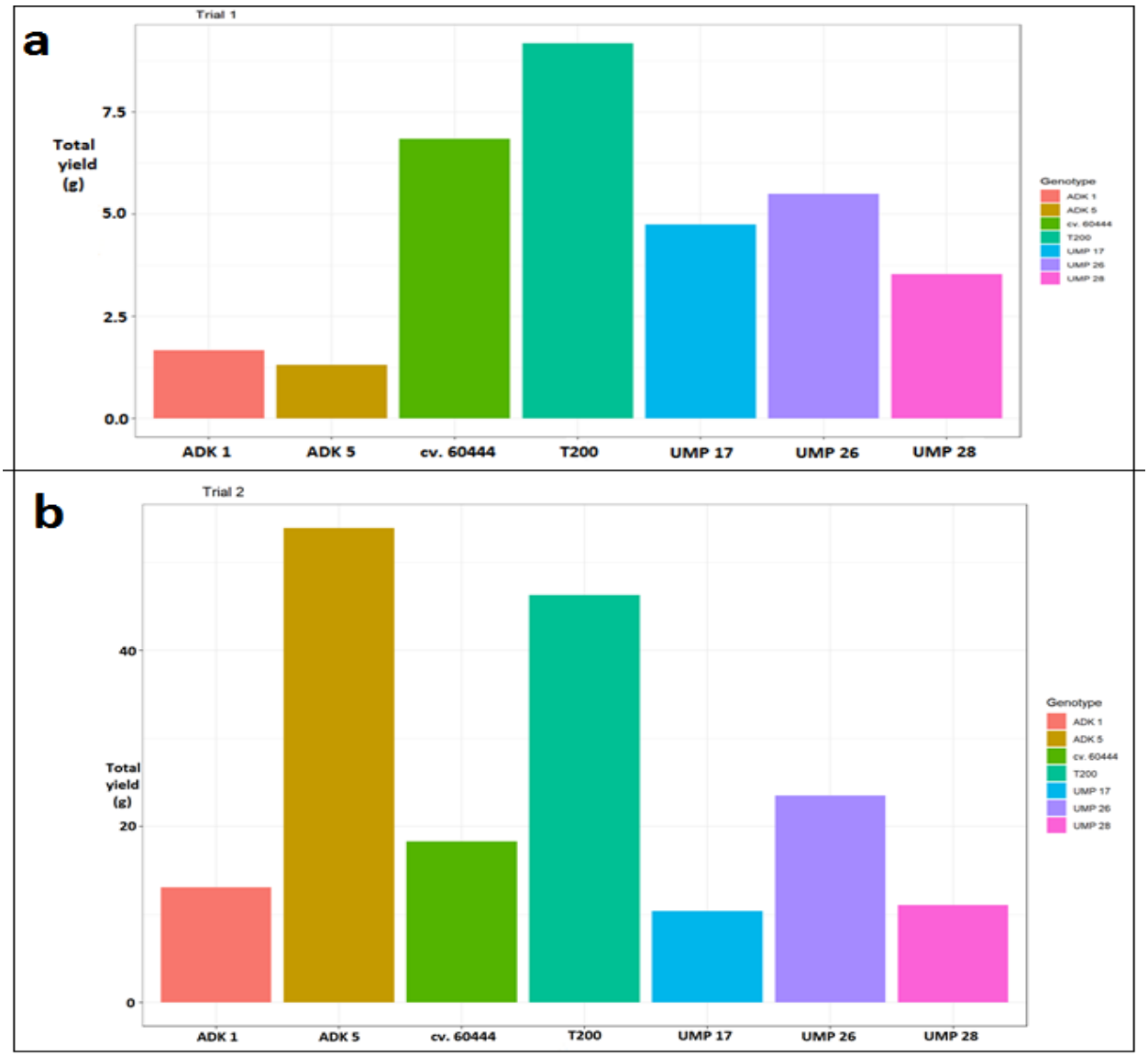


Figure 4.12: The total cassava storage root yield (a) from the controlled conditions growth chamber facility and (b) from the greenhouse facility. Each bar represents the total overall weight of all the pooled storage roots produced by each genotype.

Iodine interacts with the α 1,4 bonded amylose resulting in the iodine staining of starch (Tjaden et al., 1998). Thus, the percentage amylose can be qualitatively estimated to the extent of blue-black staining, where a darker overall iodine staining equates to higher amylose content and a

pale blue-black staining is equated to a lower amylose content in the storage roots. The colourimetric qualitative starch assay were compared to that of the cv. 60444 storage roots as a standard. The reported reduced starch of the UMP 28 line of 13.3 ± 1.0% (**Table 4.1**) correlated with the observed pale blue staining of UMP and the reduced starch content could have an added impact (**Figure 4.13**). The darker shades of blue-black staining of the storage root disks observed in cv. 60444, ADK 1, ADK 5, UMP 17, UMP 26 and T200 were a preliminary indication a higher concentration of percentage amylose content. There was no significant difference in percentage amylose content of storage roots between the untransformed cv. 60444 and the other ADK and UMP lines.

Starch contributes to the majority of the dry weight content of mature storage roots. The transgenic lines ADK 1 and UMP 28 storage roots had a low percentage dry weight of below 19% compared to the other genotypes which surpassed 22% dry weight, with notably the transgenic UMP 26 line having a peak storage root percentage dry weight of 29.4 ± 1.0%. There was some correlation (r=0.63) between the percentage dry weight and the starch content of the storage roots from trial 1, whilst in trial 2 there no correlation (r=0.3). For example in trial 2, the high percentage dry weight of the ADK 5 (25.3 ± 1.5%) and UMP 26 (29.4 ± 1.0%) lines correspondingly had high starch content of 32.3 ± 0.6g/100g and 40.2 ± 1.3g/100g respectively. The significantly low percentage dry weight of UMP 28 line (16.8 ± 0.9%) was coupled with low starch content of 13.3 ± 1.0g/100g. However, the characteristic off-white coloured storage roots of T200 which had high percentage dry weight of 28.1 ± 2.4% had below the anticipated starch content at 21.6 ± 1.2g/100g, whilst the ADK 1 storage roots which had low percentage dry weight (18.4 ± 2.7%) content had higher than anticipated starch content of 28.3 ± 1.4g/100g. The untransformed cv. 60444 and UMP 17 had dry weight content of 22.8 ± 1.6% and 22.7 ± 2.9% respectively. However, the starch content of cv. 60444 was 27.9 ± 1.3g/100g in contrast to the statistically significant low starch content of 12.8 ± 0.6g/100g of UMP 17 lines.

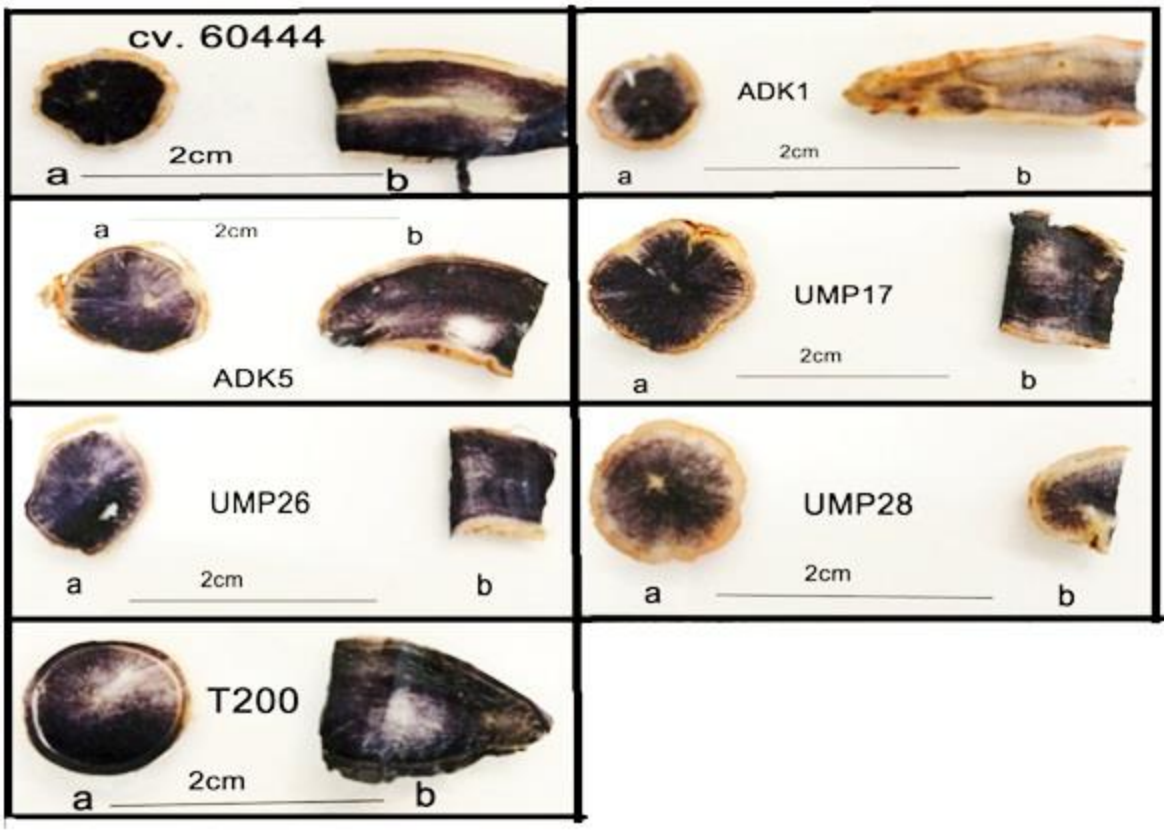


Figure 4.13: Transverse (a) and longitudinal (b) sections of storage root disks from different cassava genotypes stained with 50% (v/v) Lugol's solution to visualize presence of starch

The aggregation of starch granules as radial multi-seriate chains was observed in cv. 60444, ADK 1, UMP 17, UMP 26 and UMP 28 storage root tissue. The UMP 28 had sparsely arranged starch deposition with non-starch tissue spaces (white spaces) in-between (**Figure 4.14**). The cv. 60444, ADK 1 and UMP 26 accessions had much denser radial starch granule alignment compared to ADK 1, and UMP 28. The transgenic ADK 5 line had visually compacted non-aligned starch granules.

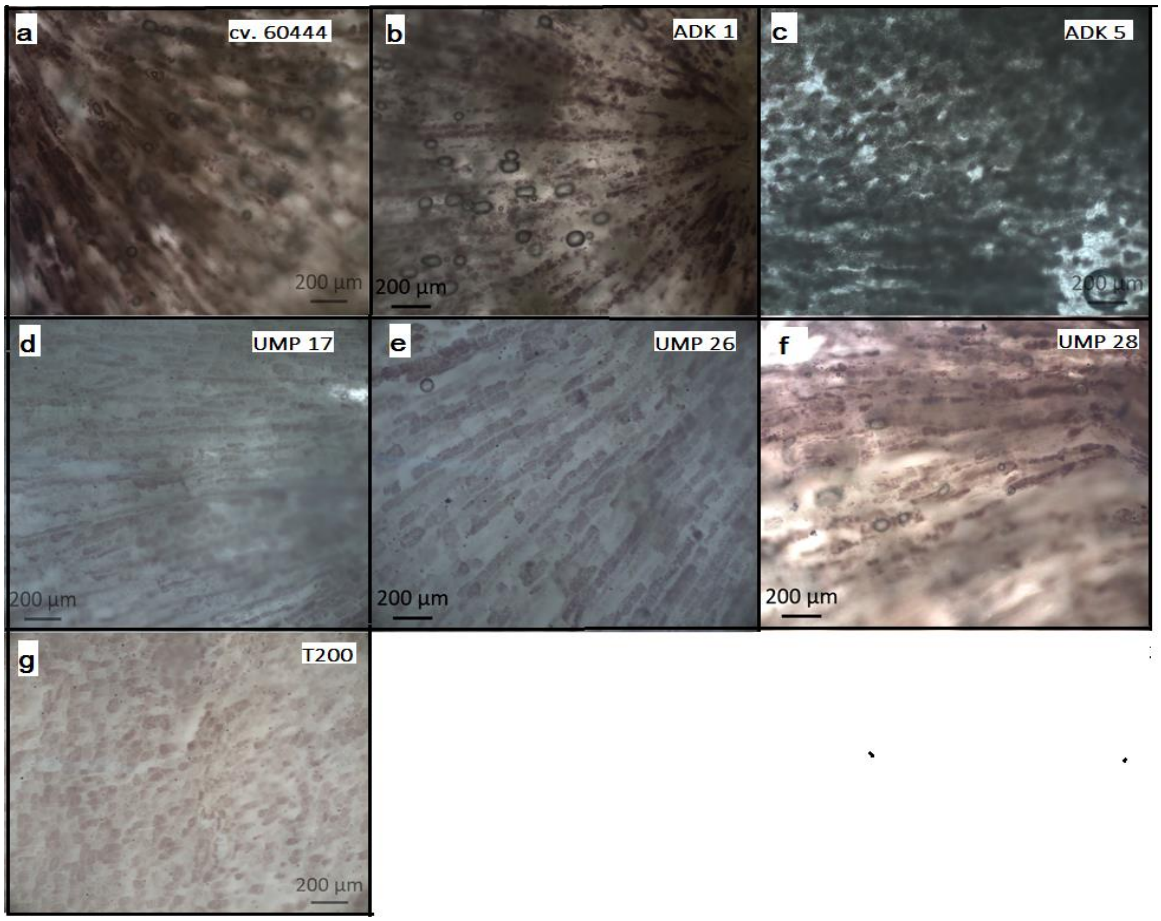


Figure 4.14: Thin hand-cut cross section of storage roots fresh tissue stained with 50% (v/v) Lugol's solution to visualize starch granule density in various cassava genotypes.

Toluidine stained the pectin-containing parenchyma tissue purple and the lignin-concentrated xylem cells developed a cyan colour (**Figure 4.15**). The roots were observed at only the final phase of storage root development (root bulking) hence it was not possible to note prior vascular bundle morphology and development at the intermediate root, fibrous root and root differentiation stages. Microscopic analysis of the storage roots showed a typical enlarged central vascular bundle surrounded by the pericycle and endodermis tissue (**Figure 4.15**). The vasculature was more compact in cv. 60444, ADK 1, ADK 5, UMP 26 and T200 compared to the UMP 17 line, where vasculature was observed to be more loosely dispersed. The phloem sieve plates (PSPs), which occur between the long sieve tube cells in the phloem form the passage of route that the photosynthetic products from leaves use to reach the roots. There was a

higher proportion of callose deposit around the PSPs of storage roots of ADK 5 and UMP 26 compared to the other genotypes.

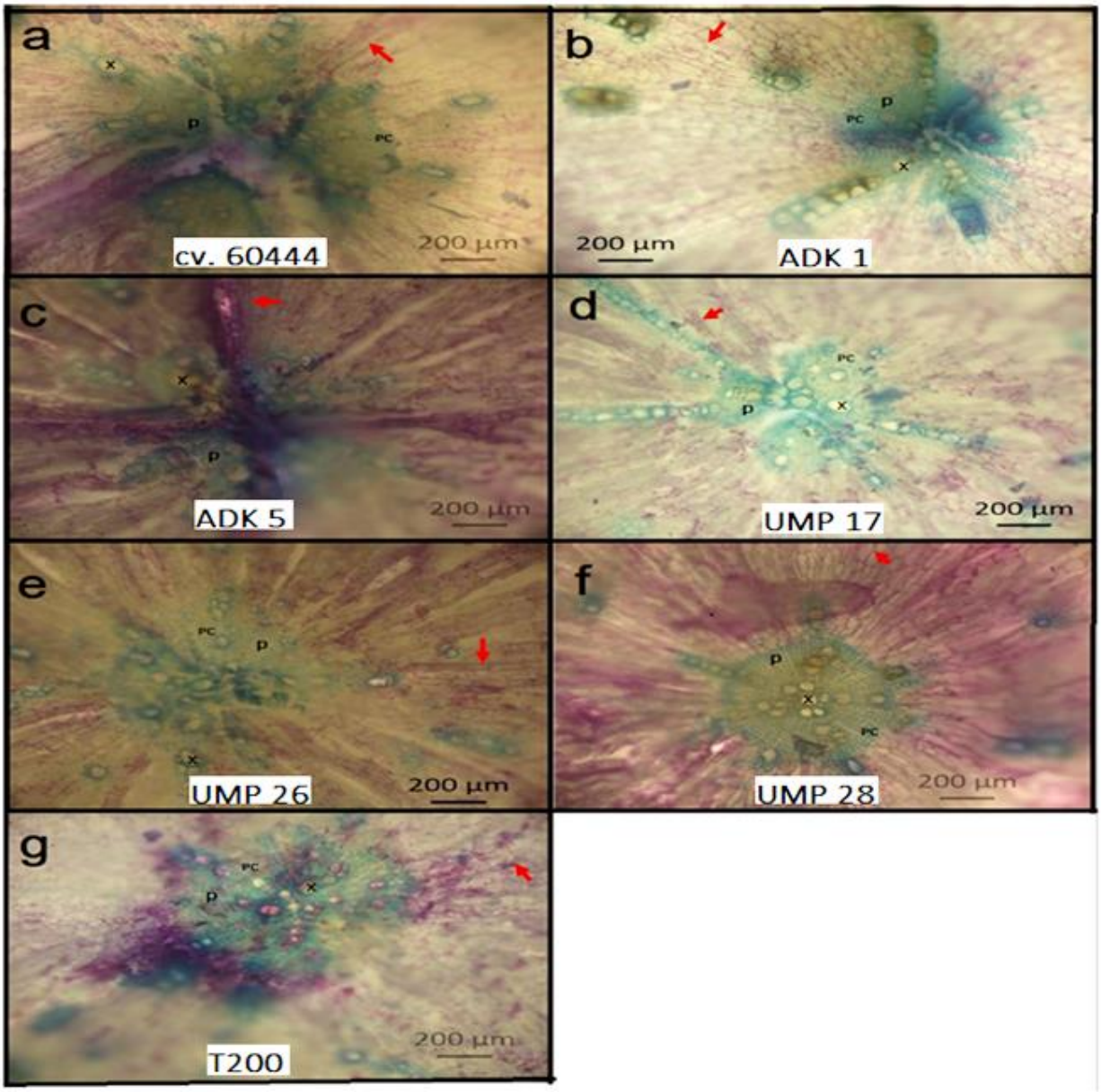


Figure 4.15: Cross section of secondary storage roots showing phloem and xylem cells in cassava storage root of various genotypes stained with 0.1% (w/v) toluidine. X - xylem, p - phloem, pc - procambium, arrowhead denotes phloem sieve plates (PSPs).

4.2.5 Gene expression study

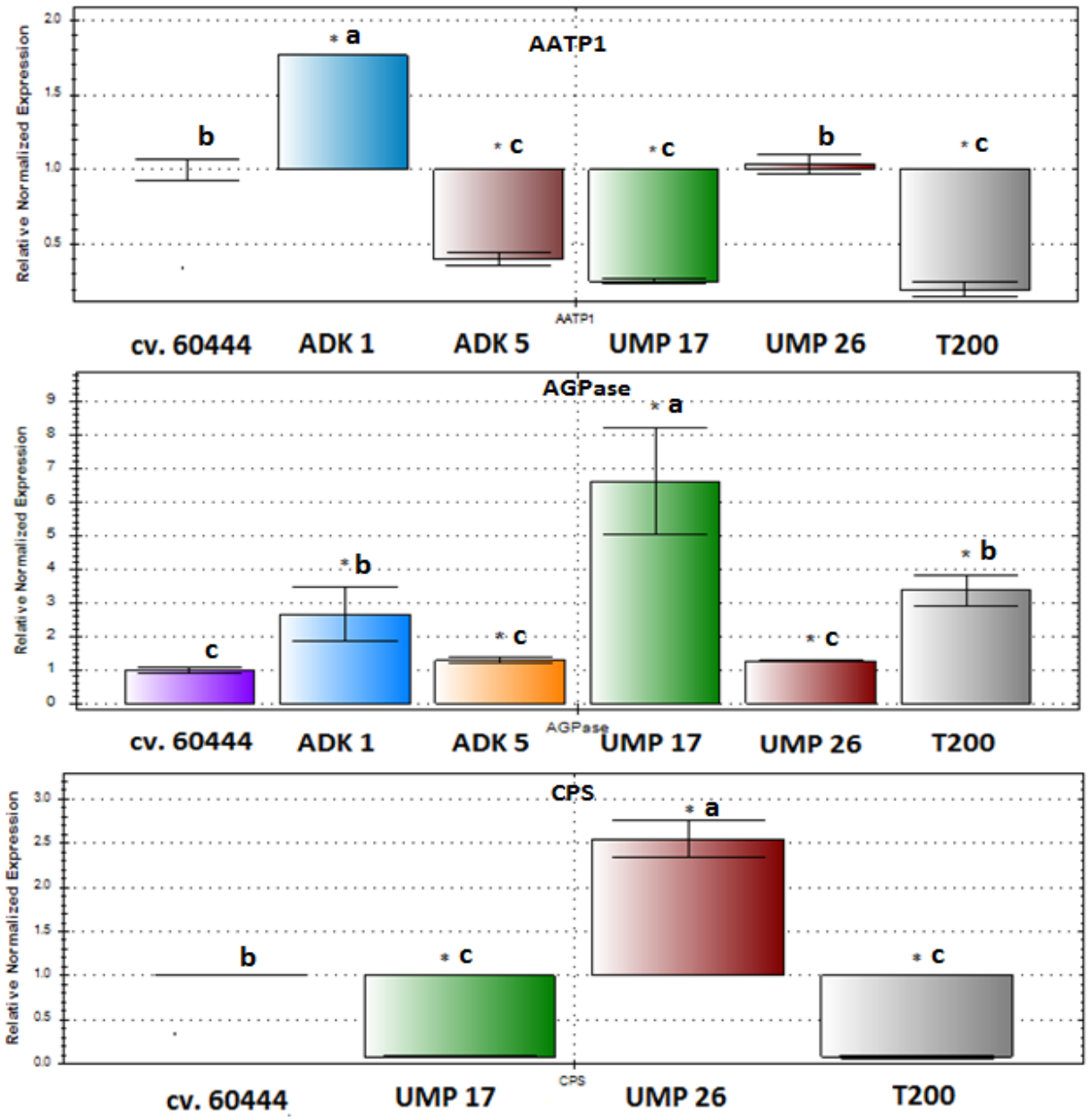
The relative expression of key starch biosynthetic enzymes (*AATP1*, *AGPase* and *CPS)* in transformed lines was evaluated by RT-qPCR relative to *UBQ10* as a reference gene and compared to the model cv. 60444. The silencing of *adenylate kinase (ADK)* in root tissue from trial 2 resulted in ADK lines (ADK 1 and ADK 5) having significantly lower expression levels of *AATP1* at 1.00 ± 0.06 and 0.98 ± 0.02 respectively in comparison to the untransformed cv. 60444 **(Figure 4.17)**. Furthermore, the transgenic UMP lines; UMP 17, UMP 28 and UMP 26 had significantly lower expression levels of the *AATP1* at 0.01 ± 0.00, 0.10 ± 0.02 and 1.84 ± 0.22 respectively. The T200 landrace had 0.90 ± 0.04 decreased significant expression level of *AATP1*. There was a contrast in relative expression levels of *AATP1* between the greenhouse-grown cassava plants (trial 2) from the plants which were grown in the controlled-conditions growth facility (trial 1). The ADK 5, UMP 17 and the T200 landrace from trial 1 had correspondingly lower significant expression level of *AATP1* of 0.41 ± 0.04, 0.25 ± 0.01 and 0.20 ± 0.04 compared to cv. 60444 (**Figure 4.16**). UMP 26 had a similar increased expression level of *AATP1* to that of cv. 60444. The transgenic ADK 1 storage root tissues achieved the higher significant expression level than cv. 60444 of 1.80 ± 0.24. The harvested UMP 28 storage root material from trial 1 was partially decomposed which made extraction of RNA of high integrity impossible. Summarily, the downregulation of plastidial *ADK* gene in the storage root tissues led to generally decreased expression levels of *AATP1* in transgenic lines of both trial 1 and trial 2.

The transgenic lines and the high-yielding T200 landrace all demonstrated upregulated expression of *AGPase* in comparison to cv. 60444 in trial 2. However, only ADK 5, UMP 17 and UMP 28 demonstrated statistically significant higher expression levels at 3.27 ± 0.16, 4.40 ± 0.72 and 5.36 ± 0.71 respectively in relation to cv. 60444 **(Figure 4.17)**. The remainder of the transgenic lines, ADK 1, UMP 26 and T200 landrace demonstrated expression levels of *AGPase* not significant cv. 60444. For trial 1, the level of expression of the *AGPase* was also significantly ($p<0.05$) upregulated across all the cassava genotypes relative to cv. 60444 (**Figure 4.16**). The transgenic line ADK 5 and UMP 26 had increased similar significant expression levels of *AGPase* of 1.24 ± 0.08 and 1.23 ± 0.04 respectively compared to cv. 60444. The transgenic ADK 1 line and T200 landrace demonstrated increased expression levels of *AGPase*. The highest

relative expression level of *AGPase* gene was obtained from the UMP 17 genotype with 6.72 ± 1.56.

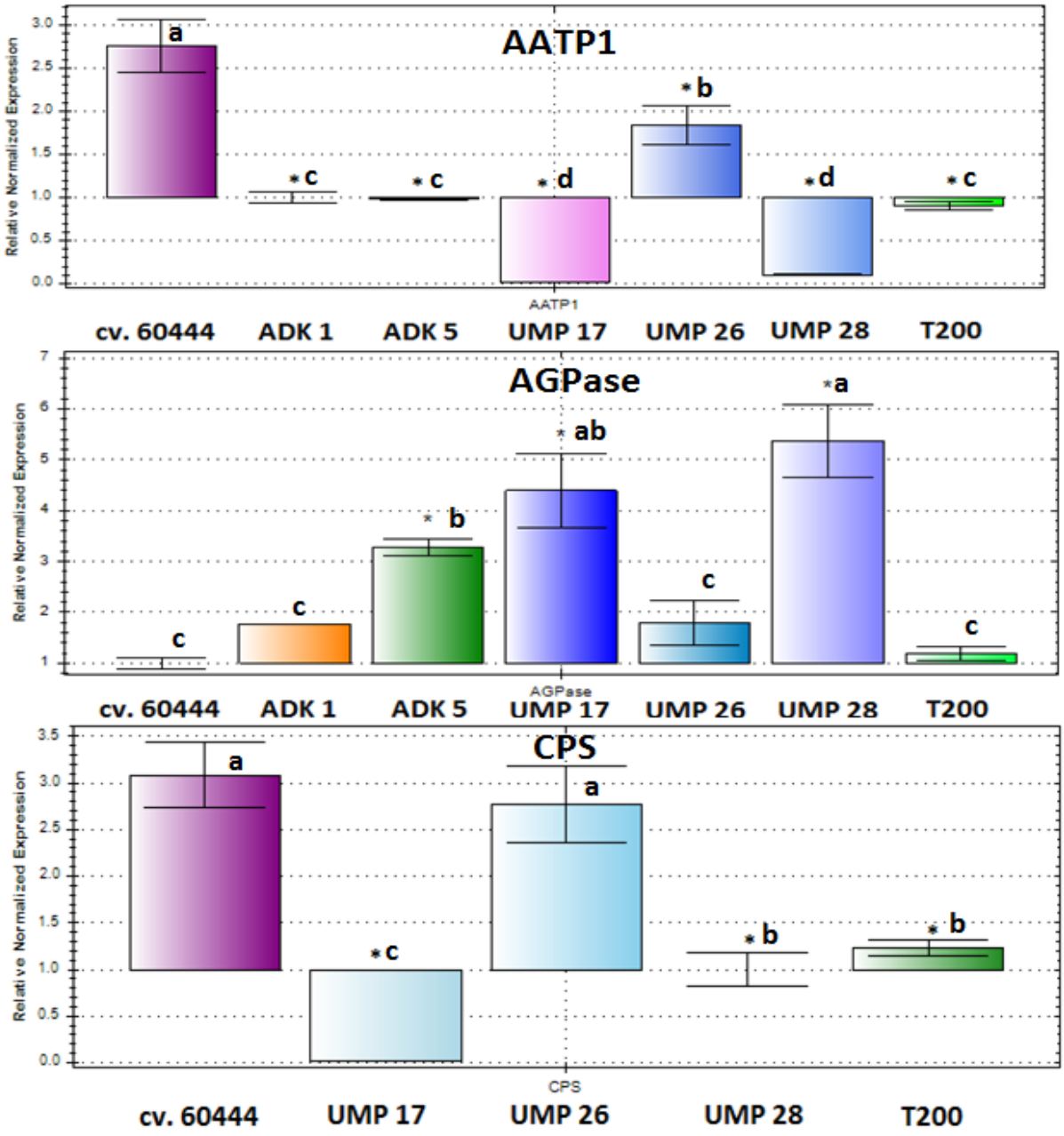
The silencing of *UMPS* gene in root tissues in UMP lines had the overall impact of lower relative expression of *CPS* gene in UMP transgenic lines for trial 2. The UMP 17, UMP 28 and T200 had significantly reduced expression levels of *CPS* of 0.03 ± 0.01, 1.00 ± 0.17 and 1.23 ± 0.08 respectively in comparison to the control cv. 60444 **(Figure 4.17)**. UMP 26 had relative expression levels of *CPS* of 2.77 ± 0.40, which was not significant. From trial 1, UMP 26 was the sole transgenic line from which recorded raised expression levels of *CPS* of 2.54 ± 0.21 compared to cv. 60444 which attained 1.00 ± 0.03 (**Figure 4.16**). Both the UMP 17 and T200 landrace attained equal lower significant relative expression values of *CPS* of 0.08 ± 0.01 compared to cv. 60444 of 1.00 ± 0.03.

When taken together, generally, there were significant differences in the reduced relative expression levels of the plastidial adenylate transporter, *AATP1* in all of the transgenic lines from both trials, in comparison to untransformed cv. 60444, except for only ADK 1 in from trial 1 which had 1.8 fold higher relative expression of *AATP1* than cv.60444. There was an overall increased relative expression level of *AGPase* across all the transgenic lines in the two trials. Significant increased levels were recorded in all the transgenic lines from trial 1 compared to cv. 60444, whilst only ADK 5 and UMP 17 had significant increased relative expression of *AGPase* in relation to cv. 60444 from trial 2. The expression level of the pyrimidine pathway gene, *CPS* had reduced expression in all the transgenic lines of trial 2, with significantly lowered expression levels in UMP 17, UMP 28 and T200 compared to cv. 60444. However, reduced significant expression levels of *CPS* were recorded in only UMP 17 and T200, whilst UMP 26 had significant raised expression level of *CPS* of 2.5 times in trial 1 compared to cv. 60444.



*Asterisks denote statistically significant results (*p<0.05) and error bars indicate mean standard error and letters indicate the results of Tukey HSD test comparing values among genotypes in the same gene (p<0.05).

Figure 4.16: Relative gene expression of starch biosynthesis-associated genes; *ATP/ADP antiporter* (*AATP1*), *adenine diphosphate glucose pyrophosphorylase* (*AGPase*) and pyrimidine biosynthesis-linked gene *carbamoyl phosphate synthas*e (*CPS*) of mature cassava storage roots in trial 1.



Asterisks denote statistically significant results (*p<0.05) and error bars indicate mean standard error letters indicate the results of Tukey HSD test comparing values among genotypes in the same gene (p<0.05).

Figure 4.17: Relative gene expression of starch biosynthesis-associated genes; *ATP/ADP antiporter* (*AATP1*), *adenine diphosphate glucose pyrophosphorylase* (*AGPase*) and pyrimidine biosynthesis-linked gene *carbamoyl phosphate synthas*e (*CPS*) of mature cassava storage roots in trial 2.

4.2.6 Pearson's correlation

The Pearson's correlation analysis was conducted to determine if there was a correlation between total starch (g/100g) and percentage dry weight of the storage roots. There was weak, but significant correlation (r=0.3, p=0.042) in trial 2 (**Figure 4.18)**, whilst trial 1 had moderate significant (r=0.63, p=8.4 x 10^{-6}) correlation (**Figure 4.19)**. This illustrates that storage roots with higher dry weight percentage had higher total starch content and storage roots with lower dry weight percentage had a lower total starch content. Small-sized cassava storage roots tend to possess a greater fibre to total starch ratio, whilst larger storage roots have a lower fibre to total starch ratio, hence a higher total starch content (Vasconcelos et al., 2017). In general, trial 2 yield had a much wider range of small size to very large storage roots hence the larger variance and low correlation observed compared to trial 1, where the storage roots were comprised of almost equal sizes.

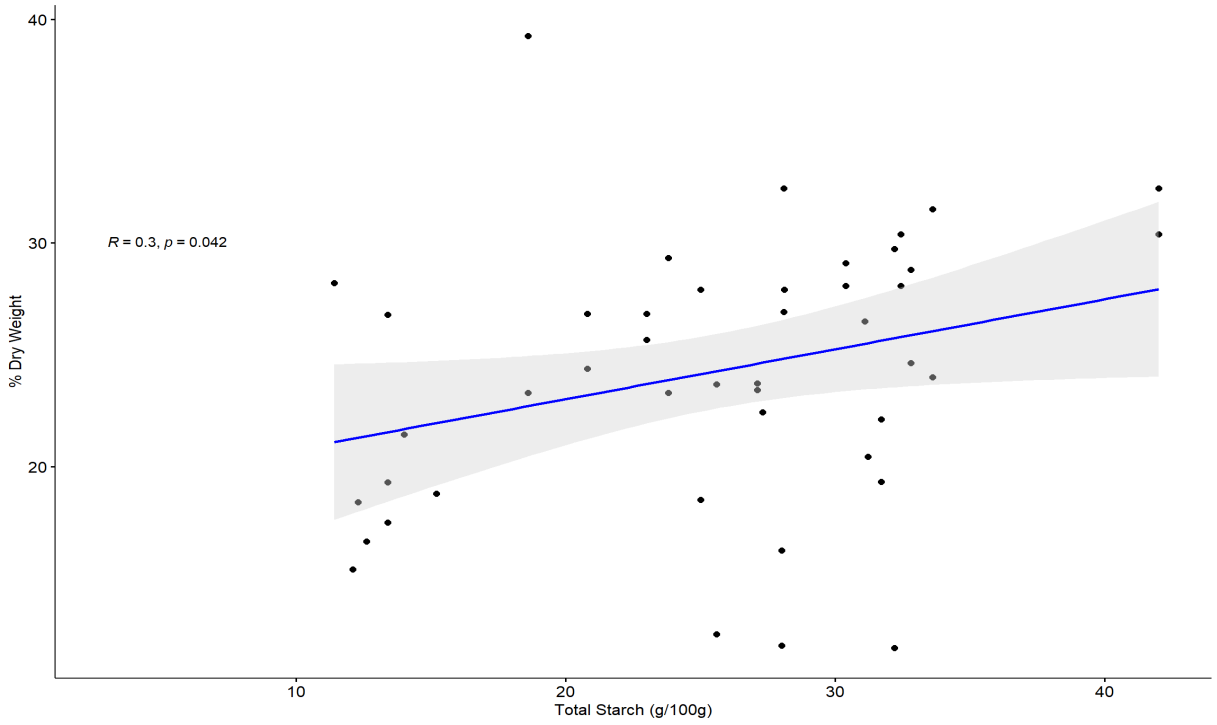


Figure 4.18: Scatter plot with trend line displaying the correlation (Pearson's) between total starch and percentage dry weight of storage roots from trial 2

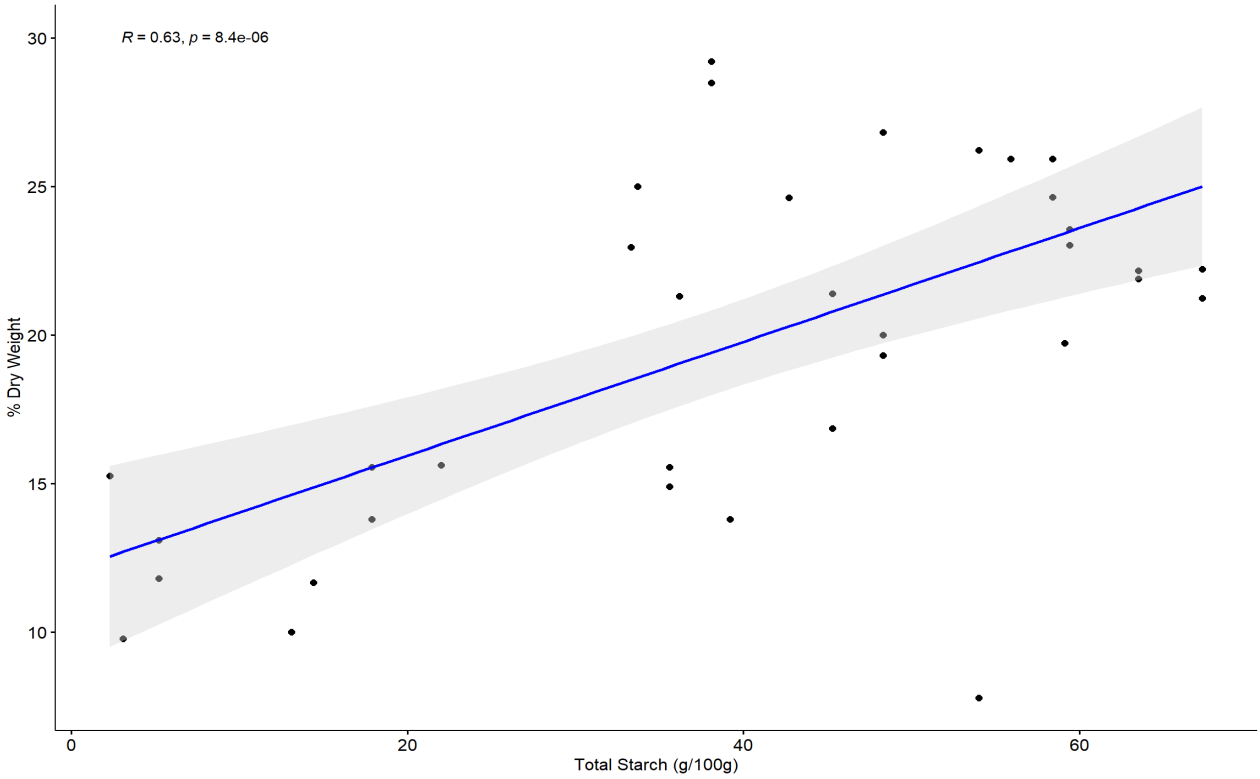


Figure 4. 19: Scatter plot with trend line displaying the significant Pearson's correlation between total starch and percentage dry weight of storage roots from trial 1

4.2.7 Principal component analysis (PCA)

The principal component analysis (PCA) of storage root traits (*AATP1* expression, *AGPase* expression, starch content, number of storage roots per plant, fresh weight yield per plant, percentage dry weight of storage roots) and plant height of all transgenic lines and untransformed controls in groups for trial 2 was performed. Results demonstrated an overlapping arrangement of genotypes with clear separation of UMP 17 and UMP 28 lines from the other genotypes, and were closer together signifying that they are much more similar to each other (**Figure 4.20**). There was little variation among the cv. 60444, ADK 1, ADK 5, UMP26 and T200 genotypes from trial 2. The relative expression of the *AATP1* was highly correlated to the total starch content of the storage roots, whilst the expression of *AGPase* was inversely proportional to percentage dry weight of the storage roots. The PCA of trial 1 showed clear segmented separation amongst the cassava genotypes with much less intersection amongst the groups. As has been illustrated with the scatterplot (**Figure 4.21)**, there was a high correlation relationship between percentage dry weight and total starch (**Figure 4.19)**. Plant height and total starch

content were major contributors to the separation of genotypes in trial 1 but total starch made only minor contributions in trial 2. Principal component 1 (PC1) was responsible for 93.5% and 98.4% of variation observed in trial 1 and trial 2 respectively. Such high variations could point to presence of outliers in the datasets, perhaps the addition of more repeats, number of plants and including parameters such as enzyme activities, metabolites and phytohormones levels could circumvent such discrepancies in the analysis.

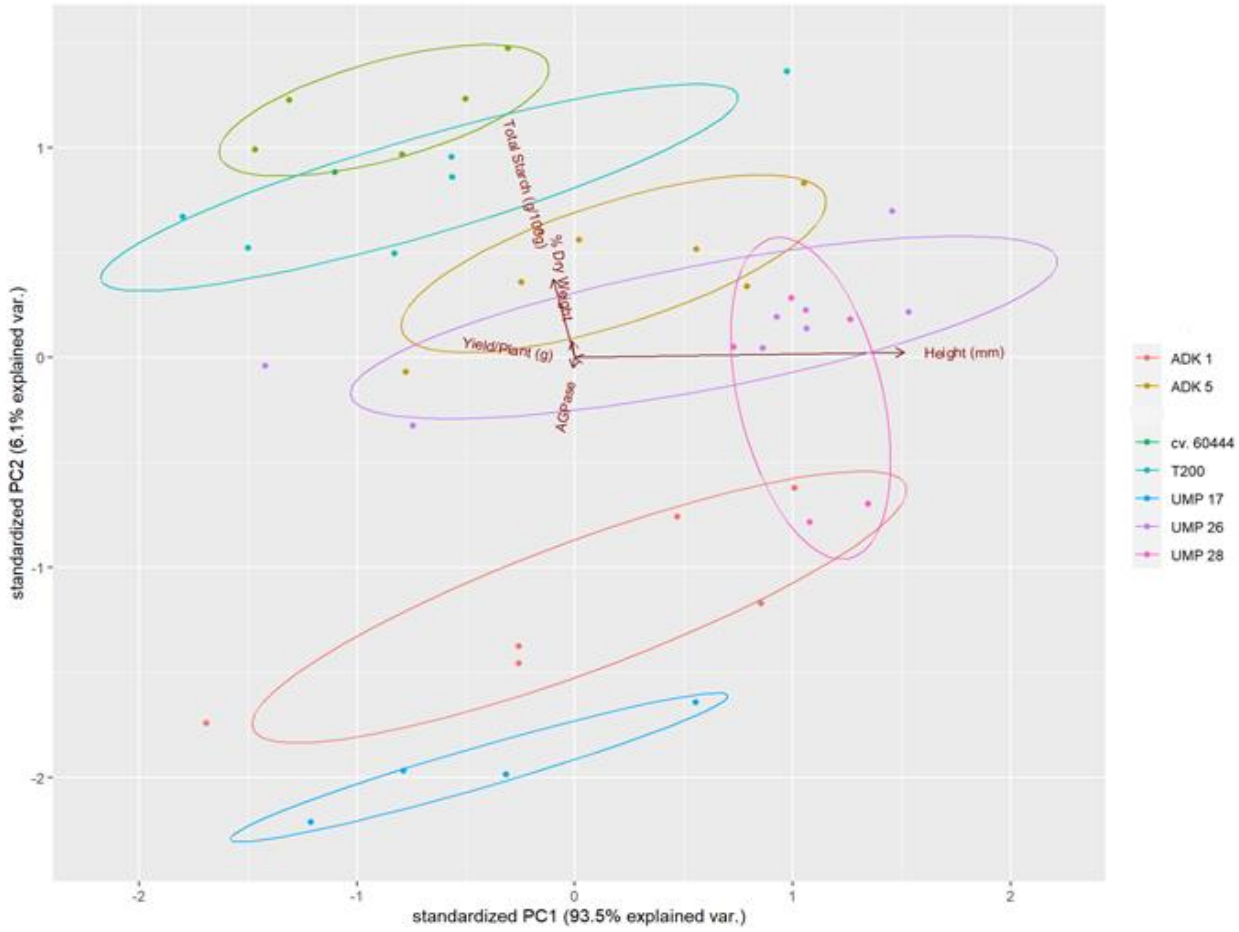


Figure 4. 20: Principal component analysis (PCA) of storage root traits and plant height in trial 2 and respective loading points including the variables that contributed the most in the separation in PC1 and PC2.

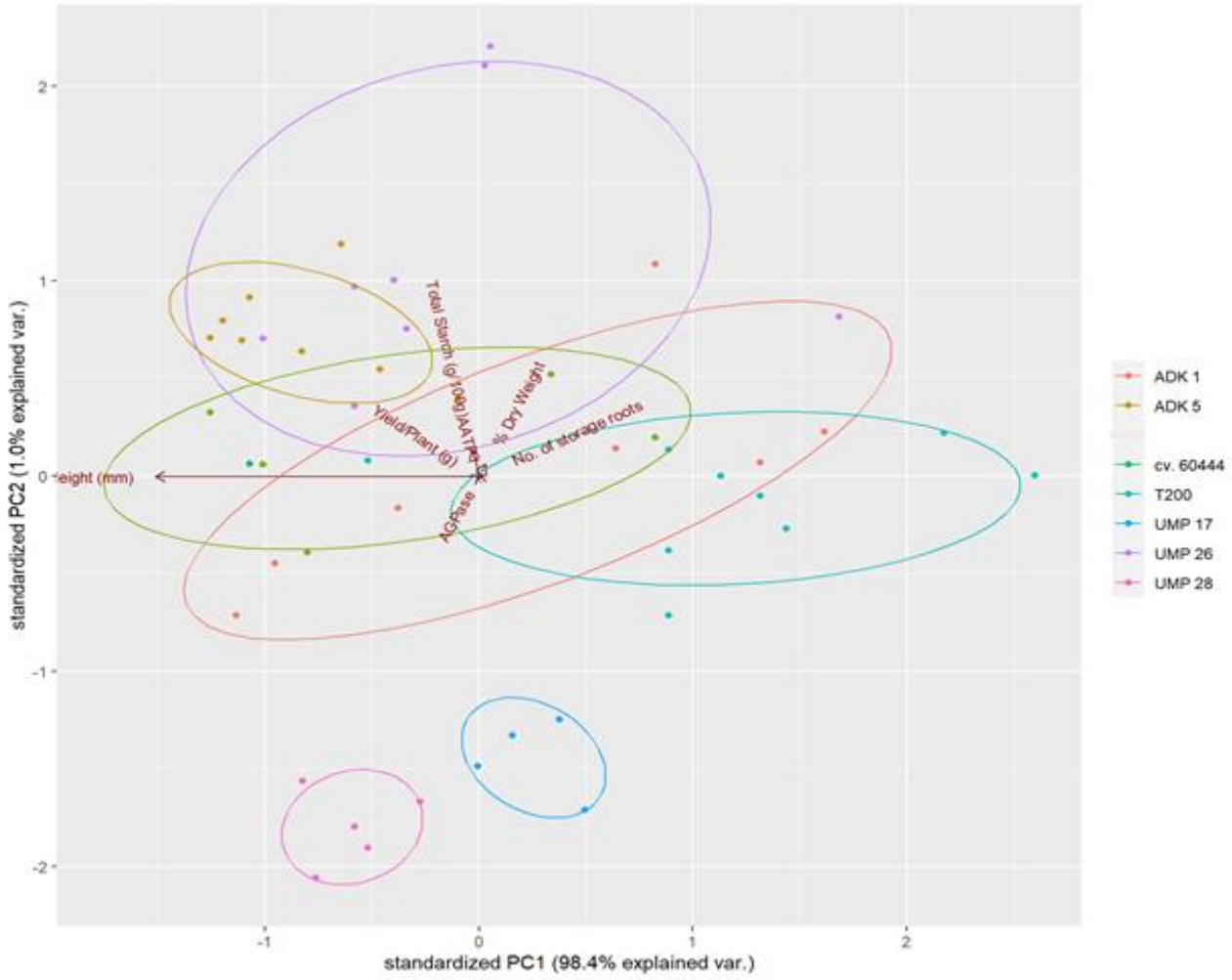


Figure 4. 21: Principal component analysis (PCA) of storage root traits and plant height measured in trial 1, and respective loading points including the variables that contributed the most in the separation in PC1 and PC2.

Chapter 5: Discussion

Genetic engineering has yielded very little benefits for the strategic vegetatively propagated crops like cassava, yams and plantains towards securing economic and food security for sub-Saharan populations (Narayanan et al., 2020). This can be partly attributed to the difficulty in transformation of cassava (Elegba et al., 2020, 2021; Lentz et al., 2018) and crop biotechnology which lags behind to genetically engineer useful stacked traits such as disease resistance, nutritionally supplemented and high-yield cassava into local farmer-preferred landraces and hybrids (Narayanan et al., 2020). Two of the most important traits for cassava on the African sub-continent are yield and virus disease resistance. There is widely documented evidence that various cassava genotypes harbour innate immunity to CMD originating from three characterized sources namely CMD1, CMD2 and CMD3 type resistance mechanisms (Kuria et al., 2017). While this germplasm has been used in breeding programs, CMD resistance remains elusive. One other effective approach to limit the spread of CMD in the field would be to identify naturally CMD-tolerant landraces (Rabbi et al., 2014). Ultimately, one of the major aims in future is to stack both starch yield and CMD resistance traits into farmer-preferred cultivars/landraces.

5.1 CMD resistance

The results presented in this study show different responses to SACMV challenge in the tested local South African cassava germplasm ranging from high level of resistance to severely symptomatic plants. The origin of Ukulinga germplasm is poorly described, but they have been described as drought resistant landraces (Melis, 2021). Often drought and disease resistance can be attributed to abiotic and biotic stress tolerance (Bakayoko et al., 2009; Garcia-Oliveira et al., 2020; Ou et al., 2018; Thalmann and Santelia, 2017). The Ukulinga 8 genotype exhibited complete immunity to SACMV infection and plants did not exhibit any form of symptoms. The high resistance and high tolerance to SACMV observed in Ukulinga 8 and Ukulinga 2 respectively, correlated with significantly suppressed viral load. The mild symptoms and high level of CMD resistance of field grown cassava plants has been previously known to be synonymous with the synergistic association between CMD1 and CMD2 loci, in addition to the highly efficient CMD3 resistance mechanism (Okogbenin et al., 2012). The Ukulinga 12 demonstrated the highest relative viral titre at the initial 14 dpi timepoint; however by 32 dpi it

had suppressed relative viral titre much lower than both the susceptible cv. 60444 and the known CMD tolerant-recovery landrace TME3. In future studies deep sequencing could be used to characterize populations of virus derived small RNAs (vsRNA) which may be targeting the geminivirus genome in these SACMV tolerant/resistant Ukulinga germplasm through post transcriptional gene silencing (PTGS) mechanism that is closely associated with recovery and tolerance (Kuria et al., 2017; Narayanan et al., 2020). The vsRNA reads will contribute towards the elucidation of both the resistance and tolerance mechanisms especially during this critical phase. Furthermore, DNA methylation of the viral genome also forms a principal plant host defence component inhibiting the proliferation and assembly of DNA geminiviruses, culminating in the recovery of the host (Butterbach et al., 2014; Raja et al., 2014; Rogans et al., 2016). For example, the hypermethylation of the tomato yellow leaf curl virus (TYLCV) DNA genome spanning the promoter region and 5′ end of the coat protein (AV1) region, through cytosine methylation of viral genomes demonstrate that *Ty*-1 provides strong resistance to different species of geminiviruses including tomato severe rugose begomovirus (ToSRV) (Butterbach et al., 2014). There is no evidence to suggest that methylation-mediated resistance could be associated with QTL-mediated resistance mechanisms in cassava (Kuria et al., 2017). The T200 landrace also has shown positive results for some of these markers (SSRY40, NS169 and NS198) linked to CMD1, CMD2 and CMD3 resistance but yet is highly susceptible, and many cassava accessions that have one or more of these markers also show variability in response to CMD (Keelan, 2022). Therefore, other molecular mechanisms are involved, and further investigation is warranted.

The known tolerant CMD2-positive TME3 accession had gradual development of the systemic CMD symptoms and exponential relative viral load increase until the 32 dpi time point, thereafter the relative viral load was reduced considerably between 32 dpi and 65 dpi timeframes. The severity of CMD symptoms on the leaves did not a recede between these two specified timeframes in this particular study, but it has been shown that post 60-67 dpi symptom recovery can be observed (Allie et al., 2014). Recovery is often influenced by other environmental factors, in particular temperature, which can impact RNA silencing known to be one factor involved in recovery (Vanitharani et al., 2005). Pepper golden mosaic virus (PepGMV)-infected pepper plants show a recovery phenotype, which has been associated with the presence of virus-derived small RNAs (Rodríguez-Negrete et al., 2009). PepGMV is

targeted by both post-transcriptional and transcriptional gene silencing mechanisms. Several different accounts have reported the loss of CMD2-mediated resistance in TME3 due to repeated propagation via somatic embryogenesis, and that suggests triggering of the methylation of regulatory DNA, possibly associated with CMD2 (Beyene et al., 2015a, 2015b; Narayanan et al., 2020). However the CMD2 locus has not been equivocally associated with methylation. Two peroxidases and one thioredoxin have been associated with the CMD2 locus (Wolfe et al., 2016) but again more experimental data is required to identify the source of resistance. The relative viral load of the susceptible cv. 60444 was relatively much lower than both the tolerant TME3 and Ukulinga 12 genotypes at 14 dpi, however thereafter the viral load increased exponentially to a very high load. Despite the disparate relative viral load throughout the study period between cv. 60444 and the two genotypes, TME3 and Ukulinga 12, they had similar mean symptom severity scores from 21 dpi onwards. It can be hypothesized from these observations that the high tolerance and resistance levels observed with Ukulinga 2 and Ukulinga 8 genotypes, respectively, could potentially be either due to the synergistic inter-crossing between the CMD1 and CMD2 loci in the wild or the highly effective CMD3 quantitative trait locus, that has been previously observed in the open field with highly resistant Western African landraces TMS 97/2205 and TMS 98/0505 which exhibit minimal CMD incidence and mild symptoms (Kuria et al., 2017; Okogbenin et al., 2012). There little information available on these Ukulinga landraces, except the observation by farmers that they are drought resistant. Whilst systemic virus infection can still take place in symptomless leaves, the asymptomatic stages of CMD are likely due to the host plant's capability to impede virus replication (Naseem and Winter, 2016). The difference in viral response between the highly resistant Ukulinga 8 and highly tolerant Ukulinga 2 could be influenced by other underlying hereditary resistance factors in conjunction with the CMD2 resistance mechanism (Okogbenin et al., 2013), and RNA silencing as mentioned previously. It will be of interest to further evaluate these SACMV-resistant and highly tolerant local landraces against other geminiviruses species infection which are dominant in the African landscape such as the ACMV, EACMV and mixed infections, that often manifest in the field (Naseem and Winter, 2016).

Within our study group, a separate project is currently underway to characterize the genetic diversity and map the qualitative CMD resistance mechanisms using an array of simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) molecular markers in an effort to screen

cassava germplasm for CMD1-3 QTL. Genomic selection thus plays a critical role towards advancing the resistance to CMD (Wolfe et al., 2016), and agronomic desirable traits such as drought-tolerance and CMD-resistance provide a solid foundation to further conventional breeding and genetic modification (GM) efforts. Furthermore, the advent of genome editing (GE) tools such as the clustered, regularly interspaced short palindromic repeats-CRISPR associated 9 (CRISPR-Cas9) mediated resistance for transgenic plants shows huge potential, however has not been commercially or legally regularized in most countries in the world, including South Africa. Early work has shown cassava (*Manihot esculenta*) to be recalcitrant to GE (Mehta et al., 2019), however this challenge will be overcome when the technique is adjusted and optimized specifically for cassava. *Manihot glaziovii* (common name ceara rubber tree) which naturally produces large storage roots has been poised to be one of the target species of CRISPR-Cas9 for *de novo* domestication (Zsögön et al., 2017).

The correlation between relative viral load and mean symptomatic score was weak ($r = 0.37$). This highlights the wide variation in relative viral load that exists in different genotypes in relation to their corresponding mean symptomatic scores. For instance among the cv. 60444, Ukulinga 12 and TME3, there were not much distinct phenotypic differences in average symptomatic scores, however the viral load accumulation or suppression at the three time points for each of these genotypes were significantly different. CMD symptoms generally correlate to viral DNA load and virus specific small RNAs targeting the geminivirus DNA genome in both polarities (Kuria et al., 2017). However these attributes are also related to the degree of resistance or tolerance in different genotypes (Kuria et al., 2017), that may be associated with resistance genes or genes involved in geminiviral suppressors of silencing (Beam and Ascencio-Ibanez, 2020). While some of these genes have been identified in cassava and other crops, potential naturally occurring resistance genes in wild relatives such as Ukulinga germplasm need to be explored in future. The low correlation index of viral load to symptomatic score could be due to varying levels of resistance, tolerance, susceptibility due to the targeted selection of the genotypes. For example, Ukulinga 2 and TME3 were able to suppress viral accumulation and effect recovery responses to infection by SACMV at 65 dpi, whilst Ukulinga 12 and cv. 60444 did not show viral suppression at any time point. Again, the mean symptomatic scores and virus loads at different time points were weakly correlated ($r = 0.26$) across all the genotypes screened,

confirming that viral response varied in asymptomatic, mildly symptomatic, and highly symptomatic genotypes in this study.

The results from the height measurements revealed that within each genotype, overall similar changes in height with time were observed, and thus there were no significant differences ($p<0.05$) among the SACMV-infected plants, healthy plants and mock-inoculated plants throughout the study period. This highlights that the viral infection by SACMV does not impede vertical plant growth or result in stunting, despite the observation that leaf area indices were visibly impacted by leaf deformation and mosaic in cv. 60444, Ukulinga 12 and TME3. SACMV is essentially a recombinant virus with its origins in eastern Africa and South-West Indian Ocean islands (Berry and Rey, 2001) and is less pathogenic compared to EACMV (Berrie et al., 2001; Kuria et al., 2017; Naseem and Winter, 2016).

5.2 Pot trials on storage root yield

5.2.1 Plant Growth Conditions

Storage root differentiation and fibrous root formation normally commences after 8 weeks from planting in the field (Olasanmi et al., 2017). Results showed that natural greenhouse-grown cassava plants had higher storage root yield from those grown in the growth chamber. For trial 1, cassava plants grown in the controlled conditions growth chamber facility, tended to allocate resources more to above-ground aerial parts of the plant (total leaf area index) in favour of below-ground biomass (storage roots). Cassava grown in pots under controlled conditions environments produce fewer storage roots as the source-sink balance of the plants differs in natural biomass distribution to observed in field grown plants (Sonnewald et al., 2020). All the tested transgenic lines from trial 1 had minimal overall storage root yield harvest that was lower than untransformed cv. 60444. The majority of plants did not form storage roots or produced a small quantity of very tiny storage roots in relation to the yield obtained overall in trial 2. This could, in part, be attributed to high total leaf area indices (dense plant canopies), indicating that most resources were directed to the leaves. The high-yielding T200 landrace attained the highest total storage root yield of below 10g.

Due to the differences in growth conditions between the two trials, while it was possible to observed general trends, it was not feasible to directly compare results meaningfully (statistically). Trial 2 was carried out under the same watering and fertilization regimen as the

controlled growth facility, but was in the greenhouse facility under natural sunlight and ambient spring temperatures. This led to a higher storage root production. The lowest total storage root yield harvest from trial 2 of 11.1g was achieved by UMP 28. Trial 2 overall produced significantly higher storage root yield harvests in comparison to trial 1, which was attributed to the more natural growth conditions in the greenhouse. There were no observed unintended effects on transgenic cassava leaf developmental growth as a result of hp-RNA inteference construct, likely due to utilization of the root specific promoter (Rocha-Sosa et al., 1989).

At the end of trial 2 (6 MAP), all of the cassava genotypes tended to priorized more resource allocation to below-ground roots (sink region) relative to trial 1. The overall plant canopy that is the total leaf area index (LAI) in the trial 1 was visually observed to be greater compared to that of trial 2 due to periodic leaf shedding in most of the genotypes from trial 2 except UMP 17 and T200. This is attributed to limited resources allocated to above ground (source region) ahead of below ground (sink region) in trial 1 compared to trial 2. This agrees with the suggestion that growth factors which encourage the higher photosynthesis in leaves (source) causes the cassava plant to be in a state where it is sink limited hence hindering optimal storage root development (De Souza and Long, 2018).

5.2.2 Gene expression of *AATPI* and *AGPase* associated with starch biosynthesis pathway efficiency

The strategies used to improve the initial rate-limiting reaction step of starch biosynthesis pathway efficiency have focused on increasing the supply of substrates (ATP and/or glucose 1-phosphate) to the enzyme *AGPase*; bolstering *AGPase* activity, or overexpressing starch synthases (Smith, 2008). In this study, the intraplastidial *ATP/ADP transporter* protein (*AATP1*) gene expression in the roots was significantly downregulated in all transgenic lines in both trials relative to untransformed cv. 60444, with different levels of expression in between the transgenic cassava lines in both trials. A decrease in supply or transport of ATP (which was not directly measured in this particular study) could limit the activity of *AGPase* (Geigenberger et al., 2001). ATP supply is critical for the starch biosynthesis rate-determining reaction catalyzed by *AGPase*. Despite a decrease in *AATP1* gene expression in transgenic lines, the expression of the *AGPase* gene was significantly upregulated relative to cv. 60444 in all of the genotypes in trial 1 and also most of the trial 2 genotypes. However, the increased expression of *AGPase* did not correlate

with an increase in starch yield in the transgenic lines, example in trial 1 expression of *AGPase* was upregulated significantly across all the transgenic lines relative to cv. 60444 in trial 1. There is need to perform enzyme activity of *AGPase* to relate starch to gene expression in a meaningful way. From trial 2, only ADK 5 and UMP 26 obtained significant increases in starch per unit weight for upregulated *AGPase* expression. The majority of the plants did not produce storage roots and a few that did, produced tiny storage roots (<2g) in trial 1. It is speculated this could be due to limited ATP supply as a result of the relative down regulation in *AATP1* in all the transgenic cassava lines in both trials as has been explained above. ATP is needed to efficiently drive the crucial energy-requiring and the rate-limiting ADP glucose formation step of the starch biosynthesis catalyzed by *AGPase* (Tjaden et al., 1998). In dicotyledonous plants, *AGPase* activity within plastids is limited by energy supply (Geigenberger et al., 2001), suggesting that increasing ATP supply would elevate both the starch content and yield in the sink (root) region. Significant upregulation of *AGPase* expression with concurrent significant repressed expression of *AATP1* in the roots may highlight the limitation of either of these vital components (ATP availability and *AGPase* activity) have on inability of cassava to optimally synthesize and accumulate starch in storage tissues and leading to substantial yield increase. This challenge of repressed expression of *AATP1* and hence limited ATP supply/transport in potatoes was solved through overexpression of plastidial *AATP1* (Geigenberger et al., 2001). Both *AGPase* activity and ATP supply have been shown to influence starch accumulation and that the importance of each may depend on tissue, developmental stage and environmental conditions.

Whilst *AGPase* expression was generally upregulated and *AATP1* downregulated in all the transgenic lines in both trials, trial 2 had significant higher total yield harvest amongst transgenic lines compared to trial 1. Thus, it is assumed other key variables such as light intensity and temperature could have a contributing effect towards yield increase since cassava is source limited (Sonnewald et al., 2020). The study was limited in that in only focused on one particular locus found on chromosome 15 out of the seven known chromosome distributions. It would be reasonable and conclusive to carry out enzyme determinations of *AGPase* as there is likelihood of existence of distinct *AGPase* isoforms. This would be useful in substantiating that the increase in starch content observed in a number of lines was related to the altered gene expression.

5.2.3 Starch content in UMP and ADK lines

The plastidial *ADK* knockdown using hp-RNA constructs led to increases in starch content of roots 1% and 16% in transgenic ADK 1 and ADK 5 lines, respectively, compared to the untransformed cv. 60444 plants from the greenhouse-grown plants (trial 2). The UMP 17 and 28 lines where *UMPS* expression was targeted using hp-RNA constructs, there was a significant reduction of 46% and 47% in starch content, respectively. The UMP 26 cassava line had the highest percentage increase of starch content in storage roots of 44% above that of the cv. 60444. The highest increase of starch content were observed in transgenic potato with antisense inhibition of the plastidial *ADK*, with 60% gain in starch compared to that of wild-type potatoes (Regierer et al., 2002).

In both trials, the rate-limiting *CPS* gene was highly expressed in both trials by the UMP 26 compared to other UMP lines (UMP 17 and UMP 28). There was no significant difference in expression of *CPS* between UMP 26 and cv. 60444 in trial 2, while in trial 1 there was significant higher expression of *CPS* in UMP 26 compared to the control cv. 60444.

Greenhouse-grown cassava UMP 26 line showed a 28% greater total storage root yield compared with the cv. 60444 control, and this was comparable to that achieved in potato plants. Transgenic potato plants expressing an antisense orientated cassette targeting *UMPS* driven by a patatin root-specific promoter attained a 25% higher tuber yield to wild-type potato plants (Geigenberger et al., 2005b). UMP 17 and 28 attained total storage root yield below that of the cv. 60444 in both trials. Downregulation of *UMPS* under the control of the root-specific promoter was intended to trigger the uridine salvaging pathway leading to upregulation of the *CPS* gene involved in first committed step of *de novo* pyrimidine biosynthesis. Upregulation of *CPS* should increase uridine nucleotides in the sink (root) region leading to increased breakdown of sucrose and increased carbon supply to starch and cell wall synthesis (Geigenberger et al., 2005b), leading to increased accumulation of starch content in the roots. This further illustrates that the downregulation of *UMPS* may regulate starch biosynthesis in cassava, as the UMP 26 line grown in the greenhouse also had the significantly ($p<0.05$) highest starch content per 100g of all the tested cassava genotypes. Overall, the relative differences in expression of the genes in transgenic lines could be due to the site of integration of the transgene into the host genome, or variations in the copy number of inserted *ADK* or *UMPS* genes (Zhang et al., 2000).

UMP 26 had the highest percentage dry weight of storage roots of 29.4 ± 1.0% in comparison to cv. 60444 and T200 which had 22.8 ± 1.6% and 28.1 ± 2.4% respectively. UMP 28 had the lowest percentage dry weight of storage roots of 11.9 ± 0.3%. The remainder of the tested transgenic lines had percentage dry weight of storage root in the range of 18.4 ± 2.7% and 25.3 ± 1.5%. Cassava storage root dry weight varies per genotype and is typically in the spectrum of 17% - 47%, and genotypes with percentage dry weight of greater than 30% are regarded as elite (Barimah et al., 1999). Dry weight is the most accurate indicator of the actual yield of a crop (Teye et al., 2011).

5.2.4 Morphological analyses of starch granules

The morphological analysis of starch granules demonstrated that the transgenic storage roots had altered shape and diameter of the starch granules. ADK 1 had greater proportion of smaller diameter of spherical granules compared to cv.60444 whilst the ADK 5 had average-sized truncated granules. The knockdown of plastidial *ADK* to alter the supply of ATP in the amyloplasts of storage root tissue is known to influence the percentage amylose, starch content and the overall shape of potato tubers (Tjaden et al., 1998). However, in this study there were no significant differences in amylose content between cv. 60444 and the two ADK lines, though there was a noted significant difference between the starch content of ADK 5 storage roots and cv. 60444 in the greenhouse trial. UMP 28 was the only transgenic with significant change in amylose content, with the lowest amylose content of 11.9 ± 0.3% compared to cv. 60444 and T200 which was 17.4 ± 1.4% and 16.0 ± 0.6%. The low amylose content observed in UMP 28 could be attributed to a change in the expression profiles of *granule-bound starch synthase 1* (*GBSS1*) and other *soluble starch synthases* (*SSS*) which impart the overall physicochemical structure of starch and its possible end-use (Raemakers et al., 2005; Šárka and Dvořáček, 2017). The remainder of the transgenic lines had amylose content which was unaltered and in the range of 14.3 ± 0.8% and 16.8 ± 0.3% which is consistent with other different studies. Amylose varies between 13.6% and 23.8% of total cassava starch contributing distinct characteristics vital for starch quality (Munyikwa et al., 1997)

There were no significant differences in starch granule sizes between the UMP lines and the untransformed cv. 60444; however all the UMP lines displayed spherical granules whilst cv. 60444 had both spherical and truncated starch granules. Lugol's staining of storage roots tissue

revealed strong and dense staining of starch granules from cv. 60444, ADK 1, UMP 17 and UMP 26, which aggregated radially. The UMP line 28 had sparsely arranged starch granules deposition, with significant non-starch tissue spaces.

The xylem vascular bundles were more compact in the cv. 60444, ADK 1, ADK 5, UMP 26 and T200 compared to the UMP 17 which presented as more loosely and disperse in form. The tight organization of the vasculature encourages high flow rates that leads to optimal metabolites exchange between different plant organs (Li et al., 2016), contributing to improved delivery of substrates of starch biosynthesis into the amyloplasts leading to higher starch content in the roots. There was a higher callose deposition around the phloem sieve plates (PSPs) of storage roots of ADK 5 and UMP 26 compared to the other genotypes. It was demonstrated in *Arabidopsis*, that callose lining associated with PSPs had a dual function of encouraging optimal smooth flow on the plate pore and also in maintaining the structural integrity of walls of sieve plates from folding in (Barratt et al., 2011). Efficient phloem loading will improve carbohydrates transport, oxygen delivery to the roots and *AGPase* activity which could result in high starch accumulation in the parenchyma cells (Ihemere et al., 2006).

The growth of storage roots was observed at 6 MAP. It would have been revealing to measure storage root growth phases prior storage root bulking phase to analyze the changes in starch content, metabolites and root gene expression at the various stages of root thickening, root bulking and root differentiation to gain a deeper understanding of into cassava root development. It is also noteworthy to acknowledge the contributory roles of phytohormones and the crosstalk amongst the phytohormones that exist during cassava storage root development (Utsumi et al., 2020). Furthermore, phytohormones, such as cytokinins and auxins have been well documented to promote and tuber formation in potato (Wang et al., 2018). However, we cannot speculate on the role of phytohormones in transgenic cassava lines versus control cassava plants. The correct concentration and distribution has to be determined to yield optimal effect, for instance different levels of jasmonic acid application can either enhance or hinder tuberization in potatoes (Abdala et al., 2002).

Chapter 6: Conclusion

The average cassava yield attained by subsistence farmers in sub-Saharan Africa has remained largely stagnant over the years due to lack of research and development against the projected exponential increase in population. The high resistance and tolerance to SACMV observed in the South African cassava genotypes, Ukulinga 8 and Ukulinga 2, respectively, in this study could play an immense role in expanding the knowledge on resistance genes through further molecular research on geminivirus resistance. These germplasm could also be adopted into plant breeding programs, and also be researched to identify essential alleles/QTLs associated with CMD tolerance. Gene editing or stacking of useful gene by genetic modification of these two cultivars could also be improved to develop other desired agronomical traits. There are already known western African cassava landraces like the TMS97/2205 which is highly resistant to some of the endemic cassava geminivirus species which can be used in interbreeding programs. Drought tolerant South African cassava landraces which are resistant to SACMV and have high yield and starch content can also be genetically manipulated to improve traits such as micronutrient deficiencies and starch quality. The frequent *El Niño* dry spells are also amongst the major challenges to nutritional and food security in sub-Saharan Africa.

The downregulation of plastidial *adenylate kinase (ADK)* gave rise to significant increases in starch content, and an overall substantial increase in cassava storage root yield harvest, in particular the ADK 5 transgenic line grown in the greenhouse. This is further proof of concept that plastidial *ADK* is a good target for genetic manipulation, and which has been previously observed in different plant species such as potatoes. This is the first transgenic genetic manipulation of plastidial *ADK* to improve root starch yield in the orphan crop cassava. The decreased expression of *uridine monophosphate synthase (UMPS)* led to significant gains in percentage dry weight and starch content of cassava storage roots in the second trial in the greenhouse, as well as slight increase in yield harvest of storage roots in UMP 26 line. There is still much to learn in understanding the process of starch biosynthesis and storage roots bulking in cassava, for example the contribution of factors such as temperature, natural light, phytohormones and the effect of using pots and their sizes. These types of pot-based trials need to be improved as they are a convenient means to study storage roots in cassava. It would be of much interest in future to carry out confined field trials to assess the agronomic performance of

these particular promising transgenic lines. Field trials are often expensive, and for genetic modified (GM) trials require permits and also agricultural land in suitable cultivation areas in South Africa.

The interconnected relationship of source and sink metabolism in cassava will simultaneously have to be taken into consideration to generate the highest yield improvement strategies that will strengthen and stabilize food security. Also, the world is gradually shifting focus and embracing low carbon footprint sustainable production and green energy as part of the global Paris Agreement Treaty. Besides being a vital staple crop in developing countries, a robust high yielding, drought tolerant and CMD resistant cassava holds untapped potential to be transformed and adopted for full scale commercial exploitation as a cash crop due to demand surges which exist within the starch industry and biofuel energy sector. The present study describes efforts towards delivering CMD resistant and high-yielding transgenic cassava engineered to meet the requirements for industrial exploitation and profitability which will facilitate the uptake of cassava farming by commercial enterprises.

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Supplementary data

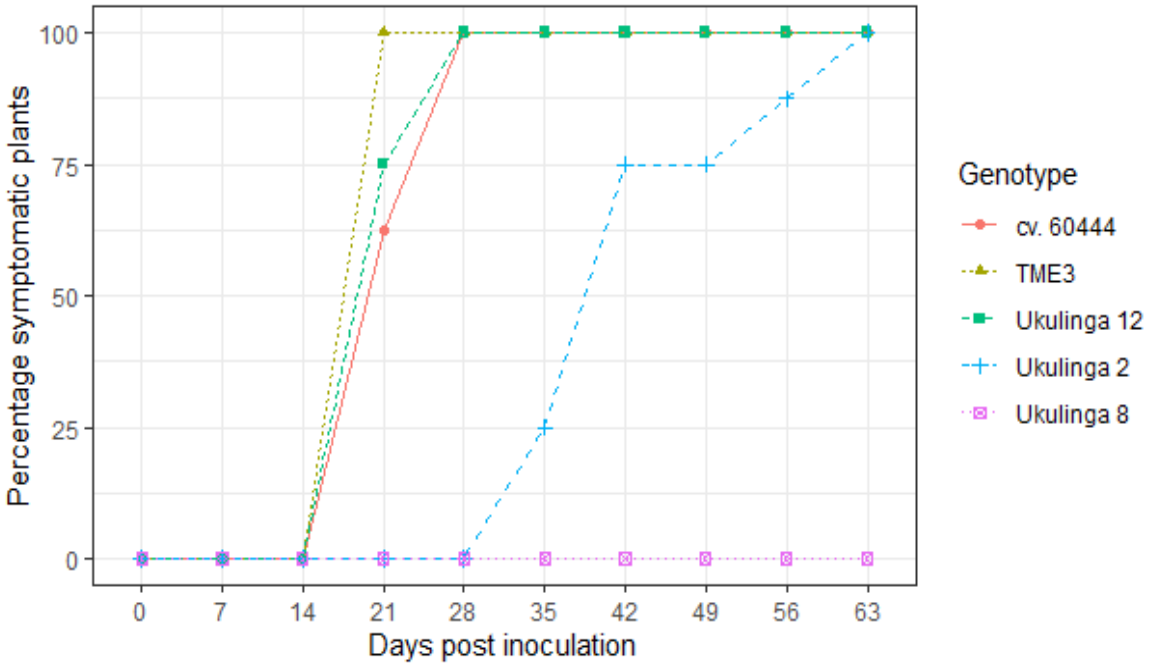

Figure *S1:* The percentage of cassava plants from each genotype that developed systemic CMD symptoms after infection with SACMV.

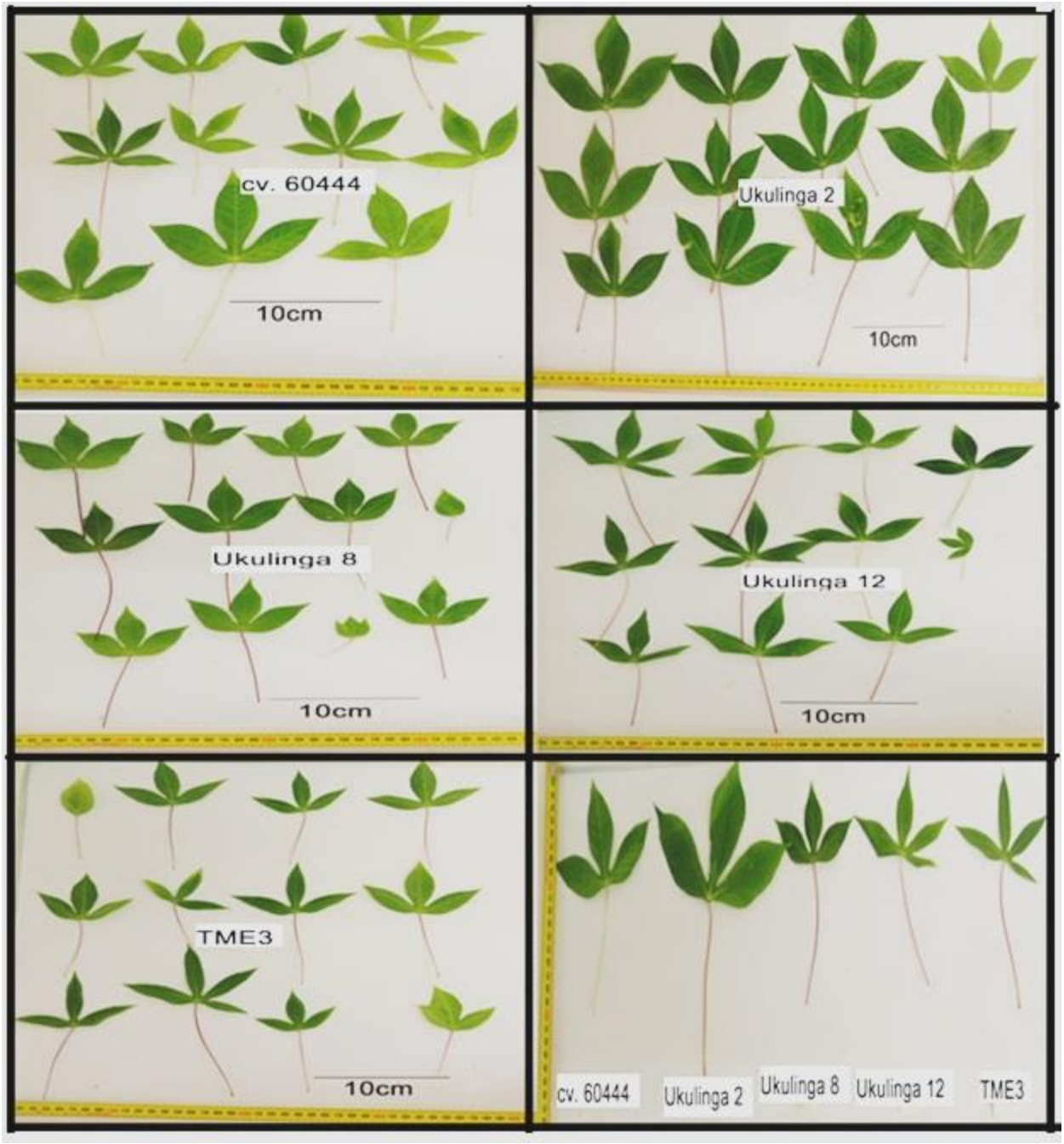

Figure *S2*: Morphology of apex cassava leaves from different cassava genotypes, and their corresponding petioles lengths, before inoculating with the infectious SACMV clones.



Figure *S3*: Mid-stem cuttings from different cassava genotypes showing internodal length at 65 dpi.

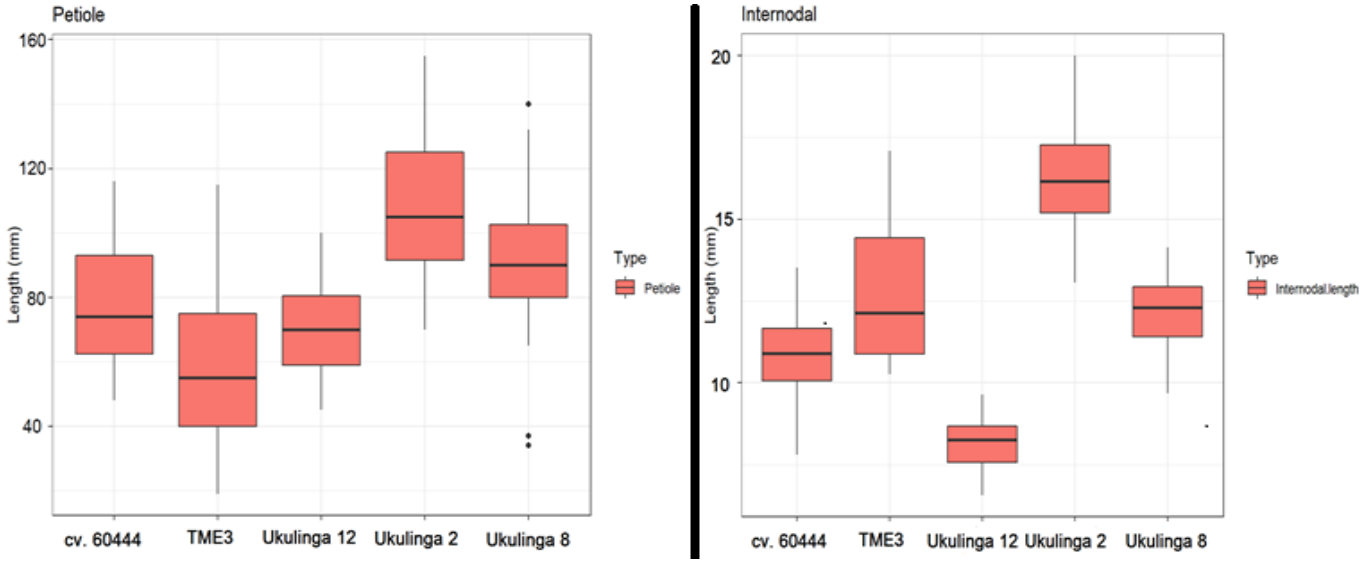

Figure *S4*: Boxplot of the distribution of petiole and internodal lengths in various cassava germplasm and in the model cultivar cv. 60444. The bottom whisker denotes the lower extreme, the top whisker denotes the upper extreme. The bottom and top of the box denote the lower quartile and upper quartile respectively, and the middle of the box is the median.



Figure *S5*: Canopy and leaf loss (senescence) from different cassava transgenic lines and landrace grown in the greenhouse facility at 4 MAP; h is the height from the ground to the lowest node-bearing leaf.

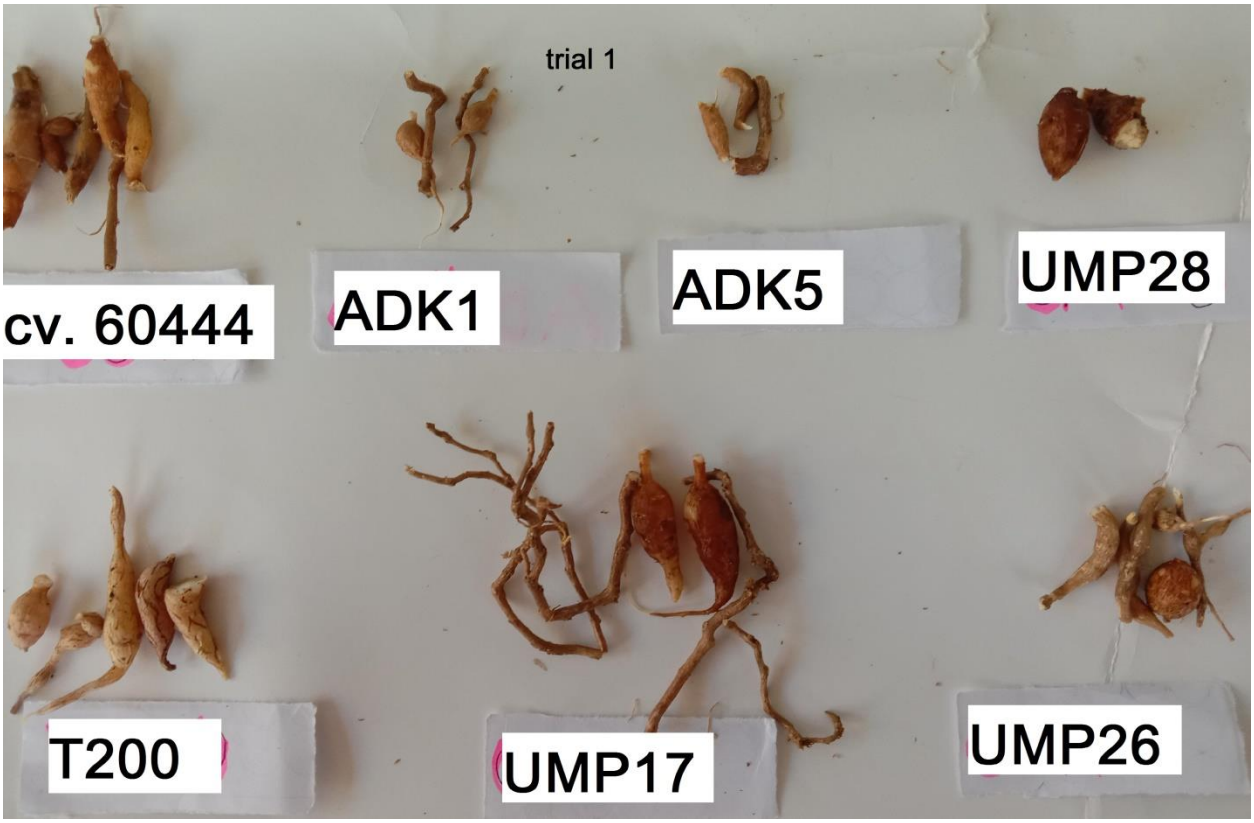

Figure *S6*: Storage root morphology and yield harvest from controlled conditions growth chamber facility-grown transgenic cassava lines, cv. 60444 and T200 landrace in trial 1.