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Effect of RNAi down-regulation of three lysine-deficient kafirins on the seed lysine content of sorghum [Sorghum bicolor (L.) Moench]

By

ANDILE W GROOTBOOM

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Department of Plant Science

Forestry and Agricultural Biotechnology Institute (FABI)

In the

Faculty of Natural and Agricultural Sciences

University of Pretoria

Supervisors: Dr R Chikwamba

Prof KJ Kunert

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DECLARATION

I, Andile W. Grootboom declare that the thesis, which I hereby submit for the degree Philosophiae Doctorate, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: …….

DATE: …….

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CHAPTER 3 TRANSFORMATION OF SORGHUM FOR SUPPRESSION OF SELECTED SEED PROTEINS

ABSTRACT

Sorghum (*Sorghum bicolor* L. Moench) ranks fifth worldwide in production among cereals. It is a major staple food for millions in Africa and Asia, and a major livestock feed grain in developed countries. However, the sorghum grain is poor in lysine content, limiting its value as food and feed. In this study, I hypothesize that reduction of some of the major storage proteins that are inherently poor in lysine through *in vitro* manipulation will result in the enhanced expression of proteins with a better lysine profile and, thus, increased overall grain lysine content. Sorghum genotypes were screened for *in vitro* amenability and a sorghum genotype-tissue culture medium combination that yielded the highest somatic embryo callus formation and regeneration potential, was identified. This resulted in the establishment of a sorghum biolistic transformation method with a transformation efficiency of 3.36%, the highest reported to date. Using genetic engineering tools, the enhancement of the nutritional quality of grain sorghum was achieved by increasing the seed lysine content. An RNAi co-suppression strategy was employed and resulted in 45.23 and 77.55% increase in whole seed and endosperm lysine increase, respectively. The co-suppression RNAi constructs targeted the endosperm specific suppression of three lysine-poor storage proteins, namely δ-kaf-2, γ-kaf-1 and -2, and an enzyme that catalyzes seed lysine degradation, lysine keto-gluterate reductase (LKR). Seven independent transgenic events displayed successful transgene integration for both the selectable marker gene and the target constructs. However, the Southern blot hybridization analysis revealed two transgenic events that displayed transgene re-arrangement at the 5'promoter end, thus resulting in a lack of suppression of target proteins. Variations in target proteins co-suppression was observed with Western blot analysis and RT-PCR for both the target kafirins and LKR suppression, and no lysine improvement was observed where no kafirin suppression occurred. The transgenic co-suppression of the target kafirins resulted in the endosperm structural change from a hard,

corneous endosperm to a soft, floury endosperm, consistent with γ-zein suppression in the Opaque-2 maize mutant.

THESIS COMPOSITION

This thesis comprises of five chapters of a PhD study that aimed at improving the nutritional value of sorghum grain for food and feed consumption. **Chapter 1** is an introduction to the morphology and physiology of the sorghum plant, its commercial and domestic usage. This chapter also reviews the nutritional deficiency of sorghum grain due to its inherently low content of the essential amino acids lysine and methionine. The chapter concludes by formulating a genetic engineering strategy that aims at improving the seed lysine content. The first technical effort towards achieving the main aims is covered in **Chapter 2**. This involves *in vitro* screening of five sorghum genotypes in three tissue culture solid media formulations. This served to identify the most amenable genotype for subsequent transformation efforts. The second transformation optimization step involved a comparison of two transgenic tissue selection systems (**Chapter 3**). Also covered in **Chapter 3** was the application of the optimized transformation conditions to generate stable transgenic sorghum plants expressing the RNAi construct for targeted endosperm proteins suppression. This is followed by characterization of the transgenic lines for target protein suppression and amino acid content analysis to examine seed lysine improvements (**Chapter 4**). **Chapter 5** is a global discussion on the impact this study exerts in cereal nutrition. At the end of each chapter, the list of references cited is provided. Finally the **Annexure** covers recipes of solutions and tissue culture media contents that were used in this thesis.

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ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	Degree Celcius
µg	Microgram
µl	Microlitre
2.4-D	2,4-dichlorophenoxyacetic acid
2n	Diploid number of chromosomes
ABS	Africa biofortified sorghum
ADH-1	Alcohol dehydrogenase-1
$AgNO_3$	Silver nitrate
BASTA	Herbicide brand name by Bayer CropScience
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CIM	Callus induction medium
CMM	Callus maintenance medium
cp	Copies
CRE	Casas root elongation medium
CROOT	Casas rooting medium
CSE	Callus shoot elongation medium
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphates

DTT	Dithiothreitol
E. coli	*Escherichia coli*
EST	Expressed sequence tags
g	Grams
GFP	Green florescent protein
GUS	β-*glucoronidase*
hIR	Homologous inverted repeats
HPLC	High pressure liquid chromatography
hrs	Hours
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IZE	Immature zygotic embryos
Kaf	Kafirin
kb	Kilo base pairs
kDa	Kilo Daltons
kPa	Kilo Pascals
L/l	Litre
LKR	*Lysine ketoglutarate reductase*
Lys/K	Lysine
M	Molar
MgCl	Magnesium chloride
min	Minutes
ml	Millilitres
mM	Millimolar
MTCs	Minimal transgene cassettes

NAA	1-Naphthalene acetic acid
NaCl	Sodium chloride
Ng	Nanogram
NH_4NO_3	Ammonium nitrate
Nos	Nopaline synthase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PCR	Polymerase chain reaction
Pmi	*Phosphomannose isomerise*
Pro	Promoter
PVDF	Polyvinylidene di-fluoride membrane
RNA	Ribonucleic Acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	Rotations per minutes
RRM	Regeneration and rooting medium
RT-PCR	Reverse transcription-PCR
SDH	Saccharopine dehydrogenase
SDS	Sodium dodecyl sulphate
Sec	Seconds
SSC	Sodium chloride-, trisodium citrate
Ubi	Ubiquitin
WT/wt	Wild-type

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The PCR results for 14 of the 61 putative transgenic plants produced by co-bombardment of IZEs with pNov3604 and pABS MTCs. A 1.2% agarose gel electrophoresis was loaded and labelled as follows: (M) 100bp ladder molecular size marker; (N) negative control without template DNA added in a reaction mix; (P) corresponds to 1ng of pABS target construct added as the only template DNA used as a positive control; (W) 50ng wild-type P898012 genomic DNA added as template used as a second negative control; (PS) wild-type P898012 genomic DNA spiked with 1ng of target construct used as a spiked positive control. Fourteen putative transgenic events resulting in 61 plants were produced. Transgene presence was observed in all samples that were analyzed.

The Southern blot hybridization analysis of 22 of the 61 T_0 P898012 plants produced after co-bombardment with pNOV3604 and pABS constructs. (A) The first 11 putative transgenic samples (left panel i.e. A, B and C) were probed for the 5' promoter region, and (B) re-probed for a 3' transgene confirmation region. (C) The last probe used was specific for *pmi*. The last 11 samples (C, D and E – right panel) were analyzed on a second membrane to confirm the complete transgene integration. The same order of probe binding was employed, i.e. (D) for 5' promoter region, (E) for the confirmation region and (F) for the *pmi* gene. A total of 61 plants which resulted in 10 transgenic events were produced. Sample labelling was as follows: (W) wild-type P898012 genomic DNA sample representing the negative control; (PA) wild-type genomic DNA spiked with 2 copies of the pNov3604 and pABS constructs and digested with B*am*HI used as positive control; (PB) wild-type genomic DNA spiked with 10 copies of the pNov3604 and pABS constructs and digested with B*am*HI used as a second positive control. Events 5 and 7 (lane numbers labelled in blue) were bombarded with closed, circular plasmid.

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