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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

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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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To God, thank You very much for the strength to endure this trying period of my life. Through Your guiding hand, I have had a pleasure of taking my life and career to the next level. KumaReledwana, ndithi nangamso!

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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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Chapter ONE

Introduction

1.1 The sorghum plant

1.1.1 Sorghum - Origin and history

Sorghum bicolor L. Moench (2n = 20) ranks fifth in commercial production among cereals in the world; in order of commercial production, sorghum is behind the following cereals: rice, maize, wheat and barley. It is believed that sorghum originates in Sub-Saharan Africa and the crop is well adapted to the semi-arid and subtropical conditions of this area. However, the distribution of the genus *Sorghum* is world-wide. There is strong belief that this was due to the continental drift of the Pangaea that took place more than 220 million years ago (Doggett, 1988). There is a strong belief that this crop originally belonged to the Congolese tribe called Andropogoneae who lived in the tropical regions of Africa. Three species were identified, i.e. *S. bicolor*, *S. halepense* and *S. propinquum*. Different classification systems for sorghum were provided by a few authors (Harlan and De Wet, 1972; De Wet, 1978). An in-depth review of the three species was provided by De Wet (1978). These classification systems included observation for different shapes, sizes and colour of the grains, glumes, panicles, leaf blades and the whole plant. *S. bicolor* is an annual plant that forms tillers, with an erect plant stature, ranging from 0.5 m to over 5 m in height and with long leaf blades up to 90cm and 12cm wide. The fruit forms either an open or closed panicle that is 5-60cm long and 3-30cm wide.

A B

Figure 1.1 Sorghum plants cultivated in the greenhouse at the CSIR Biosciences, Pretoria, South Africa. A: Full-grown plants. B: Panicle with developing seeds.

The cultivated sorghum of today is strongly believed to have arisen from the wild *Sorghum bicolor* subspecies *arundinaceum* (Mann *et al*., 1983). The Ethiopian and Sudanese Sorghum Conversion Programs possess the widest range of sorghum germ-plasm database and this further indicates the origin of the crop. This also suggests that human selection in these areas has been in existence for a very long time.

1.1.2 Agronomic attributes

1.1.2.1 *Vegetative morphology*

S. bicolor is an annual plant that forms tillers, has an erect plant stature and can grow up to 5 m in height. The stem is generally hardened but the centre is usually spongy. The lowest nodes form roots and the tall varieties have roots higher up the lowest node. This ensures a good

support structure for the plant, e.g. against strong winds. The growth rings that usually form at each node are capable of differentiation when needed, e.g. when the plant has been floored by strong winds, an upright position may be regained through these growth rings. Each node also possesses a single bud that can either develop into tillers or branches when required. The branches and tillers usually form as a result of stress caused by stem borers or other environmental damage (Doggett, 1988).

Tillers generally form as a way of plant survival. Tiller formation in sorghum is subject to variety; certain varieties tiller early while others tiller post flowering. Temperature and photoperiod also play a role in tiller formation with lower than 18°C temperatures promoting formation of tillers while short photoperiods do not. These tillers enable certain varieties to survive for a number of years (Duncan *et al*., 1981).

The number of leaves may vary from 7-24 and mature leaves vary between 30-135 cm in length and between 1.5–13cm in width. Protection against shoot-flies is conferred by the presence of trichomes (prickle hairs) on the abaxial surface of the leaves. During drought stress, motor cells enable the leaves to roll inwards. Sorghum also has an adventitious root system with a number of branched lateral roots and they can extend up to 1.5 m deep into the soil. This enables very good nutrient absorption.

1.1.2.2 *Temperature*

There are three classified groups of sorghum based on their adaptation to temperature, i.e. cold-tolerant, temperate and lowland tropical sorghum. The cold-tolerant sorghums have the ability to

tolerate both day and night cold temperatures of 17°C and below. They are also known as high-altitude sorghum because of their general ability to grow on elevated landscapes. In the USA, numerous studies have concentrated on the temperate sorghum due to substantial interest in its development. This group can tolerate cold temperatures both at the initiation and at the end of the growing season with the exception of the flowering period. They grow best during very warm days and cool nights (Doggett, 1988). The lowland tropical sorghum prefers both warm days and warm nights (25-34°C) throughout the growing season. They are also best suited for lower altitudes.

1.1.2.3 *Physiology*

The genotype-environmental relationship involving the interactions of environmental factors on the genetic make-up of a plant often selects for the physiology and subsequent product or phenotype. Conversely, this means that the optimum cultivation conditions for a particular plant select for that particular plant against others. Temperature and photoperiodicity are two important abiotic factors that influence this relationship.

1.1.2.4 *Photoperiodicity*

The cold-tolerant and lowland tropical sorghums are photoperiod sensitive while the temperate sorghum is not (Doggett, 1988). The studies on the temperate sorghums revealed a strong interaction between genotype, photoperiod and growing temperature. The time of flowering was significantly affected by this relationship (Miller *et al*., 1968).

1.1.3 Cultivation advantages and soil pH

Sorghum has the ability to better withstand drought conditions and tolerate a wider range of soils than other cereal crops. Due to a lack of resources in these areas, farmers in the developing world are usually forced to grow cultivars with the ability to produce under harsh conditions that include drought stress, pests and disease. The pH range suitable for growth is 5.5-8.5. Tolerance to poor drainage and saline soils has been displayed as well (Doggett, 1988).

1.2 **Grain utilization**

In the developed world, sorghum is grown mainly for grain and forage mostly to feed livestock, but the developing world uses sorghum products as staple food. Millions of people in Africa and Asia process and consume the grain in various ways. The plant stems can also be used for brooms and in the sweet varieties, they are chewed and processed into other products due to the high sugar content. More recently, the sweet-sorghums have become increasingly important as a potential source of ethanol for biofuel. Grain usage includes the following:

- traditional home-made meals,
- immature grain foods,
- boiled grain meals,
- roasted grains,
- popped grains,
- fermented meals,
- sorghum breads,
- stiff porridges, and
- sorghum beers.

1.3 Genetic engineering of plants

Plant genetic engineering is the application of recombinant DNA technology in plants to study and introduce foreign genetic material. This technology also focuses on the interface of all aspects of cell biology and molecular biology. The problems faced by plant breeders, such as costly and prolonged breeding methods, have led to technologies that were designed to both assist and solve plant breeding obstacles. The low probability of achieving a product that displays multiple foreign phenotypic characters gave birth to the establishment of genetic engineering tools to facilitate precision insertions of specific multiple traits in a desired genetic background. These include recombinant DNA technology, *in vitro* tissue culture and plant transformation, somaclonal and gametoclonal variation.

The first genetically modified species was the bacterium *Escherichia coli* in 1973 (Cohen *et al*., 1973) that expressed a *Salmonella* gene. The development of the methods to extend the principle of inter-species gene expression began in 1983. In the midst of ethical and complex issues surrounding the production of genetically engineered (GE) plants, transgenic crops production has since become a major scientific research and commercial component. This is supported by the data in Table 1.1, wherein the transgenic crops take up a significant fraction of the total planted land in each of the top 18 countries that grow these GE crops.

The major GE crop varieties commercialized since 1996 have been designed to help control insects using *Bacillus thuriengensis* toxins and herbicide-based weed management systems using glyphosate. The genetic modification of crops have also been accomplished in fruits such as

banana (Ságí *et al*., 1994) and citrus (Vardi *et al*., 1990); in trees such as *Casuarina glauca* Sieb. Ex Spreng (Smouni *et al*., 2002); and in beverages such as coffee (Perthuis *et al*., 2005).

Table 1.1 Worldwide status of transgenic crop production - 2005

Rank	Area	Area (mil. Ha/A.)	Crop
1	USA	49.8/723.0	soybean, maize (corn), cotton, canola, squash, papaya
2	Argentina	17.1/42.2	soybean, maize, cotton
3	Brazil	9.4/23.2	soybean
4	Canada	5.8/14.3	canola, maize, soybean
5	China	3.3/8.2	cotton
6	Paraguay	1.8/4.4	soybean
7	India	1.3/3.2	cotton
8	South Africa	0.5/1.2	maize, soybean, cotton
9	Uruguay	0.3/.7	soybean, maize, cotton
10	Australia	0.3/.7	cotton
11	Mexico	0.1/.2	cotton, soybean
12	Romania	0.1/.2	soybean
13	Phillipines	0.1/.2	maize
14	Spain	0.1/.2	maize
15	Colombia	<0.1/.2	cotton
16	Iran	<0.1/.2	rice
17	Honduras	<0.1/.2	maize
18	Portugal	<0.1/.2	Maize

1.4 Genetic engineering of sorghum

A suggestion was aired by Birch and Bower (1994) that one of the most important aspects for successful particle bombardment in generating transgenic plants was an efficient selection for transformants. In sorghum, for a long time, the selectable marker gene *bar*, an herbicide resistance gene, was used in most sorghum transformation reports. The *hpt* (hygromycin phosphotransferase) gene was only used once by Hagio (1991). The *pmi* (phosphomannose isomerase) gene was only recently reported by Gao *et al*., (2005).

1.4.1 *In vitro* regeneration of cereals

Cereals, woody trees and legumes were classified as highly resistant to *in vitro* manipulation and tissue culture regeneration until 1982 (Barton *et al*., 2000, patent). Although callus cultures of rice (*Oryza sativa*) were first achieved in 1964 (Oono, 1983), there were no reports of reproducible and sustained production of fertile and normal plants of genotype. A number of breakthroughs took place in the early 1980s and these formed the basis for the regeneration of almost all the cereals by *in vitro* tissue culture.

The breakthroughs were as follows: (a) For *in vitro* tissue culture, immature embryos, young inflorescences and the bases of immature leaves can regenerate into fertile plants; (b) this process involves a simple nutrient medium with high concentration of a strong auxin like 2,4-dichlorophenoxyacetic acid (2,4-D); (c) plant regeneration takes place mainly through somatic embryogenesis; and (d) totipotent protoplasts are a result of embryogenic cell suspension cultures derived from embryogenic calli. A number of factors influence the tissue culture

amenability of cereals, i.e. choice of explant, medium composition, culturing and regeneration conditions, and these were elucidated (Vasil, 1994; Vasil and Vasil, 1992, 1994).

1.4.2 *In vitro* regeneration of sorghum

The first sorghum tissue culture and plant regeneration was reported by Masteller and Holden (1970). High frequency plant regeneration from cultured explant material is crucial for the successful transformation of most cereal crops (Rachmawati and Anzai, 2006).

1.4.2.1 *Explant*

The choice of explant has been identified as one of the most important factors for cereal *in vitro* regeneration - this includes the physiological and developmental state of this plant material. In sorghum, immature zygotic embryos (Gamborg *et al*., 1977; Thomas *et al*., 1977; Cai *et al*., 1987; Dunstan *et al*., 1978, 1979; Brar *et al*., 1979; Ma and Liang, 1987), mature embryos (Thomas *et al*., 1977; Cai *et al*., 1987), immature inflorescences (Brettell *et al*., 1980; Boyes and Vasil, 1984; Cai and Butler, 1990; Kaeppler and Pederson, 1997), seedlings (Masteller and Holden, 1970; Brar *et al*., 1979; Davis and Kidd, 1980; Smith *et al*., 1983), leaf fragments (Wernicke and Brettell, 1980) and anthers (Rose *et al*., 1986) have been used as explants. In cereals, the immature zygotic embryos' scutellum tissue has been a reliable target for somatic embryo formation and, hence, easier uptake of DNA for generation of transgenic plants, e.g. in barley (Wan and Lemaux, 1994), in maize (Brettschneider *et al*., 1997), in oat (Somers *et al*., 1992), in rice (Christou *et al*., 1991), in rye (Castillo *et al*., 1994), and in wheat (Vasil *et al*., 1992). In sorghum, immature zygotic embryos or the callus derived from them has been an

explant of choice (Casas *et al*., 1993; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002; Gao *et al*., 2005).

1.4.2.2 *Media composition*

In vitro culture of cereals shows strong genotype dependence and production of the ideal cultures is generally limited to selected genotypes. The first stable transgenic sorghum plants were regenerated on callus initiation medium containing MS salts (Murashige and Skoog, 1962), modified (without calcium pantotenate) B5 vitamins, and 8g/l agar (Casas *et al*., 1993). This was supplemented with asparagines (150µg/ml), 10% coconut water, 30g/l sucrose and 2mg/l 2,4-D. For several sorghum genotypes tested in the CSIR's plant biotechnonolgy laboratory (Pretoria, South Africa), the medium based on Casas' yielded very low regenerants per explants values – the value calculated by dividing the number of individual fertile plants by the number of explants cultured on initiation medium (data not published).

1.4.3 Sorghum transformation

Among the direct gene transfer methods used in cereals, four are reliably used routinely to produce transgenic plants, i.e. protoplast transformation, tissue/cell electroporation, silicon carbide fibre vortexing and particle bombardment, also known as biolistics transformation. For sorghum, there are mainly two approaches currently in use for introducing foreign genes in cereals. The first one, called particle bombardment or biolistics, involves a physical method to transfer genes into target tissue. The second method, *Agrobacterium*-mediated transformation,

involves a natural plant pathogenic bacterium (*Agrobacterium tumefaciens)* that is genetically modified to transfer a segment or segments of DNA into plant cells.

In cereals, much of the early transformation methods were limited to direct gene transfer techniques because early work using the *Agrobacterium* failed to produce stable transgenic cereal crops (Shewry *et al*., 2001). However, Zhao *et al*., (2000) were the first to report on stable sorghum transgenic plants using the *Agrobacterium* method. In sorghum, the *Agrobacterium*-mediated transformation has recently been a preferred method of choice over biolistics. This is mainly due to simpler integration events that this method tends to yield and the perceived lower transformation efficiencies by biolistics in comparison to *Agrobacterium*-based methods (Table 1.2). However, Table 1.2 provokes an argument over this perceived superior transformation efficiency of the *Agrobacterium*-based method. This is because the transformation efficiencies are closely comparable. Using biolistics, Girijashankar and colleagues (2005) reported a superior value of 1.5 over Howe and colleagues' 1% (2006), who used the *Agrobacterium* method. Furthermore, no comparison of the two methods' transformation efficiencies has been reported in sorghum.

However, particle bombardment has also been used to successfully produce one of the most commercially important transgenic events called the *Mon810* or Yieldgard in *Zea mays L.* (maize). This transgenic event confers resistance to European Corn Borer (*Ostrinia nubilalis*) through the expression of a *Bacillus thuringiensis* Cry1AB protein (Sanders and Patzer, 1995). This transgenic event displayed a simple integration of the transgene (one copy), suggesting that

biolistics can also be used in sorghum for the production of transgenic plants with agriculturally important traits.

Sorghum has been classified as a recalcitrant cereal with regards to tissue culture regeneration and transformation (Kresovich *et al*., 1987; Zhu *et al*., 1998). It is ranked lower than important cereals such as maize, rice, wheat and barley and as such has not been a priority for improvement using biotechnological techniques.

Table 1.2 List of stable sorghum transformation reports to date.

Transformation method	**% Transformation efficiency (# expl.)**	**Authors**
Biolistics	0.08	Casas *et al*., 1993
Biolistics	0.33	Casas *et al*., 1997
Biolistics	0.09	Zhu *et al*., 1998
Biolistics	1.00	Able *et al*., 2001
Biolistics	0.18	Emani *et al*., 2002
Biolistics	1.30 (375)	Tadesse *et al*., 2003
Biolistics	1.5 (200)	Girijashankar *et al*., 2005
Agrobacterium	2.10 (6175)	Zhao *et al*., 2000
Agrobacterium	2.50 (2463)	Gao *et al*., 2004
Agrobacterium	2.91 (310)	Gao *et al*., 2005
Agrobacterium	1.00 (4511)	Howe *et al*., 2006

Selection for sorghum transgenic tissue after particle bombardment is crucial because the mechanical damage on explants requires careful *in vitro* culturing to recover and regain tissue totipotency. Bialaphos is a tripeptide antibiotic, which consists of the active ingredient phosphinothricin (PPT). PPT is a glutamate analogue and possesses the inhibiting effect on the enzyme glutamine synthetase (GS). This enzyme is responsible for detoxifying ammonia accumulation to toxic levels in cells, thus the inhibition effect of GS leads to tissue death. The *bar* gene encodes the enzyme phosphinothricin acetyl transferase (PAT) that detoxifies PPT by acetylation (D'Halluin *et al*., 1992). In contrast, the *pmi* selection system is considered a more tissue friendly system as the transgenic tissue is privileged with the ability to metabolize a different carbon source. Therefore, the *pmi* selection system's superiority over the *bar* selection system is believed to be attributed to its mode of action. In short, the *bar* system aims at killing cells while the *pmi* system starve the non-target tissue while promoting cell proliferation of the target cells.

1.5 Nutritional quality of the grain

Cereal grains are among the most important dietary sources of proteins, carbohydrates, B vitamins and minerals in the world. The nutritional quality of the sorghum grain is determined by the content of its constituents, i.e. proteins, carbohydrates, and lipids. The biological processing of proteins from any dietary source depends on their amino acid composition, secondary structure and the interactions of such proteins with any other matrices (tertiary and quaternary structures) which have implications for bio-digestibility and overall nutritional value. For all cereal grains, lysine is the first essential amino acid that is limiting for the diets of humans and monogastric animals (Table 1.3). Because of this, sorghum seed proteins are only partly used as

energy source in feed. An excess of certain amino acids combined with a shortage of others may result in deleterious effects of certain proteins that are essential for metabolic processes (Eggum *et al*., 1981).

The *Sorghum* seed has a nutritional profile similar to maize and other cereals (Shewry and Halford, 2002). The other major factor that limits sorghum nutritional quality is the seed protein digestibility. Sorghum seed protein digestibility, in comparison with other cereals, decreases significantly upon wet cooking (Duodu *et al*., 2002). This is attributable to the disulphide bond formations between protein polymers that contain sulphur in their side chains, e.g. cysteine (Hamaker *et al*., 1987; Rom *et al*., 1992; Duodu *et al*., 2003). Oria and colleagues (1995b) cited the cysteine-rich β- and γ- kafirins cross-linkages with themselves and with other matrix proteins as responsible for lowered protein digestibility. Granted that this postulation was accurate the β- and γ- kafirins, which are mainly on the periphery of the protein bodies, form a barrier that renders the more abundant α-kafirins on the inside unreachable by proteolytic enzymes such as pepsin.

A general structural organization of the sorghum seed was thoroughly reviewed (Hoseney, 1994). The generalized longitudinal structure is displayed in Figure 1.2. The estimated distribution by weight is generally as follows: endosperm - 84%, embryo/germ - 10% and pericarp - 6%.

Table 1.3 Sorghum kafirin amino acid content (mole %).

Amino acid	α-Kafirin	β-Kafirin	γ-Kafirin	Total Kafirin
Asparigine	6.0	3.3	0	4.8
Aspartic acid	0.4	N/A	0	N/A
Threonine	4.0	4.6	4.7	2.8
Serine	6.0	4.6	5.2	4.7
Glutamine	24.6	17.8	11.9	20.0
Glutamic acid	0.4	N/A	1.0	N/A
Proline	7.7	9.7	23.3	11.2
Glycine	1.6	6.8	8.8	2.7
Alanine	14.9	13.4	5.7	15.6
Cysteine	0.4	4.9	7.8	0.7
Valine	4.4	5.2	6.2	5.6
Methionine	0.8	5.7	1.0	1.7
Isoleucine	5.6	2.3	2.6	4.1
Leucine	15.3	12.0	8.3	15.4
Tyrosine	2.8	3.0	2.1	3.0
Phenylalanine	2.4	1.9	1.6	4.7
Histidine	1.2	0.9	7.8	1.6
Lysine	0	0.5	0	0.2
Arginine	0.8	2.7	2.1	1.2
Tryptophan	0.4	N/A	0	N/A

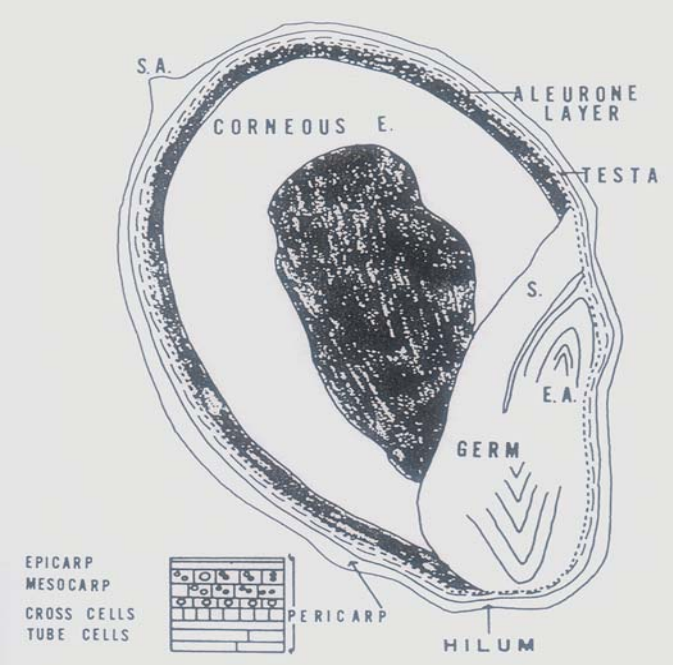

Figure 1.2 The longitudinal cross-section of the sorghum seed structure (Hoseney, 1994).

1.5.1 Seed carbohydrates

Starch is a major carbohydrate in sorghum and an average of 73% per seed dry weight is recorded (Olson and Frey, 1987). Starch occurs in granules which are both spherical and polygonal. These granules vary from 4-24µm in diameter. While 21-34% of this starch is amylase, the rest is amylopectin. Altogether 1.2-5.2% cellulose is mainly accounted for in the pericarp.

1.5.2 Seed protein

Seed protein content ranges from 7-14%. Most of the protein that accumulates during seed development is in the form of storage protein, but a small pool of amino acids is free amino acid

which account for about 10% or often less of the total amino acid content. The storage proteins have the following characteristics:

- they have no enzymatic activities,
- their synthesis is mainly during seed development,
- they are packaged in spherical structures called protein bodies, and
- they provide nitrogen, carbon and sulphur for the germinating seedling.

The content of storage proteins determines the seed quality and end use properties. Most variations are caused by environmental effects. Because the endosperm is a major part of the seed, its composition determines the seed nutritional quality. Two major types of storage proteins occur in seeds, i.e. the globulins and the prolamins. Globulins predominate in dicotyledons, but also in the aleurone layers and embryo of cereals, while prolamins are only found in the endosperm of all cereals.

1.5.2.1 *Sorghum storage globulins*

The globulin storage proteins are soluble in dilute saline solutions and are mainly packaged and stored on the embryo and aleurone layer. These storage proteins are rich in asparagines and glutamine and very poor in the sulphur-containing amino acids cysteine and methionine. Methionine is one of the essential amino acids and when it is limiting in diets, cysteine becomes essential because it is synthesized from methionine.

Two structural groups of storage globulins make up most of the globulins. These are distinguished by their sedimentary co-efficiency of 7S and 11-12S. The 7S globulins appear to

function only as protein bodies and are not involved in normal seed function. This was evident in maize where a mutant lacking these proteins displayed normal germination and general seed development (Kritz and Wallace, 1991). In comparison with the starchy endosperm, the embryo and the aleurone layer are richer in protein content but these proteins do not make a difference on the end usage in terms of seed quality. This is because they are usually lost during milling (wheat), polishing (rice), pearling (barley) and decortication in sorghum (Shewry and Halford, 2002).

1.5.2.2 *Storage prolamins*

The prolamins of sorghum are also called kafirins while in maize, a close relative of sorghum, they are called the zeins. They are rich in amino acids proline and glutamine but deficient in lysine, tryptophan and threonine. Based on their alcohol solubility, the kafirins were initially divided into 4 groups, i.e. α-, β-, γ- and δ-kafirins (Shewry and Halford, 2002). However, although some prolamins occur in alcohol-insoluble polymers, they are all alcohol soluble in a reduced state.

The most abundant α-kafirins are mainly polypeptides of 19-24kDa. These kafirins contain one or two cysteine residues per molecule, much less than the other four minor groups. The β-kafirins are polypeptides of 14-16kDa that are lower in proline and glutamine than the α-kafirins. These kafirins are rich in proline and cysteine. Two major γ-kafirins are polypeptides of 27 and 16kDa, while a minor γ-kafirin is 50kDa in size (Xu and Messing, 2008). The δ-kafirins are a minor fraction of 10 and 18kDa polypeptides that are extremely rich in sulphur-containing amino acid cysteine.

The kafirins are believed to be synthesized in the rough endoplasmic reticulum (ER) and transported from the ER via the Golgi apparatus and into the protein storage vacuole (Taylor *et al*., 1985; Kermode and Bewley, 1999), but the actual mechanism is still not well understood. However, a number of reports attempting to elucidate this mechanism in species such as maize (Coleman and Larkin, 1999; Muench *et al*., 1999), rice (Yagamata and Tanaka, 1986; Krishnan *et al*., 1986), oats (Lending *et al*., 1989), and wheat and barley (Galili, 1997; Shewry 1999) have been provided, therefore, more studies should focus on clarifying this process to fully understand the stereo-chemistry of protein bodies.

1.5.2.3 *Dietary lysine*

Lysine is one of the ten essential amino acids for mammals, with the molecular formula $C_6H_{14}N_2O_2$. Its three and one letter codes are Lys and K respectively, while the nucleotide translation codons are AAA and AAG. Table 1.4 lists the ten essential and ten non-essential amino acids for mammals. In plants and micro-organisms, lysine is synthesized by a complex cycle that involves aspartic acid as a substrate (Figure 1.3). Methionine and threonine are also synthesized by this pathway (Sakai *et al*., 2003; www.genome.ad.jp/kegg/pathway/map/map00300.html).

Table 1.4 List of 10 essential and 10 non-essential amino acids in mammals

Essential amino acids	**Non-essential amino acids**
Arginine	Alanine
Histidine	Asparagine
Isoleucine	Aspartate
Leucine	Cysteine
Lysine	Glutamate
Methionine	Glutamine
Phenylalanine	Glycine
Threonine	Proline
Tryptophan	Serine
Valine	Tyrosine

The daily lysine nutritional requirement in humans is 1-1.5g and Table 1.5 shows a comparison of the average essential amino acid composition for both maize and sorghum seeds. Foods rich in lysine include legumes, dairy products and meat products such as fish and red meat. L-lysine is a crucial building block for proteins in the body. It also plays a major role in calcium absorption; muscle protein building; damaged tissue recovery; and proper production of the body's antibodies, hormones and enzymes. It is also believed that lysine plays a healing role in herpes simplex viral infections. The alleviation of cold-sores is lysine controlled as a speedy healing process is observed after lysine supply (Griffith *et al*., 1978).

Table 1.5 Comparison of the essential amino acid contents of sorghum and maize whole grain (mg/g crude protein)*.

Amino acid	Maize	Sorghum
Lysine	33.9	25.2
Histidine	30.4	21.4
Threonine	45.7	42.7
Valine	59.7	56.3
Isoleucine	50.4	56.3
Leucine	142.8	132.0
Methionine + Cysteine	48.6	50.1
Phenylalanine + Tyrosine	98.4	67.0

* = values based on crude protein contents of 8.5 and 10.3% for maize and sorghum respectively (Scherz and Senser, 1989)

Lysine bio-synthesis branch point depends on two feedback regulated enzymes called the aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS). In 1999, Mazur's group (Mazur *et al*., 1999) achieved an increment of total lysine content of up to twofold, this after expressing a feedback-insensitive DHPS in both the aleurone layer and the embryos of the maize seed. However, Mazur's group was unsuccessful in improving the lysine content in the starchy endosperm.

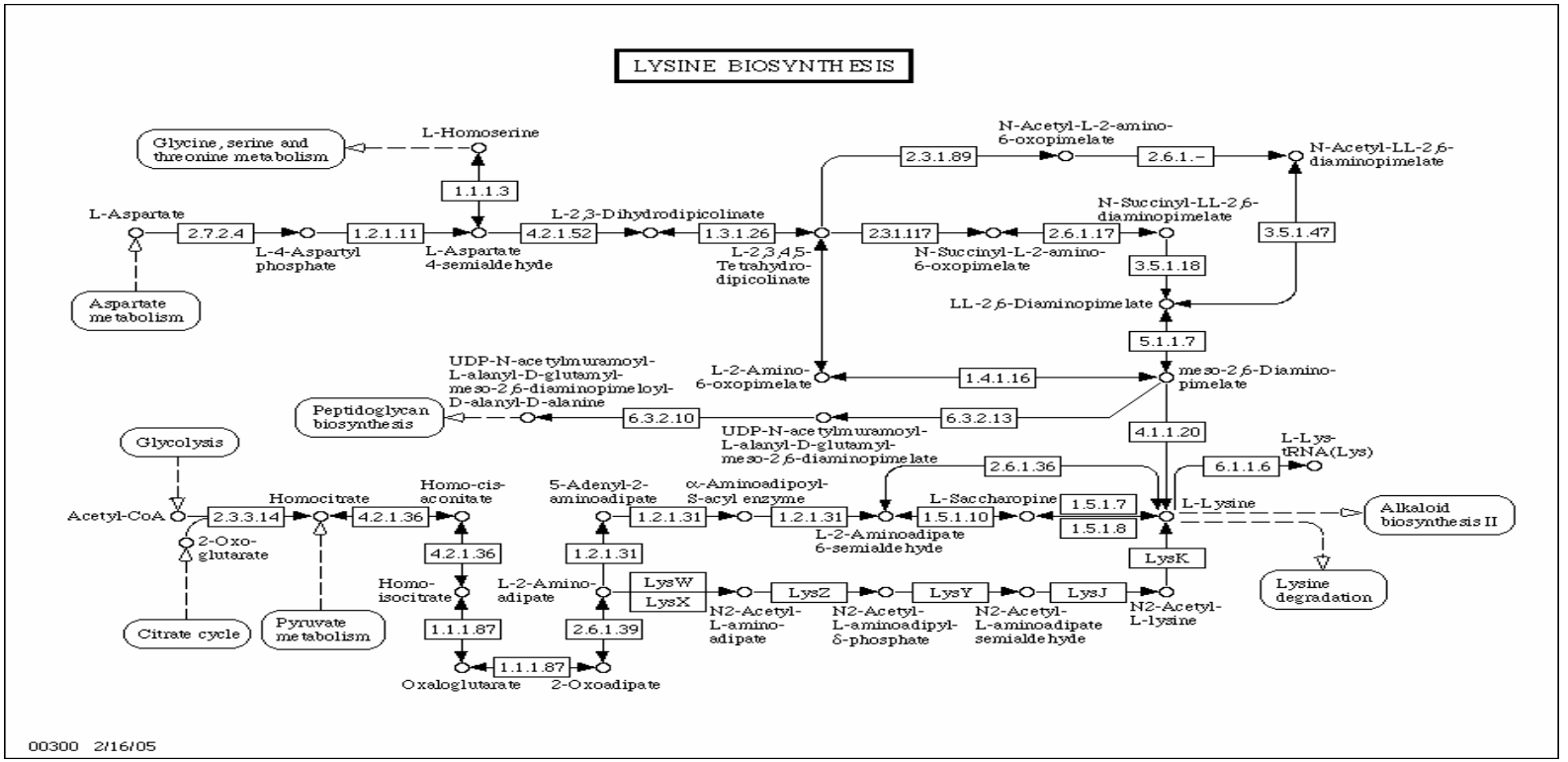

Figure 1.3 The lysine biosynthesis pathway. Numbered rectangular boxes represent substrate and product molecules (www.genome.ad.jp/kegg/pathway/map/map00300.html).

1.5.3 Seed protein digestibility

Protein bio-digestibility is a measure of how easy proteolytic enzymes catabolize a protein of interest. Proteins with superior digestibility have better nutritional quality because they yield more amino acids for absorption by the digestive system. Duodu (PhD thesis, 2000) rightly made mention of the fact that reported *in vitro* protein digestibility of uncooked sorghum and maize does not differ significantly. However, sorghum seed protein bio-digestibility significantly drops upon wet cooking in comparison with other cereals (Axtell *et al*., 1981; Hamaker *et al*., 1986; Hamaker *et al*., 1987). Duodu also suggested that wet cooking reduces bio-digestibility in sorghum via a re-conformation of α-helical structure of prolamins which could be a result of disulphide cross-links to form anti-parallel β-sheet formations. This was particularly observed with the high cysteine containing β- and γ-kafirins (Oria *et al*., 1995) suggesting more disulphide bond formations.

A few factors believed to be responsible for protein bio-digestibility include the following:

- the relationship between seed proteins and other seed components can reduce seed protein digestibility,
- phenolic compounds, especially tannins, can bind to proteins and render them less digestible,
- phytic acid chelates protein and minerals such as zinc and iron, thereby reducing the protein digestibility, and
- protein-to-protein cross-linkage assisted by wet cooking to form disulphide bonds.

1.6 Strategies for improving plant nutritional value: advantages of RNAi

Generally, initial efforts to improve plant nutritional quality were mainly directed at plant breeding. In the last two decades, genetic engineering for transgenic plant production has been an approach of choice (see section 1.4). Among other advantages for genetic engineering in plants is a wider scope and types of mutations that can be introduced. This is further strengthened by the ability to control the spatial and timing of transgene expression. This is vitally important as the plant's energy is conserved for other organs' metabolic functions and minimal disruptions are afforded to enable proper cultivation of the transgenic plant. In contrast, a lack of this spatial and timed manipulation is likely to possess deleterious effects. An example of this was the efforts to improve lysine content in plants by introducing a genetic mutation in the enzyme DHPS. It was deduced that targeted expression of transgenic DHPS in seeds as a result of driving expression by a seed-specific promoter avoids undesirable and deleterious effects in vegetative organs (Mazur, 1999).

RNA interference (RNAi) has been used in plants to improve the nutritional quality by targeted suppression of enzymes and other proteins that lead to the accumulation of desirable metabolites. Figure 1.4 shows a schematic diagram depicting the mechanism of RNAi gene silencing.

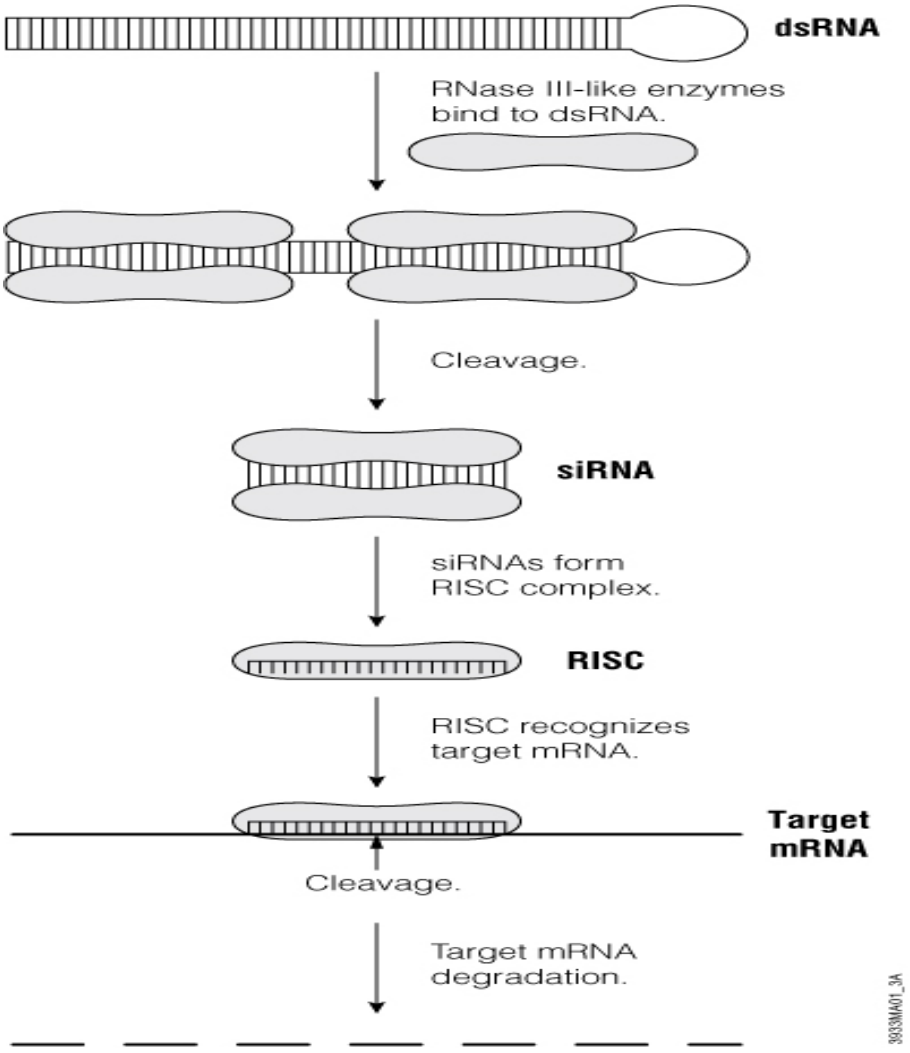

Figure 1.4 The principle of RNAi silencing. The small RNA molecules, microRNA (miRNA) and small interfering RNA (siRNA) bind to target RNAs and decrease their activity by preventing a messenger RNA from producing a protein. This process is instigated by the enzyme Dicer, which cuts long double-stranded RNA molecules into short fragments of ~20 nucleotides. This is called RNA-induced silencing complex (RISC). Photo taken from Promega website: http://www.promega.com/paguide/chap2.htm.

Using RNAi, lysine has been improved by targeting the suppression of the enzyme/s that effect lysine catabolism in seeds (Mazur *et al.*, 1999; Falco *et al.*, 1995). The RNAi technology has

also been used to reduce caffeine content in coffee plants by targeted suppression of the caffeine synthase gene (Ogita *et al*., 2003). A lysine-poor 22kDa alpha zein storage protein was successfully knocked out to improve the lysine content (Segal *et al*., 2003). In cotton, RNAi was used to improve the fatty acid quality content by co-suppression of two desaturase genes for the targeted improvements in the essential oleic and stearic fatty acids (Liu *et al*., 2002).

1.7 The rationale of the study

1.7.1 Problem Statement

Cereal seeds are generally poor in lysine and methionine and sorghum seeds, in particular, are very poor in the essential amino acids lysine and methionine contents as shown in Tables 1.3 and 1.5 (Scherz and Senser, 1989; Taylor and Belton, 2002). This deficiency in essential amino acid content is one of the two major determinants of sorghum's poor nutritional value, the other one being the low protein digestibility on wet cooking. The amino acid lysine is a first limiting essential amino acid for mammals in the sorghum seed. The endosperm accounts for most of the seed (80%) and it is the main storage compartment for seed storage proteins. Because prolamins comprise most of the endosperm proteins (Taylor and Schüssler, 1986), most of the attempts to improve the essential amino acid contents of cereals are directed at the prolamin fraction of seed proteins.

The major prolamins are α-kafirins, which comprise 70% of total prolamins. The α-kafirins are major contributors of the low lysine and methionine contents of the grain (Shewry and Halford, 2002). The second most abundant kafirin proteins are the γ-kafirins that appear on the outside of the protein body. These kafirins also contain very few lysine and methionine residues, and

therefore, are the second most contributors to the low seed lysine content. However, due to their hydrophobicity, their peripheral location on the protein body structure and their cysteine-rich nature resulting in increased di-sulphide bonds formation, in this study the γ-kafirins are identified as the major contributors to low seed lysine.

There are a few reports on attempts to enhance essential amino acid contents of cereal seed. These attempts include the suppression of the lysine catabolism enzyme LKR (Houmard *et al*., 2007) via the RNAi strategy in maize. Another approach reported involves the expression of an exogenous or heterologous protein that is rich in the amino acid of interest. One example was the expression of a γ-zein protein in various organs of the tobacco plant (Coleman *et al*., 1996). However, expression levels were very low. Technically, using this approach, it is imperative to produce single copy integration transgenic plants to effectively express the transgenic protein. There is also more demand on the seed protein resources due to the added protein expression required.

Yet another approach involved the manipulation of the lysine metabolic pathway to provide an increased source of the target amino acid, as was the case with the expression of a bacterial feedback-insensitive dihydrodipicolinate synthase (DHPS) in potato, tobacco, canola, soybean, barley, *Arabidopsis* and maize (Perl *et al*. 1992; Shaul and Galili, 1993; Falco *et al*. 1995; Brinch-Pedersen *et al*., 1996; Zhu and Galili, 2003; Huang *et al*., 2005). More recently, this approach revealed that the introduction of changes in the aspartate biosynthesis pathway often leads to changes in expression of non-targeted amino acids, especially those amino acids that are derived from the same precursor (Thu *et al*., 2007).

This study therefore attempts to address the sorghum grain's low lysine content, by using the RNAi approach to suppress two γ-kafirins, one δ-kafirin and the endosperm LKR protein suppression as a new and combined approach to improve the lysine content in sorghum. Because this approach is aimed at suppressing endogenous protein, the seed protein expression process will potentially be relieved for the expression of more valuable proteins. The LKR gene silencing aspect will ensure reduced lysine catabolic activity of the intended lysine increase.

Technically, sorghum has been classified as a recalcitrant cereal with regards to tissue culture regeneration and transformation (Kresovich *et al*., 1987; Zhu *et al*., 1998). For this reason, very few laboratories have reported successful sorghum tissue culture and transformation based manipulations in comparison to other cereal crops (Masteller and Holden, 1970; Cai *et al*., 1987; Ma and Liang, 1987; Cai and Butler, 1990; Casas *et al*., 1993, 1997; Kaeppler and Pederson, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000, 2003; Able *et al*., 2001; Emani *et al*., 2002; Tadesse *et al*., 2003; Gao *et al*., 2005; Howe *et al*., 2006). This presents a technical barrier to the use of genetic modification as a method of improving sorghum. This study addresses this barrier through improvement of the biolistics method of transformation.

1.7.2 Hypothesis

The application of RNAi technology to down-regulate selected seed lysine deficient endosperm kafirins, will result in the improvement in the nutritional quality of sorghum grain by increasing the seed lysine content. I postulate that removal of the select kafirins will avail amino acids for the accumulation of other, more beneficial, and therefore, an overall lysine content improvement.

1.7.3 Aims and objectives

This study aims to improve the seed lysine content by targeted suppression of kafirins that confer poor grain lysine content. Towards achieving the aim, improvements in the transformation process will have a major impact towards improving the sorghum plant. To make a meaningful research impact, the transformation efficiency and the ease of recovering stable transgenic plants were targeted to increase the sorghum manipulation effort *in vitro*. Four of the five sorghum genotypes that were chosen for the transformation experimentation were never tested for tissue culture amenability before.

To achieve the aims stated above, the following objectives were set as follows:

- evaluation of tissue culture amenability of five sorghum genotypes,
- sorghum biolistic transformation protocol optimization and generation of independent transgenic events with a selected vector
- characterization of transgenic plants for kafirins suppression and seed lysine improvements.

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Chapter 2

Screening of sorghum genotypes for tissue culture amenability

2.1 Abstract

Sorghum genotype responsiveness to *in vitro* culture and manipulation is essential to establish a routine transformation protocol. This entails identification of those genotypes that display desirable tissue culture response, in particular somatic embryogenesis coupled with whole plant regeneration in a defined tissue culture medium. The regeneration capacity of five sorghum genotypes on three different solid nutrient media was investigated, i.e. new genotype X medium combination. The most responsive genotypes were identified based on their ability to form totipotent callus cultures that give rise to plantlets with normal shoots and root systems within the shortest culture period *in vitro*. It was found that regeneration of plants was mainly through somatic embryogenesis via type I callus. The genotype P898012 on culture medium previously designated medium J gave the best callus induction of 98%, the highest regeneration potential of 6.13 regenerants/explants and the shortest tissue culturing period of 74 days. This genotype and medium combination has been therefore selected for subsequent transformation and regeneration of sorghum transgenics with improved traits.

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2.2 Introduction

Somatic embryogenesis is defined as a process in which somatic cells undergo bipolar development resulting in whole plants through the development of globular structured embryos. These embryos differentiate into plants that are genetically identical to their parental somatic embryo cells. The resulting plants display growth patterns that are similar to that of seed-derived plants.

High frequency plant regeneration from cultured explant material is a prerequisite for successful transformation of most cereal crops. One of the key limiting steps in the development of genetic engineering protocols for the improvement of cereal crops through biolistic and *Agrobacterium*-mediated transformation is the *in vitro* plant regeneration process. *In vitro* culture of cereals shows strong genotype dependence and production of the appropriate culture is generally limited to selected genotypes. There is also strong evidence to suggest that sorghum is no exception to this genetic control (Tomes and Smith, 1985; Morocz *et al*., 1990).

This part of the study addressed the problem of callus initiation and regeneration potential of five sorghum genotypes that originate from major sorghum growing areas in Africa, cultured on specific nutrient media formulations. The objective was to identify the best genotype-nutrient medium combination that results in satisfactory regenerability for subsequent transformation activities.

2.3 Materials and methods

2.3.1 Sorghum genotypes and explant

Five sorghum [*Sorghum bicolor* **(L.)** Moench] genotypes chosen for tissue culture amenability screening were Kapaala (Indian), Kadaga (Ghanaian), SA 2861, SA 4322 (both South African genotypes) and P898012 (American). The genotypes were chosen on the basis of agronomic and functional attributes ranging from good food and brewing qualities (Kapaala and Kadaga) to stem borer and aphid resistance (SA 2861 and SA 4322, respectively) and transformability (P898012).

2.3.2 Plant material and nutrient media

Immature zygotic embryos (IZEs) ranging from 0.8-1.2 mm in length were used as explants. These were derived from sorghum seeds harvested 12-15 days post anthesis. The immature seeds were surface-sterilized in 70% (v/v) ethanol for 3 min, and for 15 min in 2.5% sodium hypochlorite solution containing 0.1% Tween-20 before a thorough rinse with sterile distilled water. Tissue culture experiments were performed under aseptic conditions. Callus cultures were transferred to fresh callus induction medium (CIM) every two weeks until the onset of somatic embryogenesis. The IZEs were placed on CIM with the scutellum cells facing upwards and the embryogenic axis in contact with the CIM for somatic embryo formation. After somatic embryo formation, the calli were transferred to callus maintenance medium (maturation medium) before transfer to regeneration medium for plantlet production. In total, 100 to a 150 IZEs (10 embryos per petri dish) were cultured per genotype per tissue culture medium (Table 2.4). The IZE explants consisted of three biological replicates, i.e. independent panicles and harvest dates, with 15-30 explants per replication.

Tables 2.1 to 2.3 list a summary of the nutrient media contents and the culturing conditions for each solid medium used in this study. For tissue culturing on medium J (O'Kennedy *et al*., 2004), the CIM contains L3 based salts and vitamins (see Annexure), 2.5 mg l^{-1} of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), the carbon source maltose, 4 g/l gelrite as the gelling agent and 20mM L-proline. In the root and regeneration medium (RRM), 2,4-D and L-proline were not included. A 4-week culture on CIM was followed by a two-week period on callus maturation medium, prior to regeneration and rooting regimes/phases. The maturation medium contained double the amount of carbohydrate, which in this case was maltose (Table 2.1).

For tissue culturing on Tadesse's medium, somatic embryogenic calli formed within 4 weeks of culture on CIM were transferred to a modified CIM with reduction in 2,4-D (2.5 – 2.0mg/l) and increment in kinetin (0.2 – 0.5 mg/l) until somatic embryos were ready to germinate (Table 2.2). The somatic embryos were then transferred to shoot induction medium (SIM) until shoots developed (Tadesse *et al*., 2003) and subsequently to root induction medium (RIM).

The CAPD medium was described by Casas *et al.* (1993). This medium was used with the following modifications: 1 g/l asparagine and 2 mg/l 2,4-D was added in the CIM. After 14 days on CAPD2 and 7 days on CAPD1 to initiate somatic embryoids, the cultures were transferred to callus maintenance medium (CCM) for 4-7 days, followed by subsequent culture for 2-6 weeks on regeneration medium. The callus regenerating medium (CMR) was responsible for shoot formation within 14-28 days. The callus shoot elongation (CSE) medium cultures take 10-14 days before shoots are cultured on Casas Rooting Medium (CROOT) for 14 days. Finally,

transfer and culturing of rooted shoots on Casas Root Elongation (CRE) for 7-14 days was performed (Table 2.3).

2.3.3 Data collection and statistical analysis

The incubation conditions for all cultures were at 24-25˚C under low-light conditions (1.8 $\mu Em^{-2}s^{-1}$) except for regeneration of shoots (≥ 5cm), which were incubated under light conditions of 18 $\mu Em^{-2}s^{-1}$. The rooted plantlets with a height of 5-10 cm were considered as 'regenerants'. A random selection of regenerants was hardened off to be assessed for fertility in the greenhouse. Data on percentage callusing and regeneration ratio was entered into Excel and statistical analysis was carried out using the MINITAB software Release version 12.21 (MINITAB Inc., 1998). The experiment was a two-factor factorial experiment (genotype and media) in a Completely Randomized Design with replication where the genotype and media are the factors. The Tukey's Test was used to perform a pair-wise comparison of the means of the genotypes.

Table 2.1 Composition of tissue culture medium J.

Nutrient	**J CIM**	**J Maturation medium**	**Medium J RRM**
L3 macro- and micro elements	+*	+*	+*
MS-Fe source	+*	+*	+*
HL2 Vitamins	+*	+*	+*
2,4-D	2.5mg/l	-	-
Maltose	30g/l	60g/l	30g/l
pH	5.8	5.8	5.8
Gelrite	4g/l	4g/l	4g/l
L-Proline	20mM	-	-

* = see Annexure

Table 2.2 Composition of Tadesse's tissue culture medium.

Nutrient	**Tadesse's CIM**	**Tadesse's modified CIM**	**Tadesse's shoot induction medium**	**Tadesse's root induction medium**
MS macro- and micro elements	+*	+*	+*	½ strength
MS-Fe source	+*	+*	+*	½ strength
Jacobs Vitamins	+*	+*	+*	+*
2,4-D	2.5mg/l	2mg/l	-	-
Kinetin	0.2mg/l	0.5mg/l	0.5mg/l	-
IBA	-	-	-	0.5mg/l
NAA	-	-	-	0.5mg/l
Sucrose	30g/l	30g/l	30g/l	20g/l
pH	5.8	5.8	5.8	5.8
Agar	8g/l	8g/l	8g/l	8g/l

* = see Annexure

Table 2.3 Composition of CAPD2 tissue culture medium.

Nutrient	CAPD2	CAPD1	CCM	CMR	CSE	CRoot	CRE
MS Macro-, micro- & Fe source	+*	+*	+*	+*	+*	½ strength	½ strength
B5 modified Vitamin (Ca-pantothenate)	+*	+*	+*	+*	+*	+*	+*
2,4-D	2mg/l	1mg/l	1mg/l	-	-	-	-
Kinetin	-	-	0.5mg/l	0.5mg/l	0.5mg/l	-	-
IAA	-	-	-	1mg/l	1mg/l		
IBA	-	-	-	-	-	0.5mg/l	-
NAA	-	-	-	-	-	0.5mg/l	-
NH_4NO_3	3.3g/l	3.3g/l	-	-	-	-	-
Proline	2g/l	2g/l	-	-	-	-	-
Asparagine	1g/l	1g/l	-	-	-	-	-
$AgNO_3$	-	-	-	10mg/l	-	-	-
Coconut water	100ml/l	100ml/l	-	-	-	-	-
Sucrose	30g/l	30g/l	30g/l	30g/l	30g/l	20g/l	20g/l
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Agar	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l	8g/l

* = see Annexure

2.4 Results

Sorghum IZEs proliferated to produce two types of calli, a very soft and watery non-embryogenic callus (Figure 2.1 A), and a highly embryogenic totipotent type I callus that is harder and white in appearance (Figure 2.1 B). Table 2.4 shows the regeneration data for the genotypes under different media conditions. Using analysis of variance (ANOVA) for nine replicated experiments, data showed that the response to both callus induction and regeneration were influenced independently by medium and genotype. Thus their interaction had a stronger effect (probability values in Table 2.5, last column) where P values below 0.05 indicate significant differences. Overall, pair-wise comparison ranked the genotypes in the order of P898012>SA2861>Kadaga>SA4322 for callus induction on the three media, with genotype P898012 producing 98% somatic embryos on medium J.

The coefficient of variation for the callus induction and regeneration (18.8% and 6.1%) suggested that the experiments were reproducible with low experimental error. The coefficient of variation value above 40% points to low reproducibility of the data. This value is indicative of a variation of data points in comparison to the mean value. Therefore the values attained in this part of the study, i.e. below 20%, indicate that the data is reliable. A dissection of the influence of media on the genotypes also revealed a significant medium formulation effect; this was displayed by the probability values (Table 2.5 A and B; last column). Again, the P-values below 0.05 suggest a significant effect of the medium composition on both somatogenesis and regeneration. A probability value above 0.05 suggests a strong probability of no significant difference caused by the parameter tested. The significant effect caused by the genotype was further indicated by the results for Kadaga. Fifty percent of Kadaga IZEs proliferated and formed

somatic embryos on medium J, while genotype SA 4322 IZEs did not proliferate on the same medium J.

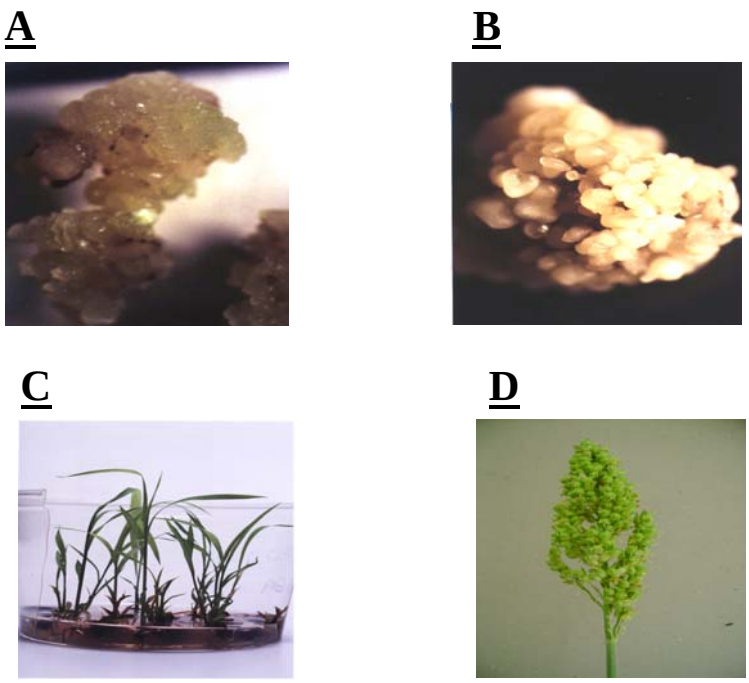
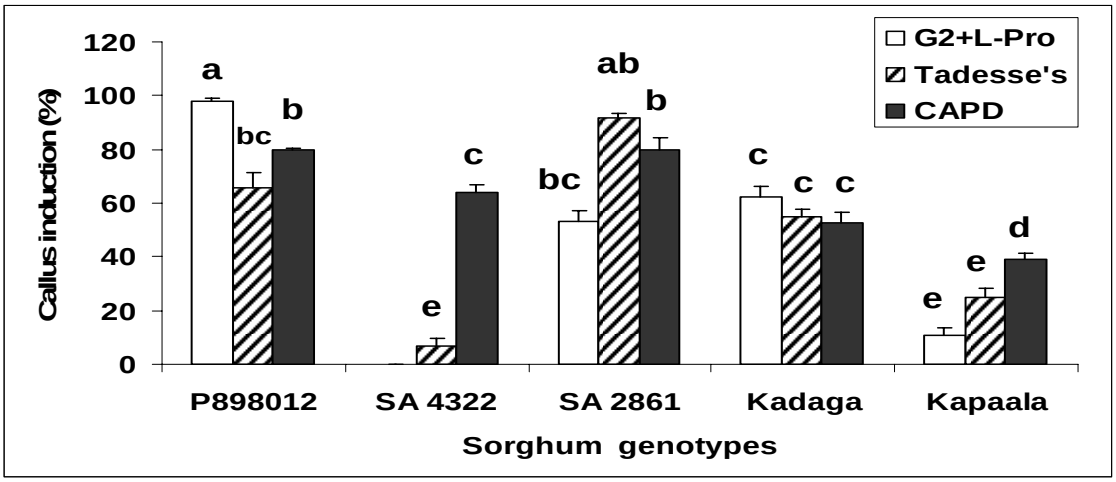


Figure 2.1 *In vitro* plant regeneration from immature zygotic embryos of sorghum. (A) Non-regenerable, soft and watery callus produced/formed by some embryos. (B) White, compact embryogenic type I tissue derived from cultured IZEs of genotype P898012 on tissue culture medium J within two weeks. (C) Sorghum genotype P898012 plantlets shooting and rooting on regime J regeneration medium. These plantlets resulted in fertile F_0 plants. (D) Mature sorghum head from tissue culture plants.

The regeneration potential of the five genotypes was calculated as the number of regenerants divided by the number of explants cultured and results are shown in Figure 2.2 and Table 2.4. Overall, the genotype responsiveness was ranked in the order of P898012>SA 2861>SA4322>Kapaala>Kadaga in all three media formulations tested. On medium J, P898012 was found to be the most regenerable genotype at 6.13 regenerants/explants (reg./expl.), followed by genotype SA 2861 at 1.01 reg./expl. On Tadesse's medium, P898012 was also found to be superior in comparison to the other four genotypes, yielding 1.4 reg./expl. On CAPD medium, the two South African genotypes were the best performers, yielding 3.56 reg./expl. for SA 4322 and 1.71 reg./expl. for SA 2861. Kapaala showed some regenerability (between 0.01 and 0.17 reg./expl.) while no regeneration was obtained for Kadaga in all three media formulations tested. Visual screening revealed that all F_0 sorghum genotypes that were hardened-off showed phenotypes with unchanged vegetative growth and viable F_1 seeds after self fertilization. Another observation was that genotype P898012 on medium J seemed to produce plantlets much faster (75-90 days) when compared to the other two (100-140 days).

A



B

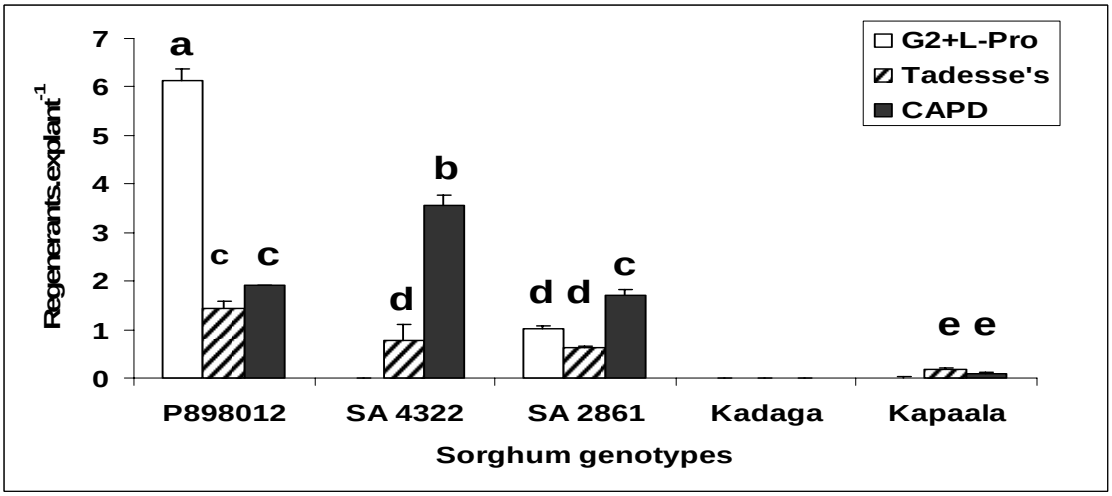


Figure 2.2 The effect of culture media on callus induction (A) and plant regeneration (B) of five sorghum genotypes from immature zygotic embryos. The bars represent the mean of nine individual experiments with ±SE (standard error). The most totipotent calli were produced by P898012 on J medium which resulted in 6.13 regenerants per explant. Bars with the same letter are not significantly different (P>0.05).

Table 2.4 Results of plant regeneration observed for each of the genotypes on the three culture media tested.

Genotype	Medium	Number of IZEs	Number of somatic embryos produced	Number of reg./expl. (expl. calculated from number of somatic embryos formed)	Days on tissue culture
P898012	J	146	143	6.13	74-100
SA 4322	CAPD	135	86	3.56	100-140
SA 2861	CAPD	134	107	1.71	100-140
P898012	Tadesse's	113	70	1.4	100-144
SA2861	J	150	80	1.01	120-140
SA4322	Tadesse's	135	9	0.77	100-144
SA2861	Tadesse's	132	121	0.63	120-144
Kapaala	Tadesse's	147	37	0.17	120-140
Kapaala	CAPD	100	39	0.10	120-140
Kapaala	J	147	16	0.02	120-140

Table 2.5 ANOVA for regeneration statistics (**A**) callus induction in sorghum genotypes, and (B) regeneration in sorghum genotypes. Nine individual experiments were carried out. * shows statistical significance at 5% level.

A

Source	Degrees of Freedom	Mean Square	*F* value	Probability
Replication	9	69.9	0.72	0.686
Genotype	4	20153.8	208.53	0.000*
Media	2	4141.6	42.85	0.000*
Genotype x Media	8	3750.9	38.81	0.000*
Error	113	96.6		

Coefficient of variation: 18.8%

B

Source	Degrees of Freedom	Mean Square	*F* value	Probability
Replication	9	0.148	0.84	0.582
Genotype	4	44.169	250.32	0.000*
Media	2	10.274	58.23	0.000*
Genotype x Media	8	22.315	126.47	0.000*
Error mean square	113	0.176		

Coefficient of variation: 6.1 %

2.5 Discussion

This study tested the response of five genotypes, of which two were from South Africa, cultured on three different media. The sorghum genotypes displayed different responses to *in vitro* culture where P898012 showed the highest regeneration capacity on medium J, supplemented with L-proline. These varied responses show clear interactions of the genotypes with the different nutrient media. Observed genotype-medium interactions are consistent with what has been observed for other cereals (Khanna and Raina, 1998). This has resulted in the identification of model genotypes for transformation purposes in other cereals, such as genotypes T309 and IR72 in rice (Christou, 1994), the A188 in maize (Ishida *et al*., 1996; Locatelli *et al*., 1992), Golden Promise in barley (Jacobsen *et al*., 1999; Tingay *et al*., 1997), and Bobwhite genotypes in wheat (Altpeter and Varshney, 2001; Wu *et al*., 2003).

In this study, P898012 on medium J gave an embryogenic callus percentage of 97.9%, the highest reported for sorghum IZE as explants. This embryogenesis potential value indicates a further improvement to what had already been reported, i.e. 81% for genotype P898012 cultured on different tissue culture media containing naphthalenacetic acid and an increased nitrogen and potassium phosphate contents (Sato *et al*., 2004).

This report also states a specific regeneration potential of 6.13 reg./expl. value for P898012 on medium J. Most sorghum regeneration reports do not provide such reg./expl. values. Instead, they report on somatic embryo callus formation potential as a measure of tissue culture amenability (Tadesse *et al*., 2003; Casas *et al*., 1993). This reg./expl. value is, however,

important for *in vitro* regeneration of the sorghum IZEs, since the callus formation potential is not always directly related to the regeneration potential. This is due to medium composition used for callus formation and regeneration purposes. Both medium compositions are different processes involving different medium formulations. This was reported first by Khanna and Raina (1998) while investigating the genotype X nutrient media interactions effects on regeneration in three indica rice cultivars. They concluded that regeneration percentage was influenced by the genotype, callus induction medium, regeneration medium and interaction between these parameters. The present study extends this knowledge since the genotype SA2861 X CAPD medium gave a 79.9% callus formation rate. This was inferior to the 91.7% rate achieved by the genotype SA2861 on Tadesse's medium. Furthermore, the former combination also had a superior reg./expl. value of 1.71 in comparison to the 0.63 value for the latter combination. The regeneration process should, therefore, be treated independently where the regeneration medium can be altered independent of the CIM.

In this study, the regeneration capability was measured in addition to the callus formation potential of five sorghum genotypes on three media formulations. This extends the knowledge on sorghum tissue culture performance as a first step to genetically engineer this cereal crop. The different callus responses and subsequent plant regeneration potential of different genotypes on identical nutrient medium is likely due to the genetic (genotype) X environmental (medium) interaction as stated by Khanna and Raina (1998). Also, the same environmental conditions (medium) can result in different callus responses and this could be a result of different genetic control factors (Tomes and Smith, 1985; Morocz *et al*., 1990). Other factors, such as the size and physiological state of the explants that could affect callus totipotency were eliminated by

experimental design in this study. All explants were carefully chosen to exclude explant sizes that could either be too small or too big. The best embryo size was chosen as 0.9-1.2mm long. Each seed batch of a particular genotype was equally divided by culturing on all three nutrient media. This ensured that a uniform callus response from explant to explant and medium plate to medium plate was achieved (Table 2.5).

The period of *in vitro* culturing, indicated by days to regeneration, is also a useful indicator for rapid tissue proliferation because it is a measure of how fast the callus tissue divides to reach the regeneration stage. The most regenerable line also produced the shortest *in vitro* culturing period, i.e. earliest plantlets produced after 74 days with P898012 on medium J. This is especially crucial in the production of healthy transgenic plants as the stress imposed by tissue culture is minimized, and so is the opportunity for somaclonal variation due to lengthy tissue culture conditions. Somaclonal variation is defined as the phenotypic variation that is often observed in plants as a result of the tissue culture stress and is suspected to be caused by chromosomal re-arrangements.

In conclusion, the sorghum genotypes tested showed a variation of genotypic responses to *in vitro* culture. This further extends our knowledge that was previously reported in indica rice that the callus formation and regeneration potential is dependent on the genotype and medium composition. From the results in this study, the chosen sorghum genotypes for transformation experiments were, in order of priority, P898012 on medium J, followed by SA 4322 and SA 2861 on CAPD. These combinations displayed superior sorghum regeneration potential and it is anticipated that it should significantly increase the probability of producing transgenic sorghum

plants. In the following chapter, the optimization of the transformation selection system and sorghum transformation was carried out by using genotype P898012 on medium J.

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Chapter 3

Transformation of sorghum for suppression of selected seed proteins

3.1 Abstract

Sorghum [*Sorghum bicolor* (**L.**) Moench] is recalcitrant to gene manipulation, thus, few laboratories have reported reliable and reproducible transformation methods. Because of biosafety concerns for using the herbicide and antibiotic resistance genes, the selection of a selectable marker for selection of transgenic tissue and plants is very important. Two tissue culture selection systems that employ the *bar* gene, encoding bialaphos resistance, and the *manA* gene, for phosphomannose isomerase, both under the control of maize ubiquitin promoters and nopaline synthase terminator sequences were compared to improve the transformation efficiency of sorghum via particle bombardment using a particle inflow gun. A total of 3 and 27 transgenic plants were recovered for bialaphos and mannose selection, respectively. This translates to transformation efficiencies of 0.11% and 0.77% for the *bar* and *manA* genes, respectively. The expression of the *bar* gene in the three transgenic plants was demonstrated by the BASTA leaf painting assay. Results further indicated that 75% of putative bialaphos resistant plants were escapees, while none was recorded for *manA* gene. Using the mannose selection system, the RNAi approach was selected as a strategy to suppress a subset of seed endosperm proteins that are implicated in poor lysine content and protein digestibility. The target genes, all carried on the same expression cassette, were co-expressed as an RNAi construct into the sorghum genome by particle bombardment using the Bio-Rad gun (Biolistic PDS-1000/He). A total of 11 independent transgenic events were generated at an average transformation efficiency of 3.36%. The PCR and Southern blot hybridization analyses of seven selected transgenic events revealed a 100% stable co-integration of the selectable marker gene with the target expression construct. Transgenic lines 2 and 4 displayed multiple transgene insertions, i.e. more than five copies, while events 1, 3 and 5 displayed simpler transgene integrations, i.e less than five copies. Two transgenic events,

namely 6 and 7 show transgene rearrangement or truncation at the 5' promoter end of the transgene cassette.

3.2 Introduction

A suggestion was aired by Birch and Bower (1994) that one of the most important aspects for successful particle bombardment in generating transgenic plants was an efficient selection of transformants. In sorghum, for a long time, the selectable marker gene *bar*, an herbicide resistance gene was used in most sorghum transformation reports. The *hpt* (hygromycin phosphotransferase) gene was only used once by Hagio (1991). The use of the *pmi* (phosphomannose isomerase) gene was only recently reported by Gao *et al*. (2005). In optimizing the biolistics technique, there are a number of parameters that have to be considered in order to maximize the transformation efficiency output. These include the bombardment distance, the preculture period, the explant type, the DNA concentration and helium pressure. As already reviewed in Chapter 1, the *bar* gene was isolated from *Streptomyces hygroscopicus*. It encodes the enzyme PAT that confers resistance to the herbicides BASTA and bialaphos. This gene has been used to select for stable transgenic sorghum plants (Casas *et al*., 1993, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002).

The *E. coli pmi* gene enables plant cells to metabolize mannose (Miles and Guest, 1984). The mannose selection system is considered superior to the bialaphos selection system due to the selection process mode of action. The *pmi* selectable marker gene has been successfully used in plant transformation (Joerbo *et al*., 1998; Negrotto *et al*., 2000; Wright *et al*., 2001; Lucca *et al*., 2001; Wright *et al*., 2001; O'Kennedy *et al*., 2004; Gao *et al*., 2005) and there is strong

indication that this selection system results in higher transformation efficiency in comparison to the bialaphos selection system. The use of the biolistics method employing the Bio-Rad and the mannose selection system will yield a transformation efficiency that exceeds 2.5%.

To date, 9 of the eleven reports for stable sorghum transformation included a reporter gene and/or visual marker gene expression. This includes expression of the *gus* and *gfp* genes (Hagio *et al*., 1991; Casas *et al*., 1993 and 1997; Able *et al*., 2001; Emani *et al*., 2002; Godwin and Chikwamba, 1994; Zhao *et al*., 2000; Gao *et al*., 2005; Howe *et al*., 2006). Only two reports involve genes for agronomically important traits, viz. *chitinase*, which impacts pathogen resistance (Zhu *et al*., 1998), and *Cry1Ac*, which confers insect resistance (Girijashankar *et al*., 2005). Both reports formed a strong basis for improving sorghum cultivation for agricultural purposes. In addition, only one report involves the use of the positive selectable marker gene *ManA* (Gao *et al*., 2005) via *Agrobacterium*-mediated transformation and resulting in the highest reported average transformation efficiency of 2.91%, ranging up to 3.3, to date has been reported using this method. This chapter is divided into two parts. The objective for the first part was a comparison between the *bar* and *manA*-based selection systems to optimize the sorghum transformation using particle bombardment. The objective for the second part was to transform sorghum with a dual trait expressing DNA construct (RNAi vector) for the improvement of seed nutritional value through the elevation of the lysine content as well as enhancement of protein digestibility via down-regulation of a subset of kafirins and the lysine ketoglutarate reductase (LKR) genes. The RNAi hairpin construct used targets the co-suppression of the enzyme LKR and selected kafirins (δ-kaf-2, γ-kaf-1 and -2) to improve lysine content and seed protein digestibility.

3.3 Materials and Methods

3.3.1 Explant preparation and transformation

Sorghum public line P898012 was grown in the greenhouse in a soil mix of red soil, rough sand and compost (1:1:1) until flowering. Seeds were harvested 12-14 days after pollination and surface-sterilized in 70% (v/v) ethanol for 3 min and 15 min in 2.5% sodium hypochlorite solution containing 0.1% (v/v) of the surfactant Tween 20 before a thorough rinse with sterile distilled water. Immature zygotic embryos (IZE), 0.8-1.2 mm in length, were excised from these seeds and used to initiate type-I embryogenic callus cultures as described by O'Kennedy *et al*. (2004). For this, the IZEs were placed on CIM with the scutellum side facing upwards and the embryogenic axis in contact with the medium for 6-8 days prior to transformation. The CIM used prior to selection contains L3 based salts and vitamins, 20 mM L-proline, 2.5 mg/l of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), the carbon source maltose, and 4 g/l gelrite as the gelling agent (see Chapter 2 and annexure).

3.3.2 Plasmids

Two plasmids were used for transforming sorghum. Plasmid pAHC25 (Christenson and Quail, 1996) contains the bialaphos resistance gene *bar* (Figure 3.2 A). The plasmid pNOV3604-ubi, obtained from Syngenta, USA, carries the *man*A gene (O'Kennedy *et al*., 2004) which confers resistance to mannose selection (Figure 3.1 A). Both genes are driven by the maize ubiquitin promoter and the nopaline synthase terminator (Nos-ter). All plasmid DNA preparations were carried out using the Qiagen Maxiprep Kit (Southern Cross Biotechnologies, South Africa) according to the manufacturer's recommendation.

The plasmid pABS encoding the RNAi co-suppression cassette is shown in Figure 3.1 A, B and C. This was generously supplied by Drs Rudolf Jung and Kimberly Glassman from Pioneer Du Pont, Iowa, USA (members of the ABS consortium). The seed endosperm specific promoter from maize 19GZ was used to drive the co-suppression of the LKR, δ-kaf-2, γ-kaf-1 and -2 genes. The rice ADH1 intron was used as a hairpin part of this double stranded DNA transgene. The target genes were isolated from a cDNA library of a developing seed. cDNA clones from a developing seed were analyzed and sequenced to generate expressed sequence tags (EST). These ESTs were classified on the basis of sequence homology to known protein sequences. Domains for silencing targeted genes were selected from the EST sequences (Jung, 2007) and the 5'-3' sense strand sequences are displayed in Figure 3.1 C. Selected domains of the LKR and kafirin genes were cloned in tandem and designed into a hairpin construct in which the tandemly cloned domains and the inverted versions are separated by the ADH-1 intron to form a loop and cloned into a binary vector through Gateway cloning (Invitrogen, USA).

The γ-kaf-1 gene sequence translates into a 186 amino acid long protein that contains only one lysine residue and a total of 12 sulphur containing cysteine residues (see NCBI protein sequence database, Accession number AAS73290. The γ-kaf-2 protein is a 211 amino acid long preprotein that comprises one lysine and 13 cysteine residues (Accession number CAA44347). The δ-kaf-2 protein is 187 amino acid long containing 2 lysine and 14 cysteine residues. This δ-kaf-2 protein sequence has not been identified and reported into any database to date. These three target proteins are implicated as major contributors to the seed lysine content deficiency problem and

the low seed protein digestibility due to their high content of cysteine, thus, increasing the di-sulphide bond formation potential with other proteins.

To create plasmid pABS (Figure 3.1 B) minimal transgene cassettes (MTCs), the full plasmid was prepared by endonuclease digestion with E*co*RI to remove the backbone carrying the kanamycin resistant gene. For the *pmi* MTC, a double digest of A*sp* 718 and H*ind* III released the backbone carrying the ampicillin resistance gene. These kafirins were identified and chosen on the basis of their inherently poor lysine content and contribution to poor protein digestibility as a result of cross-linkages with other polymers in grain endosperm.

3.3.3 Transformation

3.3.3.1 *Transformation with bar and pmi*

The particle inflow gun (PIG) was used for bombarding IZEs that were pre-cultured for 6-8 days on CIM (0-1 cm diameter) and then placed in the middle of a 9 cm petri plate containing CIM supplemented with 0.2 M D-sorbitol and 0.2 M D-mannitol for 3-4 hrs as described by Vain *et al*. (1993). Bombardment mixtures were prepared by the precipitation of plasmid DNA on 1 µm tungsten particles with 2.5 mM $CaCl_2$ and 0.1 M spermidine-free base as described by O'Kennedy *et al*. (1998). Sixteen hrs post bombardment, all calli were transferred to CIM without D-sorbitol and D-mannitol. For plasmid DNA delivery the following parameters were used: helium pressure of 900 kPa, 0.16 µg/shot plasmid DNA, 500 µm nylon mesh screen placed 8 cm above the target tissue and a vacuum of approximately -87 kPa was applied with a timer duration of 50 millisec.

3.3.3.2 *Transformation with RNAi construct*

The Bio-Rad gun (Biolistic PDS-1000/He) was used for transformation of IZEs. Bombardment experiments were carried out with both the selectable marker and target constructs introduced into the same bombardment mixture. Each co-bombardment mixture contained the target and selectable marker genes (RNAi and the *pmi*) of 22 ng and 18 ng, respectively. A total of 222 and 105 IZEs were co-bombarded with MTCs and closed circular plasmid constructs, respectively. Bombardment mixtures were prepared by the precipitation of plasmid DNA on 0.7 µm gold particles with 2.5 mM $CaCl_2$ and 0.1 M spermidine-free base. All IZEs were placed on osmoticum medium (0.2 M D-sorbitol and 0.2 M D-mannitol) for 3-4 hrs before bombardment. Sixteen hrs post bombardment, all calli were transferred to CIM without osmoticum. For plasmid DNA delivery, a Helium pressure of 900 kPa and a total plasmid DNA of 0.09 µg/shot were used, i.e. 0.05 µg target genes + 0.04 µg selectable marker gene. Bombardments were carried out by following the manufacturer's (Bio-Rad) recommended method with a chosen distance of 7.5 cm between the macro-carrier and the target tissue. Proliferating IZEs subjected to bombardment treatment were placed back on CIM for seven days before the selection stage.

3.3.4 Selection and regeneration of transformants

3.3.4.1 *Bialaphos*

Bialaphos selection was initiated seven days after bombardment by placing the formed embryogenic calli on bialaphos (2 mg/l) containing CIM for four weeks. Thereafter, cultured embryos that produced somatic embryos were transferred to medium J callus maturation, cultured for two weeks (2 mg/l) and subsequently on medium J RRM (2 mg/l) with a two-week sub-culturing intervals until rooted plantlets formed.

3.3.4.2 *Mannose selection*

Similarly, mannose selection was initiated at seven days after bombardment by transferring calli to CIM containing 9 g/l mannose and 12 g/l maltose for four weeks. For callus maturation, they were subcultured onto medium J maturation for two weeks with the same mannose concentration but with double the maltose concentration (24 g/l), followed by a two-week period with the initial selection regime (9 g/l mannose and 12 g/l maltose) on RRM. For both selection systems the growth chamber temperature and light conditions for both callus induction and regeneration were 25°C and 1.8 µE/m^2s^1, respectively. However, regenerating shoots (≥ 1 cm long) were placed under light (18 µEm/$^2s^1$). Rooted plantlets (4-6 cm long) were hardened off in the greenhouse and analyzed by PCR. Due to the phenolic compounds produced by some sorghum cultures, sub-culturing was performed every seven days as required.

3.3.5 DNA extraction

Genomic DNA was extracted from putative transgenic sorghum leaf material using the mini extraction procedure of Dellaporta *et al.* (1983).

3.3.6 PCR analysis

The PCR reaction mix consisted of: 50 ng genomic DNA or 1ng plasmid (pABS) DNA, 1/10 of the final reaction volume of a 10x PCR buffer (Bioline, Inqaba Biotech, SA), 1.5 mM MgCl, 160 µM dNTPs, 400 nM forward primer, 400 nM reverse primer, 4 units *Taq*-DNA-polymerase (Bioline, Inqaba Biotech, SA), and H_2O to a final volume of 25µl.

For PCR analysis of putative bialaphos-resistant plants the primers: BAR forward: 5′-CATCGAGACAAGCACGGTCAACTTC-3′ and BAR reverse: 5′-CTCTTGAAGCCCTGTGCCTCCAG-3′ were used to amplify the *bar* gene, while the primers: PMI forward: 5′-CGTTGACTGAACTTTATGGTATGG-3′ and PMI reverse: 5′-CACTCTGCTGGCTAATGGTG-3′ were used to amplify the *manA* gene. The primer annealing temperatures for the *bar* and *pmi* genes were 64°C and 60°C, respectively.

For PCR analysis of the pABS transgene, a 503 bp fragment was amplified using the following primers: forward 5'-GTTACGTGACCCGGACCGAA-3' and reverse 5'-ACGCCGAAGATCGCCTGGTA-3'. This sequence region is the 3' end of the transgene, hereby called the confirmation region (c-r) region (PCR 2 region in Figure 3.1 B).

A thermo-block from PE Applied Biosystems (GeneAmp PCR System 9700) was used to perform PCR reactions using the following parameters: Denaturation of DNA at 94°C for 5 min, followed by 10 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 65°C for 45 sec and DNA extension at 72°C for 59 sec, followed by 25 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 55°C for 45 sec and DNA extension at 72°C for 59 sec and a final DNA extension at 72°C for 7 min, after which the samples were held at 4°C until analyses by gel electrophoresis.

3.3.7 Southern blot analysis

The Southern blot analysis of putative plants for the *bar* and *pmi* genes was carried out as follows: 5 µg of sorghum genomic DNA were digested with the restriction enzyme *Hind*III and

the digests were separated on 0.8% agarose gel and blotted as described by O'Kennedy *et al*. (2004). A PCR digoxigenin (DIG)-labelled probe (*bar* or *manA* gene cassette) was prepared using the PCR DIG probe synthesis kit using the PCR primers and conditions already stated in section 3.3.6 as described by the supplier (Roche Diagnostics, SA). Following separation by 0.8% agarose gel electrophoresis at 60V for 3-4 hrs, DNA was transferred and fixed onto positively charged nylon membranes from Roche (Sambrook *et al*., 1989), pre-hybridization for 3-4 hrs (5 X SSC, 50% formamide, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 2% Bio-Rad blocking reagent). Probe hybridization was performed for 16 hrs at 42°C. Following membrane washes at 65°C (0.5 X SSC, 0.1% SDS), membrane blocking was done for 45 min with blocking buffer (1% blocking reagent, 0.1M maleic acid, 0.15M NaCl pH 7.5). The unbound probe was thoroughly washed-off with wash buffer (0.1M maleic acid, 0.15M NaCl pH 7.5, 0.3% Tween-20). Probe-target DNA fragment binding was detected by using the Bio-Rad CDP-Star secondary antibody (Bio-Rad, South Africa). The membranes were exposed on X-ray films (Kodak, USA) and developed at room temperature for visualization.

For the RNAi construct southern blot analysis, two different probes were prepared. The z-k target sequence mentioned above and the c-r targeting the 3' end (see the PCR 2 region in Figure 3.1). The c-r probe was prepared using the primers stated in section 3.3.6. The DNA probe for the z-k region was PCR DIG-labelled using the following primers: forward 5′-ACAATGATGAGCCTCCTATG-3′; and reverse 5′-CCTTTATGTTGGCATCAAAA-3′. The PCR reaction mixture was the same as mentioned in section 3.3.6 but the cycle parameters used were as follows: Denaturation of DNA at 94°C for 5 min, followed by ten cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 60°C (pABS) for 45 sec and DNA extension

at 72°C for 59 sec, followed by 25 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 50°C for 45 sec and DNA extension at 72°C for 59 sec and a final extension at 72°C for 7 min, after which the samples were held at 4°C until analyses by gel electrophoresis.

After the autoradiography step, the z-k probe was stripped off from the membranes using a stripping buffer (0.1 X SSC, 0.1% SDS) at 90-95°C for 10 min. The membranes were re-probed with the c-r specific probe to investigate the extent of transgene integration, i.e. to determine if any DNA re-arrangements took place on the transgene in the process of integration. This is believed to be especially prevalent when using MTCs instead of full circular plasmid constructs (Fu *et al*., 2000; Kohli *et al*., 1998; Pawlowski and Somers, 1998). After stripping off the c-r probe, the membranes were re-probed for the *pmi* gene.

3.3.8 BASTA leaf painting assay

Putative bialaphos-resistant lines were confirmed to be transgenic and expressing the *bar* gene using a BASTA leaf painting assay. Seedlings were assayed when 4-5 leaves had fully emerged from the plant. A solution of 1% (w/v) BASTA®, 0.1% (v/v) Tween 20 was applied to the upper and lower surfaces of the first fully emerged leaf. The plants were scored three days after painting. Negative plants show yellowing and necrosis on the leaves, while the leaves from the resistant lines remain green and healthy. This indicates not only that the *bar* gene in plasmid pMON19477 was expressed in these plants, but also that the expression levels were high enough to confer resistance to the herbicide BASTA® at the plant level.

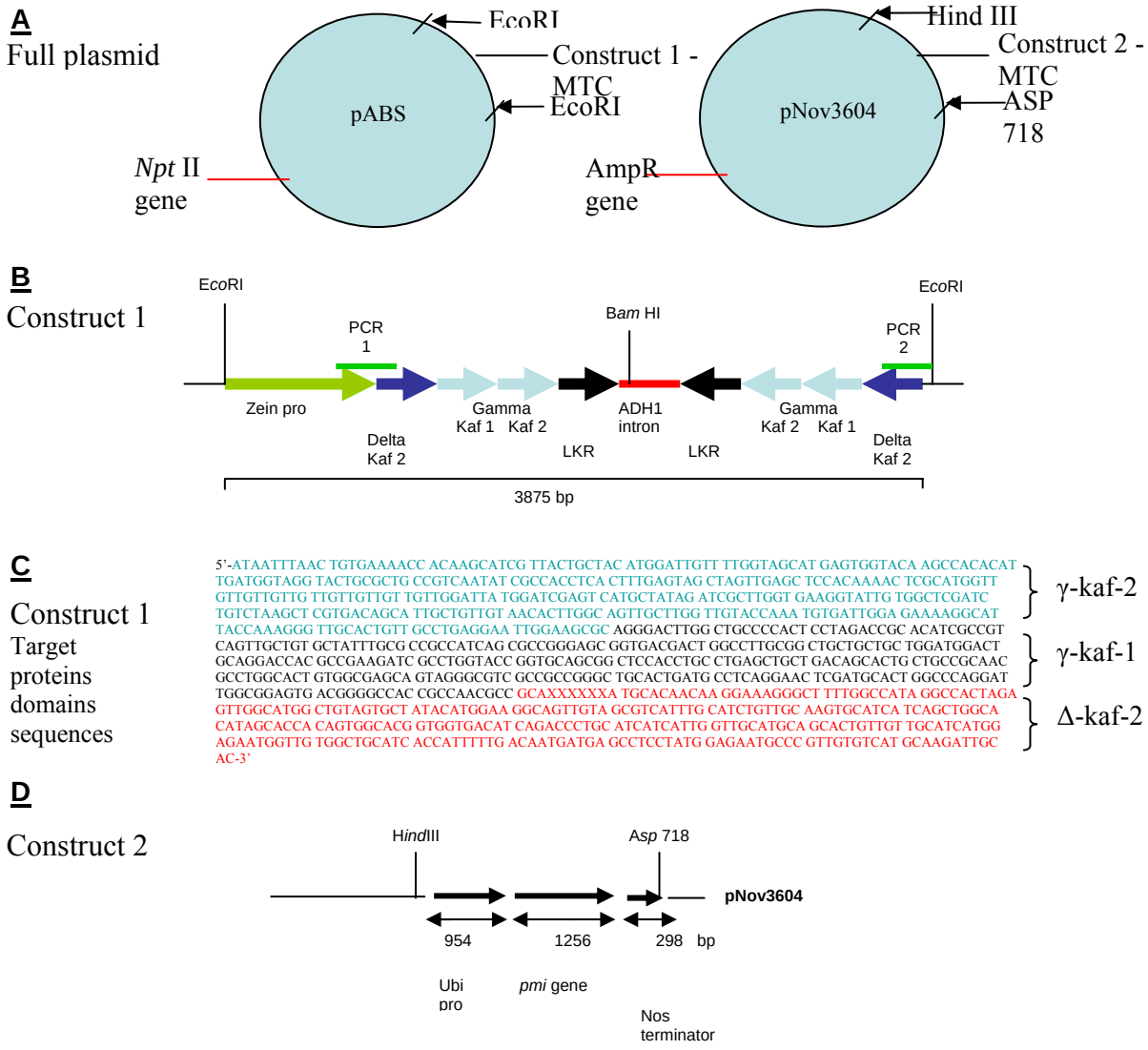

Figure 3.1 Maps of constructs used for transformation of sorghum P898012 IZEs. (A) Whole, circular, closed plasmid versions with backbone DNA sequences present. (B) The MTC construct for the co-suppression of 3 kafirins (1 δ- and 2 γ-kafirins) and LKR proteins, (C) The selected target gene domains sequences to generate siRNAs for the 3 kafirin genes, and (D) selectable marker gene, *pmi*.

3.4 Results

3.4.1 Transformation with *bar* and *pmi* genes

In this part of the study, the public line P898012 was used to generate transgenic plants using particle bombardment and two selection systems: bialaphos or mannose selection. Out of a total of 2609 IZEs (Table 3.1) that were bombarded for bialaphos selection, 12 plants survived the bialaphos selection but only three plants were PCR positive showing insertion of the *bar* gene (Figure 3.2 B). Results from the BASTA® leaf painting assay showed that the leaves from the three PCR positive plants were green and healthy after being exposed to BASTA®, while leaves from the other nine plants were burnt and yellowish (Figure 3.3). This result confirmed that the expression level of the *bar* gene was high enough to confer resistance to the herbicide BASTA®. This data display a transformation efficiency of 0.12% and 75% escapes. Also, the embryos cultured on bialaphos selection produced more phenolic compounds compared to embryos cultured on medium without selection or on mannose selection. This necessitated more frequent subculturing of every 7 days.

In comparison to the bialaphos selection system, the 27 putative transgenic events (Table 3.1) recovered with the mannose selection were all PCR positive (0.77% transformation efficiency). No false positive plants escaped the selection system. These plants displayed different gene integration patterns when analyzed by Southern blotting, while some showed identical integration patterns (Figure 3.4).

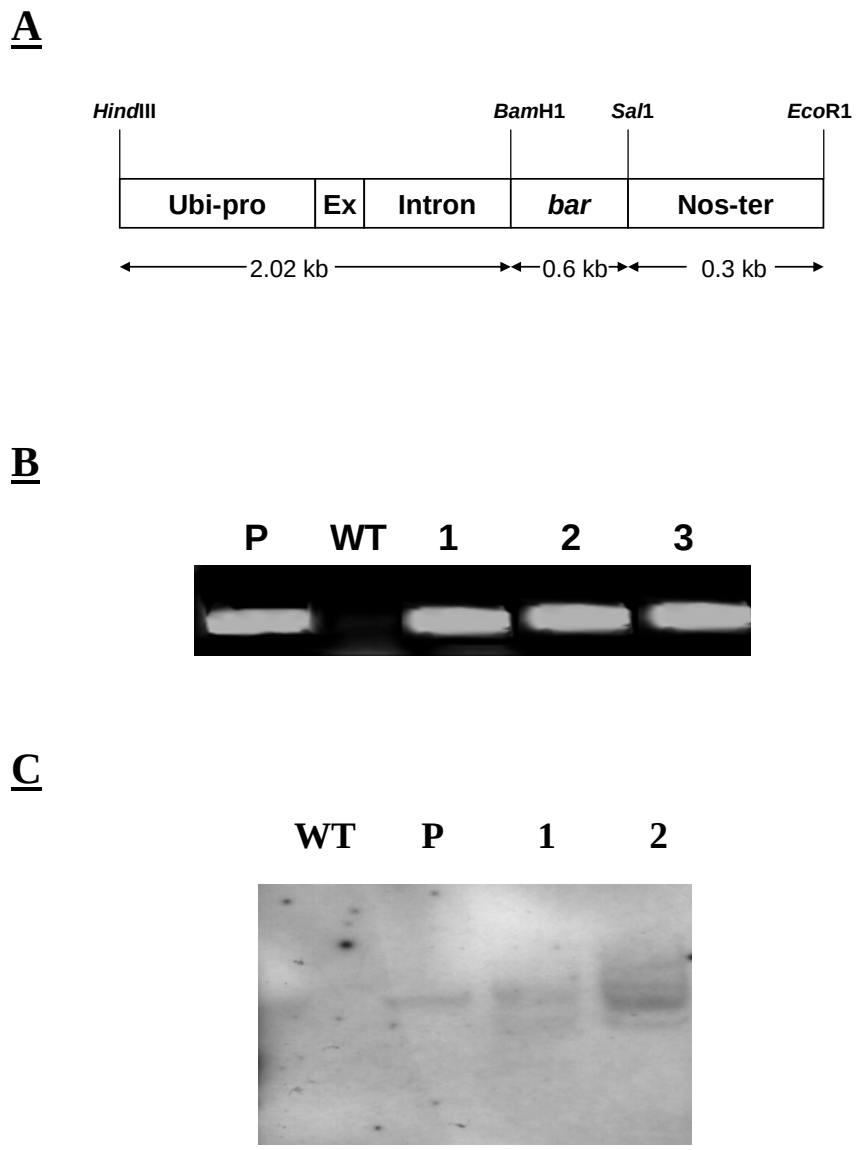

Figure 3.2 Molecular analysis of independent mannose resistant T_0 plants. (A) Schematic diagram of plasmid pAHC25 construct used for sorghum transformation. The pAHC25 (9706 bp) plasmid contains the *bar* gene, encoding BASTA® resistance under the control of the maize Ubi1 promoter (Ubi-pro), first exon (*Ex*), the first intron and the nopaline synthase terminator (Nos-ter). (B) PCR analysis of T_0 plants. Lanes (P) - positive control (pAHC25 plasmid DNA), WT represents wild-type sorghum plant DNA, and 1 to 3 – putative transgenic plants. (C) Southern blot analysis of T_0 plants. The blot was hybridized with DIG-labeled PCR *bar* DIG-labelled probe. Plasmid DNA representing two copies of the introduced transgene was mixed with *SacI* digested genomic DNA from WT plant (WT), *Sac*I digested transgenic T_0 plant DNA (5 µg/lane) from 2 of the 3 transgenic plants. Lane P represents positive control (pAHC25).

C T_0 T_0

Figure 3.3 T_0 transgenic plant leaves displaying resistance to a 2% BASTA® painting in comparison to a control plant leaf (C). Susceptibility or resistance was determined five days after painting with the herbicide BASTA®.

Table 3.1 Efficiency of biolistic transformation of sorghum using either bialaphos or mannose selection.

Parameters	**Bialaphos**	**Mannose**
Number of IZE	2609	3499
Number of resistant plants	12	61
Number of transgenic events	3	27
Number of escape plants	9	0
Transformation efficiency %	0.11	0.77

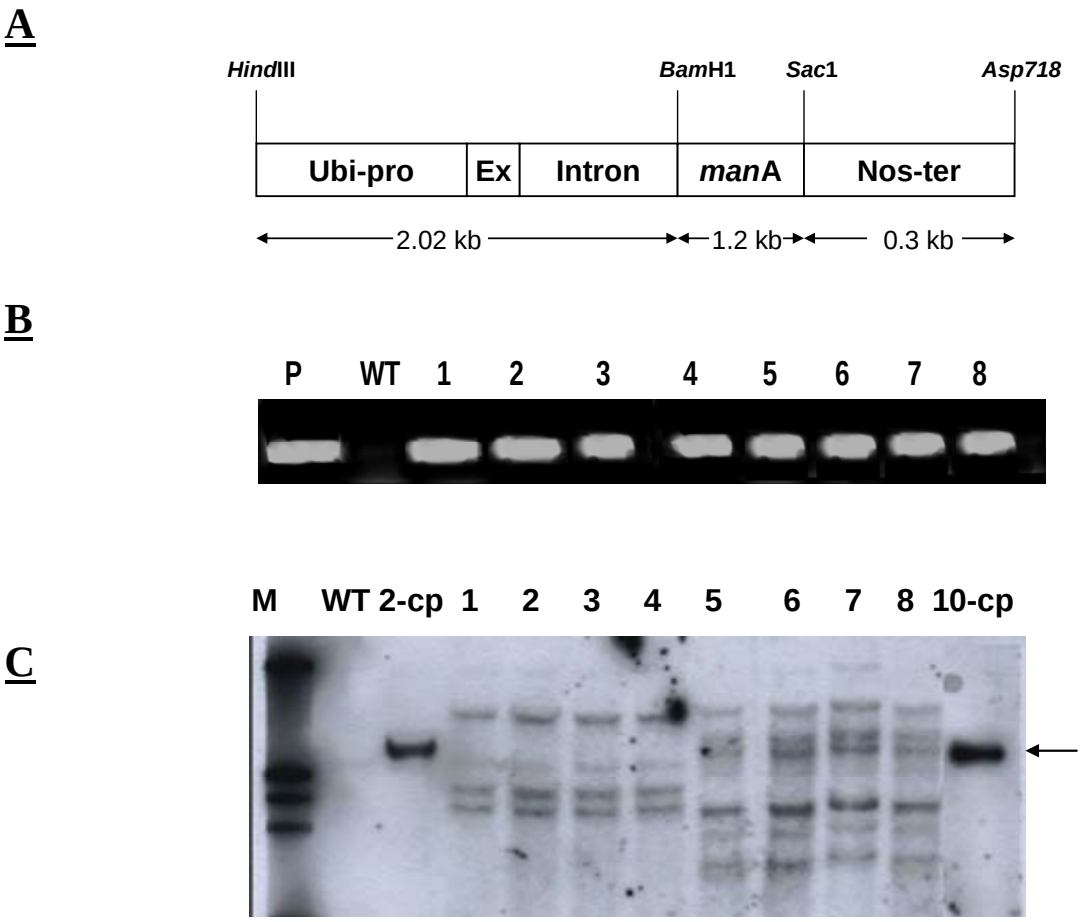

Figure 3.4 Molecular analysis of independent mannose resistant T_0 plants. (A) Schematic diagram of pNOV3604ubi construct used for sorghum transformation. The pNOV3604ubi (6210 bp) plasmid contains the *man*A selectable marker gene under the control of the maize Ubi1 promoter (Ubi-pro), first exon (*Ex*) and the first intron and the nopaline synthase terminator (Nos-ter). (B) PCR analysis of T_0 plants. (C) Southern blot analysis of eight T_0 plants, representing two transgenic events. The blot was hybridized with DIG-labelled PCR *man*A DIG-labelled probe. Plasmid DNA representing two (2-cp) and ten copies (10-cp; arrow) of the introduced transgene was mixed with *Hind*III digested genomic DNA from wild-type plant (WT), *Hind*III digested transgenic T_0 plant DNA (5 µg/lane) from eight *pmi* expressing plants (1-8). Lane P represents positive control (pNOV3604) and lane M represents DNA size marker. Approximately four to eight *ManA* gene copy integrations were observed.

Figures 3.2 and 3.4 (B and C) show PCR and Southern blot analysis results of a representative two *bar* and eight *pmi* expressing transgenic plants, respectively. Mannose selection resulted in a 6.4-fold improvement in transformation efficiency compared to bialaphos selection. Moreover, the mannose selection pressure gave a 100% inhibition on non-transformed callus cultures six weeks after selection. Further, the transgenic plants generated using the two systems did not show any visible phenotypic differences.

3.4.2 Transformation with RNAi construct

3.4.2.1 *Recovery of transgenic plants*

Two different transformation experiments were conducted. In the first experiment, MTCs, both target and selectable marker genes, were used in co-bombardment experiments. The second experiment involved closed, whole circular plasmid constructs (see Table 3.2 and Figure 3.1) in a mixture with the selectable marker gene. Fertile T_0 plants were produced through successful type I callus formation that resulted in rooted plantlets within a period of 150 days (Figure 3.5). The definition of transformation efficiency is based on the percentage of the number of independent events recovered per explant number bombarded. Using MTCs, 7 events resulting in 19 plants at a transformation efficiency of 3.2% were recovered while 4 transgenic events, resulting in 42 plants, were produced at 3.8% transformation efficiency for full plasmids (Table 3.2).

3.4.2.2 *PCR and Southern blot hybridization*

Based on plant and seed availability, seven transgenic events were chosen for further analysis. The PCR and Southern blot hybridization results of these events are shown in Figures 3.6 and

3.7, respectively. Until the Southern blot hybridization results were obtained that displayed different integration patterns for these plants, they were assumed identical (result from same callus cell) and were assigned with the same event number. Figure 3.6 shows PCR analyses results of 7 transgenic events, with some having at least 2 plants from the same event analyzed subject to leaf material availability. Transgene presence in the genome was detected by PCR in all chosen samples. For quality control purposes, the negative controls (lanes N and W) displayed no visible bands as expected. The positive controls (lanes P and PS) showed the expected reference DNA product of 503 bp as expected from the primer pairs used.

The Southern blot hybridization results confirmed transgene integration into the genome. The probes used were DIG-labelled by PCR and targeted three transgene regions. One of these targets for probe hybridization was the *pmi* internal fragment sized 965 bp (Figure 3.7, C and F). This was to confirm the selectable marker gene integration. The two target DNA sequences for pABS construct were named the z-k (Figure 3.7, A and D) and c-r (Figure 3.7, B and E). The former forms part of the 5'- end flanking the promoter and the first target kafirin, the δ-kafirin-2. The c-r forms part of the 3'- end of the gene cassette, i.e. the antisense oriented δ-kafirin-2. This approach was adopted to investigate any possible re-arrangements that could have taken place in order to understand the molecular process of gene integration and expression. Transgene re-arrangement at the promoter-end was observed in transgenic events 6 and 7 and these two transgenic events did not show any cross-reaction between genomic DNA fragments and the z-k DNA probe, i.e. no bands in Southern blot analysis. This demonstrated that transgene re-arrangement occurred at the 5'- end of the transcription cassettes.

Table 3.2 Transformation results displaying successful production of transgenic sorghum plants via particle bombardment. Transformation by using MTCs was supplemented by using closed, circular and longer version of the transcription cassettes. Eleven transgenic events were produced at an average 3.36 % transformation efficiency.

Experiment	**Number of explants**	**Number of transgenic events**	**Number of plants**	**Trans-formation efficiency**
Minimal transgene cassettes bombardment	222	7	19	3.2%
Closed, circular plasmids bombardment	105	4	42	3.8%

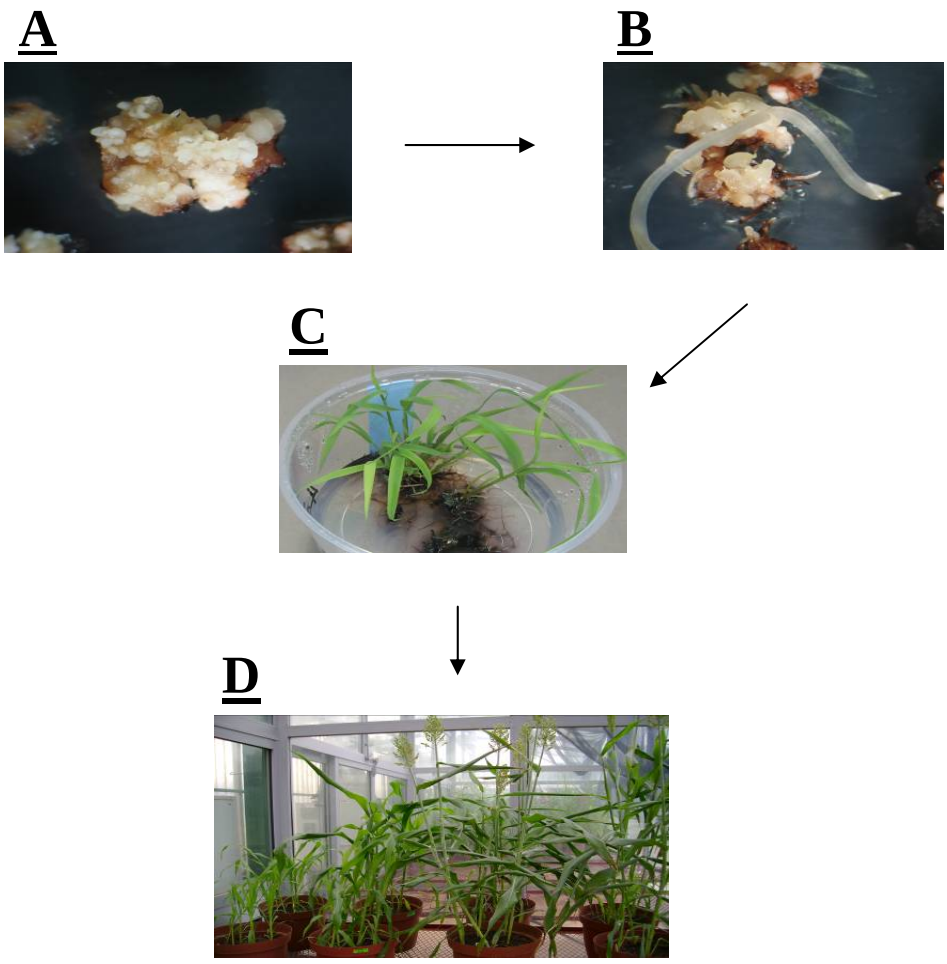

Figure 3.5 Tissue culture and regeneration of putative transgenic plants selected on mannose. (A) Selection of somatic embryos produced by type I callus 28 days after bombardment. (B) Shoot and root formation of mature transgenic callus on regeneration medium 54 days after bombardment. (C) Transgenic plantlets with defined roots and shoots 71 days after bombardment. (D) Fertile T_0 transgenic plants resembling a wild-type plant in the greenhouse 141 days after bombardment.

The effectiveness of the probe stripping process was confirmed by comparison of the binding profiles of the pABS probes (top and middle panels) with the pNov3604 probe (bottom panel, C and F). The profiles were clearly different for most transgenic events. This was confirmed by different integration of the selectable marker gene to the target genes. Therefore, successful probe stripping and re-probing were achieved.

Regarding the copy number determination, the results should be considered as qualitative because only estimated values could be determined. Because the c-r region is duplicated due to the sense and antisense nature of the RNAi construct, it is assumed that the c-r probe will bind both the sense and antisense target sequences, i.e. two times. An estimation of 3 to 12 copies of transgene integration was observed. The copy number estimation takes into account, the two times probe binding for a single transgene insertion. Accurate copy number determination can be performed via a real-time PCR technique.

Contrary to expectations when using MTCs (Fu *et al*., 2000; Kohli *et al*., 1998), events 2 and 4 displayed multiple integrations, while events 1 and 3 show slightly simpler banding patterns. The simplest integration pattern was displayed by one of the two closed, circular plasmid construct, i.e. event 5 (blue lanes). Event numbers 6 and 7 displayed no probe binding for the 5'- end z-k region (D) indicating possible re-arrangement of the transgene cassette's promoter region. However, the 3'- end confirmation region was present in the two events' samples (E). The *pmi*-specific probe also positively confirmed transgene presence.

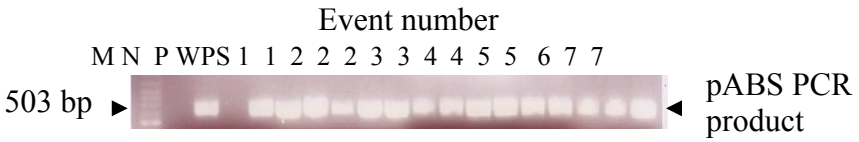

Figure 3.6 The PCR results for 14 of the 61 putative transgenic plants produced by co-bombardment of IZEs with pNov3604 and pABS MTCs. A 1.2% agarose gel electrophoresis was loaded and labelled as follows: (M) 100bp ladder molecular size marker; (N) negative control without template DNA added in a reaction 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.

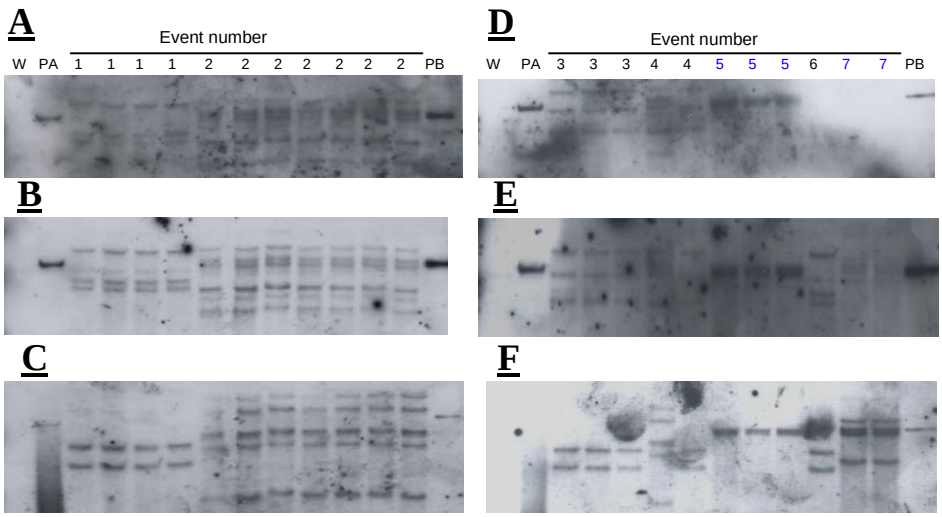

Figure 3.7 The Southern blot hybridization analysis of 22 of the 61 T_0 P898012 plants produced after co-bombardment with pNOV3604 and pABS constructs. (A) The first 11 putative transgenic samples (left panel i.e. A, B and C) were probed for the 5' promoter region, and (B) re-probed for a 3' transgene 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 a 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.

3.5 Discussion

The potential risk of the transfer of antibiotic and herbicide resistance genes from genetically modified crops into the environment or gut of microbes (Gao *et al.*, 2005) has necessitated the shift towards positive selection systems. This is especially important for sorghum, a crop with several wild relatives in Africa. Positive selection systems are callus tissue friendly as their mode of action is not poisonous but acts on starving non-transgenic tissue. In this study, a comparison was made between two selection systems for generating transgenic sorghum via particle bombardment. Although the *bar* gene has been so far the commonly used selection marker for sorghum transformation via biolistics, using this gene has resulted in variable transformation efficiencies ranging from 0.08% (Casas *et al*., 1993) to 1% (Able *et al.,* 2001). The result in this study of 0.12% transformation efficiency from the experiment is therefore within the range already described in the literature. However, the *bar* gene creates a leaky selection which results in a high number of escapes (Gao *et al*., 2005). This was also confirmed in this study because 75% of putative transgenic plants were false positives. Moreover, there is a general concern that the *bar* gene can be transmitted via pollen to wild relatives of sorghum, thus, producing herbicide resistant weeds such as Johnsongrass and shattercane. Consequently, using the *bar* gene in commercial sorghum might not pass the regulatory hurdle in South Africa.

The study further explored the use of a positive selection system based on the *ManA* gene. A 0.77% transformation efficiency was achieved, which is a 6.4-fold increase when compared with the bialaphos selection system. In addition, no escapees were found when the mannose selection was applied. The importance of reducing escapees in the transformation systems cannot be

overemphasized. Reduction in number of escapees has a concomitant effect on saving time and resources necessary for the analysis of transformed plants (O'Kennedy *et al*., 2004).

However, the transformation efficiencies achieved in this study are still below the transformation efficiencies already reported for *pmi* in sorghum. Using *Agrobacterium*-mediated gene transfer, Gao and *et al*. (2005) reported an average transformation efficiency of 2.91, with a range of up to 2.28 and 3.3% using genotypes Pioneer 8505 and C401, respectively; this reported transformation efficiency is superior when compared to 0.77% found in this study. However, using the Bio-Rad gun, an improvement of this value to 3.36 was found. This was produced via transformation with an RNAi construct plasmid targeting the introduction of two traits, namely, enhanced seed lysine content through suppression of LKR and seed protein digestibility improvements through down-regulation of δ-kafirin-2, γ-kafirins-1 and -2.

Transgene re-arrangement for transgenic events 6 and 7 was observed. These two transgenic events were produced with MTC and closed circular plasmid bombardment, respectively. However, the extent of this re-arrangement was not established in this study and further sequence probe analysis would be required to elucidate the effects this might have on transgene expression. Transgenic events 6 and 7 were carefully observed in the subsequent transgene expression analyses. This is because the seed-specific promoter used here is expected to direct successful transcription of the double-stranded RNA molecule. Truncation in these transgenic lines is likely to lead to unsuccessful transcription due to the loss of the RNA polymerase II identification site and subsequent binding to kick-start transcription.

MTCs were used in one experiment because they reportedly result in higher transformation efficiencies and simpler transgene integration patterns (Fu *et al*., 2000; Kohli *et al*., 1998). However, closed circular plasmid constructs were also used. Another reason for using full plasmids is the possibility of DNA cleavage or damage at the MTCs DNA ends. Although particle bombardment is extensively used to introduce genes into crop plants, the mechanism of transgene integration is still not well understood. However, a few reports have shed some light on the mechanism of transgene integration (Pawlowski and Somers, 1996; Riggs and Bates, 1986; Bates *et al*., 1990; Finer and McMullen, 1990; Kartzke *et al*., 1990; Kohli *et al*., 1998; Pawlowski and Somers, 1998).

In general, direct DNA delivery frequently results in multiple, intact, and rearranged transgene copies at one genomic locus. For example, in oats, Pawlowski and Somers (1998) reported on the interspersion of transgenic DNA with host genomic DNA at a number of loci and attributed this transgene clustering in one locus to a number of DNA replication forks at that particular locus. The consequence of this is, likely, the lack of successful segregation between the individual transgenes by recombination. This multiple transgene copy integration is therefore the main reason that particle bombardment is considered inferior to *Agrobacterium* method.

During the biolistic transformation procedure, two stages are believed to provide the opportunity for transgene re-arrangement. The first stage is just before the integration into the nucleic genomic DNA and the second stage is the mechanical shearing or damage during particle preparation and bombardment processes. In the former stage, the host cell's nuclease activities are believed to be responsible for these re-arrangements. Due to DNase degradation of

transgenes from the DNA ends, it is expected that whole plasmids would be better protected from mechanical shearing and bombardment process. The endonucleases would also have to act a little longer on whole plasmids than on MTCs with free ends due to longer plasmid DNA sequences, thus, lessening the damage on the transgenes in full plasmids.

For the purposes of this study, the number of integrated transgene copies is not important to pursue. This is due to the endogenous gene silencing approach employed here, instead of a transgene expression approach. The multiple transgene copy integration in the latter approach would likely result in minimal expression due to unintended multiple transgene silencing, while no negative expression effects would be observed in the former approach. In general, information available on sorghum transformation, *Agrobacterium*-based or biolistic, is very limited and efforts to rectify the situation are commendable. This study generated transgenic sorghum plants using *bar* and *pmi* genes and demonstrated that the latter produces better transformation efficiencies and has a more appealing biosafety profile. To the best of my knowledge this is a first report of the use of *pmi* in combination with biolistic transformation of a public sorghum line P898012.

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Chapter 4

Characterization of transgenic plants for suppression of target kafirins and LKR

4.1 Abstract

Eleven transgenic events displaying enhanced total lysine generated through down-regulation of selected kafirins and LKR were recovered. Down-regulation was achieved through application of RNAi co-suppression strategy. This involves the use of a double-stranded RNA molecule to trigger gene specific silencing targeting the lysine catabolism gene and a specific subset of storage proteins. The targeted kafirins include δ-kaf-2, γ-kafirins-1 and -2. The Western analysis confirmed an almost complete suppression of the target γ-kafirins-1 and -2 proteins, while no δ-kaf-2 protein expression was observed in transgenic T_1 seeds. SDS-PAGE analysis also showed a γ-kafirin-1 knock-out migrating at approximately 25kDa. In transgenic events 2, 3 and 5, the δ-kaf-2 antibody used showed significant cross-reaction with a non-targeted 21kDa protein suspected to be α-A-1 type. This was confirmed by Western analysis for the α-kaf A1 type protein expression that was partially suppressed. However, no significant α-kaf A1 suppression was observed in transgenic events 1 and 4. RNAi for the LKR and the selected kafirin genes resulted in the highest seed lysine content improvement by up to 45.2% in transgenic event 3. Transgenic event 3 also displayed 77.6% endosperm lysine content increase while whole seed proline content was reduced by 18.7%, a reflection of reduction in expression of proline-rich γ and δ-kafirins. The promoter-less transgenic event, as a result of transgene re-arrangement, showed neither target protein suppression nor lysine improvement. The transgenic endosperm structure also showed a shift to a soft, floury phenotype in comparison to wild-type seed. The six transgenic events that were analyzed had normal seed morphology and germination.

4.2 Introduction

In plants, RNAi is usually achieved by transformation with transgene constructs that are designed to promote the transcription of a double-stranded RNA (dsRNA), a substrate for RNase III-type enzymes named Dicer-like enzymes. The activity of these enzymes results in short interfering RNA (siRNA) molecules that serve as specific markers for targeted cleavage of homologous mRNAs (Baulcombe 2004; Xie *et al*., 2004; Tomari and Zamore, 2005; Bordersen and Voinnet, 2006). Three known forms of RNAi include antisense suppression, sense co-suppression and homologous inverted repeats (hIR) silencing. The latter method is very efficient in achieving targeted gene silencing and it involves multi-cloning steps that include an intron separating the hIRs (Waterhouse *et al*., 1998; Smith *et al*., 2000; Wesley *et al*., 2001; Helliwell and Waterhouse, 2005; Filichkin *et al*., 2007).

The hIR strategy for the transgene open reading frames where the inverted repeats are separated by an intron was used in this study. Transgene expression leads to intron splicing which results in a dsRNA molecule which triggers co-suppression (Lee and Carthew, 2003). The RNAi approach has been successfully used to achieve decreased expression of target genes in transgenic plants (Vaucheret *et al*., 2001; Zamore, 2004).

In spite of the gene silencing phenomenon being conserved across the plant and animal kingdoms, the mechanisms are different between some species (Baulcombe, 2004). A possible drawback for using RNAi strategies includes the occurrence of transitive RNAi. This is a secondary, unintended silencing of mRNAs that are found in the surrounding regions of the primary target dsRNA inducer sequence (Sijen *et al*., 2001; Vaistij *et al*., 2002). In maize, a close relative of sorghum, the transgenic approach targeting the endosperm-specific

suppression of ZLKR/SDH was confined only to the endosperm, i.e. no transitive RNAi was detected (Houmard *et al*., 2007).

The objective of this part of the study was to use the RNAi technology to suppress the expression of the LKR and three lysine poor kafirins genes, namely, the δ- kafirin-2, γ-kafirins-1 and -2, and to investigate whether this directed co-suppression will result in a change in the lysine content. The suppression of these poor lysine containing proteins will result in the elevation of seed lysine content by at least 30%. Furthermore, the seed physical properties, that include seed mass and germination potential, have been investigated.

4.3 Materials and methods

4.3.1 Seed physical structure

4.3.1.1 *Determination of seed morphology and seed weight*

Whole T_1 seeds were screened for any possible abnormalities as a result of the tissue culture stress by comparison to wild-type seeds that had been subjected to the same tissue culture and bombardment stress. Ten randomly chosen seeds per event, from the same panicle, were weighed and compared to wild-type P898012 seeds that have been regenerated on the same medium J lacking mannose selection.

4.3.1.2 *Determination of seed endosperm structure*

Cross-sections of the seeds from putative transgenic sorghum and wild-type lines were carried out with a sharp blade to observe for any possible visual morphological changes in the transgenic seed endosperm. Endosperm phenotype classification includes ‘corneous’, ‘intermediate’ and ‘floury’ phenotypes (Figure 4.1). Although wild-type P898012 seeds are

classified as intermediate, for clarification purposes the seeds were classified as ‘corneous’ or ‘floury’ in this study because variants of these phenotypes were found in transgenic seeds.

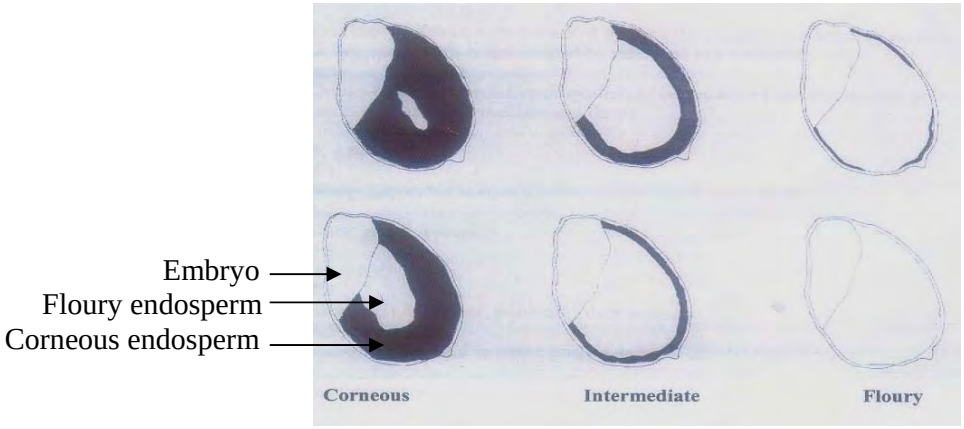


Figure 4.1 The criteria used in the classification of seed endosperm phenotype (picture, courtesy of Prof. J Taylor, University of Pretoria). The corneous seed endosperm is characterized by a dark brown or black colour caused by both the tight protein body packaging and diffusion of tannins from the aleurone layer. A floury seed is almost free of any black layer while an intermediate seed displays some degree of the black layer.

4.3.2 Seed protein expression analyses

4.3.2.1 *Protein extraction from whole seeds*

For protein extraction, mature dry T_1 seeds were ground to a fine meal using a mortar and pestle. To ensure that almost identical amounts of total protein were analyzed, 10 mg of each meal was weighed and placed into 1.5 ml microfuge tubes. To extract total protein, 400 µl of a reducing protein extraction buffer (100 mM DTT; 2% SDS; 60 mM Tris, pH 6.8), was added to each tube. The mixture was vortexed briefly and the tubes were incubated at 100°C for 8 min with intermittent vortexing. The mixture was centrifuged at 14000 rpm for 10 min

at room temperature, and the supernatant was collected and transferred into a clean microfuge tube.

4.3.2.2 *Determination of protein concentration*

To ensure that uniform amounts of protein were loaded for comparable analyses of protein expression, total protein was determined using the Bradford assay with bovine serum albumin (BSA) used as a protein standard (Bio-Rad; Bradford, 1976). The BSA concentration range chosen for the standard curve was 0, 1, 3, 5, 10, and 20 ng/µl protein.

4.3.2.3 *SDS-PAGE analysis*

Ten microgram of sample protein was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to visually confirm co-suppression of the targeted kafirins. This was performed by use of a pre-cast 12% Bis-Tris Bio-Rad Criterion gel system (Laemmli, 1970; Weber and Osborn, 1969). Protein extracts were pre-mixed at a 4:1 dilution with 4X sample loading buffer containing dithiothreitol/DTT (200 mM Tris-HCl pH 6.8; 400 mM DTT; 8% w/v SDS; 0.5% w/v bromophenol blue; 50% glycerol). The samples were incubated at 100°C for 1 min before loading onto pre-cast SDS-PAGE gels. The voltage was set at 120V for 90 min and after proper separation of the protein size marker (PageRuler™ Prestained Protein Ladder, Fermentas), protein detection followed. Protein detection was done with Coomassie brilliant blue staining (0.2% Coomassie stain, 45:45:10 methanol: water: acetic acid) for 1 hr to visualize the protein followed by destaining in 45:45:10 = methanol: water: acetic acid. For negative controls, wild-type P898012 and transgenic event 6 were used. Transgenic event 6 lacks the 5’ promoter end through transgene re-arrangement or deletion and, therefore, should display wild-type expression of the target genes because no transgene transcription is expected.

4.3.2.4 *Western blot analysis*

Four SDS-PAGE pre-phenotyped seed protein extracts were chosen for further analysis. These included one null segregant (non-transgenic) from each transgenic event after segregation was determined by SDS-PAGE and visual examination of the endosperms. Following separation of proteins by SDS-PAGE (see section above), the semi-dry Hoefer™ TE 77 transfer unit (Amersham BioSciences, UK) was used to transfer separated protein profiles onto 0.45 µm pore size polyvinylidene di-fluoride membrane (PVDF) (Sigma-Aldrich, USA). The PVDF membranes were pre-equilibrated in 99.9% methanol for 5 min and re-suspended in Towbin's transfer buffer (25 mM Trizma-Base pH 8.3; 192 mM glycine; 20% v/v methanol) for 5 min. The acrylamide gel was also pre-equilibrated in Towbin's transfer buffer for 15 min. The gel-membrane sandwich and current settings were performed according to the manufacturer's recommendations (Bio-Rad, UK). Membrane blocking was performed for 1 hr in blocking buffer (20 mM Tris pH 7.5; 150 mM NaCl; 0.05% Tween 20; 3% fat-free milk powder).

The 27kDa γ-zein-1, the 50kDa γ-zein-2, and the 18kDa δ-kafirin2 primary antibodies raised in rabbit used were generously donated by Dr Rudolf Jung (Pioneer Du Pont, Iowa, USA) and used for hybridization at a dilution of 1:10000 for 1 hr at room temperature on a shaker set at a slow speed (40-50 rpm). Unbound antibodies were washed-off by washing the membrane 3-times for 10 min each time, with TBST buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) via shaking at 180-250 rpm. The membranes were incubated for 1 hr at room temperature with a 1:10000 diluted secondary antibody (anti-rabbit conjugated horse-radish peroxidise; Sigma Aldrich, USA). The membranes were then washed 3-times for 10 min with TBST buffer. All the steps were performed at room temperature. For protein

detection, the ECL Plus™ Western blotting system (Amersham, UK) was used according to the manufacturer's instructions to detect target antibody cross-reactions with targeted kafirins. This was followed by autoradiography using the 8"x10" BioMax MR Film (Kodak, USA).

Non-targeted α-kaf A1 and B1 types were also investigated for possible suppression. The Western blot analysis technique was also used to display T_1 to T_2 stable inheritance of the transgenic trait. One transgenic seed extract from each generation was used for transgenic event 3. Null segregants served as negative controls.

4.3.2.5 *Transgenic trait – endosperm phenotype linkage and T_1 Mendelian segregation*

The SDS-PAGE, Western blot analysis and seed endosperm phenotype results were used to determine the correlation between the transgenic trait (RNAi construct expression) and the endosperm phenotype. The sectioned seeds were independently scored for endosperm phenotype, i.e. 'corneous' and 'floury'. The same seeds (10 seeds per event from the same panicle) were independently genotyped for the presence or absence of the transgenic trait by using the SDS-PAGE method (see above). The Western blot analysis on the same protein extracts was performed to confirm the seed phenotypes. A statistical correlation between the seed flouriness and transgenic trait was performed. A statistical chi-square test, at a 5% level, was also used to determine whether there was any deviation from the expected T_1 3:1 Mendelian segregation ratio transgenic : non-transgenic seeds.

4.3.2.6 *Reverse-transcription PCR (RT-PCR)*

Total seed RNA was isolated at 15 days post anthesis using the Sigma-Aldrich (USA) total RNA isolation kit following the supplier's instructions. The first strand cDNA synthesis kit (Roche, South Africa) was used for reverse transcription of total mRNAs from 1µg total RNA. The PCR reactions were performed as explained in Chapter 3 with the reaction conditions as follows: denaturation at 94°C for 2 min, followed by denaturation at 94°C for 45 sec, annealing at 64.6°C for 30 sec (both γ-kafirin-2 and LKR) and extension at 72°C for 45 sec repeated for 25 cycles and a final extension at 72°C for 3 min, after this the samples were held at 4°C until analysis by gel electrophoresis. The γ-kafirin-2 and the LKR transcripts expression were directed by using the following specific primers: γ-kafirin-2 Forward 5'-TGGTAGCATGAGTGGTACAAGCCA-3' and γ-kafirin-2 Reverse 5'-CAGTGCAACCCTTTGGTAATGCCT-3'; and LKR Forward 5'-ACCGCATTCTGACAGGTCTTCTGA-3' and LKR Reverse 5'-GGGCAATGGAGTTGTTGGGATTCT-3', respectively.

Detection of genomic DNA in cDNAs

For quality control purposes, genomic DNA contamination or interference was investigated by RT-PCR of transgenic events 2 and 3 samples targeting the transgenic zein promoter amplification. Complementary DNAs (cDNAs) were used as template DNA in a PCR reaction using the following zein promoter specific primers: zein pro Forward 5'-CACATCTCAGTCCTTGTGCTTGTGC-3' and zein pro Reverse 5'-CACAACTCCTTACTTCCTCCGCTTC-3'. The following amplification reaction conditions were used: denaturation at 94°C for 2 min, followed by denaturation at 94°C for 45 sec, annealing at 66.2°C for 30 sec and extension at 72°C for 45 sec repeated for 25 cycles and a

final extension at 72°C for 3 min, after which the samples were held at 4°C until analysis by gel electrophoresis.

As a second quality control standard, the 18S ribosomal protein that is involved in the protein synthesis machinery, was selected as an internal standard, i.e. a non-targeted protein. This internal standard served to show that the RNAi transgene expression did not interfere with other seed protein expression and that the cDNA template amount used in a PCR reaction was constant from seed to seed. The abundance nature of this protein required a slight modification of the PCR parameters. Using the same template cDNA as the experimental samples, the crossing point value (Cp) determined by real-time PCR was 9 cycles. Therefore, to ensure that the PCR reactions were still in the exponential phase of product amplification, 13 cycle repeats for the PCR reaction were used. The following 18S primer pairs were used: 18S forward primer 5'-GCCATCGCTCTGGATACATT-3', and 18S reverse 5'-TCATTACTCCGATCCCGAAG-3'. The following reaction conditions were used: denaturation at 94°C for 2 min, followed by denaturation at 94°C for 45 sec, annealing at 63°C for 30 sec and extension at 72°C for 45 sec repeated for 13 cycles and a final extension at 72°C for 3 min, after which the samples were held at 4°C until analysis by gel electrophoresis.

4.3.3 Seed amino acid content analysis

For analysis, meal pools of eight randomly selected seeds per event from the same panicle were sent to the University of Pretoria's Biochemistry Department for amino acid analysis that excludes the cysteine content determination. The HPLC method used was based on the PICO-TAG method (Bidlingmeyer *et al*., 1984). More accurately, transgenic events 3 and 6 were analyzed for lysine and proline content after seed phenotyping by Western blot analysis

of 32 transgenic seeds. To carry out the analysis, cross-sections of seeds in half were performed. One half of each seed was used for phenotyping, i.e. to determine target kafirins and LKR suppression in transgenic seeds by Western blot analysis. The transgenic half-seeds for each transgenic event were pooled and grounded into meal samples and submitted for amino acid analysis. Transgenic endosperm data were generated by pooled meals that excluded the embryos. The total seed lysine, proline and protein content were determined. Excluded was the cysteine amino acid content determination.

4.3.4 Determination of seed germination potential

A small-scale germination potential test was performed. The brown paper germination method was used (Torres *et al*., 2003). Fifteen randomly selected seeds were gently scratched on the sides away from the embryo using a sharp blade to determine the endosperm flouriness for genotyping purposes. The germination paper was moistened with sterile distilled water and the germination paper was wrapped around. This was placed in a vertical position into a 2 L beaker and incubated in a germination chamber at 25°C in the dark. After three days, the number of germinating seeds was counted for root and coleoptile development.

4.4 **Results**

4.4.1 Target proteins expression analysis

4.4.1.1 *Mature seed analysis*

Figure 4.2 shows the BSA standard curve that was used to quantitate total protein. The r^2 value of 0.9991 indicates significant linear correlation of the BSA concentration and the absorbance reading at 595nm, i.e. very close to the value 1. This standard curve therefore represents a reliable measure for the determination of total protein concentration by extrapolating the absorbance readings (y-axis) with the protein concentration (x-axis).

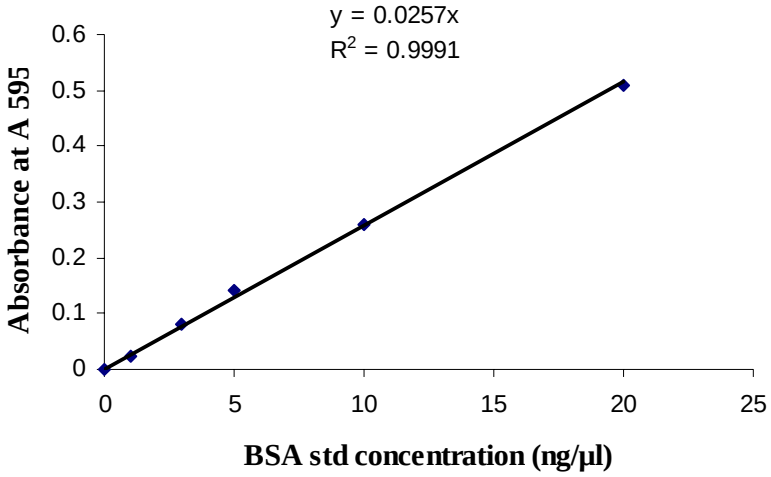
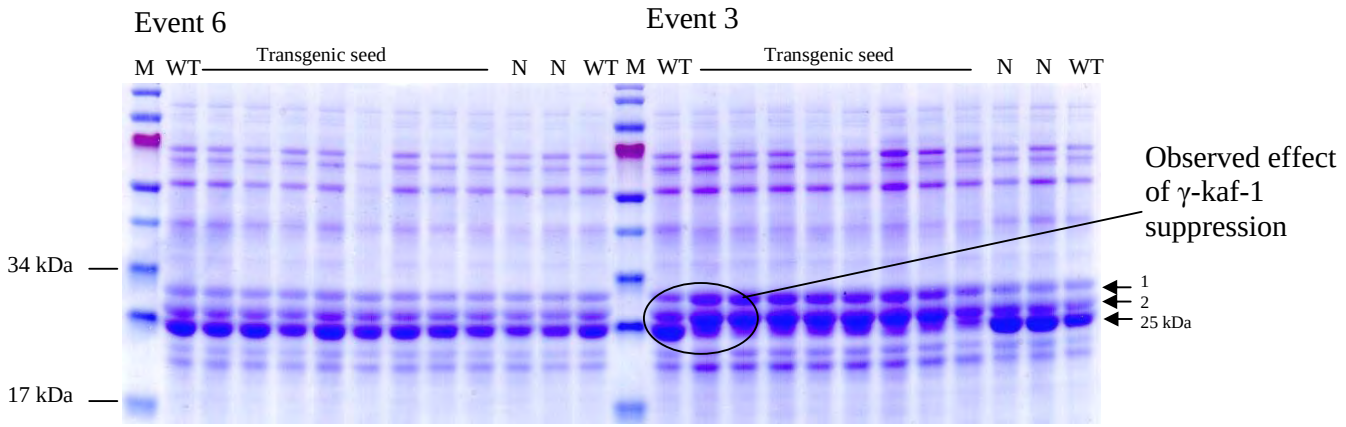


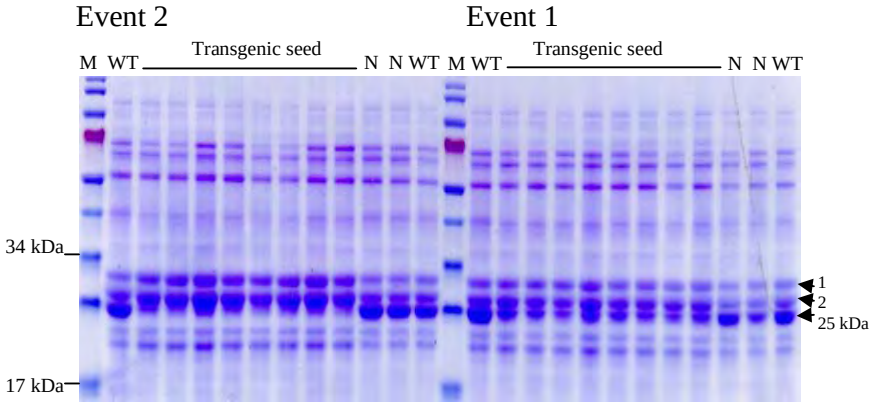
Figure 4.2 Bovine serum albumin standard curve where absorbance was measured using a WPA Lightwave spectrophotometer (Labotec Ltd, South Africa).

Figure 4.3 represents results obtained by SDS-PAGE analysis of 10 individual seeds from a single panicle per event. These samples represent a segregating T_1 population that should follow a 3:1 (transgenic : wild-type) ratio for each transgene insertion, provided that each is heterologously integrated into one locus for each genome. The pre-cast 12% Bis-Tris mini-gel system (Figure 4.3) provided adequate separation of the target γ-kaf-1 protein from other kafirins. This allowed separating transgenic seeds from non-transgenic seed. In comparison to wild-type P898012 and null segregant seed proteins, the most abundant co-suppressed target protein band, γ-kaf-1, was resolved at a size of 25 kDa (Figure 4.3). This was slightly smaller than the reported maize protein of 27 kDa. Two further proteins were visually up-regulated and resolved at 30 and 32 kDa (Figure 4.3). Transgenic events 2, 3 and 5 displayed a more pronounced γ-kaf-1 protein band suppression than transgenic events 1 and 4. This variation was further confirmed by lower up-regulation of the two non-target proteins (30 and 32 kDa).

No co-suppression of the target γ-kaf 2 (50 kDa) and δ kaf-2 (18 kDa) was detected by SDS-PAGE.

The chi-square test of the 60 seeds that were analyzed revealed a statistically insignificant difference between the 3:1 ratio and the 49:11 transgenic : null segregant ratio. A chi-square of 1.422 (P = 0.2330) was calculated which confirmed that the observed ratio did not statistically deviate from the Mendelian segregation of 3:1.


Event 6
Event 3
M WT Transgenic seed N N WT
M WT Transgenic seed N N WT
34 kDa
17 kDa
Observed effect of γ-kaf-1 suppression
1
2
25 kDa


Event 2
Event 1
M WT Transgenic seed N N WT
M WT Transgenic seed N N WT
34 kDa
17 kDa
1
2
25 kDa

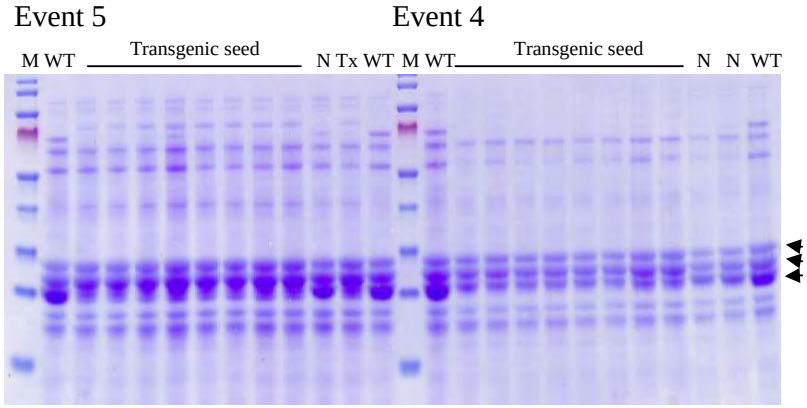

Event 5
Event 4
M WT Transgenic seed N Tx WT
M WT Transgenic seed N N WT

Figure 4.3 SDS-PAGE analysis of transgenic seed total protein (Bio-Rad pre-cast 12% Bis-Tris gel). Single seed analysis was performed on 10 T_1 putative transgenic seeds per event, from six transgenic events. Sample labelling was as follows: WT corresponds to wild-type P898012 protein extract for negative control; N represents null segregant seed; M represents prestained protein marker. The black arrows indicate one visible target protein co-suppression at size 25kDa, the γ-kafirin-1. The target γ-kafirin-1 protein co-migrates with other proteins and a reduction in band intensity was observed in all five transgenic events, while the promoter-less event, event 6, displays a normal protein expression, i.e. similar protein profile with wild type. Other non-targeted proteins are up-regulated. The circle shows a suppression of γ-kaf-1 (25 kDa) that resulted in an up-regulation of two non-targeted proteins (arrows 1 and 2). A variation in target protein band suppression was observed, with transgenic events 1 and 4 showing partial suppression.

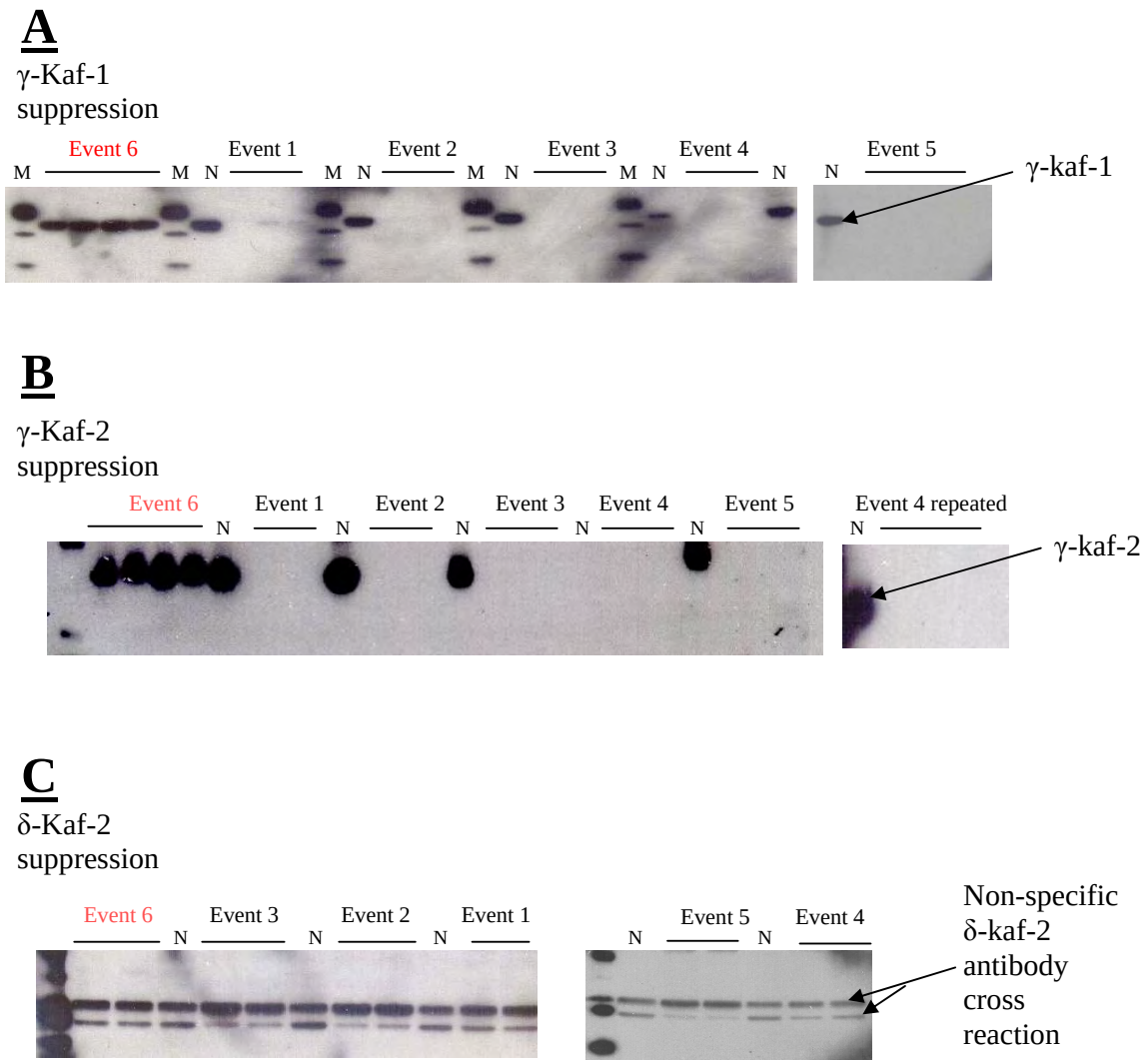


Figure 4.4 Western blot analysis of pABS transformed P898012 sorghum T_1 seeds to investigate target γ-kaf-1, γ-kaf-2 and delta kaf-2 protein suppression. Single seed analysis of T_1 segregation seeds for six transgenic events was performed with rabbit-raised antibodies specific for the following: (A) 27 kDa γ-zein-1, (B) 50 kDa γ-zein-2 and (C) 18 kDa δ-kaf-2. Sample labelling was as follows: N = null segregant seed protein extract as negative control. The δ-kaf-2 antibody cross-reacted with two non-targeted proteins that were resolved at 21 and 27 kDa.

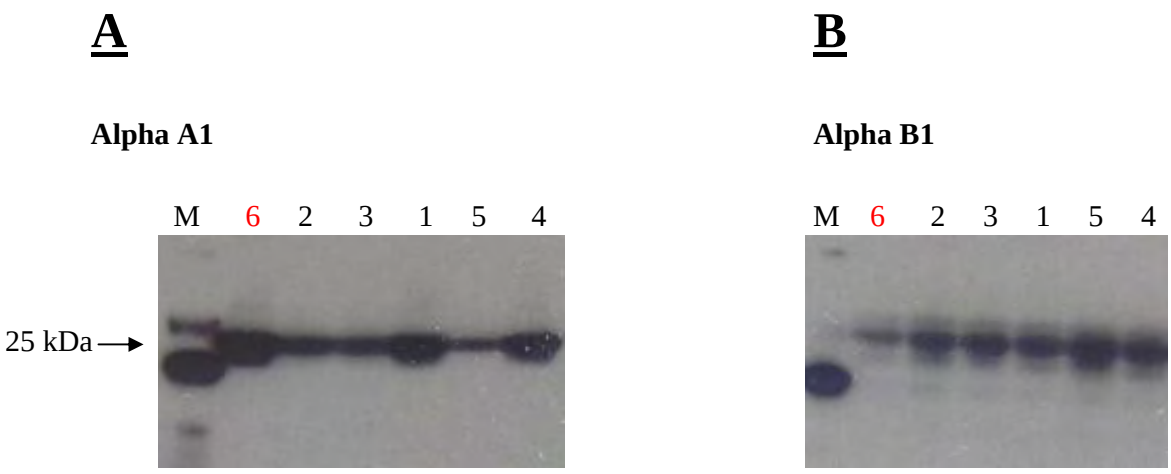


Figure 4.5 Western blot analysis of transgenic events 1-6 for α-kafirins A1 and B1 types. The non-targeted α-kaf A1 partial suppression was confirmed in events 2, 3 and 5, while transgenic events 1 and 4 displayed no significant α-kaf A1 suppression (A). However, the α-kaf B1 type proteins were up-regulated (B) in comparison to event 6. M = protein marker.

The target γ-kaf-1, -2 and δ-kaf-2 protein suppression was investigated by Western blot analysis (Figure 4.4). A 25 kDa band corresponding to γ-kaf-1 was detected in the negative control with antibody raised against γ-zein-1 (Figure 4.4). Transgenic events 2-5 showed almost complete suppression of γ-kaf-1. Transgenic event 1 showed a low γ-kaf-1 expression (Figure 4.4 A) and the promoter-less transgenic event 6 did not show, as expected, any suppression. When a γ-kaf-2 antibody was used an almost complete suppression of γ-kaf-2 was observed with the size of 50 kDa (Figure 4.4 B) in all five transgenic events. However, no suppression of γ-kaf-2 protein was observed in event 6.

The δ-kaf-2 antibody did not cross-react with the 18 kDa δ-kaf-2 protein for both the wild-type and the transgenic seeds, suggesting that δ-kaf-2 was not expressed (Figure 4.4 C). Instead, the antibody was able to bind two, unspecifically, proteins resolved at 21 kDa and 27 kDa. There was consistent reduction of the 21 kDa protein in transgenic seeds when compared to wild-type seeds (Figure 4.4 C). The extent of this protein reduction varied with different transgenic events. There was simultaneous up-regulation of the 27 kDa protein in transgenic seeds. This resulted in an apparent inverse proportion relationship between the 21 kDa and 27 kDa proteins, i.e. the lesser the 21 kDa protein expression, the more the 27 kDa protein expression. Transgenic event 3 showed the most reduced 21 kDa protein, with transgenic event 6 (lacking the promoter) showing no reduction of this protein.

Since the 21 kDa protein cross-reacted with the δ-kaf-2 antibody, it was investigated if this 21 kDa protein was an α-kaf A1 type protein. Therefore, non-targeted α-kaf A1 and B1 types were investigated for possible suppression. When antisera against the α-kaf A1 and B1 types protein were used for detection, the Western blot analysis result, using the α-kaf A1 and B1 types specific antibodies, is shown in Figure 4.5. Transgenic events 2, 3 and 5 showed partial suppression of the α-kaf A1 type protein, while the α-kaf B1 type protein was up-regulated in all transgenic events except for event 6 (Figure 4.5 A and B). Transgenic events 1 and 4 showed insignificant suppression of the A1 type, while an up-regulated B1 type protein was observed, suggesting that the α-kaf A1 protein was expressed at the level almost similar to wild-type. This was in direct correspondence with the SDS-PAGE results on these two events where the target 25 kDa size protein was partially suppressed. In contrast, the LKR Western blot analysis could not be performed due to the unavailability of an antibody

The investigation on the effect of the target kafirins co-suppression on the endosperm phenotype revealed a shift from a ‘corneous’ (wild-type) to a ‘floury’ (transgenic) endosperm (Figures 4.6). Figure 4.7 shows the correlation analysis result between successful transgene suppression of target kafirins and the floury endosperm phenotype. There was a perfect correlation between the genotype (transgenic seed) and the phenotype (seed endosperm flouriness).

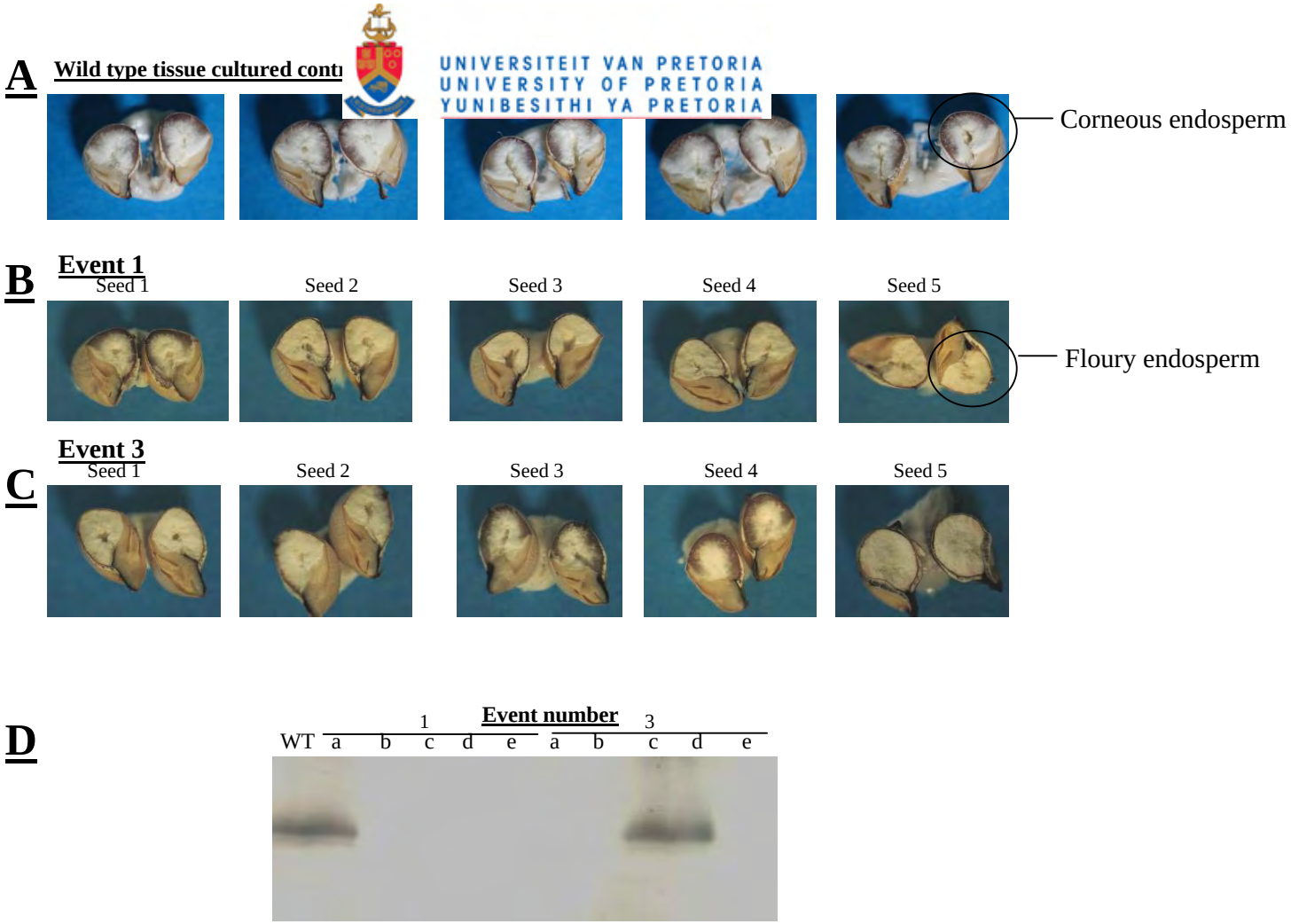


Figure 4.6 Investigation of possible linkage between kafirins suppression and seed endosperm phenotype. (A) Five randomly chosen seed endosperms from wild-type seeds. (B) Five randomly chosen endosperms from transgenic event 1. (C) Five randomly chosen endosperms from transgenic event 3. (D). The Western blot analysis results on the same segregating T_1 seeds for events 1 and 3 compared to a wild-type seed. Sample labelling was as follows: WT represents wild-type P898012 protein extract; a, b, c, d and e represent seed numbers 1, 2, 3, 4 and 5. Each transgenic seed, confirmed by Western blot analysis (D) showed a floury endosperm phenotype and each null segregant (transgenic event 1, seed 1 and transgenic event 3, seeds 3 and 4) had a wild-type corneous phenotype.

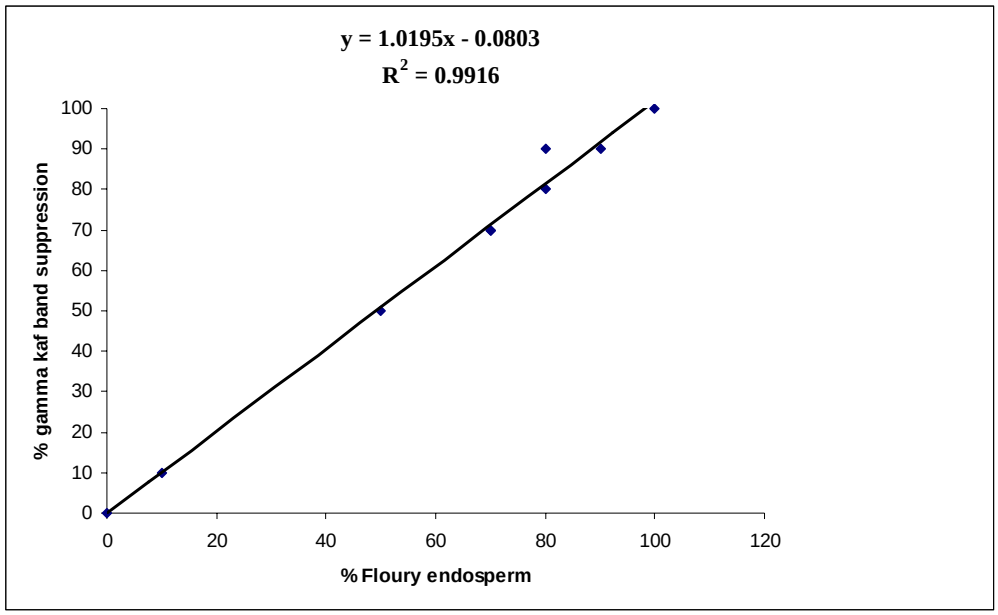


Figure 4.7 Correlation chart for suppressed kafirin expression with the floury endosperm phenotype for transgenic event 1. Eighty transgenic seeds were cross-sectioned and analyzed by SDS-PAGE and Western blot analysis. A co-efficient of 0.9916 was determined thus suggesting a significant relation between target kafirins suppression and floury endosperm phenotype.

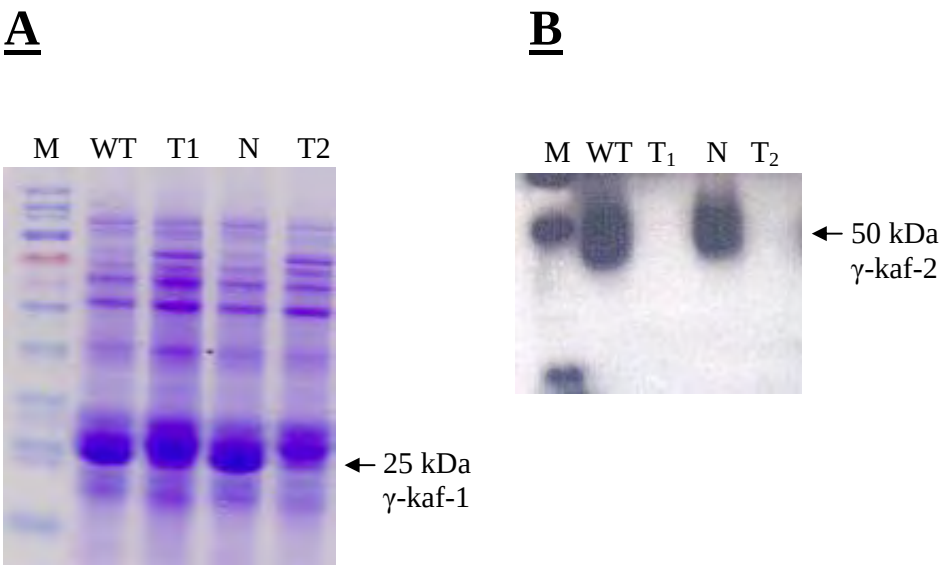


Figure 4.8 Stable T_1 to T_2 transgenic trait inheritance of transgenic event 3 seeds. (A) SDS-PAGE of proteins isolated from T_1 and T_2 seeds of transgenic event 3. (B) Western blot analysis of γ-kaf-2 expression from proteins isolated from transgenic event 3 seeds. M represents the protein size marker, WT corresponds to the wild-type seed protein, T_1 represents a transgenic seed protein extract from A T_1 generation, T2 represents a transgenic seed extract from a T_2 generation and N corresponds to null segregant seed extract. The arrow at size 25 kDa shows a γ-kaf-1 protein suppression. The arrow at 50 kDa protein size shows a γ-kaf-2 protein suppression.

The SDS-PAGE and Western analysis were also used to investigate the T_1 to T_2 stable inheritance of the transgenic trait. Stable trait inheritance from T_1 to T_2 seed generation was achieved for both the γ-kafirins 1 and 2 proteins (Figure 4.8 A and B) in transgenic event 3.

The seed amino acid content analysis by HPLC (Figure 4.9 A) demonstrated an elevation of lysine content in all six transgenic events with the smallest increase shown by transgenic

events 6 (1.6% increase), thus, contributing to improvements of the overall seed nutritional quality. Transgenic events 5 and 2 resulted in a 5.5% and 5.6% lysine increase, respectively. Transgenic event 3 had the highest increase in free lysine (30.3%) followed by transgenic event 1 (14% increase). Because only eight seeds were submitted for analysis due to limited seed availability, this result provided only a rough measure to identify the best transgenic event for free lysine improvement. The increase in lysine content in these transgenic events was conservatively determined because meals of eight randomly selected seeds per event were pooled and then analyzed. Since T_1 seeds display a 3:1 segregation ratio of transgenics : non-transgenics, a quarter of the meal still represents non-transgenic seeds pooled with transgenic seeds.

The quantitative results for transgenic events 3 and 6 (non-expressing control) for pooled (eight seeds per event) transgenic endosperm lysine content, whole seeds lysine content and endosperm proline content are shown in Table 4.1 and Figure 4.9 B, C and D. For quantitative analysis, pooled meals were prepared from 30 seed halves. This represented almost double the sample amounts used in the previous analysis outlined above. This bigger pool ensured a replicated and accurate amino acid analysis. The null-segregant pools of endosperms and whole seed data (from the same panicle) were used as controls because they represent the closest, if not the same, physiological status as the transgenic seeds. For transgenic event 3, lysine increases by 45.2% and 77.6% for the whole seed and endosperm, respectively, were found. Seed endosperm proline was reduced by 18.7% due to suppression of the proline-rich γ-kafirins. However, average total protein content was not negatively affected by the transgenic trait, as seed total protein content of transgenic seeds was 12.6% which was within the normal range of 8-13% (Figure 4.10 and Table 4.1).

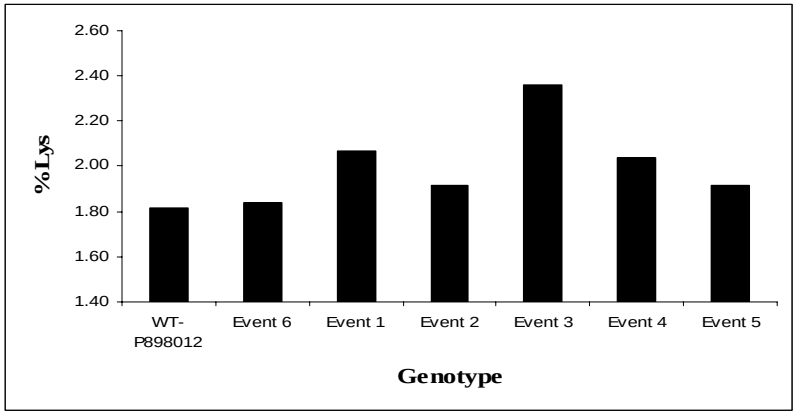
Table 4.1 Free amino acid analysis (g/100g) measured (two replicates A and B) via HPLC for total amino acid content determination of transgenic events 3 and 6.

	Event 3 transgenic endosperm		Event 3 endosperm null-segregant		Event 3 transgenic whole seed		Event 3 whole seed null-segregant		Event 6 endosperm (transgenic non-expressor)		Event 6 whole seed (transgenic non-expressor)	
	Replication A	Replication B	Replication A	Replication B	Replication A	Replication B	Replication A	Replication B	Replication A	Replication B	Replication A	Replication B
ASPARTIC ACID	0.69	0.75	0.6	0.64	0.78	0.79	0.65	0.68	0.59	0.56	0.6	0.67
GLUTAMIC ACID	2.57	2.79	2.72	3.18	2.7	2.77	2.85	2.9	2.72	2.57	2.65	2.85
SERINE	0.48	0.51	0.52	0.55	0.55	0.56	0.59	0.6	0.51	0.51	0.54	0.57
GLYCINE	0.27	0.29	0.29	0.3	0.39	0.41	0.38	0.4	0.26	0.26	0.33	0.36
HISTIDINE	0.22	0.23	0.25	0.26	0.25	0.27	0.32	0.32	0.25	0.24	0.3	0.31
ARGININE	0.37	0.38	0.33	0.36	0.59	0.64	0.51	0.52	0.28	0.27	0.44	0.49
THREONINE	0.32	0.35	0.34	0.36	0.4	0.4	0.38	0.39	0.33	0.31	0.35	0.36
ALANINE	1.11	1.2	1.18	1.25	1.2	1.23	1.21	1.26	1.22	1.15	1.16	1.22
PROLINE⚙	0.88	0.94	1.12	1.17	0.9	0.9	1.11	1.18	1.09	1.04	1.03	1.1
TYROSINE	0.5	0.5	0.5	0.53	0.51	0.51	0.52	0.57	0.45	0.42	0.47	0.5
VALINE	0.6	0.66	0.6	0.63	0.69	0.69	0.67	0.67	0.62	0.58	0.62	0.64
METHIONINE	0.21	0.23	0.22	0.21	0.22	0.23	0.24	0.24	0.16	0.17	0.2	0.2
CYSTEINE	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
ISOLEUCINE	0.49	0.51	0.48	0.5	0.49	0.49	0.52	0.51	0.48	0.43	0.47	0.5
LEUCINE	1.8	1.82	1.83	1.88	1.79	1.82	1.97	2.03	1.98	1.78	1.86	1.94
PHENYLALANINE	0.63	0.67	0.65	0.69	0.71	0.68	0.72	0.71	0.72	0.66	0.69	0.73
LYSINE⚙	0.26	0.25	0.15	0.15	0.35	0.38	0.26	0.26	0.13	0.13	0.25	0.25
TOTAL	11.4	12.08	11.78	12.66	12.52	12.77	12.9	13.24	11.79	11.08	11.96	12.69
Average Total Seed Protein	11.74		12.22		12.65		13.07		11.44		12.33	

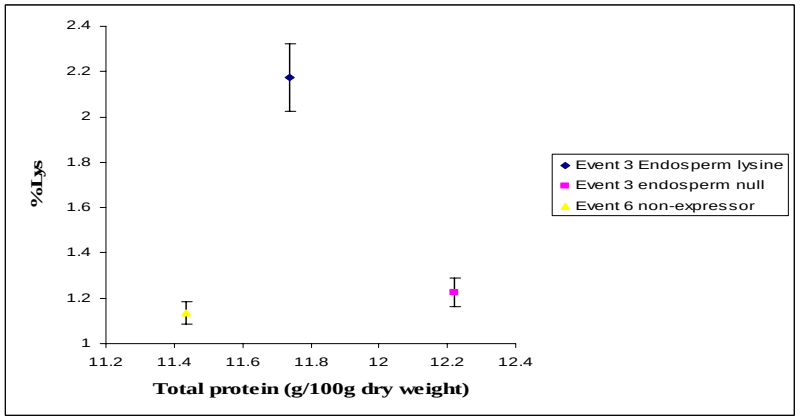
⚙**The amino acid contents of interest**

***ND = not determine**

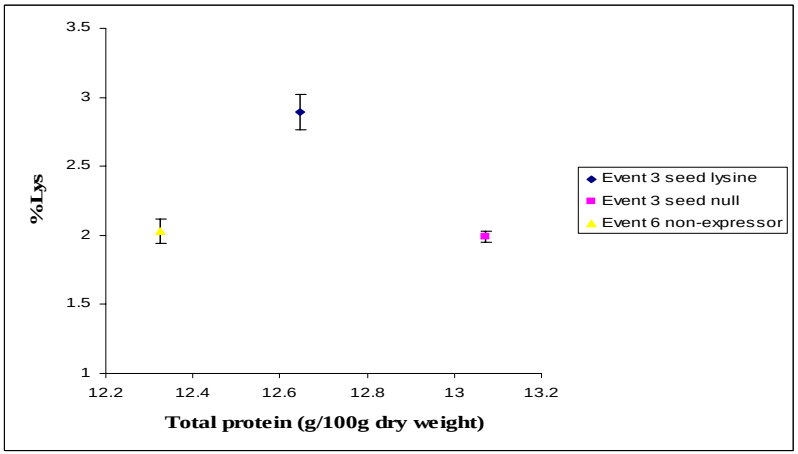
A


%Lys
2.60
2.40
2.20
2.00
1.80
1.60
1.40
WT-P898012
Event 6
Event 1
Event 2
Event 3
Event 4
Event 5
Genotype

B


%Lys
2.4
2.2
2
1.8
1.6
1.4
1.2
1
11.2
11.4
11.6
11.8
12
12.2
12.4
Total protein (g/100g dry weight)
Event 3 Endosperm lysine
Event 3 endosperm null
Event 6 non-expressor

C


%Lys
3.5
3
2.5
2
1.5
1
12.2
12.4
12.6
12.8
13
13.2
Total protein (g/100g dry weight)
Event 3 seed lysine
Event 3 seed null
Event 6 non-expressor

D

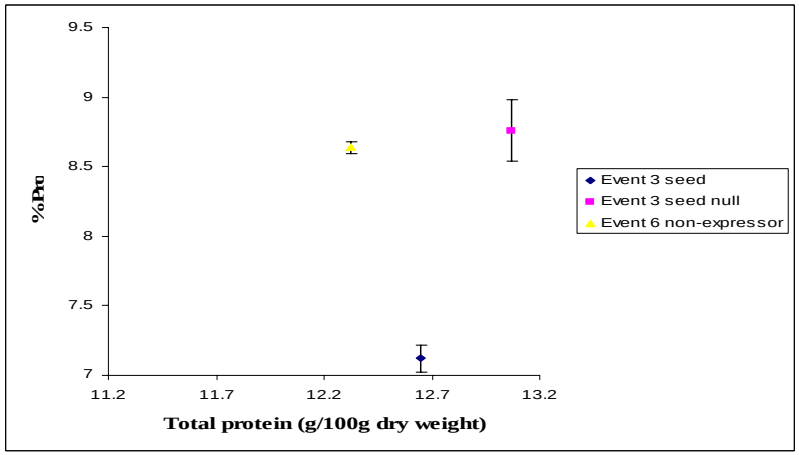

%Pro
9.5
9
8.5
8
7.5
7
11.2
11.7
12.2
12.7
13.2
Total protein (g/100g dry weight)
Event 3 seed
Event 3 seed null
Event 6 non-expressor

Figure 4.9 HPLC results for whole seed (A, C and D) and endosperm (B) lysine and proline contents. (A) Eight randomly selected seed meals per panicle per event from seven transgenic events were analyzed, i.e. segregating T_1 seeds. (B) Transgenic endosperm pooled meals for event 3, representing the highest lysine increase of 30.3% and event 6, showing no significant lysine improvement. (C) Whole seed lysine content of Transgenic event 3. (D) Whole seed proline content of transgenic event 3. Seed endosperm and whole seed lysine contents were increased by 77.6 and 45.2%, respectively, while an 18.7% decrease in seed proline was observed. Neither significant improvement of lysine content nor decrease in proline content in event 6 was observed. Each scatter plot represents means and standard deviation values obtained from two replicates.

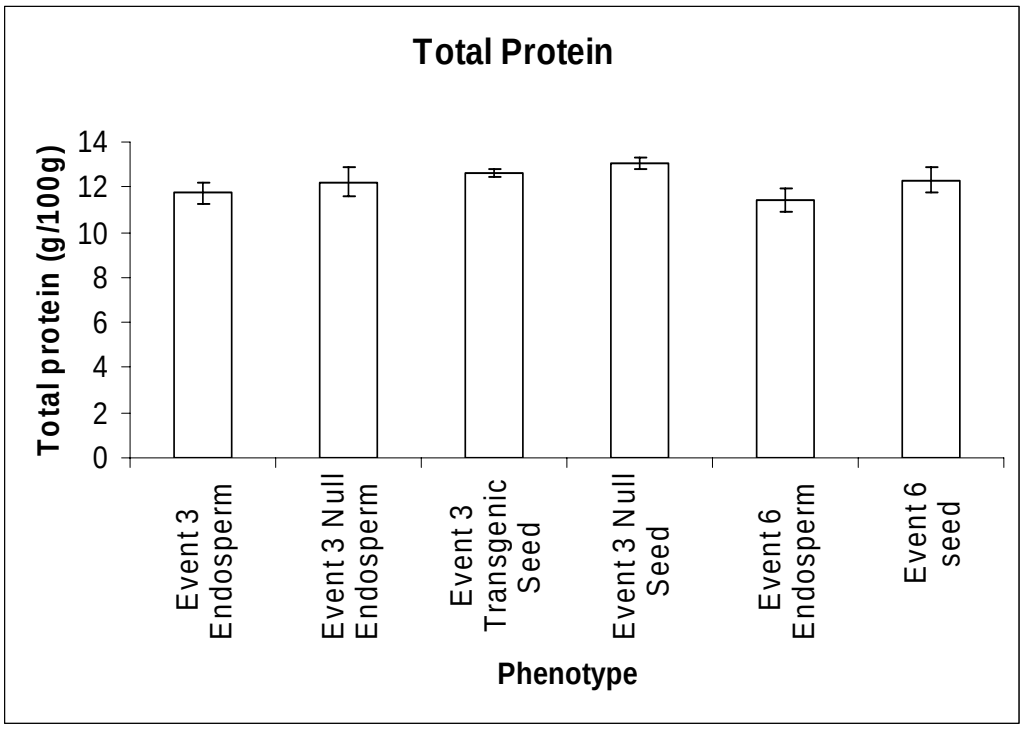


Figure 4.10 Whole seed and endosperm **t**otal protein contents of transgenic event 3 for transgenic T_1 seeds excluding cysteine. No significant difference of total protein was observed between transgenic seeds and endosperms of transgenic event 3 and control transgenic event 6.

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4.4.1.2 *Developing seed analysis*

Transgenic lines 1-6 were chosen for target transcripts expression investigations at a developing seed stage of 15 days post anthesis. For the analysis, four seeds per transgenic event were selected randomly from one panicle. Figure 4.11 shows the results obtained by RT-PCR of randomly selected developing T_1 seeds for transgenic events 1-6. After total RNA isolation and quantitation (Figure 4.11 A), cDNA was synthesized (Figure 4.11 A, right). The target amplicons showed significant co-suppression of the γ-kafirin 2 transcript in comparison to the wild-type target transcript expression (Figure 4.11 C). Since there was no

LKR antiserum available, the LKR expression was analyzed by RT-PCR. However, LKR transcript co-suppression was very low during this seed developmental stage. Neither contamination of genomic DNA nor 18S gene suppression was observed (Figure 4.11 B and C).

4.4.2 Seed morphology and germination

The average seed mass for the five transgenic events was lower when compared to wild-type seeds (Figure 4.12). Transgenic events 1 and 4 had the lowest decrease of 10%, while transgenic events 2, 4 and 5 had lower seed weights when compared to the wild-type, i.e. seed weight decreases of a 20, 40 and 26%, respectively. The promoter-less transgenic event 6 had an almost identical mass as the wild-type. The seed morphologies for all the transgenic events also resembled the wild type seeds (Figure 4.13). The germination test revealed 100% seed germination potential, similar to wild type P898012 for all five transgenic events (Figure 4.14).

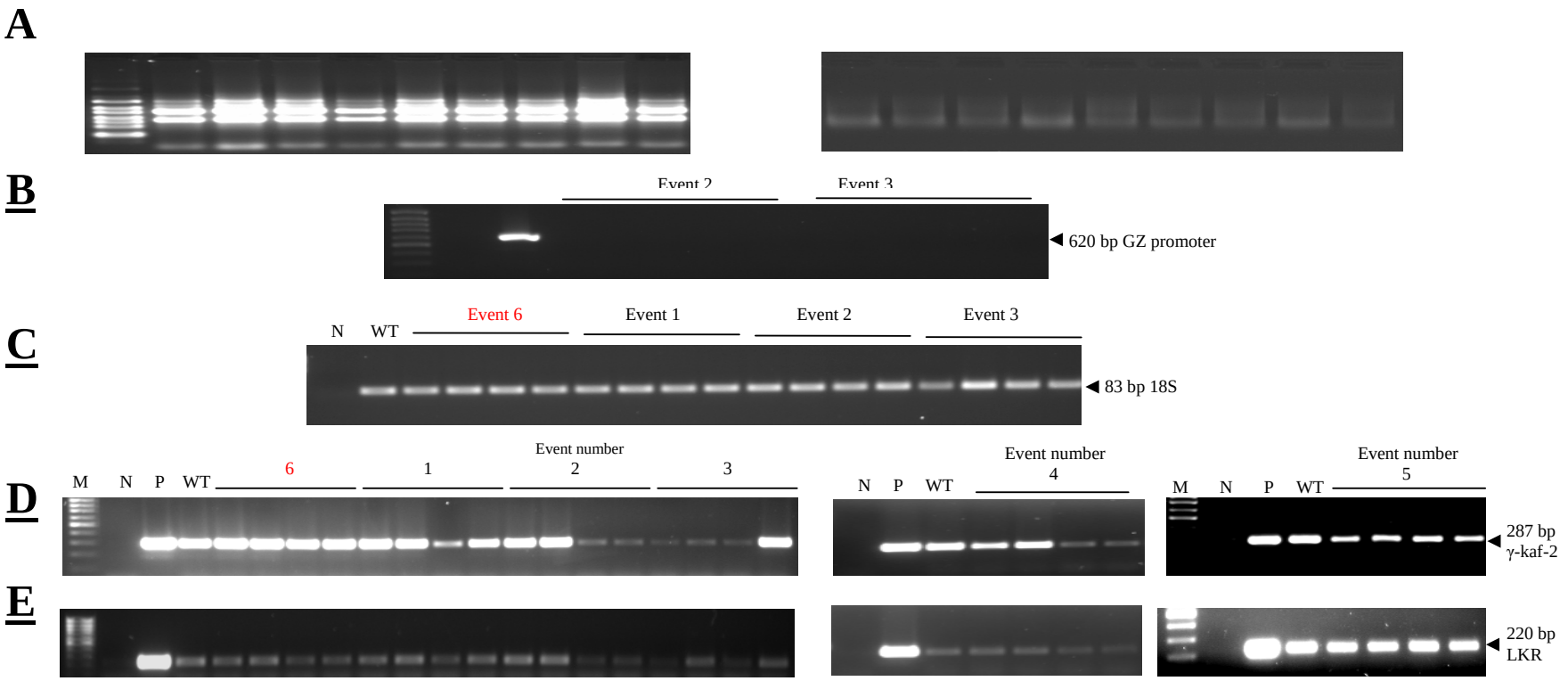

Figure 4.11 Reverse transcription PCR on developing T_2 segregating seeds at 15 days after pollination stage. (A) Integrity of total RNA (left) and cDNA formed after reverse transcription. (B) RT-PCR for 19GZ promoter product on transgenic events 2 and 3. (C) The RT-PCR of 18S gene transcript for transgenic events 1, 2, 3 and 6. (D) The γ-kaf-2 transcript analysis by RT-PCR for transgenic events 1-6. (E) The LKR transcript analysis by RT-PCR for transgenic events 1-6. After PCR amplification of 25 cycles, there was significant γ-kaf-2 suppression (arrow at 287.bp), while LKR suppression was only significant for transgenic events 2 and 3 (arrow at 220 bp). Sample labelling was as follows: WT corresponds to wild-type P898012, N represents negative control lacking template DNA, P corresponds to 1ng of transgene, i.e. positive control; M represents the molecular size marker.

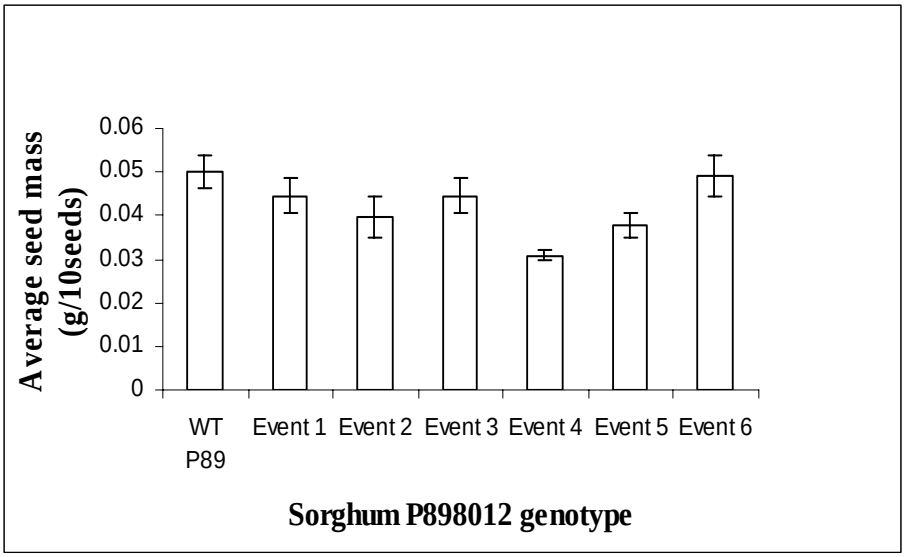

Figure 4.12 Average seed mass, in grams, for 10 T_1 transgenic seeds and wild-type P898012 seeds. The bar graph and its error bars represent mean and standard deviation values of 10 seeds per transgenic event. A general decrease in transgenic seeds was observed, while event 6 seed weights were similar to wild-type seeds. WT P89 represents the wild-type seeds.

Wild-type **Transgenic**

Figure 4.13 Seed morphology of wild-type (left) and transgenic seeds (right) from transgenic event 3. Transgenic seeds show wild-type sorghum seed morphology.

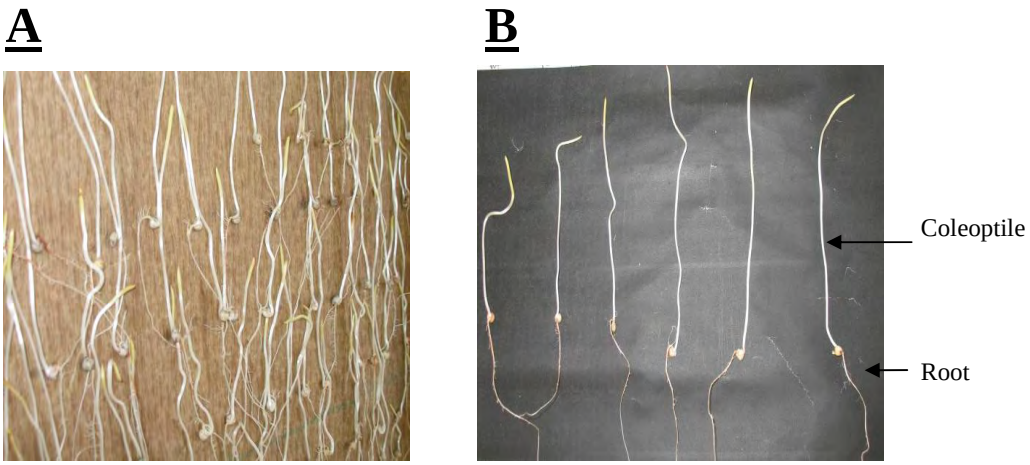

Figure 4.14 Transgenic seed germination potential. (A) A 100% germination rate was observed in all five transgenic events and wild-type seeds after three days. (B) All seedlings formed normal coleoptile and root systems.

4.5 Discussion

In this study, a dual trait RNAi construct expressing a double-stranded RNA molecule was successfully expressed in sorghum public line P898012 to suppress the seed endosperm LKR, δ-kaf-2, γ-kaf-1 and -2. This study confirms that the γ-kaf-1 and -2 suppression resulted in a significant elevation of seed lysine content in sorghum, and thus, the sorghum grain nutritional value improvement. This γ-kaf-1 protein is still referred to as a 27 kDa protein due to its gene and protein homology to the maize and millets counterparts (Xu and Messing, 2008; Shewry and Halford, 2003). However, in this study, the γ-kaf-1 protein was resolved at 25kDa by SDS-PAGE and Western blot analysis.

As a result of γ-kaf-1 and -2 protein suppression, whole seed lysine was successfully elevated by up to 45.2%, while the endosperm lysine content was increased by 77.6%. The γ-kafirins 1 and 2 are abundant proteins and their down-regulation is expected to avail a substantial amount of amino acids for the synthesis of other proteins with better nutritional value. Storage protein synthesis depends on seed nitrogen availability (Rolletschek *et al*., 2005). In this study, the up-regulation of two visible proteins resolved at 30 and 32 kDa was most likely due to increased amino acid availability for the synthesis of these proteins. Figure 4.10 displayed no significant changes in total protein for transgenic seeds, therefore, it is safe to postulate that the seed filling machinery must have compensated for these two up-regulated non-targeted proteins. Moreover, there should be other non-targeted proteins that are expressed in smaller amount, and therefore, will not be visible on SDS-PAGE. The identify and bio-safety implications of these up-regulated proteins are part of a bigger study that is currently being undertaken at Pioneer Hi-bred Int. Inc. in Johnston, Iowa, USA.

The seed proline content was reduced by 18.7% and the transgenic seed endosperm was altered to be 'floury' from a wild-type corneous phenotype. These two observations were expected because γ-kafirins are rich in proline and have more sulphur containing amino acids (cysteine and methionine) in comparison to other kafirins (Shewry and Halford, 2003). Because the proline content is abundant in sorghum, the 18.7% proline content reduction of the transgenic seeds is not a great reduction and should still meet the human and animal nutritional requirement.

The LKR suppression aspect was only shown by the RT-PCR result because there is currently no LKR antibody for Western blot analysis. Only transgenic events 2 and 3 seem to have noticeable LKR suppression. The other events show no significant suppression. The LKR protein is expressed both in the embryo and the endosperm (Tang *et al*., 1997; Malvar *et al*., 2006) and, therefore, suppression of the endosperm LKR alone is likely to increase the expression of the embryo LKR expression as was the case with other non-targeted seed protein expressions.

The endosperm is rich in tightly packed protein bodies and this gives the endosperm a specific phenotype called 'corneous', depending on how tight the packaging really is (see Figure 4.1). This is displayed by the dark brown layer below the aleurone layer. A loosely packed endosperm is referred to as a 'floury' endosperm; the phenotype here is mainly the absence of the dark brown layer. The co-suppression strategy for γ-kafirins 1 and 2, as applied in this study, was expected to disrupt this tight packaging, thus, resulting in a soft and floury endosperm phenotype. This is consistent with the high lysine and high protein digestible Opaque-2 maize mutant (Mertz *et al*., 1964; Geetha *et al*., 1991; Dannenhoffer *et al*., 1995). The 'floury' seeds present bigger challenges to the sorghum growing farmers

because they negatively affect the milling properties of the grain (Vegrains, 2001). However, these floury seeds are likely to result in the improvement of seed protein digestibility, although this aspect of the trait will not be further investigated in this study.

The δ-kaf-2 protein suppression segment of the transgene proved unnecessary as the wild-type P898012 did not express this protein. The gene expression for this 18kDa protein is currently a subject of investigation by the cereal seed protein laboratory at Pioneer Hi-bred Int. Inc. in Johnston, Iowa, USA (Jung R., 2007). The δ-kaf-2 gene is present in the sorghum genome but not expressed in some sorghum varieties, including P898012. Therefore, not including this δ-kaf-2 gene suppression would not affect the observed transgenic trait.

The transgenic trait was stably inherited by the T_2 generation. Transgenic T_1 seed morphology and germination, as expected, did not display any negative or detrimental effects as a result of the introduced trait. This is because the target proteins did not represent the majority of the storage proteins and therefore enough nitrogen and sulphur will still be available for a developing embryo. A reduction in the average transgenic seed weight was observed and further investigations for $T_2 – T_3$ seed weight need to be carried out. There was an unintended partial suppression of the α-kafirin A1 type protein in 50% of the transgenic events that were analyzed, but generally, there was an up-regulation of non-targeted seed.

Transgenic event number 6 showed wild-type like results confirming that no successful silencing of target proteins was achieved. This is in line with the likely truncation that took place in the 5'- promoter end of the transgene in this transgenic event. This is the first report on the use of RNAi technology to enhance the sorghum grain's nutritional quality.

4.6 References

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Chapter 5

Discussion

5.1 The need for establishing protocols for genetic engineering of sorghum

The need to improve the nutritional quality of the sorghum grain was reviewed in the introduction Chapter 1. Sorghum is one of the most difficult cereal crops to manipulate through tissue culture and transformation and this led to fewer reports for sorghum transformation in comparison to maize, a close relative of sorghum. This is further made difficult by the lack of extensive information clearly outlining a successful sorghum transformation process. In this study, I undertook to improve the manipulation of sorghum using genetic engineering tools. The *in vitro* protocol to successfully propagate and transform this hardy cereal crop has far reaching implications for the improvement of the sorghum crop for field performance and its usage for human and animal consumption. The advantages of using plant genetic engineering tools for crop improvements were also reviewed in Chapter 1, and emphasis on the speed, cleanliness and precision of this approach enable successful genetic modification of the sorghum plant genome for beneficial gains in agriculture.

The lysine content of the grain is considered too low to meet the human and animal requirements, and traditional breeding practices have been focussed on the development and release of high-lysine cultivars through directed cross breeding programs. Axtell and Ejeta (1990) developed a high-lysine sorghum mutant P721 that displayed a 99% improvement of the lysine content. One of the disadvantages of this mutant was the lower yields. An alternative approach to meet the lysine content for human and animal food and feed requirements is the use of synthetic lysine supplementation of sorghum-based foods. While the former approach is labour intensive and time consuming, the latter approach is expensive. This study aimed at increasing the lysine content via a genetic approach while improving the transgene transfer efficacy and stability into the sorghum genome.

5.2 Optimization of parameters for transformation

To achieve genetic enhancement of lysine in grain sorghum, a key objective was to establish a routine transformation protocol to extend the *in vitro* transformation knowledge and to improve the efficiency of introducing and expressing foreign genetic material in sorghum tissue. Three parameters were investigated in this study; genotypes, medium and the DNA delivery method (particle in-flow gun versus the Bio-Rad gun). The screening of several sorghum genotypes yielded a new tissue culture medium-genotype combination employing medium J and genotype P898012, and achieving a regeneration potential of 6.13reg./expl in this genotype-medium combination. The superiority of mannose over the bialaphos as a selection system in producing stable transgenic plants is a confirmation of what has been previously reported. The use of phosphomannose isomerise (*pmi*) gene and subsequent selection on mannose promotes transgenic cell proliferation and effectively limits the regeneration of false positive plants.

The first report that employed the mannose selection system in sorghum was in 2005 (Gao *et al*., 2005). In that study, the authors transformed one inbred line C401 and one commercial hybrid Pioneer 8505. This study represents the first report on stable transformation of the public line P898012 using the mannose selection. The Bio-Rad gun was noted to achieve better DNA delivery to plant tissue relative to the particle inflow gun (PIG), which has been noted to cause more damage to the scutellum tissue. Recent literature reports have highlighted *Agrobacterium* transformation as better in delivering higher transformation efficiencies when compared to biolistic transformation. However, the transformation efficiency using the biolistic method in this study was found to be 3.36%, the highest

reported to date. Further optimisation of the protocol reported in this study is still necessary to enhance the repeatability of the highly efficient procedure.

5.3 The RNAi approach to enhance lysine through reduction of storage proteins

The hypothesis for this study was that the RNAi strategy could be used successfully to suppress genes that encode target storage proteins, and therefore, could result in the enhancement of available essential amino acid lysine in the endosperm and, therefore, the grain. By suppression of key classes of kafirins, namely, the γ-kaf-1 and -2, I demonstrated an enhancement of seed lysine content of up to 45.2%. The observed amino acid enhancement was attributed to the suppression of storage proteins that are poor in lysine content and their subsequent expression substitution in sorghum grain by other proteins that are higher in lysine content.

Unlike introducing a heterologous protein, the use of RNAi in combination with the biolistics method is not expected to negatively impact on the expression or suppression levels due to genome integration of multiple transgenes usually observed with particle bombardment. In fact, multiple transgene integration is likely to re-inforce the suppression levels. This is due to the increased suppression activity of the transgene against themselves and against the endogenes. Thus I concluded that the RNAi strategy to suppress some storage proteins is indeed a viable approach to enhance grain lysine content, thereby enhancing sorghum grain's nutritional quality. This study therefore encourages the manipulations of other limiting essential amino acids in this cereal via the RNAi strategy. It also inspires the introduction of agronomic and other traits of international interest to be stably expressed.

The RNAi strategy was successfully used in maize to improve the lysine content through the reduction of the bi-functional enzyme LKR/SDH (Houmard *et al*., 2007). However this study presents, to our knowledge, a first report on using the RNAi strategy to engineer the sorghum grain for the improvement of the nutritional value. The variation in lysine enhancement observed between different events is attributed to the different loci for transgene integration and the differences in transgene copy numbers. Integration of genes in highly expressed regions of the genome are likely to result in better suppression of the transgenes, whereas multiple gene integrations have been reported to have a whole range of effects, but notable is the high likelihood of gene silencing when too many copies of the same sequence get integrated into the genome. This was the case with transgenic transgenic events that were produced in this study as most events showed an almost complete suppression of target proteins.

The RNAi approach targeted the suppression of specific storage proteins, and the LKR enzyme. Included in the RNAi construct were the LKR, δ-kaf-2, γ-kaf-1 and -2. These were all under the endosperm-specific promoter, the zein GZ-19 promoter. The δ-kaf-2 gene inclusion proved unnecessary as no P898012 protein expression takes place in the endosperm. The LKR suppression was not effective due to a likely up-regulated embryo LKR expression. However, the suppression of γ-kaf-1 and -2 was successful and this resulted in a simultaneous up-regulation of non-targeted proteins that presumably expressed more lysine content. The unintended protein suppression that was observed was a 21 kDa alpha A-1 type protein. This non-intended alpha protein suppression is likely to assist the intended strategy employed in this study. This is generally because the alpha subset of proteins are poor in lysine content and the suppression of this group of proteins in maize led to an increased lysine content and flouriness (Segal *et al*., 2003). This non-target silencing of the alpha

protein was not investigated in this study but the hypothesis should concentrate on the 19-GZ promoter sequence analysis with endogenous kafirin alpha promoter and proteins sequences. For regulatory de-regulation process, it would be critical to elucidate the cause of this unintended suppression. All the other regulatory tests such as the allegernicity and toxicity tests will cover the regulatory requirements. Also novel in this study was the observation that the γ-kaf-1 protein was resolved at 25 kDa and not the reported size of 27 kDa.

5.4 Limitations and product drawbacks

The tissue culture amenability and transformability of sorghum are two major hindrances for the use of genetic engineering tools to enhance sorghum grain quality and agronomic performance attributes. The sorghum transformation technology of 3.36% is far from satisfactory in terms of transformation efficiency. Transformation efficiencies in maize, a close relative of sorghum, have been reported to be higher than 40% (Zhao *et al*., 2002) using the *Agrobacterium* method. Efforts should thus concentrate on improving the sorghum transformation efficiency to be as high or better for the process to become routine. This will intensify worldwide biotechnology research activities to improve various attributes of this important African crop.

The high-lysine expressing transgenic seeds reported in this study resulted in a softer and floury seed endosperm in comparison to wild-type seed. The inferior field performance and other undesirable attributes of a similar non-transgenic maize line, the Opaque-2 mutant, raise concern that the same may be observed in the high-lysine sorghum as well. The Opaque-2 mutant's poor traits include reduced total protein content yield, reduced processing potential and increased disease and insect susceptibility (Ufaz and Galili, 2008). The T_1 transgenic seeds produced in this study display an unchanged protein content. However, other possible

drawbacks present major product challenges. As a result, the farmers' lack of confidence in high-lysine maize mutant may be transferred to the high-lysine sorghum line. However, the genetic basis for the floury phenotype between Opaque-2 maize mutant and transgenic sorghum reported in this study are quite different because a combination of proteins were suppressed in this study compared to only γ-zein 2 non-expression in the O2 mutant. Therefore transgenic sorghum may perform agronomically different and may or may not display normal or acceptable processing attributes in comparison. The data I have generated to date suggest a normal phenotype in terms of plant physical appearance and grain density, and the same is expected when this germplasm is crossed into the final destination germplasm, which would be preferred in various geographical regions.

The public acceptance aspect of transgenic products will form a major part of a marketing and adoption strategy for any commercial genetically modified (GM) sorghum product release. To allay possible negative perception around public acceptance of GM crops, as in other GM crop products, a strict regulatory protocol of approval is generally followed. This entails very rigorous analysis not only of field performance but also of grain quality to establish the absence of any untoward effect such as potential allergenicity of proteins that may have been unintentionally introduced. In this case, I reduced the expression of the kafirin storage proteins but other endosperm proteins were up-regulated to compensate for the removal of the kafirins. The nature and identity of such proteins will thus need to be identified as part of the regulatory process prior to product release. Thus the technology must address and meet the regulatory requirements to show substantive equivalence to the natural product in all but the aspects that were the targets of change.

An important aspect is that the new varieties arising from this work must still perform well or better compared with traditional varieties, because it may be difficult to convince farmers to adopt a nutritionally valuable variety if it will have yield penalties. The challenge of developing a high-yielding, hardier and high-lysine sorghum variety may be overcome by generating a large number of transgenic events to obtain lines that have high lysine but not the negative attributes on seed hardiness. This coupled with breeding the high lysine trait into other preferred germplasm will contribute to the development of sorghum lines with a high probability of adoption by the farmers and ultimately consumers.

5.5 **Future research**

In this study, seed lysine was significantly improved through the reduction of storage proteins. The next step would be to produce these sorghum seeds commercially. This would require transgenic events production with simple transgene integration to lower the copy numbers, which would ensure commercial and biosafety deregulation success. This can be achieved by either producing larger numbers of events to improve the chances of simple integration displaying events and/or the use of the *Agrobacterium*-mediated transformation method. The latter approach has been reported to produce simpler patterns of transgene integration.

The general up-regulation of non-targeted seed proteins is one aspect that requires a thorough investigation. The abundant expression of the targeted γ-kaf-1 and -2 proteins resulted in poor lysine content, conversely, the improved expression of certain lysine-rich proteins can result in other negative effects. The regulatory processes will require a display of the law of substantive equivalence wherein proteins that exceed a certain level of expression would have

to be identified and a demonstration of their effects should be proven safe for the environment, human and animal feed. This study is currently being undertaken at Pioneer Hi-bred Int. Inc. in Johnston, Iowa, USA. The work reported in this thesis played a fundamental role to instigate this crucial part of the regulatory process.

The second trait of interest that the RNAi strategy aimed to investigate in this study is the prevention of loss in seed protein digestibility during wet cooking. The molecular characterization for this trait was not covered because the transgenic line that was produced is a type II tannin line. Accurate analyses of the protein digestibility trait require a tannin-free seed. This is because the polyphenols, tannins, interfere with sorghum protein digestibility tests by binding to seed proteins, thus, lowering the nutritional value (Taylor *et al*., 2007). One possible solution to this problem would require an identification of a sorghum tannin-free genotype that is transformable. Unfortunately, at this stage, only the sorghum line P898012 was transformed. There is a strong requirement for future efforts to widen the transformability of the sorghum germplasm. Another solution would require a back-cross breeding process of the transgenic traits to a tannin-free sorghum line such as Macia, and then analysing the progeny of such crosses for enhanced digestibility properties.

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Annexure

Nutrient medium components

1 L3 macro-nutrients

Macro-nutrient	**g/L**
KNO_3	35
NH_4NO_3	4
$MgSO_4.7H_2O$	7
KH_2PO_4	4
$CaCl_2.2H_2O$	9

2 L3 micro-nutrients

Micro-nutrient	**g/L**
H_3BO_3	1
$MnSO_4.4H_2O$	5
$ZnSO_4.7H_2O$	1.5
$NaMoO_4.2H_2O$	0.05
$CuSO_4.5H_2O$	0.005
$CoCl_2.6H_2O$	0.005
KI	0.15

3 MS-Fe source

Reagent	**g/l**
$FESO_4.7H_2O$	2.78
$Na_2EDTA.2H_2O$	3.73

4 HL2 vitamins

Vitamin	**mg/ml**
Thiamine-HCL	2
Pyridoxine-HCL	0.2
Nicotinic acid	0.2
myo-inositol	20
L-glutamine	84

5 Jacobs' vitamins

Vitamin	**mg/l**
Glycine	7.5
Thiamine-HCl	1
Myo-inositol	100

6 B5 vitamins

Vitamin	**mg/ml**
Thiamine-HCL	2
Pyridoxine-HCL	0.2
Nicotinic Acid	0.2
Myo-inositol	20