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.

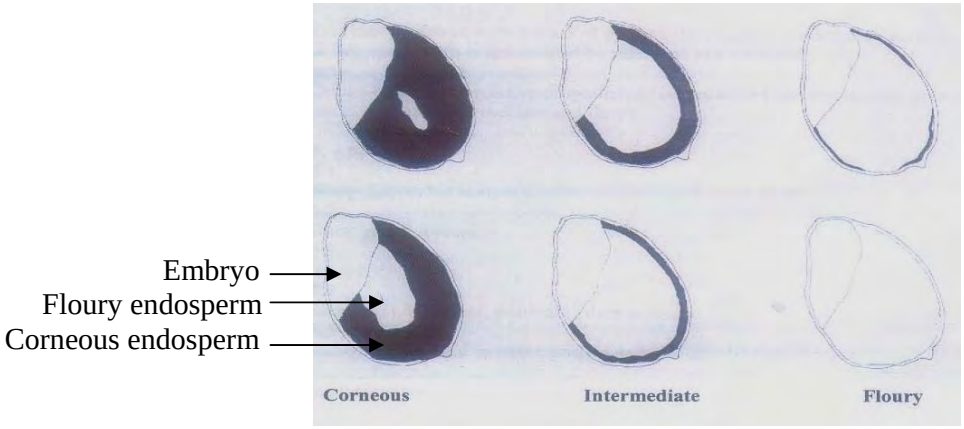


Figure 4.1 The criteria used in the classification of seed endosperm phenotype (picture, courtesy of Prof. J Taylor, University of Pretoria). The corneous seed endosperm is characterized by a dark brown or black colour caused by both the tight protein body packaging and diffusion of tannins from the 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).

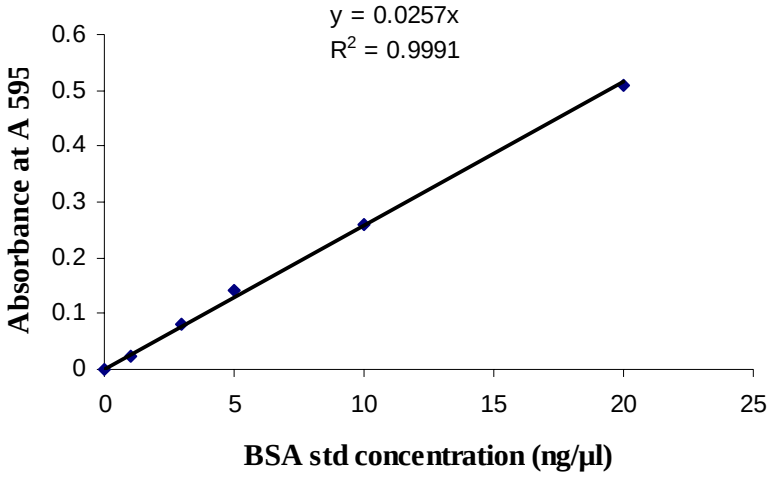
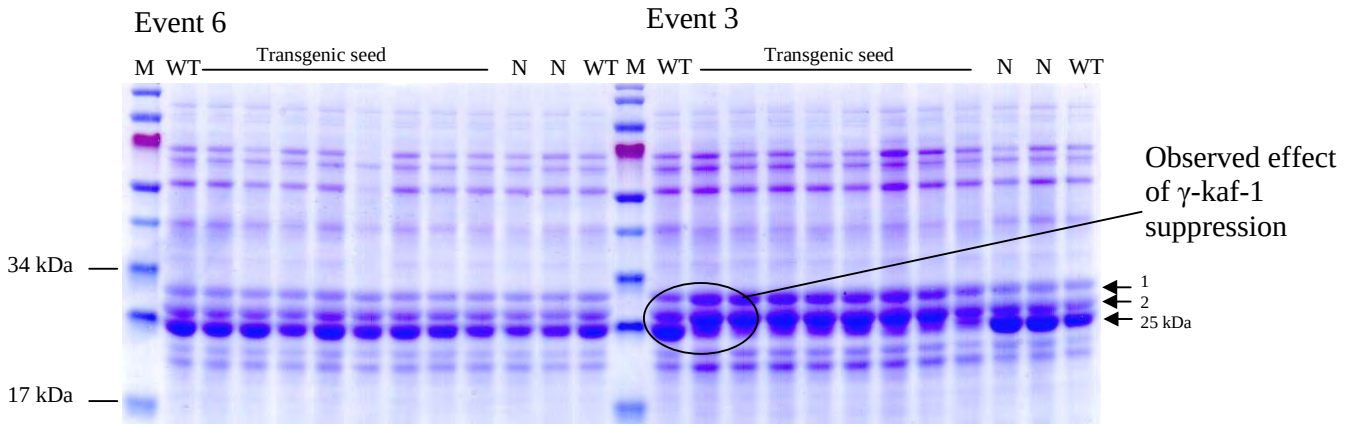


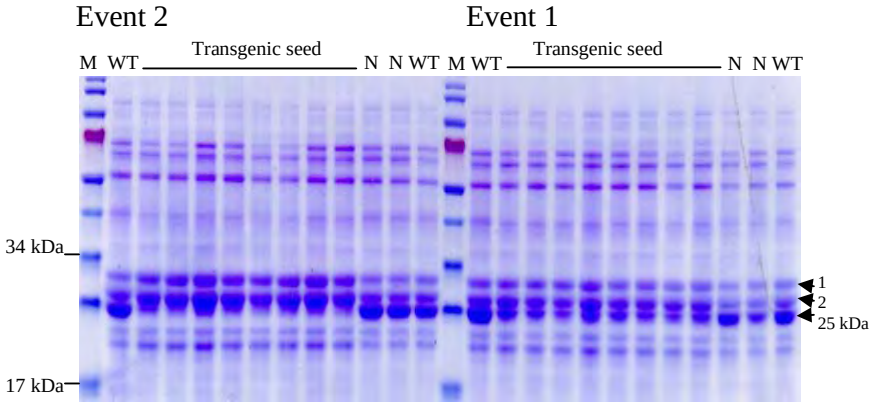
Figure 4.2 Bovine serum albumin standard curve where absorbance was measured using a WPA Lightwave spectrophotometer (Labotec Ltd, South Africa).

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

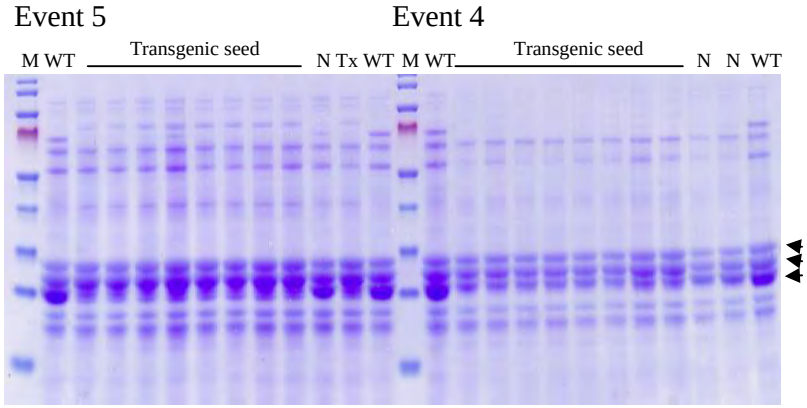

Event 5
Event 4
M WT Transgenic seed N Tx WT
M WT Transgenic seed N N WT

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.

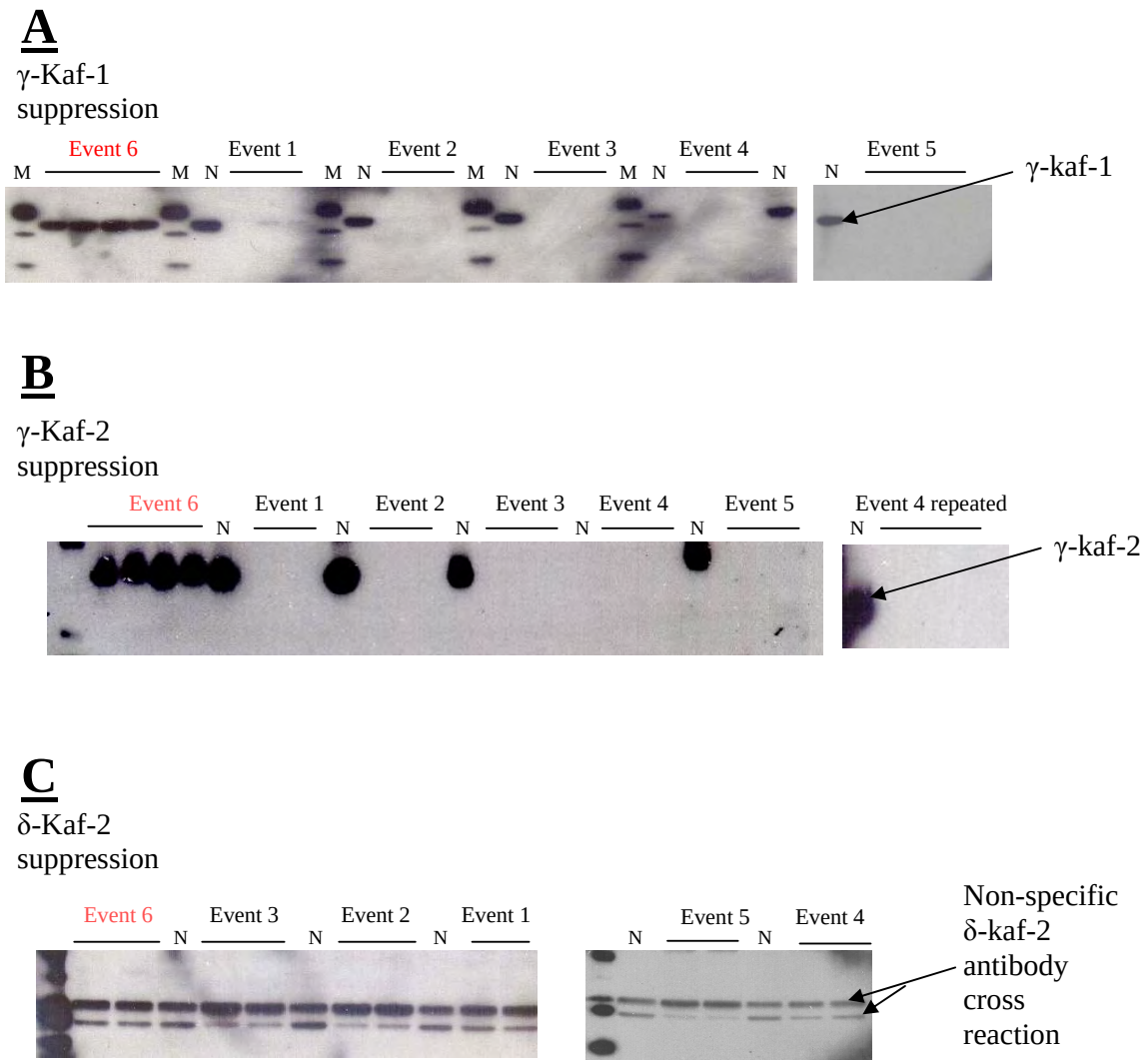


Figure 4.4 Western blot analysis of pABS transformed P898012 sorghum T_1 seeds to investigate target γ-kaf-1, γ-kaf-2 and delta kaf-2 protein suppression. Single seed analysis of T_1 segregation seeds for six transgenic events was performed with rabbit-raised antibodies specific for the following: (A) 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.

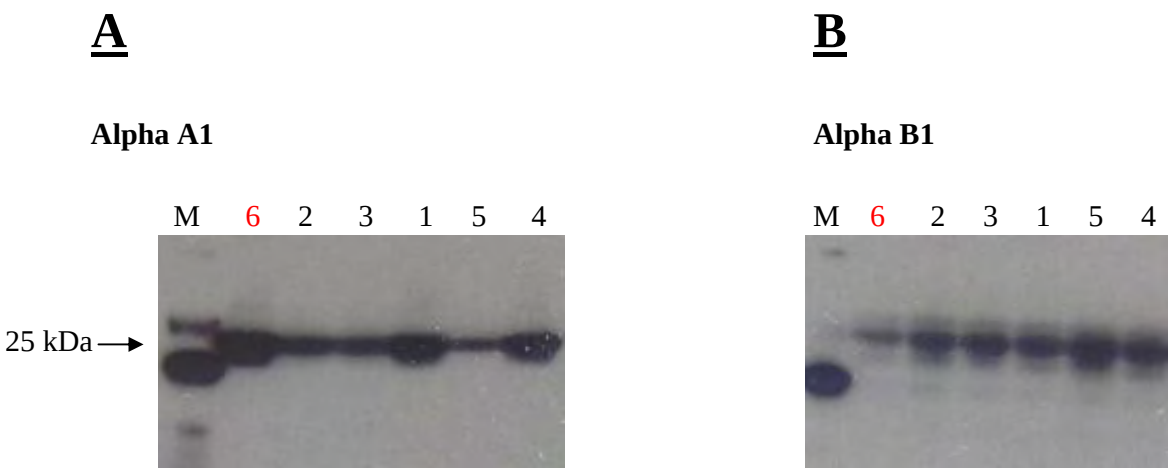


Figure 4.5 Western blot analysis of transgenic events 1-6 for α-kafirins A1 and B1 types. The non-targeted α-kaf A1 partial suppression was confirmed in events 2, 3 and 5, while transgenic events 1 and 4 displayed no significant α-kaf A1 suppression (A). However, the α-kaf B1 type proteins were up-regulated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

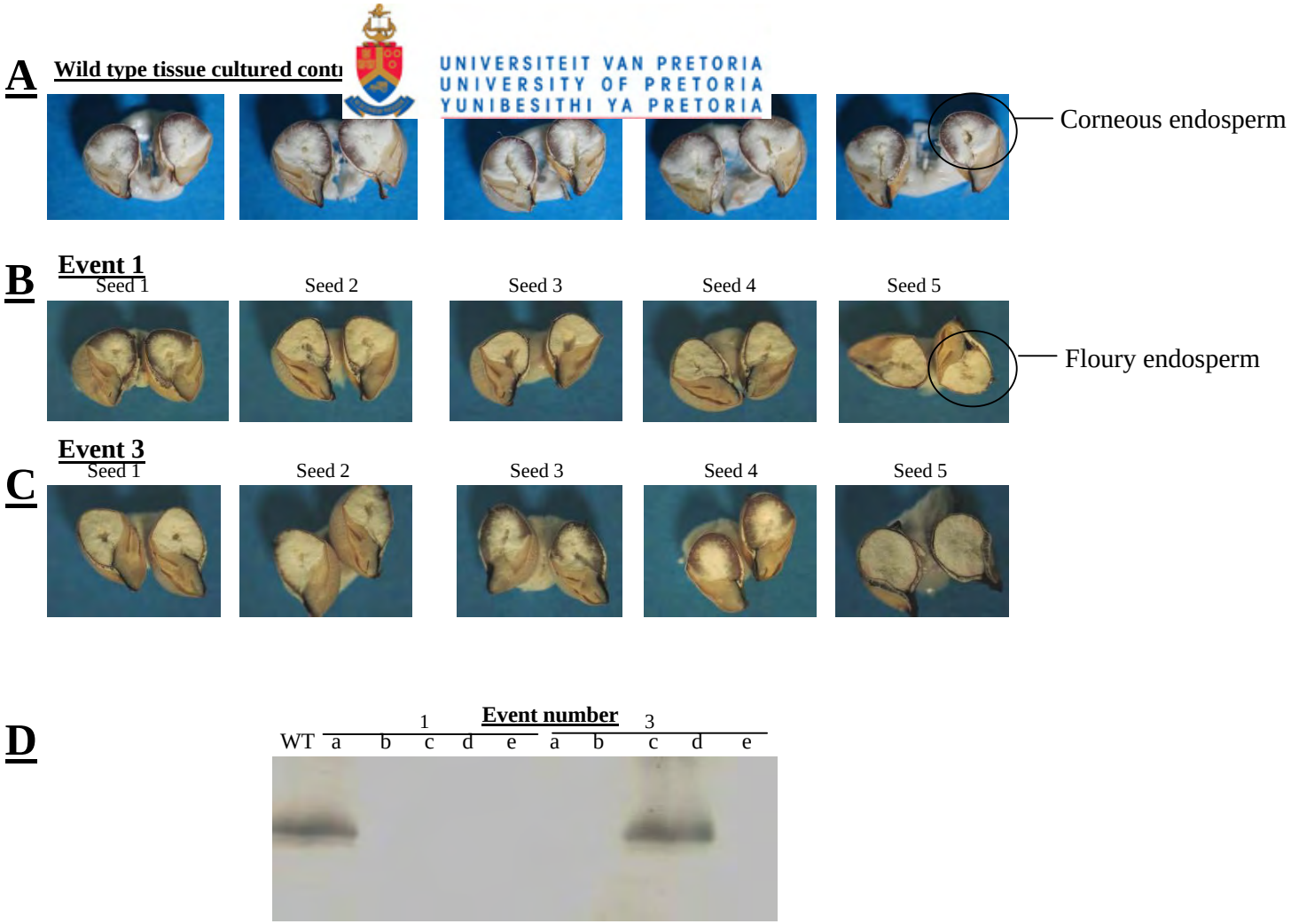


Figure 4.6 Investigation of possible linkage between kafirins suppression and seed endosperm phenotype. (A) Five randomly chosen seed endosperms from wild-type seeds. (B) Five randomly chosen endosperms from transgenic event 1. (C) Five randomly chosen endosperms from transgenic event 3. (D). The Western 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.

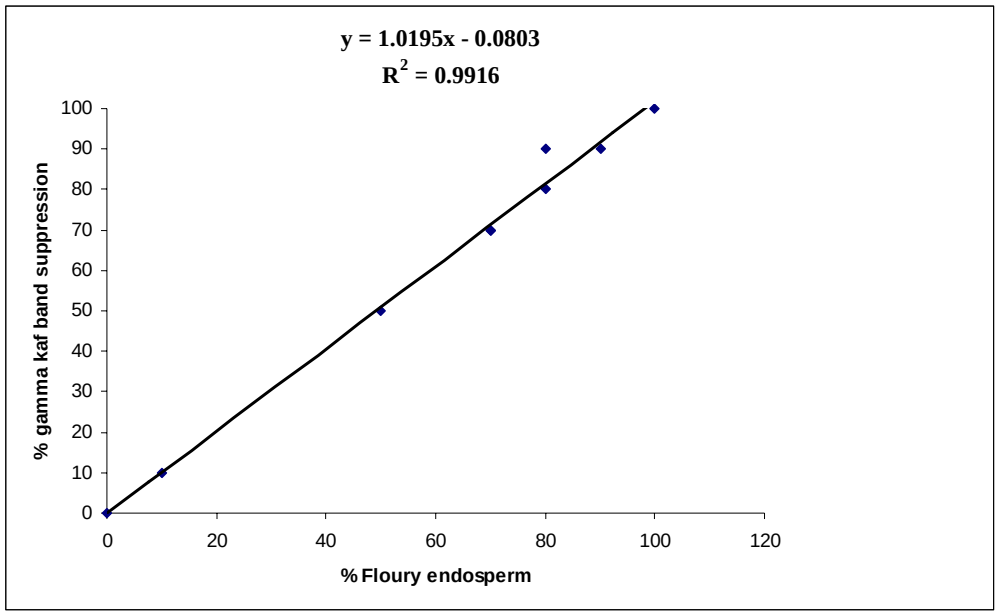


Figure 4.7 Correlation chart for suppressed kafirin expression with the floury endosperm phenotype for transgenic event 1. Eighty transgenic seeds were cross-sectioned and analyzed by SDS-PAGE and Western blot analysis. A co-efficient of 0.9916 was determined thus suggesting a significant relation between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

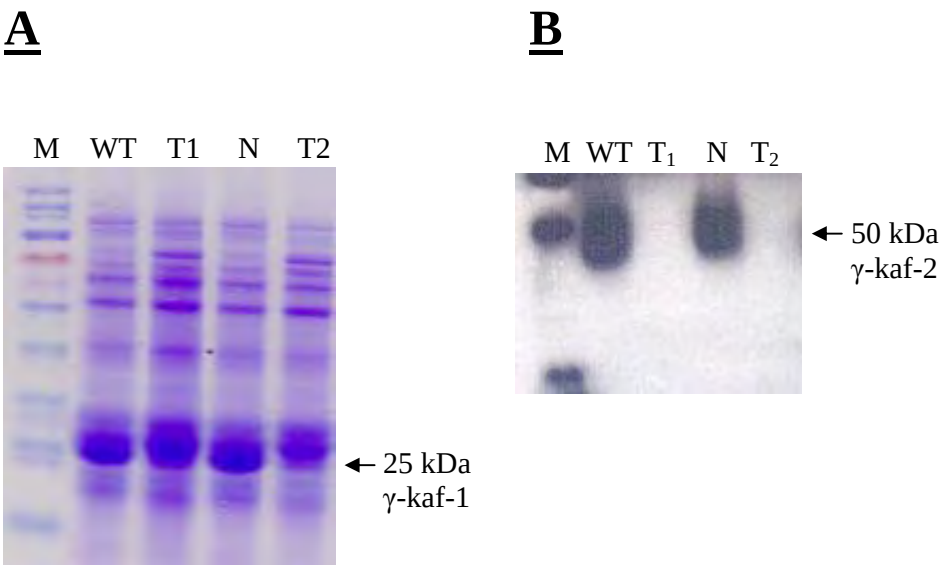


Figure 4.8 Stable T_1 to T_2 transgenic trait inheritance of transgenic event 3 seeds. (A) SDS-PAGE of proteins isolated from T_1 and T_2 seeds of transgenic event 3. (B) Western blot analysis of γ-kaf-2 expression from proteins isolated from transgenic event 3 seeds. M represents the protein size marker, 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).

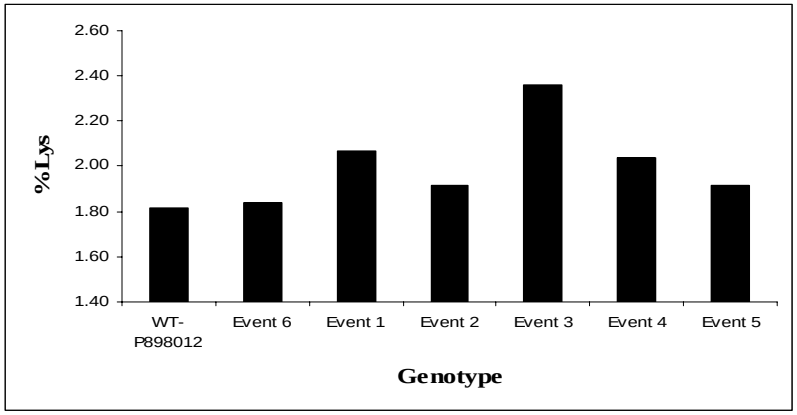
Table 4.1 Free amino acid analysis (g/100g) measured (two replicates A and B) via HPLC for total amino acid content determination of transgenic events 3 and 6.

	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	

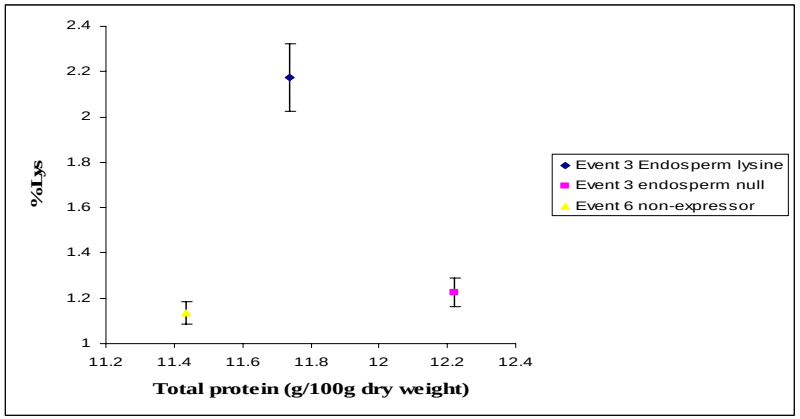
⚙**The amino acid contents of interest**

***ND = not determine**

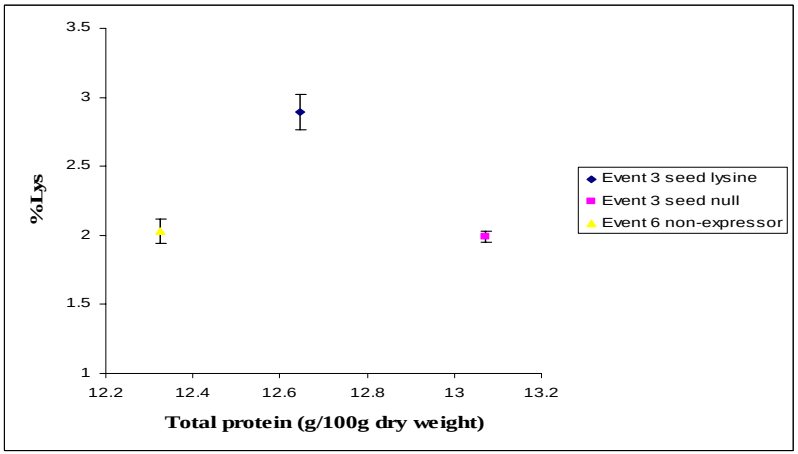
A


%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

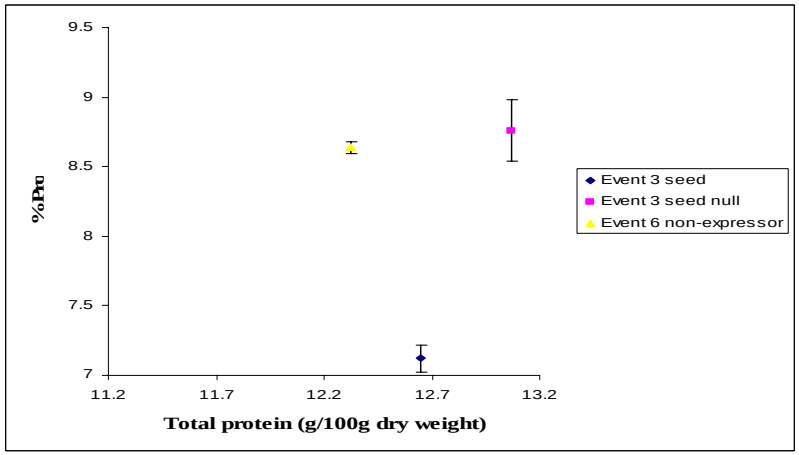

%Pro
9.5
9
8.5
8
7.5
7
11.2
11.7
12.2
12.7
13.2
Total protein (g/100g dry weight)
Event 3 seed
Event 3 seed null
Event 6 non-expressor

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.

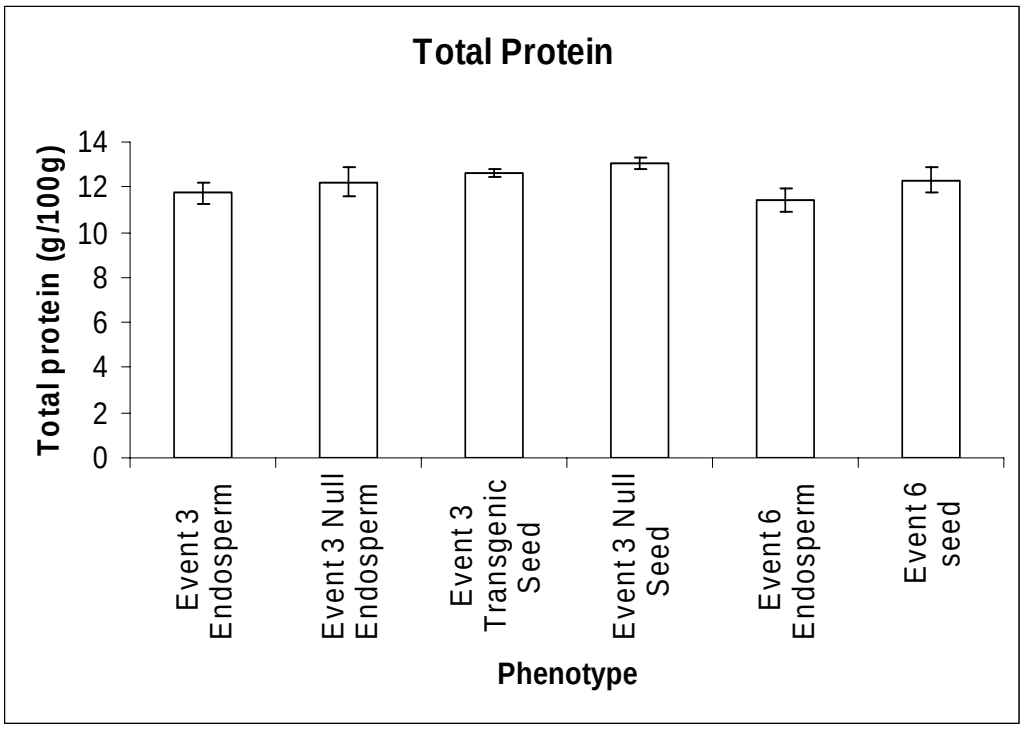


Figure 4.10 Whole seed and endosperm **t**otal protein contents of transgenic event 3 for transgenic T_1 seeds excluding cysteine. No significant difference of total protein was observed between transgenic seeds and endosperms of transgenic event 3 and control transgenic event 6.

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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).

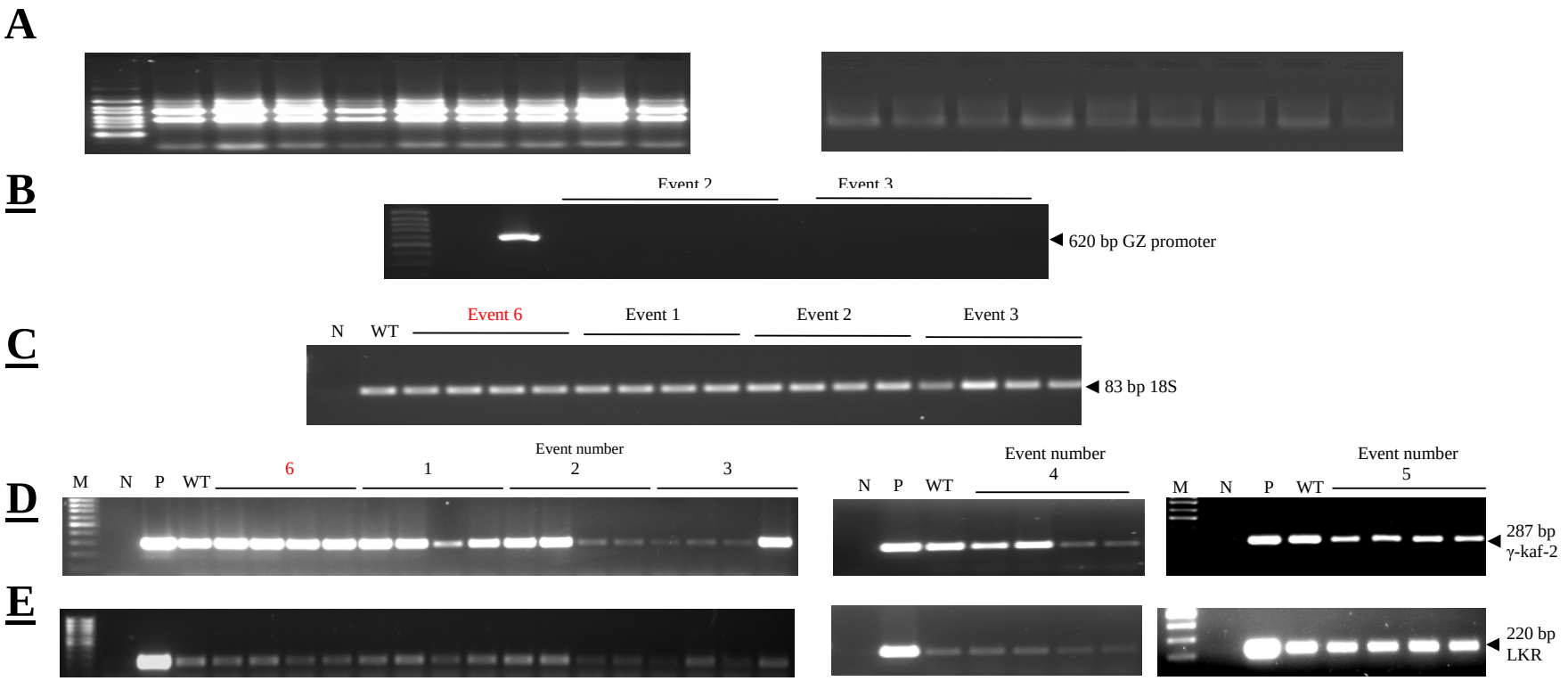

Figure 4.11 Reverse transcription PCR on developing T_2 segregating seeds at 15 days after pollination stage. (A) Integrity of total RNA (left) and cDNA formed after reverse transcription. (B) RT-PCR for 19GZ promoter product on transgenic events 2 and 3. (C) The RT-PCR of 18S gene transcript for transgenic 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.

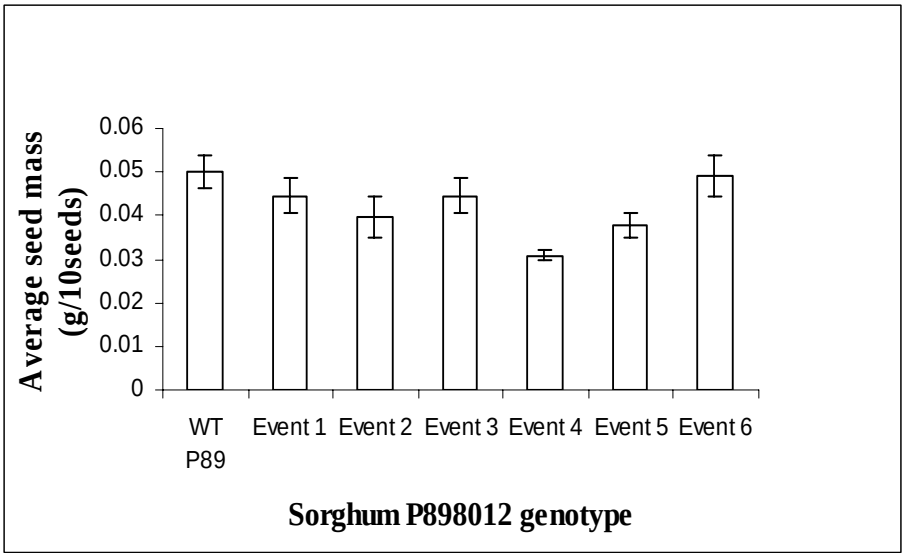

Figure 4.12 Average seed mass, in grams, for 10 T_1 transgenic seeds and wild-type P898012 seeds. The bar graph and its error bars represent mean and standard deviation values of 10 seeds per transgenic event. A general decrease in transgenic seeds was observed, while event 6 seed weights were similar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

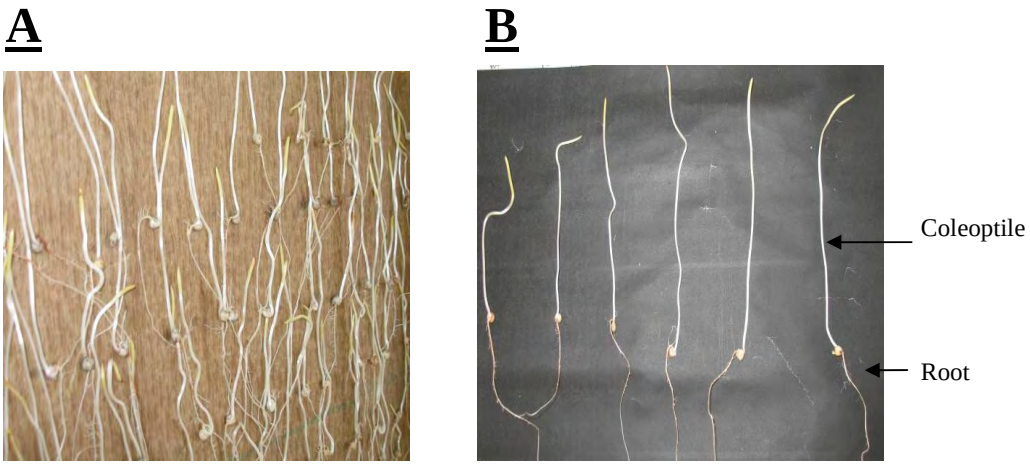

Figure 4.14 Transgenic seed germination potential. (A) A 100% germination rate was observed in all five transgenic events and wild-type seeds after three days. (B) All seedlings formed normal coleoptile and root systems.

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.

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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.

5.6 **References**

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