3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Transgenic sorghum lines with altered synthesis of the major kafirin sub-classes will have improved protein quality, specifically increased lysine content and improved protein digestibility. Non-transgenic sorghum genotypes with reduced relative kafirin content have higher lysine content (up to 60%) (Singh and Axtell, 1973; Guiragossian et al., 1978). This is due to compensatory synthesis of lysine-rich non-kafirin proteins in the grain endosperm. Some high-lysine types also have improved protein digestibility (Weaver et al., 1998; Oria et al., 2000). Improved protein digestibility has been attributed to changes in the shape of the kafirin protein bodies from spherical to invaginated (Oria et al., 2000), which increases their surface contact area to protein attack. The improved protein quality of transgenic sorghum with altered kafirin synthesis will be due to reduced levels of indigestible highly crosslinked kafirins. The ɣ-kafirins are responsible for the formation of highly crosslinked kafirin polymers via disulphide bonds (El Nour et al., 1998). Highly crosslinked kafirins are resistant to proteolysis (Hamaker et al., 1986).

Transforming different sorghum genotypes to have altered kafirin synthesis will have an influence on the endosperm, resulting in floury endosperm texture. Non-transgenic sorghums with altered kafirin synthesis commonly have floury endosperm texture (Singh and Axtell, 1973; Guiragossian et al., 1978). The floury endosperm is caused by low kafirin synthesis during grain development, resulting in loose association of endosperm components, where the starch and protein bodies are not tightly packed and the protein matrix is discontinuous (Shull et al., 1990; Chandrashekar and Mazhar, 1999).

3.2 Objectives

To determine the effect of altered kafirin synthesis on sorghum protein quality.

To determine the effects of altered kafirin synthesis on protein quality and endosperm structure in different sorghum genotypes.

To determine the cause of improved protein quality in transgenic sorghum with altered kafirin synthesis.

4 RESEARCH

4.1 Protein digestibility and endosperm structure of transgenic sorghum with altered protein synthesis

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4.1.1 Abstract

Transgenic (TG) sorghum with altered kafirin synthesis, to improve the protein nutritional quality of the grain, is being developed. The impact of genetic transformation on grain physicochemical properties, protein digestibility, protein body structure and endosperm texture was investigated and compared to normal and high-protein digestibility mutant (HPDM) sorghums. Some TG lines had improved lysine content (3.06 g/100 g protein) and moderate (48.9-56.2%) cooked *in vitro* protein digestibility, compared to the parent (2.05 g/100 g protein and 24.2-41.1%, respectively). Transmission electron microscopy revealed that improved nutritional traits were associated with floury endosperm texture and irregular shaped protein body structure (2-3 µm, with few to numerous invaginations), compared to the intermediate endosperm texture and spherical protein bodies of normal sorghums. The internal concentric ring structure of normal protein bodies was apparently also absent. The protein bodies of the transgenic lines resembled the invaginated protein bodies of the HPDM, but HPDM protein bodies were smaller (1 µm). It appears that reduced kafirin synthesis, specifically co-suppression of α- and ɣ-kafirin synthesis, has a major effect on the structure of sorghum protein bodies, possibly due to reduced levels of crosslinked kafirins, which in turn alters the protein digestibility and endosperm structure.

4.1.2 Introduction

In Africa and Asia, sorghum is an essential food crop, often being the primary source of energy and protein for millions of the world's poorest people (FAO, 2002; ICRISAT, 2009). Compared to other major cereals, sorghum has the distinct advantage of being drought-tolerant, thus making it the cereal of choice for many subsistence farmers living in these arid regions. However, a number of nutritional constraints with regard to its protein nutritional quality, namely limited lysine (essential amino acid) content and poor protein digestibility of cooked sorghum, are of particular concern in areas where sorghum is used as a major source of protein (Duodu et al., 2003).

The low lysine content of sorghum of approximately 2 g lysine per 100 g protein, is due to the major storage protein, kafirin (70 to 80% of the total endosperm protein (Taylor, Schüssler and Liebenberg, 1985), being essentially free of lysine (Taylor and Schüssler, 1986). However, exceptions include two high-lysine native Ethiopian cultivars (IS11167 and IS11758) (Singh and Axtell, 1973) and a chemically (diethyl sulphate) induced high-lysine mutant (P721 opaque) (Guiragossian et al., 1978, Weaver et al., 1998). These lines are known as "low prolamin" mutants in which the proportion of kafirin is reduced to about 50% (O'Kennedy et al., 2006). This results in compensatory increases in other more lysine-rich proteins and free amino acids, resulting in 40-60% more lysine in the grain.

The poor protein digestibility of sorghum has been attributed to the formation of disulphide crosslinked kafirin oligomers and polymers during protein body formation (Mazhar and Chandrashekar, 1993), as well as during wet cooking (Hamaker at al., 1986). These crosslinked kafirins result in the formation of enzyme-resistant structures that retard protein digestion (Duodu et al., 2003). In normal sorghum, kafirins (α-, β- and ɣ-kafirins) are packaged into individual protein bodies (0.5 to 3.5 µm in diameter) (Taylor et al., 1985), with α-kafirin (major kafirin) being located centrally with most of the β- and ɣ-kafirins being located at the bodies periphery (Shull et al., 1992). Upon grain maturity, β- and ɣ-kafirin proteins become crosslinked, forming a distinct border between individual protein bodies (Mazhar and

Chandrashekar, 1993). However, there is evidence that mutant sorghums with modified protein body structure are more digestible, even after cooking, than normal sorghums (Oria et al., 2000; Weaver et al., 1998). The protein bodies of these mutant sorghums are highly invaginated (containing deep folds) with ɣ-kafirin being located at the base of the folds. Apparently this results in increased exposure of the α-kafirin to digestive enzymes, and thus improved protein digestibility is observed (Weaver et al., 1998, Oria et al., 2000).

Advances in sorghum tissue culture and transformation research have led to the development of the first nutritionally improved transgenic sorghum (Zhao et al., 2003). These nutritionally improved sorghum lines are being developed by the Africa Biofortified Sorghum (ABS) Project, funded by the Bill and Melinda Gates Foundation (Grand Challenges in Global Health No. 9) (ABS Project, 2009). Different genetic engineering strategies have been employed to increase the protein nutritional quality of the sorghum grain, namely increased lysine content and improved protein digestibility (O'Kennedy et al., 2006, Henley et al., 2010). However, it is of paramount importance that any changes in the chemical composition of the grain achieved through grain transformation, should not affect grain functionality and agronomic performance negatively. Nor should they compromise traditional and modern food processing ability and consumer acceptance of the final product.

The objectives of this study was to determine what effect different grain transformations via particle bombardment, suppressed synthesis of only ɣ-kafirin, or both ɣ- and α-kafirins, have on the protein nutritional quality and endosperm structure of various transgenic sorghum lines.

4.1.3 Materials and Methods

4.1.3.1 Grain samples and whole grain flour preparation

Five different transgenic (TG) sorghum lines, plus a non-transgenic null control (NC), were developed by the CSIR Biosciences, South Africa, using particle bombardment according to the method described by Grootboom et al. (2010) (Table 4.1.1). Parent line (P898012, a purple plant, type II low-tannin sorghum) was used for the different transformations. Two different gene constructs (ABS042, and ABS044) each designed to suppress the synthesis of different kafirin sub-classes within the grain endosperm were used, namely: ɣ-kafirin-1 (27 kDa) and -2 (50 kDa) for ABS042, and α-kafirin-A1 (25 kDa), ɣ-kafirin-1 (27 kDa) and -2 (50 kDa) for ABS044. The NC was a non-transgenic grain obtained from a hemizygous transgenic plant, and is the best counterpart to the TG for transgene performance assays. The HPDM sorghum lines (HPDM1 to 7) were either original lines from Purdue University, as described by Oria et al. (2000) or crosses between HPDM and normal lines from Texas A&M University. The parent lines (normal protein digestibility sorghums) (NPD1 to 5), either parent or progeny lines, were all tan-plant, non-tannin sorghums. Macia, (developed from SDS 3220, ICRISAT SMIP) cultivated at Makoro Lands, Central District, Botswana, 2004, is a popular improved tan plant, non-tannin variety commonly cultivated and used for food preparation in southern Africa.

Where indicated, small samples were received as 10 to 15 single half or crushed kernels, and bulk samples (3-15 kg) were received as whole grains, or whole crushed grains. For chemical analyses, whole or crushed bulk samples were milled into flour using a hammer mill fitted with a 500 µm opening screen. Small samples were milled by hand using a mortar and pestle. All samples were stored at ±8°C until use.

Table 4.1.1 Transgenic, non-transgenic null control, parent lines, high protein digestibility mutant and normal sorghum lines studied.

Sorghum lines	Project Code	Sample type	Source
Transgenic (type II tannin)			
ABS042 (AGNM 42-2B)	TG-ABS042-1	H/CG	Pretoria (2007)[1]
ABS042 (AGNM 42-5C)	TG-ABS042-2	H/CG	Pretoria (2007)[1]
ABS044 (AGNM 44-1A)	TG-ABS044-1	H/CG	Pretoria (2007)[1]
ABS044 (AGNM 44-2G)	TG-ABS044-2	H/CG	Pretoria (2007)[1]
ABS044 (AGNM 44-3A)	TG-ABS044-3	H/CG	Pretoria (2007)[1]
Controls (type II tannin)			
P898012 (null control tissue culture)	P898012-TC	H/CG	Pretoria (2007)[1]
P898012 (parent)	P898012-H/CG	H/CG	Pretoria (2007)[1]
P898012 (parent)	P898012-Bulk	Bulk (WCG)	Johnston, Iowa (2007)[2]
High Protein Digestibility Mutants (HPDM) (non-tannin)			
HD parent PI851171 WES	HPDM1	Bulk (WG)	Weslaco, Texas (2006)[3]
HD parent PI850029 WES	HPDM2	Bulk (WG)	Weslaco, Texas (2006)[3]
HD parent PI851171 LUB	HPDM3	Bulk (WG)	Lubbock, Texas (2006)[3]
HD Progeny 04CS11248-1 XTX436 WES	HPDM4	Bulk (WG)	Weslaco, Texas (2006)[3]
HD Progeny 04CS112278 X 851171 / 96GCP0124 WES	HPDM5	Bulk (WG)	Weslaco, Texas (2006)[3]
HD progeny 04CS11186-1 X 850029 / TX635 WES	HPDM6	Bulk (WG)	Weslaco, Texas (2006)[3]
HD Progeny 04CS11278 X 851171 and 96GCPO124 LUB	HPDM7	Bulk (WG)	Lubbock, Texas (2006)[3]
Normal Protein Digestibility (NPD) (non-tannin)			
LD parent 96GCPOB124 WES	NPD1	Bulk (WG)	Weslaco, Texas (2006)[3]
LD parent BTX436 WES	NPD2	Bulk (WG)	Weslaco, Texas (2006)[3]
LD parent TX635 WES	NPD3	Bulk (WG)	Weslaco, Texas (2006)[3]
LD Parent 96GCPOB124 LUB	NPD4	Bulk (WG)	Lubbock, Texas (2006)[3]
LD progeny 04CS11199-1 X 850029 / TX635 WES	NPD5	Bulk (WG)	Weslaco, Texas (2006)[3]
Macia	Macia	Bulk (WG)	Botswana (2004)

[1]CSIR Biosciences, Pretoria, South Africa. [2]Pioneer Hi-Bred, Des Moines, Iowa, USA. [3]Texas A&M University, College Station, Texas, USA. H/CG = Half/Crushed grain. WCG = Whole crushed grain. WG = Whole grain.

4.1.3.2 Physical characterisation

Thousand kernel weight was measured by weighing 1000 sound grains. Grain size (small, medium or large) was characterised by sieving 100 g of clean grain through two test sieves with mesh openings of 4.00 mm followed by 2.36 mm (Gomez, Obilana, Martin, Madzvamuse and Monyo, 1997). Hectolitre weight (Test weight) was measured according to AACC standard method 55-10 (AACC International, 2000). These analyses were performed in triplicate.

Pericarp colour (white, lemon yellow or red) and glume colour (tan, red or purple) was determined as described by Rooney and Miller (1982). Endosperm colour (white or red) and pericarp thickness (thick, intermediate or thin) was determined subjectively by viewing longitudinal sections of 20 half kernels under a stereomicroscope as described by Rooney and Miller (1982).

Endosperm texture, defined here as the proportion of corneous endosperm relative to floury endosperm in the grain was determined by viewing longitudinal sections of 20 half kernels under a stereomicroscope, and comparing them to sorghum standards (ICC, 2008). Due to the small sample size of the transgenic lines analyzed, all half kernels obtained were viewed (± 6 kernels). Light micrographs were taken of longitudinal kernel sections using a stereomicroscope (Nikon Optiphot, Tokyo, Japan) fitted with a digital camera (Nikon SMZ800, Tokyo, Japan).

Whole grains were characterised as tannin or non-tannin by determining the presence or absence of a pigmented testa of 100 sound grains using the Bleach Test as described by Taylor and Taylor (2008).

4.1.3.3 Chemical characterisation

Condensed tannin content was determined using the modified Vanillin HCl assay (1% concentrated HCl in methanol extraction) according to Maxson and Rooney (1972), with subtraction of sample blanks. Catechin hydrate (Sigma, St Louis, MO) was used as a standard, and tannin content was expressed as mg catechin

equivalents per 100 g flour (mg CE/100 g). Total protein content (N x 6.25), was determined by Dumas combustion, AACC standard method 46-30 (AACC International, 2000). Lysine was determined after defatting three times with cold hexane at a ratio of 1:5 hexane to flour, using reverse phase high performance liquid chromatography, Pico-Tag method (Bidlingmeyer, Cohen and Tarvin, 1984). Moisture content was determined by air oven drying using AACC standard method 44-15A (AACC International, 2000). For small samples (10 to 15 kernels), 10% moisture content was assumed. All analyses are expressed on a dry weight basis.

4.1.3.4 In vitro protein digestibility

In vitro protein digestibility (IVPD) was determined on whole grain flour under wet cooked conditions, using either 200 mg (large-scale, LS) or a 20 mg (small-scale, SS) flour scale using the pepsin digestibility method of Hamaker et al. (1986), suitably modified for small-scale assays (200 mg) (Taylor and Taylor, 2011). Briefly, the total protein content (N x 6.25) of whole sorghum flour was determined by the Dumas combustion method. For pepsin digestion of raw grain, samples (200 mg for LS or 20 mg for SS) were suspended in citrate-pepsin buffer (pH 2.0) (35 ml, 1.05 mg pepsin (porcine gastric mucosa, (Sigma-Aldrich, P7000) / ml for LS or 1.0 ml, 3.67 mg pepsin / ml, plus 0.75 ml citrate buffer (pH 2.0) for SS) in centrifuge or Eppendorf tubes with caps, respectively. Samples were mixed and incubated for 2 h at 37°C in a water bath with mixing every 15 min. Pepsin digestion was terminated by the addition of 2 M sodium hydroxide (2 ml for LS, or 100 µl for SS). Samples were centrifuged (3380 x g for 10 min for LS, or 7200 x g for 10 min for SS) and the supernatant was removed. The residue was washed once with distilled water (35 ml for LS, or 1 ml for SS), re-centrifuged and the supernatant removed. The residue was dried in the tubes at 100°C overnight in a forced draft oven and the protein content of the dried residue was determined by the Dumas combustion method. Protein digestibility was then defined as the percentage nitrogen solubilised under the conditions of the assay relative to flour total nitrogen.

For pepsin digestion under wet cooked conditions, samples (200 mg for LS or 20 mg plus a glass bead for SS) were suspended in distilled water (2 ml for LS, or 0.25 ml

for SS) in centrifuge or Eppendorf tubes with caps, respectively. The tubes were immersed in a boiling water bath for 15 min, and citrate buffer (pH 2.0) was immediately added (2 ml for LS, or 0.5 ml for SS). Samples were vortexed vigorously to avoid lumping of the cooked flour. Citrate-pepsin buffer (pH 2.0) (28 ml, 1.31 mg pepsin / ml buffer for LS, or 1.0 ml, 3.67 mg pepsin / ml buffer for SS) was added, vortexed vigorously and incubated for 2 h at 37°C in a water bath with mixing every 15 min. The method continued as for raw samples described above. For SS method, the glass bead was removed before centrifugation. Samples were analysed in duplicate for both assays and repeated at least once to give a total of four results per sample per assay.

4.1.3.5 Transmission electron microscopy

For transmission electron microscopy (TEM), grains were sectioned longitudinally using a sharp scalpel. The pericarp was scraped from the top of the kernel directly opposite the germ, leaving the sub-pericarp and aleurone layers intact. In brief, the preparation procedure involved taking small sections (1 to 2 mm thick) of cleaned peripheral endosperm using a sharp scalpel. Specimens were fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer, pH 7.4, for 18 h and rinsed three times (10 min each) in the same buffer, then fixed in 0.5% aqueous osmium tetroxide for 3 h, and rinsed three times in distilled water. The specimens were dehydrated in a graded aqueous acetone series, 2 h each in 50, 70, 90% aqueous acetone and twice in 100% acetone for 30 min and stored overnight in 100% acetone, before gradual infiltration with Quetol resin and polymerised at 60°C for 2 days. Ultrathin sections were cut with an ultra-microtome fitted with a diamond knife. Sections were stained with 4% aqueous uranyl acetate, then further stained in Reynold's lead citrate. Sections were examined either with a Phillips EM301 or a Phillips CM10 TEM (Eindhoven, Netherlands).

4.1.3.6 Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) at a confidence level of $p<0.05$. Means were compared by Fisher's least significant difference (LSD)

test. The calculations were performed using Statsgraphics Centurion XV (Stat Point, Herndon, VA).

4.1.4 Results and Discussion

4.1.4.1 Physical characterisation

The transgenic sorghum lines (TG-ABS042 and TG-ABS044) all had floury endosperm texture (Figure 4.1.1e-f, i-j, respectively). Their normal sorghum parent (P898012) and null controls all had intermediate endosperm texture (Figure 4.1.1a, g-h, k-l, respectively). The HPDM also had floury endosperm texture but its parent had corneous endosperm texture, as did Macia (Figure 4.1.1d, c and b, respectively). Generally, the HPDM had lower 1000 kernel weight and hectolitre weight than the normal sorghums, even though they had similar grain size (Table 4.1.2). Floury endosperm appearance is caused by a discontinuous protein matrix, smaller and fewer kafirin protein bodies and loosely packed starch granules with air-filled spaces that diffracts light (Rooney and Miller, 1982). Many of the transgenic grains derived from the constructs ABS042 and ABS044, and to a lesser extent their normal parent, P898012, showed a distinct lumen (small hole) in the centre of the endosperm (Figure 4.1.1, black arrows). It would appear that floury endosperm texture is due to target kafirin suppression, specifically the ɣ-kafirins.

4.1.4.2 Chemical characterisation

All the P898012 lines were type II tannin sorghums according to the classification given by Maxson and Rooney (1972). The parent showed typical tannin staining of the corneous endosperm (Figure 4.1.1, black dashed arrow). This is due to tannin leaching from the testa layer and binding to the proteins in the dense peripheral endosperm tissue. Tannin leaching was not visible in the transgenic sorghum lines with floury endosperm. Nevertheless, a pigmented testa layer was visible between the pericarp and endosperm layers of these grains, (Figure 4.1.1, white arrows).

The tannin content of the parent, P898012, was measured and found to contain 2.77 g CE/100 g flour, when extracted with acidified methanol (data not shown). All the other sorghum lines were classified as type I (non-tannin) sorghums as they contained no tannins, and did not have a pigmented testa (Table 4.1.2).

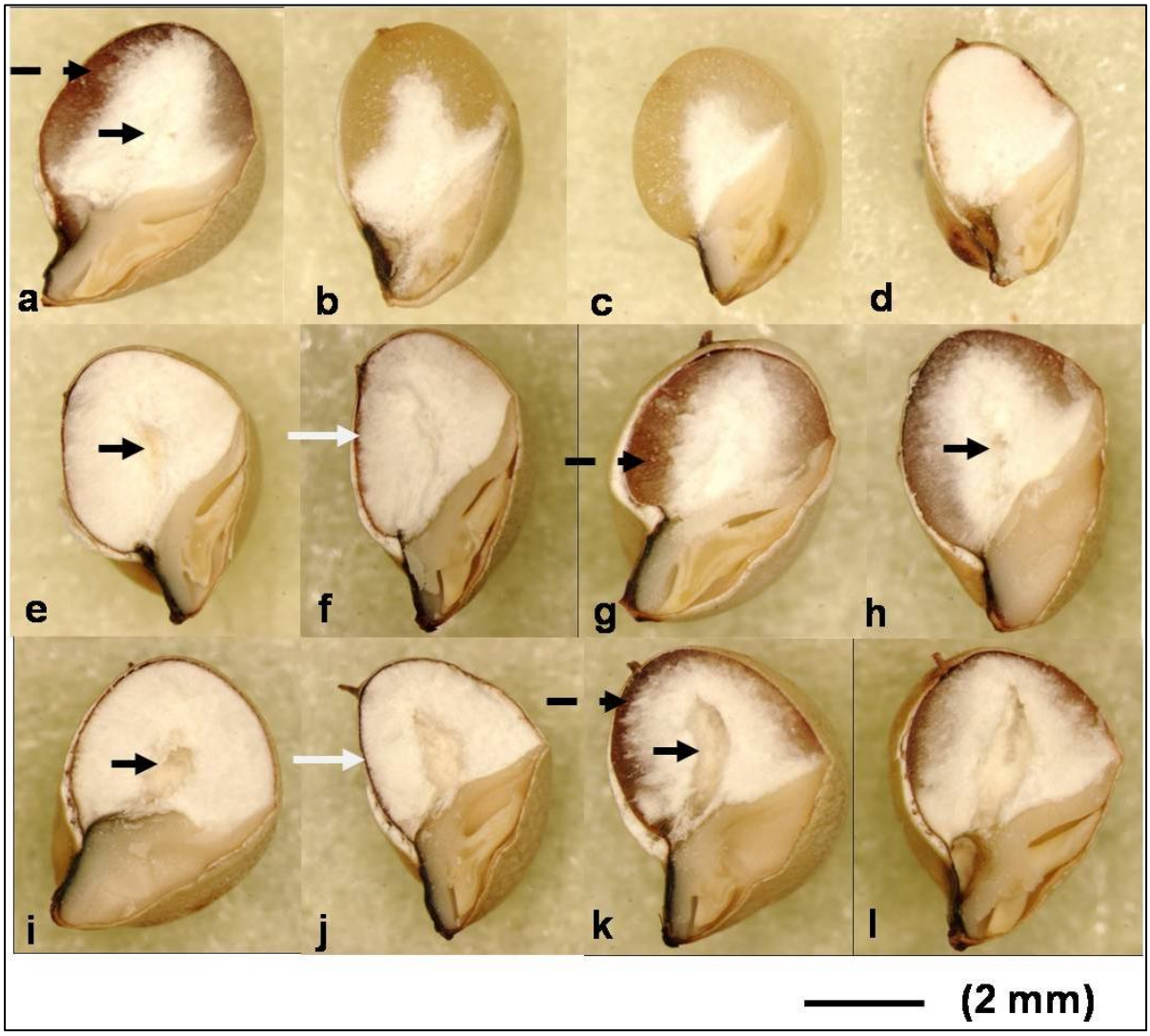

Figure 4.1.1 Longitudinal cross-sections of whole sorghum grains.
a) P898012; b) Macia; c) NPD-2; d) HPDM-1, e-f) TG-ABS042, g-h) Null controls - ABS042; i-j) TG-ABS044; k-l) Null controls - ABS044. Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining; white arrow indicates pigmented testa layer.

Table 4.1.2 Physical properties of high protein digestibility mutant sorghums compared to normal parents and Macia.

Sorghum line	Pigmented testa / (Tannin type)	Glume colour	Pericarp colour	Pericarp thickness	Endosperm colour	Endosperm texture	Grain size[1]	1000 kernel weight (g)[2]	Hectolitre weight (kg)[2]
HPDM									
HPDM-1	No	Purple	Lemon yellow	Thin	White	Floury	Medium	21.8^{d} ±0.0	73.9^{e} ±0.2
HPDM-2	No	N/D	Lemon yellow	Thin	White	Floury	Medium	24.6^{g} ±0.1	73.0^{d} ±0.1
HPDM-3	No	Purple	Lemon yellow	Thin	White	Floury	Medium	24.6^{g} ±0.1	66.4^{b} ±0.1
HPDM-4	No	Red	Lemon yellow	Intermediate	White	Floury	Medium	24.4^{g} ±0.1	70.9^{c} ±0.5
HPDM-5	No	Red	Lemon yellow	Intermediate	White	Floury	Medium	16.8^{a} ±0.0	73.2^{d} ±0.2
HPDM-6	No	N/D	Lemon yellow	Intermediate	White	Floury	Medium	21.9^{d} ±0.0	75.9^{f} ±0.3
HPDM-7	No	Tan	Lemon yellow	Thin	White	Floury	Medium	18.2^{b} ±0.1	63.8^{a} ±0.2
						Mean and Range		**21.7^{A} (16.8-24.6)**	**71.0^{A} (63.8-75.9)**
NPD									
NPD-1	No	N/D	Red	Thin	White	Corneous	Medium	27.4^{h} ±0.2	79.3^{i} ±0.2
NPD-2	No	Tan	Lemon yellow	Thick	White	Corneous	Medium	23.6^{e} ±0.1	77.0^{g} ±0.2
NPD-3	No	Tan	Lemon yellow	Thin	White	Corneous	Medium	20.8^{c} ±0.1	77.8^{h} ±0.4
NPD-4	No	Tan	Lemon yellow	Thin	White	Corneous	Medium	24.2^{fg} ±0.2	N/D
NPD-5	No	Tan	Lemon yellow	Thin	White	Corneous	Medium	31.4^{i} ±0.3	79.5^{i} ±0.0
Macia	No	Red	Lemon yellow	Thin	White	Intermediate	Medium	24.1^{f} ±0.2	75.7^{f} ±0.2
						Mean and Range		**25.3^{B} (20.8-31.4)**	**77.5^{B} (75.7-79.5)**
							Mean	23.4	74.3
							Min	16.8	63.8
							Max	31.4	79.5

HPDM-High Protein Digestibility Mutant, NPD-Normal Protein Digestibility. [1]Grain size: small <2.36 mm, medium >2.36 mm to <4.00 mm, large >4.00 mm. [2]Each value represents the grand mean and standard deviation for each cultivar, analyses were done in triplicate. Values with different letters in a column are significantly different at the 95% level using Fisher's least significant difference (LSD) procedure (small letters between cultivars, capital letters between sorghum groups).

In terms of protein content, the transgenic sorghums had a similar mean protein content (12.5%) to their controls (11.7%) and the HPDM (12.8%) and NPD sorghums (13.2%) (Table 4.1.3). These protein contents are all well within the range of 10.0 to 17.2% for native high-lysine sorghums (Singh and Axtell, 1973; Guiragossian et al., 1978), 10.2 to 14.7% for high-protein digestibility high-lysine mutant sorghums (Weaver et al., 1998; Tesso et al., 2006) and 8.1 to 16.8% for normal sorghums (reviewed by Rooney and Serna-Saldivar, 1990).

In contrast, the mean lysine content (expressed as g/100 g flour or protein) differed significantly between the different sorghum types (Table 4.1.3). The TGs had the highest mean lysine content (0.35 g/100 g flour and 2.48 g/100 g protein), compared to the NC, HPDM and normal lines. However, significant differences between the different transgenic sorghum lines were also observed, with TG-ABS044 having the highest lysine content (0.43 g/100 g flour and 3.06 g/100 g protein) compared to TG-ABS042. TG-ABS044 line had twice as much lysine as that for the normal sorghums, and was approximately 50% higher than that of the HPDM when expressed on a flour or protein basis. The lysine content of TG-ABS044 is similar to those reported for native high-lysine (0.44 to 0.54 g/100 g flour, and 3.2 to 3.7 g/100 g protein) (Singh and Axtell, 1973; Guiragossian et al., 1978) and high-lysine high-protein digestibility mutant sorghums (0.36 to 0.39 g/100 g flour, and 2.9 to 3.4 g/100 g protein) (Weaver et al., 1998; Tesso et al., 2006). The high-lysine content of TG-ABS044 is most probably due to suppressed synthesis of kafirin proteins, and compensatory synthesis of lysine-rich non-kafirin proteins. However, the increased lysine content reported here and in the literature is still below that recommended by the WHO (4.8 g lysine/100 g protein for 4-18 year olds) for foodstuffs to meet basic protein requirements (WHO/FAO/UNU Expert Consultation, 2007).

Table 4.1.3 Protein and lysine content of different transgenic and high protein digestibility mutant sorghums compared to normal sorghum parents and Macia.

Sorghum lines	Protein (g/100 g flour dry base) (N x 6.25)	Lysine (g/100 g flour)	Lysine (g/100 g protein)
Transgenic (type II tannin)			
TG-ABS042-1	10.57±0.22	0.30^{efg} ±0.02	2.36^{cde} ±0.12
TG-ABS042-2	9.07±0.32	0.32^{g} ±0.04	2.04^{e} ±0.31
TG-ABS044-1	16.03±0.01	0.43^{h} ±0.00^{1}	3.06^{f} ±0.00^{1}
TG-ABS044-2	14.85 ±0.29	Pooled^{1}	Pooled^{1}
TG-ABS044-3	12.12±0.19	Pooled^{1}	Pooled^{1}
Mean and Range	***12.53^{A} (9.07-14.85)***	***0.35^{C} (0.30-0.43)***	***2.48^{C} (2.04-3.06)***
Controls (type II tannin)			
P898012-TC	10.19±0.21	ND	ND
P898012-H/CG	13.95±3.81	ND	ND
P898012-Bulk	10.90±0.51	0.24^{cd} ±0.01	2.05^{bc} ±0.06
Mean and Range	***11.68^{A} (10.19-13.95)***	***0.24^{B}***	***2.05^{B}***
High protein digestibility mutant (HPDM) (non-tannin)			
HPDM1	10.36±0.01	0.27^{cdef} ±0.02	2.10^{bcd} ±0.12
HPDM2	13.21±0.19	0.31^{fg} ±0.04	2.41^{de} ±0.31
HPDM3	14.61±0.05	ND	ND
HPDM4	11.90±0.04	0.26^{cde} ±0.02	2.04^{bc} ±0.18
HPDM5	10.83±0.03	0.28^{defg} ±0.01	2.22^{cde} ±0.06
HPDM6	13.37±0.12	0.24^{bc} ±0.01	1.87^{b} ±0.06
HPDM7	15.02±0.01	ND	ND
Mean and range	***12.76^{A} (0.36-15.02)^{A}***	***0.27^{B} (0.24-0.31)^{B}***	***2.13^{B} (1.87-2.41)***
Normal protein digestibility (NPD) (non-tannin)			
NPD1	13.44±0.02	0.18^{a} ±0.02	1.29^{a} ±0.11
NPD2	12.16±0.05	0.17^{a} ±0.02	1.25^{a} ±0.17
NPD3	14.51±0.07	0.19^{a} ±0.02	1.37^{a} ±0.11
NPD4	15.12±0.17	ND	ND
NPD5	13.46±0.06	0.16^{a} ±0.01	1.20^{a} ±0.06
Macia	10.61±0.01	0.20^{ab} ±0.03	1.87^{b} ±0.23
Mean and range	***13.00^{A} (10.61-15.12)***	***0.19^{A} (0.16-0.20)***	***1.50^{A} (1.20-1.87)***

Values are the mean and standard deviation from at least two replicate samples. Values with different letters in a block are significantly different at the 95% level using Fisher's least significant difference (LSD) procedure. (Small letters between cultivars, capital letters between sorghum groups (transgenic, high digestibility mutants and normal)). ^{1}ABS044-1, 2 and 3 samples pooled for amino acid analysis. ND = not determined.

4.1.4.3 In vitro protein digestibility

The *in vitro* protein digestibility (IVPD) of cooked flours from the TG sorghum lines with the ABS044 gene construct was substantially higher (minimum 48.9%, maximum 56.2%) than its controls (minimum 19.4%, maximum 37.0%) (Table 4.1.4). In fact, the IVPD of ABS044 (mean 53.7%) was similar to that of the HPDM sorghums (mean 57.7%). This is despite the fact that the ABS044 sorghum contained tannins, which are well-known to reduce sorghum protein digestibility (Duodu et al., 2003). However, the IVPD of the ABS042 gene construct (minimum 27.6%, maximum 39.9%) was not significantly different from the controls.

As expected, the HPDM sorghums had substantially higher IVPD than their normal parent and progeny. However, the IVPD of HPDM was similar to that of Macia (Table 4.1.4). The cooked IVPD values of the HPDM sorghums in this present study were much lower than those of HPDM sorghums previously reported, which had cooked digestibilities of decorticated grain ranging from 72.5 to 80.8% (Weaver et al.; 1998; Tesso et al.; 2006). This is probably due to the fact that whole milled grain was used in this study. Duodu et al. (2003) in a review of factors affecting sorghum protein digestibility, presented evidence that polyphenols and phytic acid, which are both concentrated in the external layers of the grain can adversely affect protein digestibility.

Table 4.1.4 In vitro protein digestibility (IVPD) of raw and wet cooked whole grain flour of different transgenic and high protein digestibility mutant sorghum compared to normal parents and Macia.

Sorghum lines	IVPD (%) Raw	IVPD (%) wet cooked
Transgenic (type II tannin)		
ABS042-1	ND	33.5^{a} ±8.3 (27.6-39.4)
ABS042-2	ND	35.7^{a} ±6.0 (31.5-39.9)
ABS044-1	ND	55.4^{b} ±1.2 (54.5-56.2)
ABS044-2	ND	54.8^{b} ±0.8 (54.3-55.4)
ABS044-3	ND	50.9^{b} ±2.9 (48.9-53.0)
Mean and Range		***46.1^{B} (31.5-56.2)***
Controls (type II tannin)	ND	
P898012-TC	ND	29.1^{a} ±9.3 (19.4-41.1)
P898012-H/CG	ND	30.7^{a} ±6.2 (23.0-37.0)
P898012-Bulk	65.0^{e} ±7.3 (58.1-72.3)	25.6^{a} ±1.5 (24.2-27.1)
Mean and Range	***65.0^{B} (58.1-72.3)***	***28.5^{A} (19.4-41.1)***
High protein digestibility mutant (HPDM) (non-tannin)		
HPDM1	71.9^{f} ±0.8 (71.0-72.8)	60.8^{fgh} ±1.0 (59.4-61.6)
HPDM2	67.8^{e} ±0.9 (66.7-68.7)	55.2^{de} ±1.5 (53.6-57.2)
HPDM3	75.2^{fg} ±1.3 (73.8-76.7)	61.4^{gh} ±1.6 (59.5-63.3)
HPDM4	68.3^{e} ±0.8 (67.4-69.2)	51.9^{c} ±5.6 (44.0-56.9)
HPDM5	72.7^{f} ±2.2 (70.5-75.1)	58.1^{ef} ±1.6 (56.7-59.8)
HPDM6	65.6^{e} ±0.6 (65.0-66.4)	53.1^{cd} ±1.3 (51.3-54.1)
HPDM7	76.3^{g} ±1.6 (74.8-78.1)	63.1^{h} ±1.3 (61.7-64.6)
Mean and Range	***71.1^{B} (65.0-78.1)***	***57.7^{C} (51.3-64.6)***
Normal protein digestibility (NPD) (non-tannin)		
NPD1	42.3^{bc} ±1.7 (40.6-44.2)	33.3^{a} ±2.3 (30.3-35.8)
NPD2	43.9^{c} ±0.8 (43.1-44.7)	36.4^{b} ±1.7 (34.4-38.4)
NPD3	39.8^{ab} ±1.4 (38.5-41.2)	33.1^{a} ±1.6 (31.3-35.2)
NPD4	48.3^{d} ±1.8 (46.5-50.3)	36.7^{b} ±2.3 (34.2-39.8)
NPD5	36.7^{a} ±2.0 (34.7-38.8)	32.6^{a} ±1.1 (31.5-33.8)
Macia	75.0^{fg} ±1.4 (72.8-76.4)	59.8^{fg}±0.7 (58.9-60.3)
Mean and Range	***50.1^{A} (34.7-76.4)***	***39.9^{AB} (30.3-60.3)***

Values are the mean, standard deviation and (range), from at least two replicate samples. Values with different letters in a block are significantly different at the 95% level using Fisher's least significant difference (LSD) procedure.

4.1.4.4 Transmission electron microscopy

The protein body structure of the transgenics was studied to assess a possible link between endosperm texture and protein digestibility, following suppression of synthesis of ɣ- and α-kafirin proteins. The protein bodies of TG-ABS044 had modified peripheral endosperm protein body structure (Figure 4.1.2c,d), compared to the parent P898012 (Figure 4.1.3a,b). The protein bodies of TG-ABS044 were densely packed, 2-3 µm diameter and deeply folded (invaginated) (black dashed arrows). The protein bodies were similar to those of the HPDM (Figure 4.1.2e,f). However, the protein bodies of the HPDM were smaller (approximately 1 µm diameter.). The protein bodies of TG-ABS044 and HDPM were also similar in that the majority of inclusions seemed to extend radially from the protein body periphery (black dashed arrows). In addition, the characteristic internal concentric ring structure common to normal protein bodies (Figure 4.1.3, black arrows) was absent. The irregular protein body structure of the TG-ABS044 line is consistent with it having higher cooked IVPD to that of the controls (Table 4.1.4). According to Oria et al. (2000), the improved protein digestibility of mutant sorghum lines, having invaginated protein bodies, could be due to increased protein body surface area and easy accessibility of digestive enzymes to the more digestible α-kafirin proteins.

In contrast, the protein bodies from the TG-ABS042 (Figure 4.1.2a,b) appeared to be identical to the protein bodies of its normal control, P898012 (Figure 4.1.3a,b). These protein bodies of TG-ABS042 were densely packed, individual spherical protein bodies, 2-3 µm in diameter. Many of the protein bodies exhibited the internal concentric ring structure (black arrows), observed in the protein bodies of normal sorghum (Shull et al., 1992). The normal protein body structure of the TG-ABS042 line is consistent with it having the same cooked IVPD to that of the controls (Table 4.1.4). In normal sorghum protein bodies, highly cross-linked kafirin proteins (ɣ- and β-kafirins) are found at the protein body periphery and as dark-staining inclusions (in the form of concentric rings) within the protein body's interior (Hamaker et al., 1987; Shull et al., 1992; Oria et al., 1995).

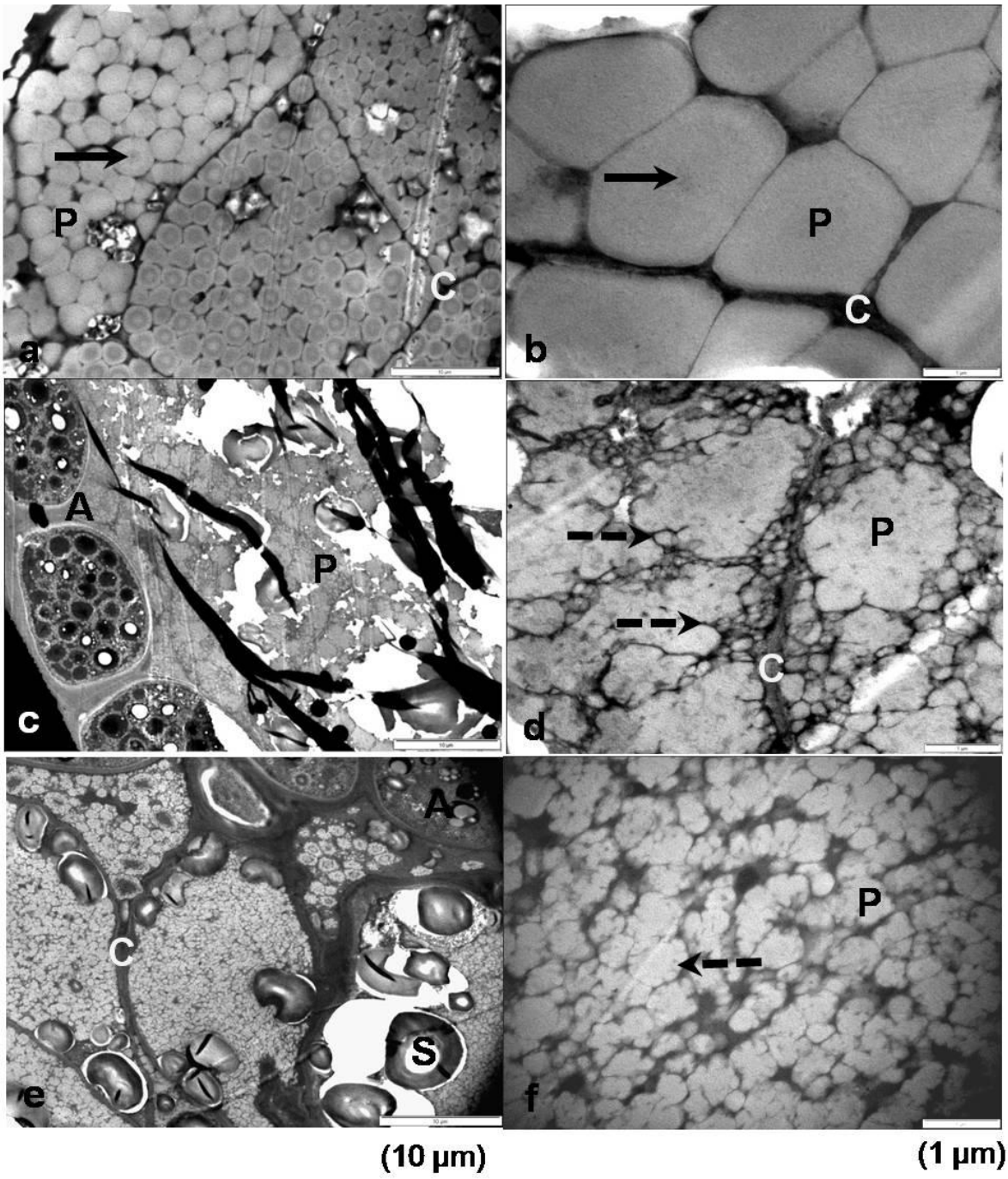

Figure 4.1.2 Transmission electron micrographs of protein bodies in the peripheral endosperm of transgenic and HPDM lines.
a-b) TG-ABS042; c-d) TG-ABS044; e-f) HPDM-1. Where indicated: A, aleurone; C, cell wall; P, protein body; S, starch granule; black arrows indicate dark-staining inclusions within protein bodies; Black dashed arrows indicate deep folds (invaginations) in protein body periphery.

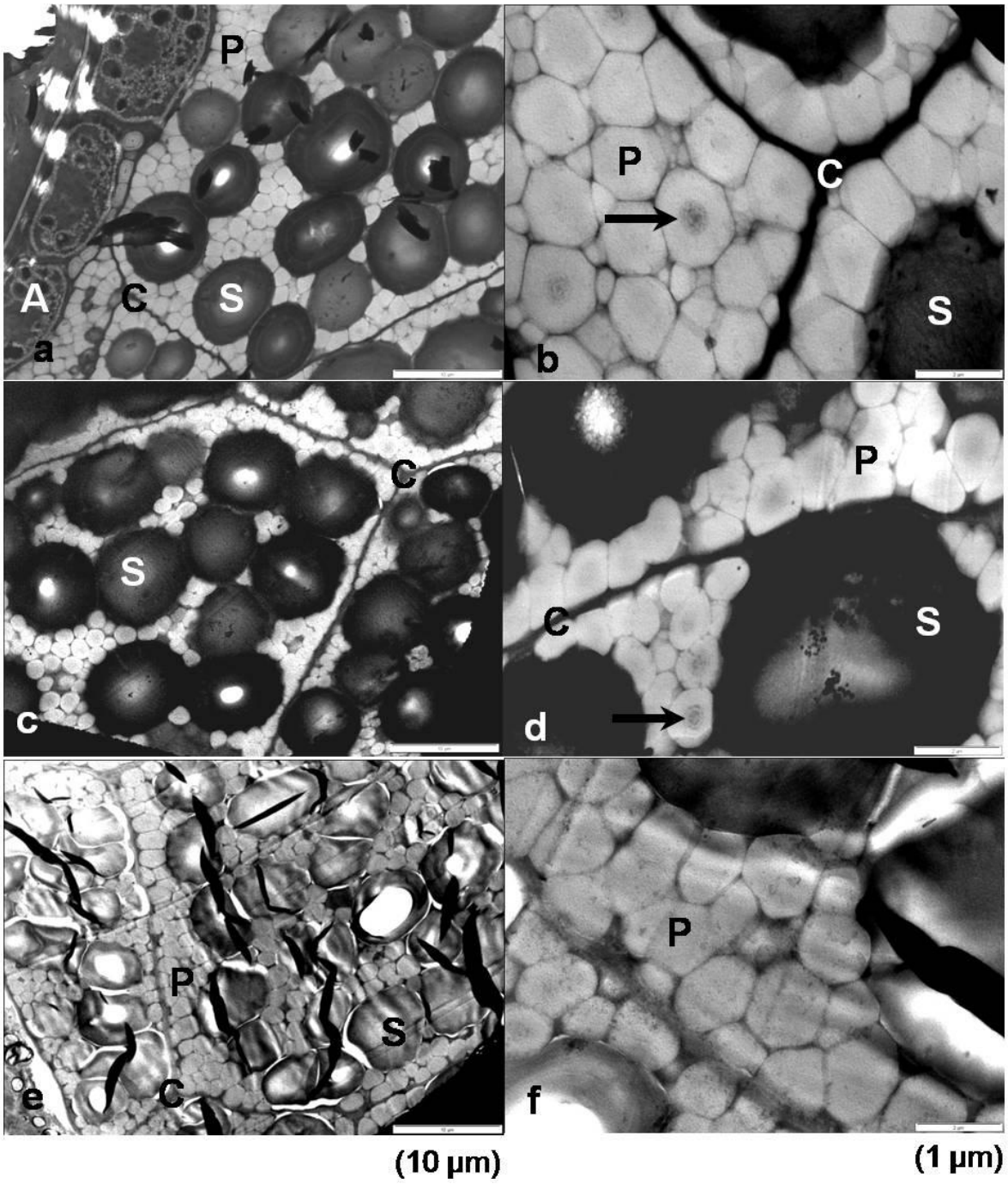

Figure 4.1.3 Transmission electron micrographs of protein bodies in the peripheral endosperm of normal sorghum lines.
a-b) P898012; c-d) NPD-2; e-f) Macia. Where indicated: A, aleurone; C, cell wall; P, protein body; S, starch granule; black arrows indicate dark-staining inclusions within protein bodies.

4.1.5 Conclusions

Significant improvements in lysine content and protein digestibility were obtained in transgenic sorghum with combined suppression of α- and ɣ-kafirin synthesis. It would appear suppressed ɣ-kafirin synthesis is responsible for floury endosperm texture, but co-suppression of α- and ɣ-kafirin synthesis results in irregular protein body structure. Irregular protein body structure, regardless of the genotype (transgenic or high protein digestibility mutant sorghum), seems to be associated with improved cooked protein digestibility. Due to the fact that the TG lines used in this present study contained tannins, the levels of digestibility obtained are potentially higher than that found, as tannins are well known to interfere with protein digestion.

4.1.6 References

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4.2 Effect of suppressing the synthesis of different kafirin sub-classes on grain endosperm texture, protein body structure and protein nutritional quality in improved sorghum lines

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4.2.1 Abstract

To improve sorghum grain protein nutritional quality, improved sorghum lines were transformed to suppress the synthesis of different kafirin sub-classes, or back-crossed into transgenic lines with improved protein quality. Co-suppression of the α-, ɣ- and δ-kafirin sub-classes and removal of the tannin trait resulted in transgenic sorghum lines with high cooked protein digestibility (±80%), improved Amino Acid Score (0.8) and Protein Digestibility Corrected Amino Acid Score (0.7) compared to the non-transgenic null controls (±50%, 0.4 and 0.2, respectively). These high-protein quality lines had a floury endosperm. They also had modified protein body structure, where the protein bodies were irregular shaped with few to numerous invaginations and were less densely packed, with a dense protein matrix visible around the protein bodies. When fewer sub-classes were suppressed, i.e. only ɣ-kafirin-1 and δ-kafirin, the endosperm was corneous with normal protein body structure but the improvement in cooked protein digestibility appeared to be less. Apparently, co-suppression of synthesis of several kafirin sub-classes is required to obtain high protein nutritional quality sorghum lines, but this seems to result in floury-type grain endosperm texture.

4.2.2 Introduction

Sorghum is a major source of protein for people in tropical and subtropical developing countries (FAO, 2002; ICRISAT, 2009). However, the nutritional quality of sorghum protein is of concern. Sorghum proteins are very deficient in the indispensable (essential) amino acid lysine, due to the kafirin storage proteins being essentially free of lysine (Shewry, 2007). Additionally, sorghum proteins have lower cooked protein digestibility compared to other cereals, reducing the bioavailability of the protein (Duodu et al., 2003). The reasons for the lower protein digestibility of cooked sorghum are multifactorial, including extensive polymerisation of the kafirins upon cooking, the location and organisation of the different kafirin sub-classes in the protein bodies, and the presence of tannins in certain sorghum lines (Duodu et al., 2003).

Efforts to address sorghum protein nutritional quality started with identification of native high-lysine sorghum genotypes from Ethiopia (Singh and Axtell, 1973) and was followed by chemical mutagenesis to develop a high-lysine genotype (P721 opaque) (Mertz et al., 1993). The different high-lysine native and mutant genotypes were found to have 50 and 60% increased lysine content, respectively (Singh and Axtell, 1973; Mertz et al., 1993). Improved lysine contents were attributed to decreased levels of kafirin proteins and increased levels of lysine–rich, non-kafirin proteins in the grain endosperm (Shewry, 2007). However, poor grain quality, especially soft and floury endosperm texture, is common in high-lysine cereals (Shewry, 2007), including sorghum (Weaver et al., 1998; Tesso et al., 2006).

Breeding using the P721 opaque (P721Q) line has been undertaken to produce high-lysine genotypes with improved grain hardness and protein digestibility after cooking (Weaver et al., 1998; Tesso et al., 2006). Electron microscopy and immunological studies of these high-lysine high-protein digestibility mutants showed their grain to have modified protein bodies (irregular shapes with deep invaginations), compared to the spherical protein bodies of normal sorghums (Oria et al., 2000). The location and organisation of different kafirin sub-classes within the

protein bodies also differed with the ɣ-kafirins being located at the bottom of folds, exposing the more digestible α-kafirins to digestive enzymes.

Advances in sorghum tissue culture and transformation research have led to the development of the first nutritionally improved transgenic sorghum (Zhao et al., 2003). Nutritionally improved sorghum lines are being developed using genetic engineering techniques by the Africa Biofortified Sorghum (ABS) Project, under the Bill and Melinda Gates Foundation Grand Challenges in Global Health initiative (ABS Project, 2009). Early transformation work was done using a tannin sorghum line (P890812), with poor end-use quality. This study describes the effect of suppressing different kafirin sub-classes on the grain endosperm texture, protein body structure and protein nutritional quality in different transgenic lines produced by *Agrobacterium*-mediated transformation and through backcrossing into improved normal sorghum lines, with the aim of developing sorghum types of improved protein quality and good functional properties.

4.2.3 Materials and Methods

4.2.3.1 Grain samples and whole grain flour preparation

Six different transgenic (TG) sorghum lines (plus six non-transgenic null controls) (NCs) developed for the ABS project using *Agrobacterium*-mediated transformation as described by Zhao et al. (2000) or backcrossing were developed by Pioneer Hi-Bred (Table 4.2.1). Three different gene constructs (ABS032, ABS166, and ABS149) each designed to suppress the synthesis of different kafirin sub-classes within the grain endosperm were used, namely: α-kafirin A1 (25 kDa) and α-kafirin B1 and B2 (which correspond to 19 kDa and 22 kDa α-kafirin classes, respectively, (Belton et al., 2006)), ɣ-kafirin 1 (27 kDa) and 2 (50 kDa), and δ-kafirin (15 kDa). ABS032 gene construct; α-kafirin A1 and ɣ-kafirin 1 for ABS166 gene construct; and δ-kafirin 2 and ɣ-kafirin 1 and 2 for ABS149 gene construct. In addition, reduced expression of lysine ketoglutarate reductase (LKR) was included in the ABS032 and ABS149 gene constructs.

Table 4.2.1 Transgenic sorghum lines, null controls (no kafirin suppression detected) and normal lines studied.

Line and Gene construct	Sample code	n	Supplied by
Macia	Macia	1	BTS, 2004
P898012	P898012 Bulk	1	PHB, 2007
P898012	P898012 H/CG	1	PHB, 2009
Tx430	Tx430	1	PHB, 2009*
Transgenic P898012 (ABS032 gene construct), greenhouse trial, T1 seed, α-, ɣ- and δ-kafirin suppression.	TG-P898012 **(ABS032)**	1	PHB, 2007
P898012, T1 seed, no kafirin suppression.	NC-P898012	1	PHB, 2007
Transgenic P898012 (ABS032 gene construct) backcrossed into Macia, greenhouse trial, F3 seed, α-, ɣ- and δ-kafirin suppression..	TG-P898012xMacia **(ABS032)-1**	4	PHB, 2009
P898012 backcrossed into Macia, greenhouse trial, F3 seed, no kafirin suppression.	NC-P898012xMacia-1	2	PHB, 2009
Transgenic P898012 (ABS032 gene construct) backcrossed into Macia, summer confined field trial, F3 seed, α-, ɣ- and δ-kafirin suppression.	TG-P898012xMacia **(ABS032)-2**	3	PHB, 2009*
P898012 backcrossed into Macia, summer confined field trial, summer confined field trial, F3 seed, no kafirin suppression.	NC-P898012xMacia-2	1	PHB, 2009*
Transgenic P898012 (ABS032 gene construct) backcrossed into Macia, summer confined field trial, F3 seed, α-, ɣ- and δ-kafirin suppression.	TG-P898012xMacia **(ABS032)-3**	3	PHB, 2009*
P898012 backcrossed into Macia, summer confined field trial, F3 seed, no kafirin suppression.	NC-P898012xMacia-3[3]	2	PHB, 2009*
Transgenic Tx430 (ABS166 gene construct), greenhouse trial, T1 seed, α- and ɣ-kafirin suppression.	TG-Tx430**(ABS166)**	6	PHB, 2009
Tx430, greenhouse trial, T1 seed, no kafirin suppression	NC-Tx430-1	6	PHB, 2009
Transgenic Tx430 (ABS149 gene construct), greenhouse trial, T1 seed, δ- and ɣ-kafirin suppression.	TG-Tx430**(ABS149)**	2	PHB, 2009
Tx430, greenhouse trial, T1 seed, no kafirin suppression.	NC-Tx430-2	2	PHB, 2009

H/CG - Half Crushed Grain. BTS – Botswana (University of Pretoria, sorghum collection). PHB - Pioneer Hi-Bred, Des Moines, Iowa. TG- transgenic grain, NC - Non-transgenic null control. n= number of samples or transgenic events received, *samples received as crushed grain (±500 g). All others received as sectioned kernels (±8 kernels).

The NCs were non-transgenic grains obtained from hemizygous transgenic plants and they are the best counterpart to the transgenics for transgene performance assays (Dr R. Jung, Pioneer Hi-Bred, personal communication). Two parent lines (P898012 and Tx430) were used for the different transformations. P898012 is a purple-plant, type II tannin (low tannin) sorghum and Tx430 is a non-tannin, tan-plant, inbred line. Macia (developed from SDS 3220, ICRISAT SMIP) cultivated at Makoro Lands, Central District, Botswana, 2004, a normal non-tannin line, tan plant improved variety popular in southern Africa, was used for backcrossing with a type II tannin TG line, P898012 with ABS032 gene construct (TG-P898012 (ABS032)), with the aim of breeding out the tannin trait and improving grain endosperm quality. Grain from both greenhouse trials and a summer confined field trial were obtained. All TG grain samples were tested and verified by Pioneer Hi-Bred for kafirin suppression. Methods used included sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using kafirin sub-class specific antibodies developed by Pioneer Hi-Bred.

The grain samples analysed in this study were in the form of single sectioned kernels (±8 kernels from greenhouse trials) or crushed whole grains (±500 g, from a summer confined field trial). All samples were milled into flour using a hand-held mill (IKA A11 Basic, Staufen, Germany) until all flour passed through a 500 µm opening screen. All milled flours were stored at ±8°C until analysis.

4.2.3.2 Grain endosperm texture

Endosperm texture, defined as the proportion of corneous endosperm relative to floury endosperm in the grain, was determined subjectively by viewing sectioned kernels (±8 kernels, with or without germ) using a stereomicroscope, and comparing them to sorghum standards. The kernels were classified as corneous, intermediate or floury (ICC, 2008). For crushed grain samples, the meal was sieved through a 1500 µm mesh screen, 8 partially crushed kernels (with or without germ) showing large sections of endosperm were selected and viewed. Light micrographs were taken of all kernel sections using a stereomicroscope (Nikon Optiphot, Tokyo, Japan) fitted with a digital camera (Nikon SMZ800, Tokyo, Japan).

4.2.3.3 Chemical characterization

Condensed tannin content was determined on all bulk samples using the modified Vanillin HCl assay (1% concentrated HCl in methanol extraction) according to Maxson and Rooney (1972), with subtraction of sample blanks. Catechin hydrate (Sigma, St Louis, MO) was used as a standard, and tannin content was expressed as mg catechin equivalents per 100 g flour (mg CE/100 g). Total protein content (N x 6.25), was determined by Dumas combustion, AACC standard method 46-30 (AACC International, 2000). Total amino acid composition was determined using reverse phase high performance liquid chromatography, Pico-Tag method (Bidlingmeyer et al., 1984). Due to small sample size, 10% moisture content was assumed and all data were expressed on a dry weight basis.

4.2.3.4 In vitro protein digestibility

In vitro protein digestibility (IVPD) using pepsin digestion was determined on whole grain flour under raw and wet cooked conditions, using either 200 mg or 20 mg flour scale using the pepsin digestibility method of Hamaker et al. (1986), suitably modified for small-scale assay. In brief, the method involved incubating the flour with pepsin (pepsin from porcine gastric mucosa, power 800 to 2500 units/mg protein, P7000-100G, Sigma-Aldrich) at pH 2.0, 37°C for 2 h. Protein digestibility is defined as the percentage nitrogen solubilized under the conditions of the assay relative to flour total nitrogen. This was measured in terms of insoluble residue by the above Dumas method.

4.2.3.5 Protein nutritional quality

The protein nutritional quality was evaluated by both the Amino Acid Score (AAS) and Protein Digestibility Corrected Amino Acid Score (PDCAAS). The AAS was calculated as g lysine (limiting essential amino acid (EAA)/100 g protein of the sorghum sample/4.8. Where 4.8 g lysine/100 g protein, is the recommendation for quality protein for 4-18 year olds (WHO/FAO/UNU Expert Consultation, 2007). The PDCAAS was determined by multiplying the AAS values with the wet cooked IVPD values obtained.

4.2.3.6 Transmission electron microscopy

Briefly, sections of cleaned peripheral endosperm (1 to 2 mm thick) were fixed in glutaraldehyde in pH 7.4 phosphate buffer (18 h) before staining with osmium tetraoxide. Samples were dehydrated sequentially in acetone. Samples were infiltrated with Quetol resin and polymerised at 60°C. Ultrathin sections were stained with uranyl acetate, and Reynold's lead citrate, and viewed with JEOL JEM 2100F field emission electron microscope (Tokyo, Japan). All images shown depict subaleurone layer endosperm cells.

4.2.3.7 Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) at a confidence level of $p<0.05$. Means were compared by Fisher's least significant difference (LSD) test. The calculations were performed using Statsgraphics Centurion XV (Stat Point, Herndon, VA).

4.2.4 Results and Discussion

4.2.4.1 Tannin content

Backcrossing the tannin-containing TG-P898012 (ABS032) line into Macia (type I, non-tannin sorghum line) was effective in breeding out the tannin trait, as a non-tannin TG line (TG-P898012xMacia (ABS032)-3) was obtained from the summer confined field trial. The non-tannin trait was confirmed by the absence of a pigmented testa layer and no tannins (Table 4.2.2). All other TG lines obtained from backcrossing TG-P898012 (ABS032) with Macia were type II tannin lines, confirmed by the presence of a pigmented testa layer (Figures 4.2.2, 4.2.3, 4.2.4, white arrows) and significant levels of tannin (2.34±0.21 mg CE/100 mg flour) was found in TG-P898012xMacia (ABS032)-2 (Table 4.2.2). The presence of a pigmented testa in tannin-containing sorghum lines is genetically controlled, requiring both B_1 and B_2 dominant genes (reviewed by Dykes and Rooney, 2006).

4.2.4.2 Endosperm texture

Visual examination of kernels from the different TG lines revealed considerable variation in endosperm texture modification compared to their respective NCs and the parent lines. This ranged from completely floury for TG-P898012 (ABS032) (Figure 4.2.2a-c) and backcrosses (TG-P898012xMacia (ABS032) -1, -2 and -3, Figure 4.2.3a-l, 4.2.4a-l, 4.2.5a-i), to corneous for TG-Tx430 (ABS149) (Figure 4.2.7a-f). P898012, TG-P898012 (ABS032) and some of the backcrosses showed a distinct lumen (small hole) in the centre of the grain (Figures 4.2.1 to 4.2.4, black arrows).

The modified endosperm phenotype of TG-P898012 (ABS032) and backcrosses appears to be a direct consequence of the co-suppression of synthesis of several kafirin sub-classes (namely α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2 and δ-kafirin 2, Table 4.2.1) within the endosperm. As stated, in sorghum certain nutritional quality traits such as high essential amino acid content and improved protein digestibility tend to be associated with soft endosperm (Tesso et al., 2006). Similarly, in high-lysine mutant maize (*opaque*-2 and *floury*-2), soft, starchy endosperm texture has been observed (Shewry, 2007). For *floury*-2 mutant, the mutation resulted in a decrease in the synthesis of all sub-classes of zeins, modified endosperm texture and the zein protein bodies being smaller than normal and asymmetrical or misshapen (Lending and Larkins, 1992). Also, the native Ethiopian high-lysine sorghum landrace identified among the world germplasm collection are found to have soft endosperms (Singh and Axtell, 1973).

In contrast to the above types, TG-Tx430 (ABS166) kernels had a modified endosperm texture with a large central floury portion with faint bands or patches of corneous-like endosperm (Figure 4.2.6a-r, black arrows), unlike the corneous endosperm texture of the NC (Figure 4.2.6s-aj) and normal parent line Tx430 (Figure 4.2.1g-i). It is likely that suppression of synthesis of only α-kafirin A1 and ɣ-kafirin 1 in ABS166 gene construct (Table 4.2.1) did not disrupt protein body synthesis substantially.

Table 4.2.2 Presence of pigmented testa, tannin and protein content, and *in vitro* protein digestibility (IVPD) of raw and cooked whole grain flour for different transgenic (TG, with gene constructs in bold) sorghum lines compared to non-trangenic null controls (NC) and normal sorghum lines.

Line	n	Pigmented testa (Yes/No)	Tannin content (mg CE/100 mg flour)	Protein (g/100 g flour)	IVPD Raw (%)	IVPD Cooked (%)
Macia	1	No	0.02^{a}±0.02	10.1±0.7	75.2^{ghi}±1.6	59.2^{fg}±0.7
P898012 Bulk	1	Yes	3.26^{c}±0.12	10.9±0.5	41.1^{bc}±2.0	25.7^{b}±1.5
P898012 H/CG	1	Yes	ND	13.9±1.2	48.9^{cd}±0.3	30.7^{bc}±6.2
Tx430	1	No	ND	10.2±0.2	65.0^{ef}±0.8	48.2^{e}±0.3
TG-P898012**(ABS032)**[1]	1	Yes	ND	12.8±0.2	ND	73.7^{h}±2.5
NC-P898012	1	Yes	ND	10.1±0.2	32.3^{a}±1.7	22.2^{a}±3.8
TG-P898012xMacia **(ABS032)-1**[1]	4	Yes	ND	10.6±1.4 (8.8-12.4)	69.4^{fg} ±8.6 (61.3-82.8)	50.0^{e}±6.2 (43.0-59.2)
NC-P898012xMacia-1[1]	2	Yes	ND	9.0±0.2 (8.9-9.1)	47.3^{cd}±5.4 (42.8-51.8)	28.7^{b}±1.4 (27.8-29.5)
TG-P898012xMacia **(ABS032)-2**[2]	3	Yes	2.34^{b}±0.21 (1.90-2.73)	11.8±0.7 (11.3-12.8)	80.2^{i}±3.1 (77.2-82.8)	58.3^{fg}±3.0 (54.1-61.0)
NC-P898012xMacia-2[2]	1	Yes	2.65^{b}±0.02	11.8±0.2	68.0^{efgh}±0.1	34.6^{bcd}±0.1
TG-P898012xMacia **(ABS032)-3**[3]	3	No	0.02^{a}±0.01 (0.02-0.03)	12.4±0.3 (12.1-12.8)	91.4^{j}±1.8 (90.4-93.0)	79.8^{i}±1.4 (78.4-80.8)
NC-P898012xMacia-3[3]	2	No	0.02^{a}±0.01	12.3±0.2 (12.4-12.4)	75.5^{hi}±2.2 (73.8-77.2)	56.4^{f}±1.9 (55.1-57.6)
TG-Tx430**(ABS166)**[1]	6	No	ND	12.6±2.3 (8.9-15.5)	77.9^{i}±7.3 (66.0-85.2)	61.3^{g}±7.3 (50.9-71.6)
NC-Tx430-1[1]	6	No	ND	12.1±2.5 (8.5-15.5)	49.9^{d}±6.9 (43.5-63.2)	40.0^{d}±4.9 (33.6-48.7)
TG-Tx430**(ABS149)**[1]	2	No	ND	13.7±2.2 (11.7-15.6)	61.5^{e}±2.3 (60.0-63.0)	41.1^{d}±0.2 (40.9-41.2)
NC-Tx430-2[1]	2	No	ND	13.6±2.0 (11.8-15.5)	40.4^{b}±2.1 (39.7-41.0)	34.4^{c}±1.3 (33.8-35.0)

Values are means ±standard deviations, values in parentheses are the range. Values of a parameter in the same column with different superscript letters were significantly different ($p \leq 0.05$). n= number of samples analysed, samples were analysed in duplicate and the analysis was repeated at least once. H/CG= Half crushed grain. [1]Greenhouse trial. [2]Summer confined field trial, tannin type. [3]Summer confined field trial, non-tannin type. ND= Not determined.

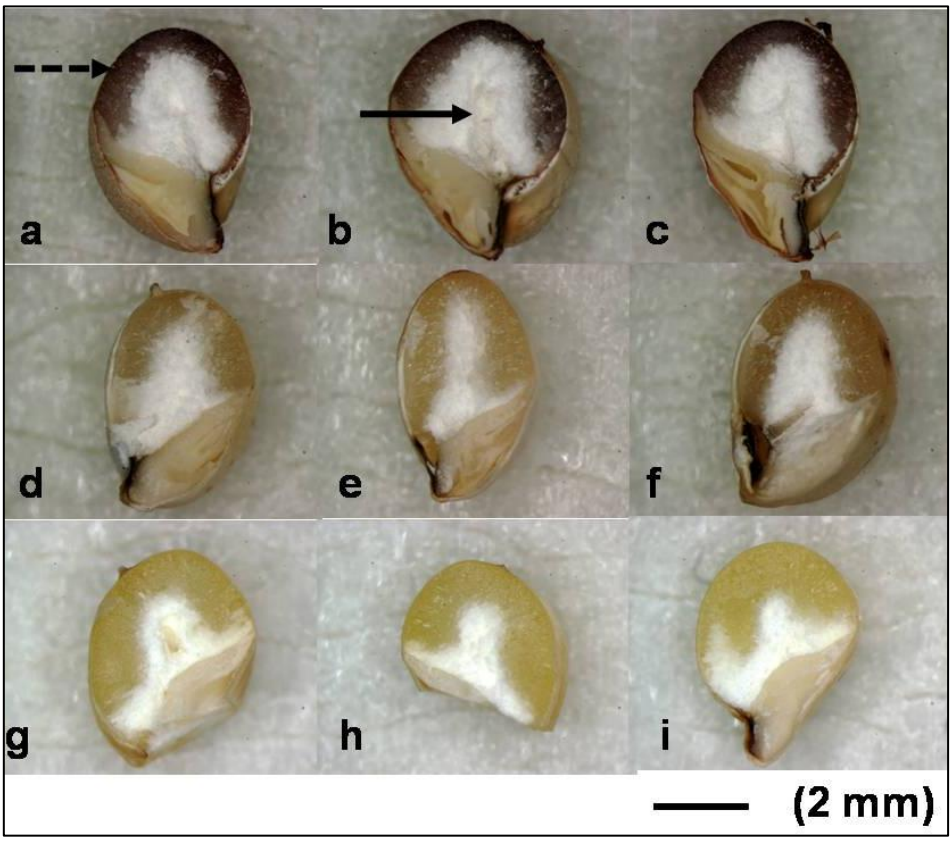

Figure 4.2.1 Sectioned kernels (with or without germ) of the normal sorghum parent lines used for transformation or backcrossing.
a-c) P898012; d-f) Macia; g-i) Tx430. Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining.

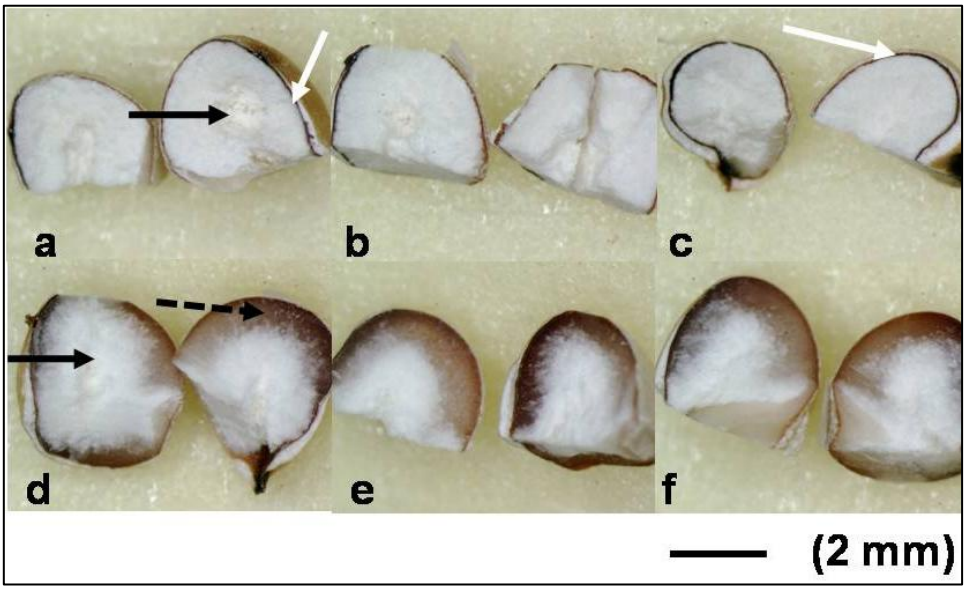

Figure 4.2.2 Sectioned kernels (without germ) of trangenic line (ABS032 gene construct) and its null control.
a-c) TG-P898012 (ABS032); d-f) NC-P898012. Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining; white arrow indicates pigmented testa layer.

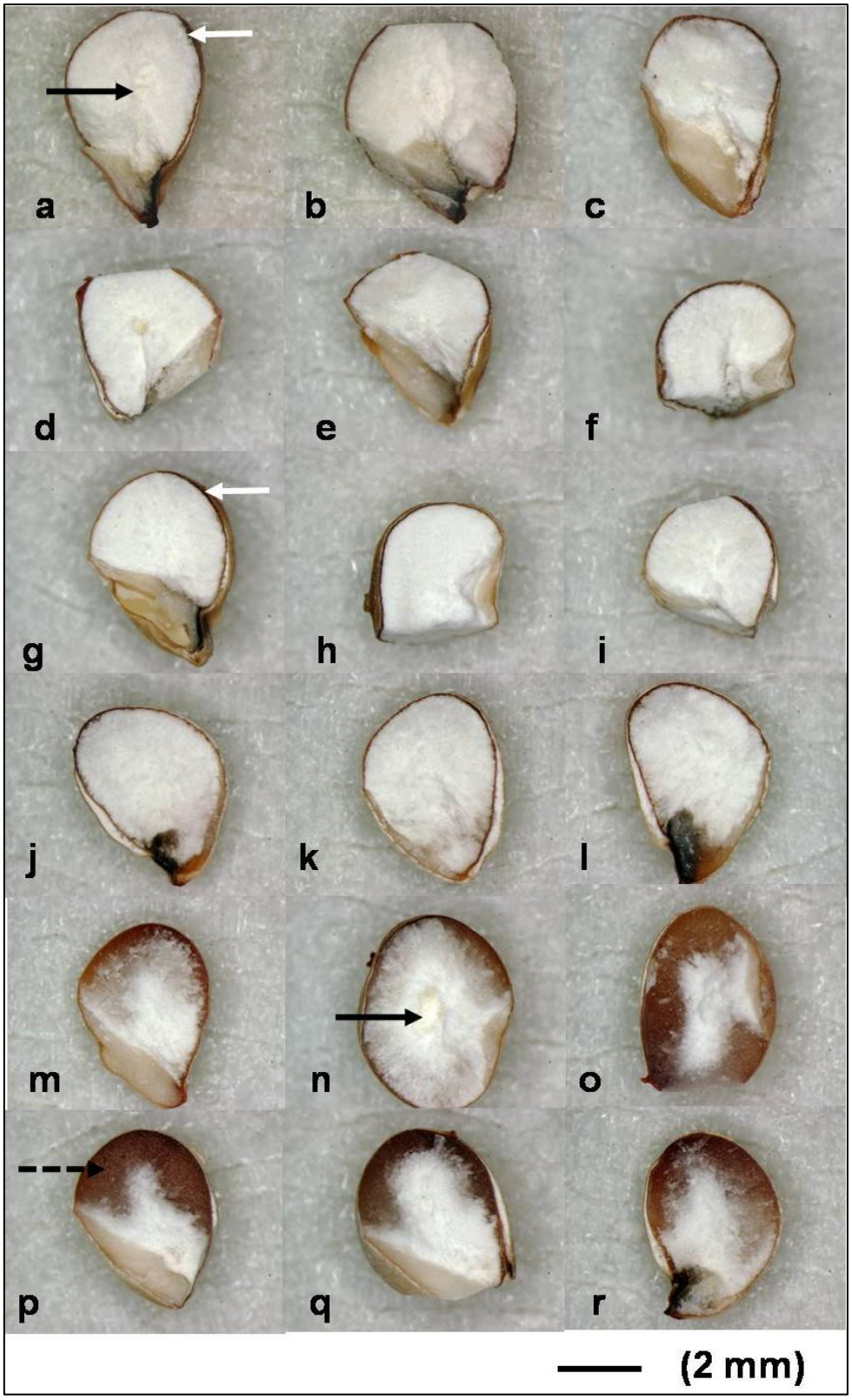

Figure 4.2.3 Sectioned kernels (with or without germ) of transgenic line (ABS032 gene construct backcrossed into Macia, type II tannin) and its null control.

a-l) TG-P898012xMacia (ABS032)-1; m-r) NC-P898012xMacia-1 Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining; white arrow indicates pigmented testa layer.

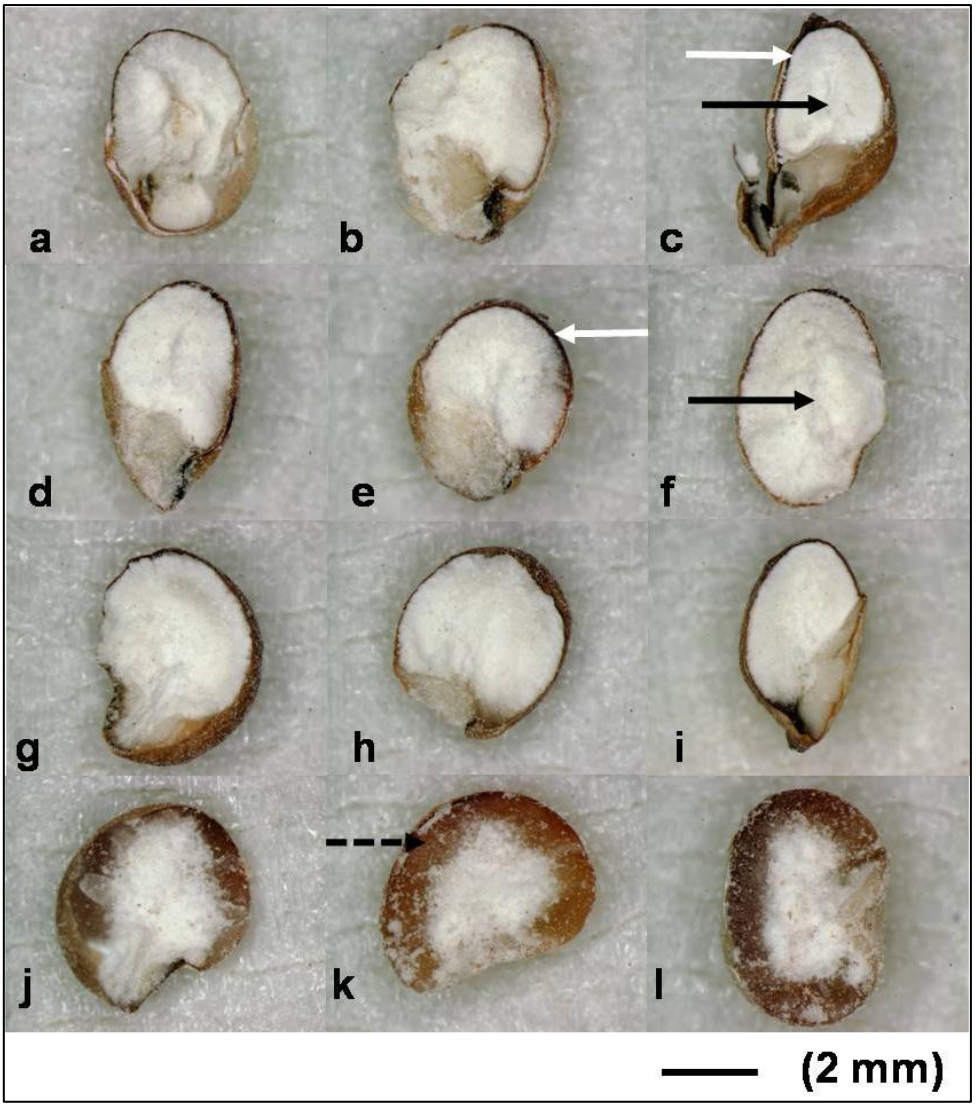

Figure 4.2.4 Sectioned kernels (with or without germ) of transgenic line (ABS032 gene construct backcrossed into Macia, type II tannin) and its null control.

a-i) TG-P898012xMacia (ABS032)-2; j-l) NC-P898012xMacia-2 Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining; white arrow indicates pigmented testa layer.

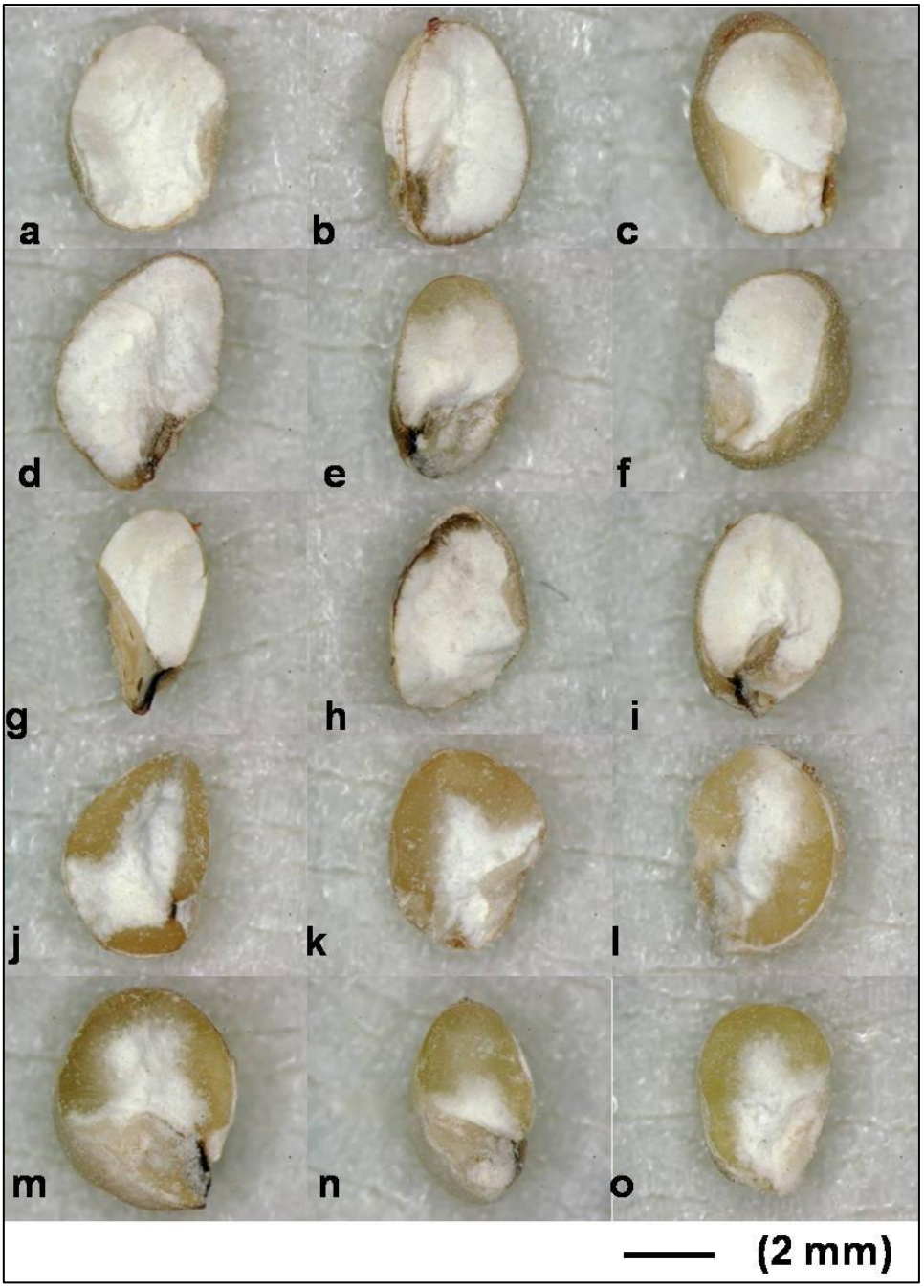

Figure 4.2.5 Sectioned kernels (with or without germ) of transgenic line (ABS032 gene construct backcrossed into Macia, non-tannin) and its null control.
a-i) TG-P898012xMacia (ABS032)-3; j-o) NC-P898012xMacia-3.

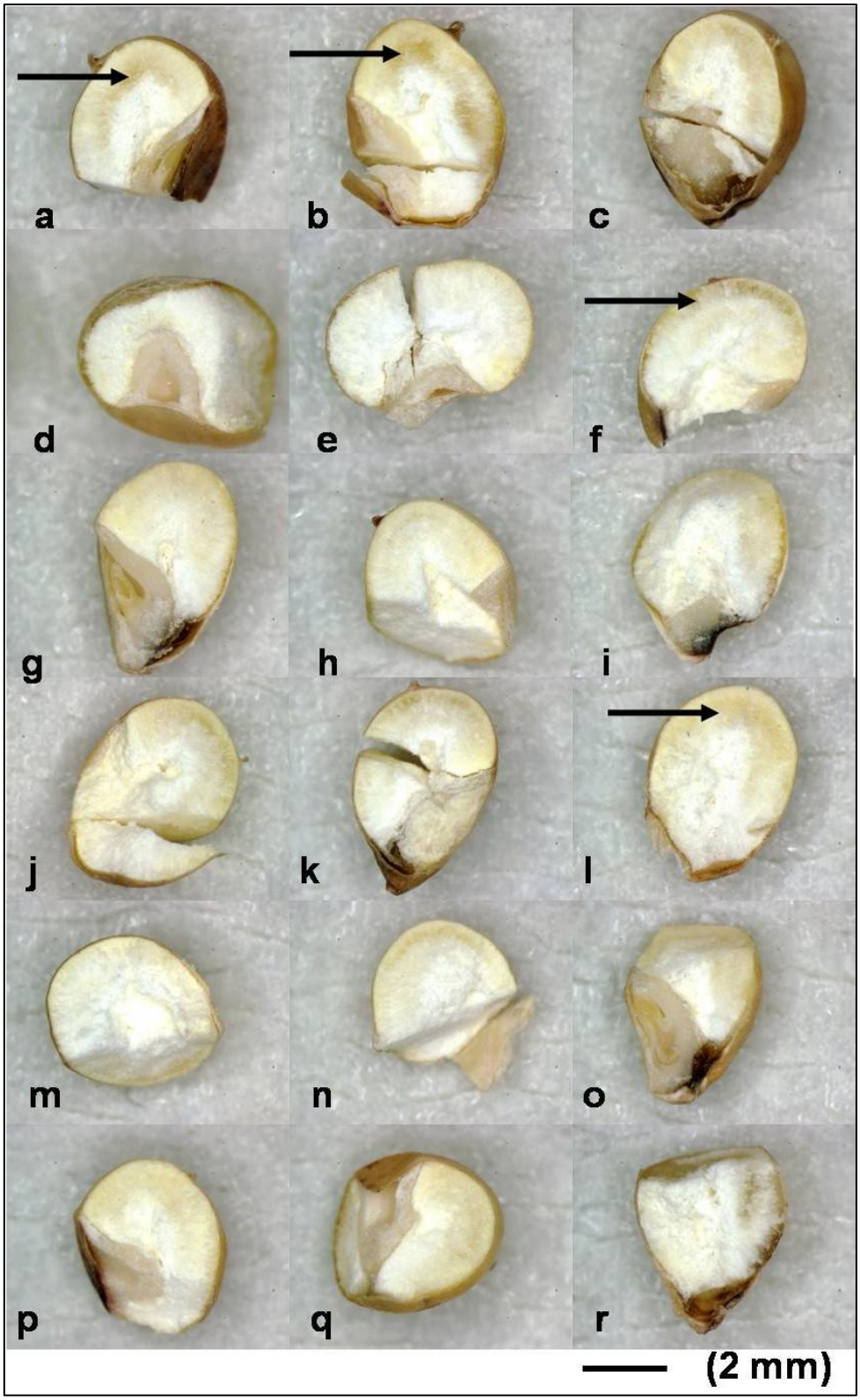

Figure 4.2.6 Sectioned kernels (with or without germ) of transgenic line (ABS166 gene construct) and its null control.
a-r) TG-Tx430 (ABS166); s-aj) NC-Tx430-1. Black arrow indicates patches of corneous endosperm texture.

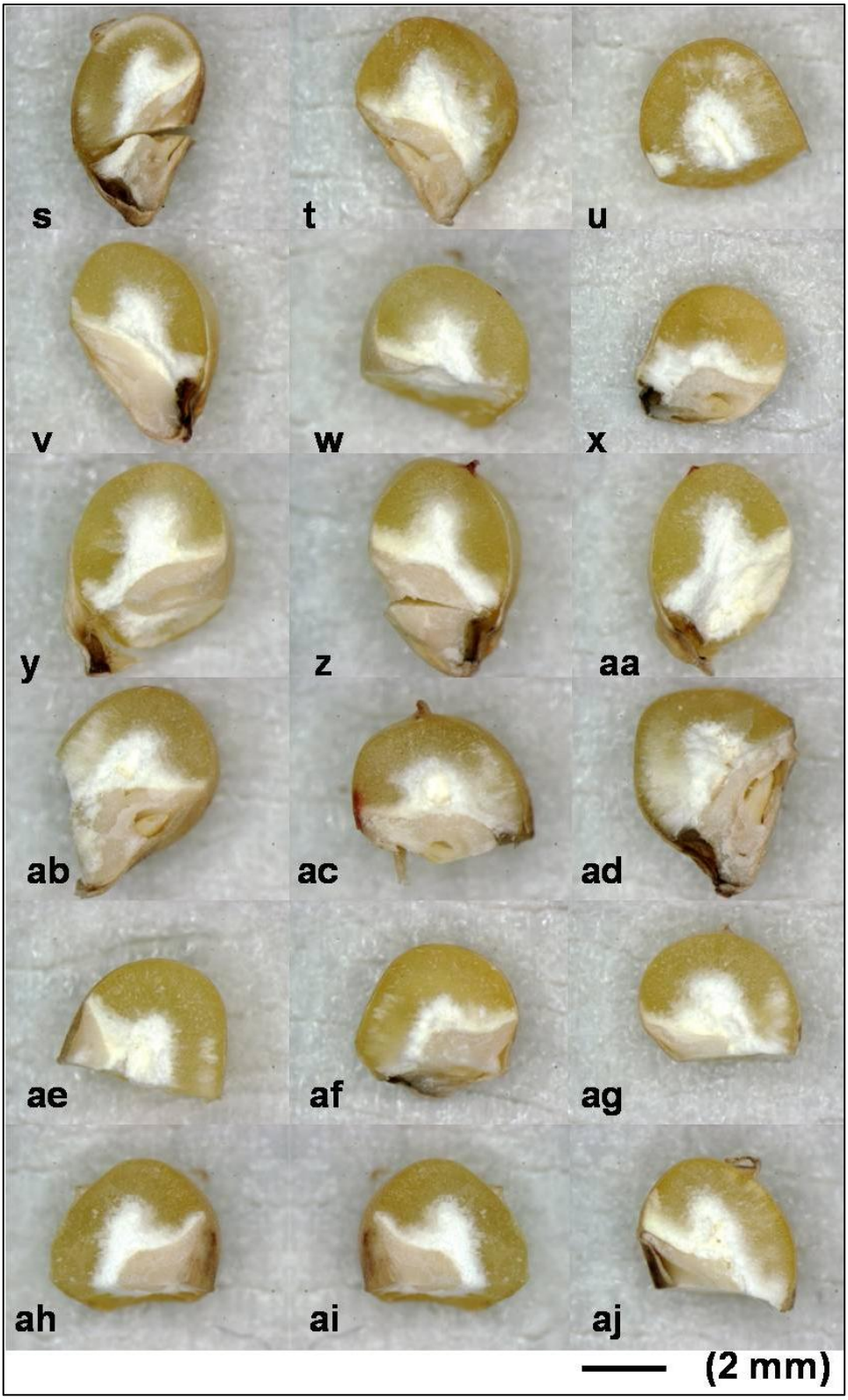

Figure 4.2.6. Continued.

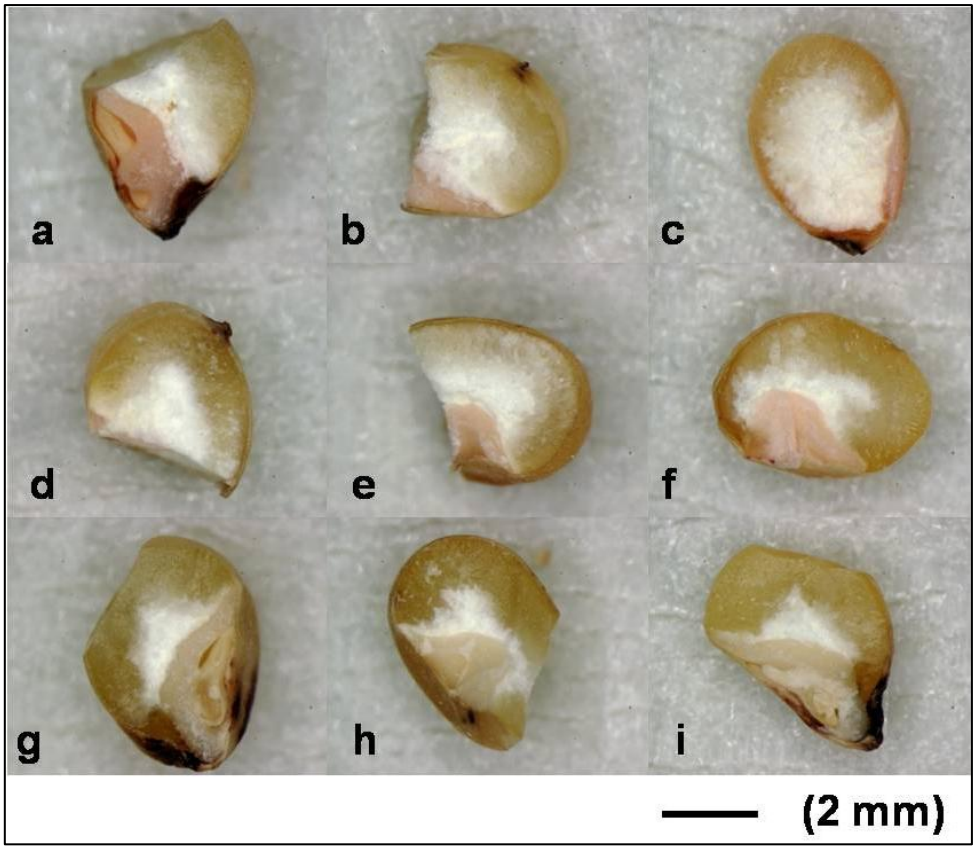

Figure 4.2.7 Sectioned kernels (with or without germ) of transgenic line (ABS0149 gene construct) and its null control.
a-f) TG-Tx430 (ABS149); g-i) NC-Tx430-2.

Hence, the co-expressed synthesis of α-kafirin B1 and B2, ɣ-kafirin 2 and β-kafirin and δ-kafirin sub-classes may have been sufficient to ensure normal protein body synthesis, and retain some of the corneous phenotype of the parent line Tx430. Studies with transgenic tobacco plants in which genes encoding one or more zein sub-classes indicate that β-zein or ɣ-zein must be co-expressed with α-zein (and δ-zein), to promote α-zein stability and retention in the endoplasmic reticulum, in order to form normal protein bodies (Coleman et al., 1996; Coleman et al., 2004). Further, the hard endosperm texture of TG-Tx430 (ABS149) suggests that the co-suppression of δ-kafirin 2 and ɣ-kafirin 1 and 2 sub-classes synthesis alone, did not have a dramatic effect on the endosperm phenotype.

4.2.4.3 Protein content and amino acid profile

The different TG lines had protein contents ranging from 10.6% (TG-P898012xMacia (ABS032)-1) to 13.7% (TG-Tx430 (ABS149)) (Table 4.2.2). The TG lines and their respective null controls were all generally very similar or even identical in protein content. The protein contents reported here are well within the range for native Ethiopian high-lysine sorghums (10.0-17.2%) (Singh and Axtell, 1973; Guiragossian et al., 1978), high-protein digestibility high-lysine mutant sorghums (10.2-14.7%) (Weaver et al., 1998; Tesso et al., 2006) and normal sorghums (8.1-16.8%) (Rooney and Serna-Saldivar, 1990).

In contrast, amino acid profile differed substantially between the different TG lines, specifically with regard to lysine content (Table 4.2.3). TG-P898012 (ABS032) and the backcrosses (TG-P898012xMacia (ABS032)-1, -2 and -3) had the highest lysine content, ranging from 3.65 to 4.1 g lysine/100 g protein, compared to TG-Tx430 (ABS166), TG-Tx430 (ABS149) and the parent lines, which had lysine contents of 2.18, 2.43 and 2.08 g lysine/100 g protein, respectively, within normal ranges for sorghum (Rooney and Serna-Saldivar, 1990). The amino acid profiles including lysine contents of the NCs were essentially the same as the parent lines (data not shown).

The lysine content of TG-P898012 (ABS032) and the backcrosses were slightly higher than that reported for native Ethiopian high-lysine genotypes (3.2-3.7 g/100 g protein) (Singh and Axtell, 1973; Guiragossian et al., 1978), and high-protein digestibility high-lysine mutant sorghums (2.9-3.4 g/100 g protein) (Weaver et al., 1998; Tesso et al., 2006). But lysine values were in the range reported for quality protein maize (QPM) cultivars (3.43 to 4.56 g lysine/100 g protein) (Zarkadas, Hamilton, Yu, Choi, Khanizadeh, Rose and Pattison, 2000). The high-lysine TG lines also showed considerable reduction in proline (24% lower), alanine (17%), glutamic acid/glutamine (15%), leucine (15%) and phenylalanine (15%), and increases in arginine (76% higher), aspartic acid (48%), methionine (42%) and glycine (32%) compared to the normal sorghums (Table 4.2.3). Similar changes in the amino acid profile of high-lysine cereals have been reported (Singh and Axtell,

1973; Guiragossian et al., 1978; Zarkadas et al., 2000; Vendemiatti, Ferreira, Gomes, Medici and Azevedo, 2008).

The altered amino acid profile observed for TG-P898012 (ABS032) and the backcrosses is probably as a direct consequence of co-suppression of the synthesis of the major kafirin sub-classes. Since the kafirins contain essentially no lysine, these results indicate a substantial increase in the proportion of non-kafirin storage proteins and non-storage proteins in the grain, which are higher in lysine content. In other high-lysine sorghum genotypes, the proportion of lysine rich non-kafirin storage proteins (albumins, globulins and glutelins) was considerably higher compared to normal sorghums (Guiragossian et al., 1978; Vendemiatti et al., 2008). While in high-lysine *opaque*-2 mutant maize genotypes, the increase in lysine content is attributed to elongation factor-1α (EF-1α), a lysine rich (11% lysine) non-zein protein (Habben, Moro, Hamaker and Larkins, 1995). Other non-zein proteins found to be over expressed in *opaque-2* mutants, include catalase-2 (7% lysine) and trypsin inhibitor (1% lysine).

The reduced suppression of LKR in TG lines with the ABS032 gene construct, may also have contributed to the increased lysine content. This is similar to the situation in *opaque-2* maize, where the activity of lysine LKR is reduced, resulting in increased levels of free lysine (Gibbon and Larkins, 2005).

Table 4.2.3 Amino acid composition (g/100 g protein), recovery, Amino Acid Score (AAS) and Protein Digestibility Corrected Amino Acid Score (PDCAAS) for different transgenic (TG) and normal sorghum lines.

	Transgenic lines with different ABS gene constructs (in bold)						Parent lines used for transformation or backcrossing		
Amino Acid	TG-P898012 **(ABS032)**[1]	TG-P898012x Macia**(ABS032)-1**[1]	TG-P898012x Macia**(ABS032)-2**[2, 4]	TG-P898012x Macia**(ABS032)-3**[3, 4]	TG-Tx430 **(ABS166)**[1]	TG-Tx430 **(ABS149)**[1]	P898012	Macia	Tx430
Non-EAA									
Glu	15.72±0.76	16.26±1.18	-	-	21.12±1.58	20.91±1.82	19.34±0.29	16.95±0.67	21.32±0.47
Asp	6.74±0.00	7.43±0.78	-	-	6.60±0.54	5.14±0.40	5.24±0.13	4.99±0.13	4.54±0.35
Ala	7.46±0.25	6.93±0.50	-	-	7.86±0.53	9.20±1.03	8.77±0.23	7.77±0.20	9.08±0.03
Pro	6.29±0.13	6.15±0.46	-	-	5.94±0.40	7.70±0.59	8.26±0.23	7.58±0.20	8.70±0.24
Arg	5.75±0.25	6.21±0.96	-	-	3.91±0.42	4.35±0.04	3.46±0.00	2.87±0.33	4.06±0.47
Ser	3.73±0.19	3.76±0.33	-	-	3.60±0.28	4.35±0.36	4.16±0.16	3.63±0.07	4.46±0.10
Gly	3.68±0.13	3.71±0.43	-	-	2.84±0.38	3.07±0.03	2.85±0.05	2.50±0.07	3.09±0.16
His*	1.71±0.13	1.80±0.24	-	-	2.38±0.32	1.76±0.13	2.10±0.05	1.55±0.07	2.21±0.19
EAA									
Ile	3.86±0.13	3.50±0.18	-	-	3.46±0.26	4.00±0.27	3.95±0.05	3.39±0.13	3.90±0.02
Leu	10.74±0.32	13.11±0.94	-	-	10.74±0.73	18.41±2.05	13.70±0.19	12.10±0.60	18.60±0.25
Met	2.20±0.19	2.38±0.22	-	-	1.57±0.21	2.14±0.07	1.61±0.14	1.51±0.13	1.82±0.03
Cys	0.00±0.00	0.00±0.00	-	-	0.00±0.00	0.00±0.00	0.09±0.11	0.00±0.00	0.04±0.05
Phe	4.72±0.32	3.86±0.27	-	-	4.18±0.30	4.82±0.35	5.29±0.05	4.24±0.27	4.70±0.03
Tyr	3.59±0.02	3.68±0.29	-	-	3.63±0.26	4.41±0.38	3.81±0.09	3.58±0.40	4.33±0.13
Thr	2.61±0.76	2.52±0.33	-	-	2.89±0.29	2.50±0.17	2.92±0.12	2.82±0.13	2.46±0.08
Val	5.21±0.13	4.92±0.39	-	-	4.48±0.35	4.93±0.24	4.96±0.08	4.47±0.20	4.95±0.07
Lys	3.28±0.06	3.65±0.47	4.1±0.2	4.1±0.5	2.18±0.37	2.43±0.07	2.10±0.05	1.95±0.20	2.18±0.47
Recovery	**87.3**	**89.9**			**87.4**	**100.0**	**81.9**	**92.6**	**100.0**
AAS	**0.68**	**0.76**	**0.8**	**0.8**	**0.45**	**0.51**	**0.41**	**0.44**	**0.45**
PDCAAS	**0.50**	**0.38**	**0.5**	**0.7**	**0.28**	**0.21**	**0.24**	**0.21**	**0.16**

Values are means ±standard deviations. - = no data available. [1]Greenhouse trial. [2]Summer confined field trial, tannin type. [3]Summer confined field trial, non-tannin type. [4]Lysine data supplied by Pioneer Hi-Bred, 2009. EAA- Essential amino acids. His*- EAA for infants. AAS-((g lys/100 g protein)/4.8), 4.8 is WHO recommendation for lysine quality protein for 4-18 year olds, and PDCAAS- (AAS x Cooked IVPD) (WHO/FAO/UNU Expert Consultation, 2007).

4.2.4.4 In vitro protein digestibility

Different TG lines gave a wide range of raw, 61.5 % (TG-TX430 (ABS149) and 91.4% (TG-P898012xMacia (ABS032)-3) and wet cooked, 41.1% (TG-Tx430 (ABS149)) and 79.8% (TG-P898012xMacia (ABS032)-3) protein digestibilities (Table 4.2.2). The differences observed were due to different kafirin sub-classes being suppressed, as well as the presence or absence of tannins within the grains. Generally, TG-P898012xMacia (ABS032)-3 the non-tannin TG line in which the major kafirin sub-classes was suppressed showed substantially higher raw (91.4%) and wet cooked protein digestibilities (79.8%) compared to the tannin-containing TG lines, with similar kafirin suppression, ranging from 69.4 to 80.2%, for raw and 50.0 to 58.3% for cooked flours, respectively (Table 4.2.2). According to Taylor and co-workers, kafirin proteins, specifically the ɣ-kafirin, bind considerable quantities of tannins, ranging from 35 to 77%, depending on kafirin composition, forming very large molecular weight (>200 kDa) aggregates of kafirin polymers and tannin molecules with reduced protein digestibility compared to unbound kafirin (Taylor, Bean, Ioerger, Taylor, 2007). In TG lines where co-suppression of fewer kafirin sub-classes occurred (TG-Tx430 (ABS166) and TG-Tx430 (ABS149)) lower protein digestibility improvement seemed to occur, even in the absence of tannins (Table 4.2.2). However, it should be noted that all three different constructs, ABS032, ABS149 and ABS166 gave improvement in protein digestibility in the TGs when compared to their NCs.

Wet cooking reduced the protein digestibility of all sorghum lines. However, the reduction in digestibility was notably less in the TG lines compared to the parent lines and NCs (Table 4.2.2). The non-tannin line, TG-P898012xMacia (ABS032)-3, showed the least reduction in protein digestibility with cooking, only 12%, while the parent and NCs showed the highest reduction, at least 40%. A possible explanation is that suppression of synthesis of the major kafirins sub-classes, as in the case of TG lines with ABS032 gene construct or cysteine-rich ɣ-kafirins in the case of TG lines with ABS166, and ABS149 gene construct, could result in lower levels of crosslinked kafirin polymers in the TG lines, compared to the NCs and parent lines.

Within the TG events there was a wide range of improvement in protein digestibility. For example, for TG-P898012xMacia (ABS032)-1 the raw digestibility ranged from 61.3 to 82.8% and the cooked digestibility ranged from 43.0 to 59.2% (Table 4.2.2). The reason for this was that different TG events from the same vector usually give different levels of transgene expression and plant performance, due to factors such as transgene insertion site, pattern and copy number (Dr R. Jung, Pioneer Hi-Bred, personal communication). Therefore the best potential TG event was ABS032-3 with a cooked protein digestibility of up to 80.8%. This compares to its NC, where the highest cooked protein digestibility was 57.6% and the best normal sorghum, Macia with a cooked protein digestibility of 59.2%.

4.2.4.5 Protein nutritional quality

AAS and the PDCAAS varied considerably between the different TG lines, as a result of the differences in both the lysine contents (Table 4.2.3) and cooked IVPDs (Table 4.2.2). Generally, the TG lines with co-suppression of the major kafirin sub-classes (ABS032 gene construct) had significantly higher AAS (0.68-0.80) and PDCAAS (0.38-0.70) compared to TG lines with co-suppression of fewer kafirin sub-classes (0.45 AAS and 0.28 PDCAAS; TG-Tx430 (ABS166) and 0.51 AAS and 0.21 PDCAAS; TG-Tx430 (ABS149), respectively) and the parent lines (0.41-0.45 AAS and 0.16-0.24 PDCAAS, respectively) (Table 4.2.3). However, the presence of tannins in some of the TG lines (TG-P898012 (ABS032) and TG-P898012xMacia (ABS032)-1 and -2) reduced the PDCAAS, by at least 40% (Table 4.2.3), because the IVPD of the tannin-containing TG lines was considerably lower (Table 4.2.2).

4.2.4.6 Endosperm ultrastructure

The peripheral endosperm texture and protein body structure of the different transgenic sorghum lines showed variable modification compared to the parent lines (Figure 4.2.8) and respective NCs (data not shown). Protein bodies of parent lines (P898012, Macia and TX430, Figure 4.2.8a-c) were typical, with tightly packed, round protein bodies, ±2 µm in diameter, with internal concentric ring structures (Figure 4.2.8a, black arrow) as described for normal sorghum protein bodies (Adams et al., 1976). The protein bodies of all the NCs were typical, and were similar to the

parent lines (data not shown). In normal sorghum protein bodies, highly crosslinked kafirin proteins (γ- and β-kafirins) are found at the protein body periphery and seen as dark-staining inclusions in the form of concentric rings within the protein body interior (Oria et al., 1995; Shull et al., 1992).

TG-P898012 (ABS032) (Figure 4.2.8d-e) and the backcrosses (TG-P898012xMacia (ABS032)-1, -2 and -3 (Figure 4.2.8f-g, h-i, j-k, respectively) showed substantial modification in peripheral endosperm texture and protein body structure. The protein bodies were sparsely packed, 2 µm in diameter, and were often surrounded by a dense continuous dark protein matrix (Figure 4.2.8, white dashed arrow). Protein body margins were slightly folded (invaginated) (Figure 4.2.8, black dashed arrow) with a proportion of the protein bodies showing occasional irregular, thick dark-staining inclusions (Figure 4.2.8, white arrows). The characteristic internal concentric ring structure of normal protein bodies was absent. However, a number of atypical concentric rings were observed around the protein body periphery of TG-P898012xMacia (ABS032)-1 (Figure 4.2.8f-g, black arrow head). The modified protein body structure of the TG lines with the ABS032 gene construct is dissimilar to that of high-lysine, high-protein digestibility mutants, where the protein bodies are described as being highly invaginated (with deep folds) (Oria et al., 2000).

It appears that the co-suppression of synthesis of the major kafirin sub-classes in TG lines with ABS032 gene construct, had a major effect on the peripheral endosperm texture and protein body structure, which may in part be responsible for the improved cooked IVPD of these TG lines. As explained, the improved protein digestibility of mutant sorghum lines, having highly invaginated protein bodies is believed to be due to increased protein body surface area and easy accessibility of digestive enzymes to the more digestible α-kafirin proteins (Oria et al., 2000). In addition, the dense protein matrix observed in the TG lines, is probably composed of more digestible lysine-rich endogenous proteins, which would further improve the cooked IVPD of these lines.

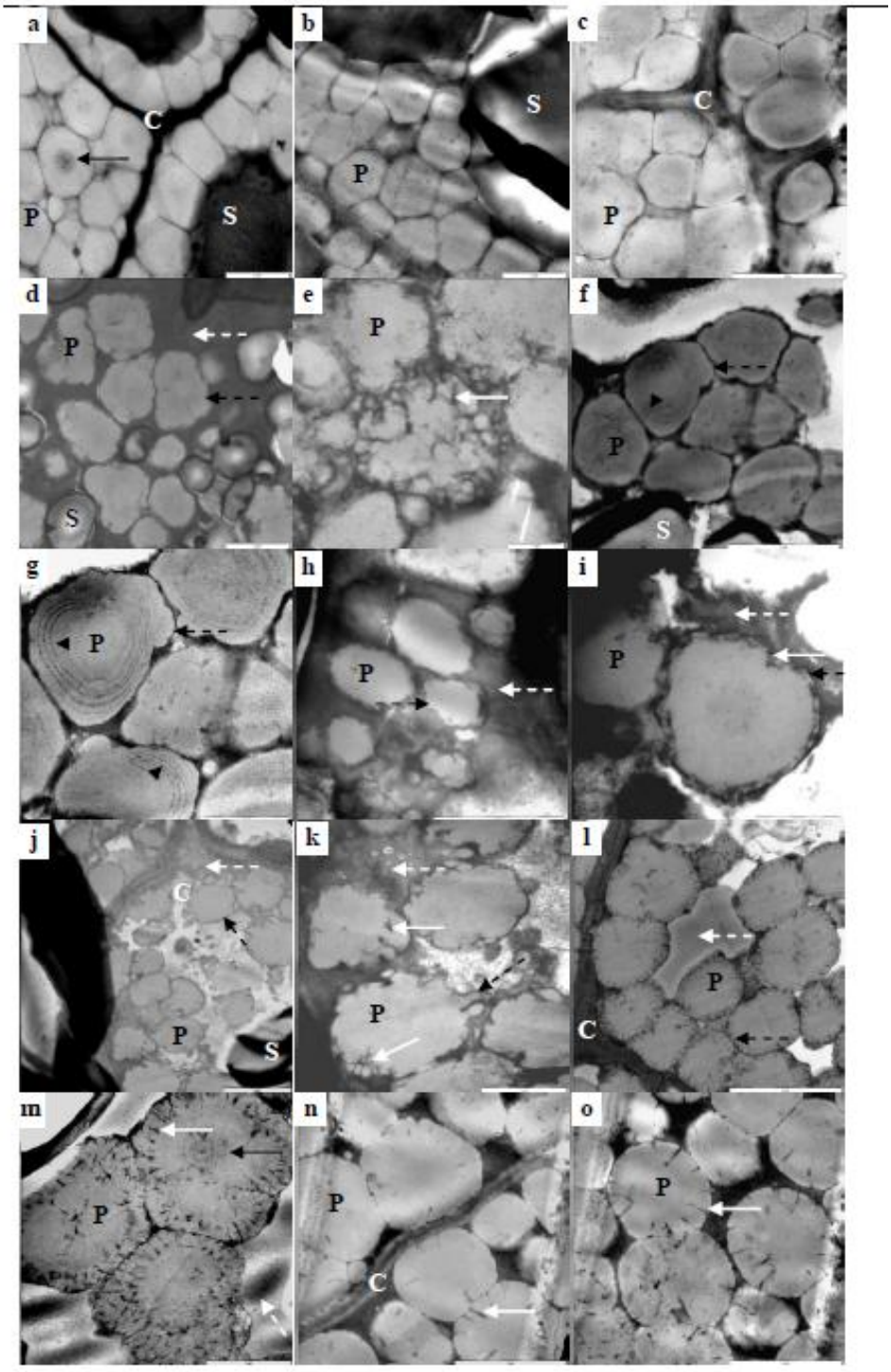

Figure 4.2.8 TEM of protein bodies in the peripheral endosperm of different sorghum lines.

Parent lines: (a) P898012, (b) Macia, (c) Tx430 used for transformation or backcrossing. Transgenic lines: (d, e) TG-P898012(ABS032), (f, g) TG-P898012xMacia(ABS032)-1, (h, i); TG-P898012xMacia(ABS032)-2, (j, k) TG-P898012xMacia(ABS032)-3, (l, m) TG-Tx430 (ABS166), (n, o) TG-Tx430(ABS149). C, cell wall, P, protein body, S, starch granule. Black arrow indicates concentric ring structure, black dashed arrow indicates irregular shaped protein body, black arrow head indicates atypical concentric ring structure, white arrow indicates dark staining inclusions, white dashed arrow indicates dark protein matrix.

In contrast, TG-Tx430 (ABS166) and TG-Tx430 (ABS149) (Figure 4.2.8l-m and n-o, respectively) had protein bodies with structure more typical of the parent line Tx430 (Figure 4.2.8c). Protein bodies of TG-Tx430 (ABS166) were generally round, ±2 µm in diameter, and the internal concentric ring structure was also present in some of the protein bodies (Figure 4.2.8l-m, black arrow). However, there were considerable levels of dark staining inclusions around the periphery of the protein bodies, giving the appearance of slight invagination of the periphery (Figure 4.2.8m, white arrow). The protein body packing density of TG-Tx430 (ABS166) also appeared to be less dense than that of the parent and patches of dark protein matrix were observed between some of the protein bodies (Figure 4.2.8l-m, white dashed arrow). The protein bodies of TG-Tx430 (ABS149) did not appear to be modified, showing typical protein body structure, packing density and size (±2 µm) (Figure 4.2.8n-o) as that of the parent Tx430 (Figure 4.2.8c). However, the dark staining inclusions around the protein body periphery would indicate that these protein bodies were also slightly invaginated (Figure 4.2.8n-o, white arrow). The observed endosperm ultrastructure of the different TG Tx430 lines was not surprising as these TG lines had IVPD and protein nutritional quality within normal ranges. This is probably due to differences in the levels and types of cysteine-rich kafirin, available to form crosslinked polymers.

According to studies conducted on the interaction of different zein proteins to form storage protein bodies, stable accumulation and aggregation of α-zein into protein bodies requires the interaction of either ɣ-zein (Coleman et al., 1996) or β-zein (Coleman et al, 2004). This is due to ɣ- and β-zein being structurally related, having similar roles in the initiation of protein bodies in the developing maize endosperm (Kim et al., 2002). Both ɣ- and β-zeins are rich in cysteine, resulting in their aggregation via inter-molecular disulphide bonds to form an insoluble protein body core, which is required for the interaction and integration of α-zein (Coleman et al., 2004).

4.2.5 Conclusions

Co-suppression of the major kafirin sub-classes, α-, ɣ- and δ-, results in improved transgenic sorghum lines with substantially improved cooked protein digestibility, improved Amino Acid Score and hence greatly improved Protein Digestibility Corrected Amino Acid Score. Breeding out the tannin trait by backcrossing into improved non-tannin sorghum lines, greatly improved the protein nutritional quality of tannin containing transgenic lines because protein tannin interaction does not take place. The substantially improved protein digestibility appears to be associated with floury endosperm texture. In turn, this seems to be related to modified protein body structure due to the suppression of kafirin synthesis. When fewer kafirin sub-classes are suppressed, i.e. ɣ-kafirin 1 and δ-kafirin 2, the endosperm is corneous, with apparently normal protein body structure but the improvement in cooked protein digestibility seems to be less.

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4.3 Transgenic sorghum with altered kafirin synthesis: Kafirin solubility, polymerisation and protein digestion

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4.3.1 Abstract

Transgenic sorghum (TG) lines with altered kafirin synthesis, particularly suppression of ɣ-kafirin synthesis, and improved protein quality have been developed. The proportion of kafirin extracted with 60% tert-butanol alone was greatly increased in the TG lines. However, the total amount of kafirin remained unchanged. Further, in the TG lines, the kafirin was much less polymerised by disulphide bonding. There was also evidence of compensatory synthesis of other kafirin proteins. Cooked protein digestibility was increased in the TG form, even after removal of interfering starch. The TG protein bodies were intermediate in appearance between the normal type and the invaginated high digestibility mutants. Hence, the increased protein digestibility of these TG lines is probably related to their lower levels of disulphide-bonded kafirin polymerisation, allowing better access of proteases. This work appears to confirm that disulphide bond formation in kafirin is responsible for the reduced protein digestibility of cooked sorghum.

4.3.2 Introduction

The poor protein nutritional quality of sorghum is due to the major storage proteins, the kafirins, being essentially free of lysine, and to the sorghum protein having low digestibility, especially after cooking (Shewry, 2007). Numerous factors contribute to the low protein digestibility of sorghum. However, crosslinking of the kafirin proteins through disulphide bonding and the organization of the various kafirin sub-classes within protein bodies, are believed to be major factors (Duodu et al., 2003; Belton et al., 2006).

Sorghum lines with improved protein quality have been developed through reduced expression of different kafirin sub-classes, using genetic engineering techniques by the Africa Biofortified Sorghum (ABS) Project, under the Bill and Melinda Gates Foundation Grand Challenges in Global Health initiative (ABS Project, 2009). We have shown that transgenic sorghum lines (TG) with co-suppression of synthesis of α-, ɣ- and δ-kafirin sub-classes and removal of the tannin trait have high cooked *in vitro* protein digestibility (IVPD) (approximately 80%), improved Lysine Score (0.8) and Protein Digestibility Corrected Amino Acid Score (0.7) (see section 4.2). The improved protein quality traits are maintained when the TG sorghum is used to produce sorghum porridge, a major African staple food (Henley et al., 2010) and a wide range of other sorghum food products (Taylor and Taylor, 2011).

A problem, however, with these high-protein quality sorghum TG lines is that they have a floury endosperm phenotype with the protein bodies irregularly shaped and surrounded by a dense protein matrix (see section 4.2). Therefore this study investigated the effect of reduced synthesis of the major kafirin sub-classes on kafirin solubility, polymerisation and protein digestibility, with the aim of understanding the mechanisms responsible for the TG sorghum lines having improved protein digestibility but modified endosperm structure.

4.3.3 Materials and Methods

4.3.3.1 Grain samples and whole grain flour preparation

The sorghum samples used were two independent transgenic events (TG1 event 5/15028 and TG2 event 6/15032) and their respective non-transgenic null controls (NC1 and NC2) (parent variety P898012, a purple-plant, Type II tannin type, 3.26 mg catechin equivalent /100 mg flour). The transgenic samples were T2 selfed seeds, 75% pure with respect to the ABS032 gene construct, that suppresses synthesis of α-kafirin A1 (25 kDa) and α-kafirin B1 and B2 (which correspond to 19 kDa and 22 kDa α-kafirin classes respectively, (Belton et al., 2006)); ɣ-kafirin 1 (27 kDa) and 2 (50 kDa), and δ-kafirin (15 kDa). The NCs were obtained from hemizygous transgenic plants, and are the best counterpart to the transgenics for transgene performance assays (Dr R. Jung, Pioneer Hi-Bred, personal communication). The transgenics and their NCs were developed using *Agrobacterium*-mediated transformation, as described by Zhao et al. (2000) and cultivated in a confined trial under the same environmental conditions at Johnston, Iowa in 2008. These were compared with a high-protein digestibility line, 07HW PRGE 103 (BTx635*P850029)-CS9-CS1-CS1 (HD), a tan-plant, non-tannin sorghum with P721 opaque (P721Q) in its pedigree, supplied by Texas A&M University, Weslaco, Texas, and a normal sorghum, Macia, (developed from SDS 3220, ICRISAT SMIP) cultivated at Makoro Lands, Central District, Botswana, 2004, a popular southern African tan plant, non-tannin variety.

Sorghum was received as whole grain or crushed whole grain. They were milled using a hand-held mill (IKA A11 Basic, Staufen, Germany) until all the whole grain flour passed through a 500 µm opening screen. The flours were stored at ±8°C until use. The samples were previously characterized with respect to tannin, protein and lysine content, and *in vitro* protein digestibility of raw and wet cooked flour (Taylor and Taylor, 2011).

4.3.3.2 Kafirin solubilization

Extraction 1 – Whole grain flour (8 g) was suspended in 40 ml, 60% (v/v) aqueous tertiary butanol and agitated for 5 h at ambient temperature, centrifuged (2000 g for 10 min) and the supernatant collected. The residue was re-suspended in the same solvent (40 ml), agitated overnight, centrifuged and the two supernatants pooled and termed kafirin-1, as defined (Duodu, Nunes, Delgadillo, Parker, Mills, Belton and Taylor, 2002).

Extraction 2 – The residue from extraction 1 was re-suspended in 40 ml 60% (v/v) aqueous tertiary butanol plus 5% (v/v) 2-ME and agitated for 3 h at room temperature, and then centrifuged and supernatant collected. The residue was re-suspended in the same solvent (40 ml), agitated for a further 3 h, centrifuged and the two supernatants pooled and termed kafirin-2 (Duodu et al., 2002). The supernatants and residues were freeze dried, weights recorded and the protein contents determined.

4.3.3.3 Isolation of protein preparations (PP) by wet milling

To study the protein-body proteins, protein preparations (PP) by wet milling were prepared (Duodu et al., 2002). In brief, defatted flour was wet milled using an Ultra Turrex (Janke & Kunkel, Staufen, Germany). The resulting slurry was passed through sieves of different opening size. The 75 µm fraction containing the protein bodies and starch was centrifuged several times to separate the protein from the more dense starch. The PP by wet milling was then freeze dried and the protein content determined.

4.3.3.4 Isolation of protein preparations (PP) by starch digestion

To study the pattern of protein digestion, PP by starch digestion were prepared. This was performed by a modification of the Megazyme Total Starch Assay Kit method (Megazyme International Ireland Limited, Wicklow, Ireland). Alpha-amylase in 3-(N-morpholine) propanesulphonic acid (MOPS) buffer (6 ml) was added to flour (200 mg) and the samples incubated at boiling temperature (95°C) for 15 min. Sodium

acetate (8 ml) and amyloglucosidase (0.2 ml) were added and the samples were further incubated at 50°C for 30 min. The samples were centrifuged and the supernatant decanted off and directly subjected to pepsin digestion.

4.3.3.5 Protein

Protein (N x 6.25) was determined by a Dumas combustion method (AACC Standard Method 46-30) (AACC, 2000).

4.3.3.6 Pepsin digestion

The *in vitro* pepsin digestibility (IVPD) method of Hamaker et al. (1986) was used, modified for small-scale assay (200 mg) (Taylor and Taylor, 2011).

4.3.3.7 Transmission electron microscopy

TEM of the PP by wet milling and PP by starch digestion after pepsin digestion was performed. Specimens were fixed in 2.5% glutaraldehyde (0.075 M phosphate buffer, pH 7.4) for 2 h, dehydrated in a graded aqueous acetone series and then infiltrated with Quetol resin. Sections were stained with aqueous uranyl acetate and then further stained in Reynold's lead citrate, and then examined using a JEOL JEM 2100F field emission electron microscope (Tokyo, Japan).

4.3.3.8 SDS-PAGE

This was carried out under both non-reducing and under reducing conditions using an X Cell SureLock Mini-Cell electrophoresis unit (Invitrogen Life Technologies, Carlsbad, CA), and pre-prepared NuPAGE 4-12% Bis-Tris gradient gels 1 mm thick, with Invitrogen Mark 12 unstained standard molecular weight markers, 2.5–200 kDa. For reducing conditions, 10% 2-ME was added to the sample buffer. Procedures were according to Gallagher (1999) and to ensure complete protein solubilization, samples were boiled for at least 15 min with vigorous vortexing every 5 min. Samples were loaded at constant protein (10 µg), unless stated otherwise. Staining was with Coomassie Brilliant Blue R250.

4.3.3.9 Statistical analysis

Samples were analysed in duplicate, twice (4 values). The data were analysed by one-way analysis of variance (ANOVA) at a confidence level of $p<0.05$. Means were compared by Fisher's least significant difference (LSD) test. The calculations were performed using Statsgraphics Centurion XV (Stat Point, Herndon, VA).

4.3.4 Results and Discussion

4.3.4.1 PP by wet milling

During the wet milling process to produce the PP by wet milling, the protein and starch fractions in the TG and HD samples separated more easily than the NCs and Macia samples. Additional washing and centrifugation steps were required with the latter to obtain PP by wet milling with similar protein contents (25-30%). The TG and HD samples had all floury endosperm texture, whereas NC had some peripheral corneous endosperm, and Macia had intermediate endosperm texture (see sections 4.1 and 4.2). The floury endosperm texture of TG and HD probably facilitated the wet milling process, allowing easier separation of the protein bodies from the starch granules because of the weaker starch-protein and protein-protein matrix, compared to grain of medium to hard endosperm texture (Xie and Seib, 2000). The relatively low protein contents of the PP by wet milling were due to the fact that a reducing agent, such as sodium metabisulphite, was not used during wet milling process, unlike in commercial wet milling (Jackson and Shandera, 1995). The reducing agent was not included so as to study the proteins in their natural state.

The PP by wet milling mainly comprised endosperm material made up of protein bodies surrounded by matrix protein, with some starch granules and cell wall material, as reported in similar work (Duodu et al., 2002). Significantly, the TG had somewhat more irregular shaped and invaginated protein bodies (Figure 4.3.1a), as compared to those of NC1 (Figure 4.3.1b) and Macia (Figure 4.3.1d). The TG protein bodies were, however, less irregular and invaginated than the characteristic HD protein bodies (Oria et al., 2000) (Figure 4.3.1c). The TG protein bodies could

be considered intermediate in form between those of the NC, Macia and HD. The protein bodies in the PP by wet milling had the same form as those observed in endosperm sections from these types (see section 4.2).

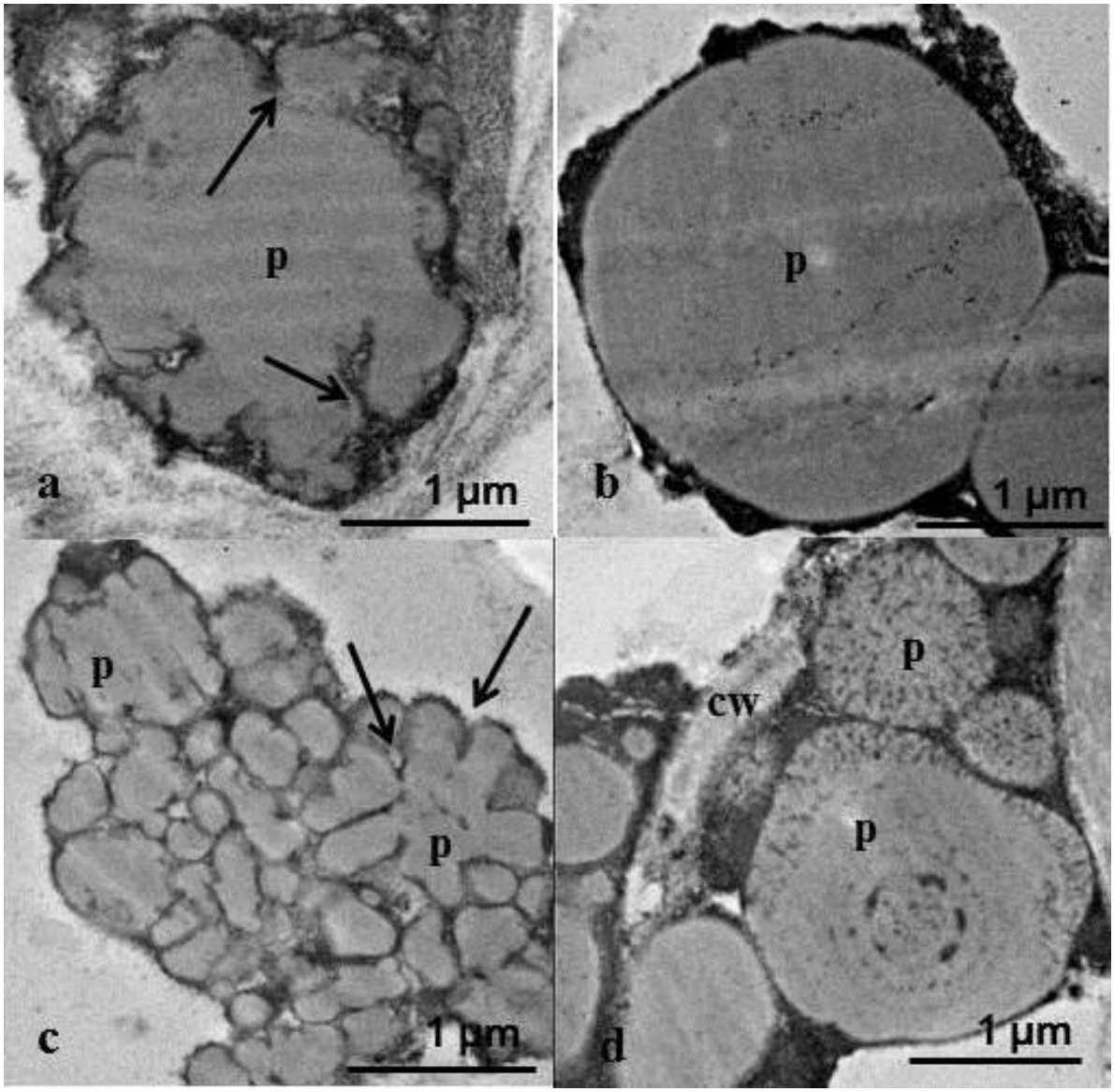

Figure 4.3.1 Representative images of TEM of sorghum protein body preparations by wet milling.
a) TG1; b) NC1; c) HD; d) Macia. cw = cell wall; p = protein body, arrows show invaginations

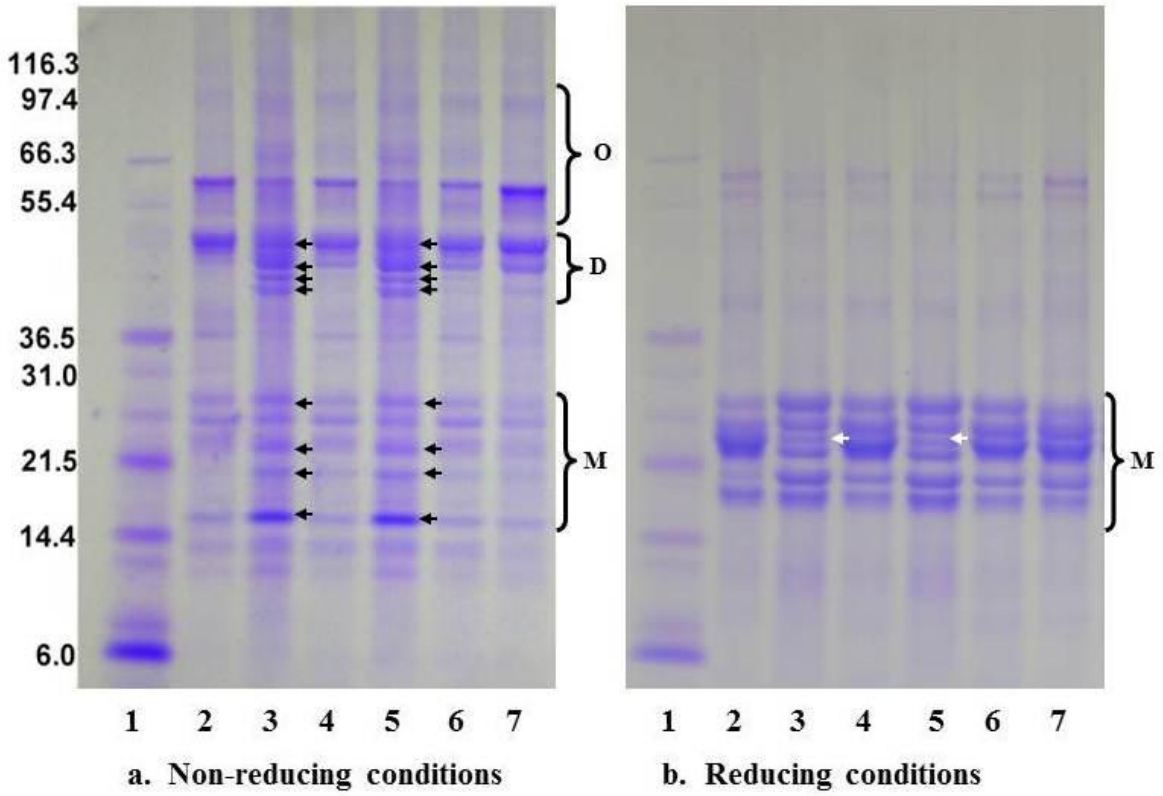

Figure 4.3.2 SDS-PAGE of sorghum protein preparations by wet milling.
1) Molecular weight markers (kDa); 2) C1; 3) T1; 4) C2; 5) T2; 6) HD; 7) Macia. M = Monomers; D = Dimers; O = Oligomers. Black arrows show high band intensity. White arrows show reduced band intensity. Loading at constant protein (10 µg).

The protein composition of the PP by wet milling was heterogenous (Figure 4.3.2), as they comprised all aqueous insoluble proteins in the grain. However, as would be expected, the kafirin proteins predominated due to their high concentration relative to other grain proteins (Table 4.3.1). Under both non-reducing and reducing conditions at least six monomeric kafirin bands with apparent molecular weights ranging from approximately 18 to 27 kDa were present, as was described by El Nour et al. (1998). The individual kafirin monomers are not identified, as unpublished work (Dr R. Jung, Pioneer Hi-Bred, personal communication) showed that the important 27 kDa ɣ-kafirin was not separated from the α-kafirins by SDS-PAGE in these sorghum lines.

Under non-reducing conditions, kafirin dimers and oligomers were also present in relatively high concentration (Figure 4.3.2a). The latter have been identified as

disulphide-bonded kafirins, pre-existing in the endosperm tissue (El Nour et al., 1998; Duodu et al., 2002; Emmambux and Taylor, 2009). Of significance is that the band intensities of the monomers and the number of resolving bands for the dimers were significantly higher in the TG lines (black arrows) compared to their NCs, HD and Macia. As the protein loading was constant for all the sorghums, this suggests that a lower proportion of the highly crosslinked polymeric kafirins (>200 kDa) (Ezeogu et al., 2005; Emmambux and Taylor, 2009) was formed in the TG lines during protein body synthesis.

Under reducing conditions, essentially only kafirin monomers were present (Figure 4.3.2b). This suggests that the kafirin polymers, oligomers and dimers were essentially all reduced, by disulphide bond reduction, into the various kafirin monomer sub-classes. In the TG lines, a major monomeric kafirin band was either absent or present, at much lower concentration, (white arrow) compared to their NCs, HD and Macia, presumably as result of suppressed synthesis of that particular kafirin protein.

4.3.4.2 Kafirin solubility and composition

Total kafirin extracted from whole grain flour of the TG lines and their NCs was in the range 47 to 50 g/100 g protein (Table 4.3.1), showing that they both had similar total kafirin content. The total kafirin content of the TG lines, fall within the normal range for whole grain flour, approximately 50-60% of the total protein in whole grain flour (Taylor, Novellie and Liebenberg, 1984). The high kafirin content of the TG lines is somewhat surprising as the ABS032 gene construct causes suppressed synthesis of α-kafirin A1, B1 and B2, ɣ-kafirin 1 and 2, and δ-kafirin 2 through RNA interference technology. Also, these TG lines were shown to contain considerably more total lysine, mean 2.65 g/100 g protein, than their NCs, mean 1.84 g/100 g protein. Also, the total protein content of the TG lines fell within the normal range for sorghum (Taylor and Taylor, 2011). Hence, the suppression of synthesis of these kafirin sub-classes also did not result in substantial reduction in total protein content. In native and mutant high-lysine cereals, their increased lysine content is attributed to a decrease in total prolamin content, with a compensatory increase in the lysine-rich

non-prolamin proteins (Shewry, 2007), as can be seen in the low percentage of total kafirin in the HD mutant, 33 g/100 protein.

Table 4.3.1 Kafirin extracted with 60% (v/v) tert-butanol 60% followed by (v/v) tert-butanol plus 5% (v/v) mercaptoethanol from transgenic (TG), null controls (NC), high protein digestibility mutant (HD) and Macia sorghum.

	Kafirin Extraction Yield (g/100 g protein)		
	Kafirin-1	**Kafirin-2**	**Total**
NC-P898012-2 (NC1)	19.9^{b}±1.5[1] (42.1)[2]	27.3^{c}±2.5 (57.9)	47.2^{c}±1.0
TG-P898012 (ABS032)-2 (TG1)	34.8^{d}[3]±1.2[1] (69.9[2])	15.0^{a}±2.3 (30.1)	49.8^{c}±1.1
NC-P898012-3 (NC2)	19.3^{b}±0.8 (40.4)	28.5^{c}±1.3 (59.6)	47.8^{c}±0.4
TG-P898012 (ABS032)-3 (TG2)	32.9^{d}±0.9 (69.1)	14.8^{a}±3.3 (30.9)	47.7^{bc}±2.4
HPDM8 (HD)	14.1^{a}±1.0 (42.8)	18.9^{b}±1.2 (57.2)	33.0^{a}±0.2
Macia	22.9^{bc}±1.4 (55.6)	18.3^{b}±1.9 (44.4)	41.2^{b}±0.5
Overall mean	**24.0 (53.3)**	**20.5 (46.7)**	**44.5**

[1]Means and standard deviations. [2]Values in parentheses indicate each kafirin fraction as a percentage of the total kafirin extracted. Kafirin-1 = protein extracted from whole grain flour using 60% tert-butanol. Kafirin-2 = protein extracted from residual whole grain flour using 60% tert-butanol plus 5% (v/v) 2-mercaptoethanol. [3]Different letters in columns indicate significant difference ($p<0.05$).

The fact that the TG lines had normal levels of total kafirin but increased protein lysine content, suggests compensatory synthesis of high-lysine non-kafirin proteins occurred at the expense of other non-kafirin endosperm proteins.

With regard to the nature of the kafirins in the TG lines, it is significant that extraction with 60% tert-butanol alone, extracted substantially more kafirin-1 (El Nour et al., 1998; Duodu et al., 2002; Emmumbux and Taylor, 2009) from the TG lines (approximately 69% of total kafirin extracted) compared to their NCs (40-42%), HD (43%) and Macia (56%) (Table 4.3.1). Since the total amount of kafirin extracted (kafirin-1 plus kafirin-2) from the TG lines was the same as their NCs and more than from HD and Macia, this indicates that the kafirin proteins in the TG lines were less crosslinked by disulphide bonding than the kafirins in normal sorghums, as was suggested by SDS-PAGE (Figure 4.3.2a).

SDS-PAGE of kafirin-1 under non-reducing conditions (Figure 4.3.3a) showed that the kafirin-1 of the TG lines comprised much higher concentrations of oligomers, dimers and certain monomers (black arrows) compared to their NCs, HD and Macia. As the protein loading was the same for all the sorghum types, this indicates that the kafirin-1 of the TG lines contained a lower proportion of kafirin polymers (>200 kDa) than the other sorghums. With SDS-PAGE under reducing conditions (Figure 4.3.3b) all the sorghum types essentially only exhibited kafirin monomers, which were at much higher band intensity than under non-reducing conditions (Figure 4.3.3a). This was due to the kafirin polymers, oligomers and dimers essentially all being reduced into kafirin monomers. Together, these results clearly show that the kafirin-1 in the TG lines was much less polymerised than that of their NCs, HD and Macia (Figure 4.3.3a). This can be attributed to suppression of synthesis of the cysteine-rich ɣ-kafirins, which are required for polymerisation through disulphide bonding (El Nour et al., 1998).

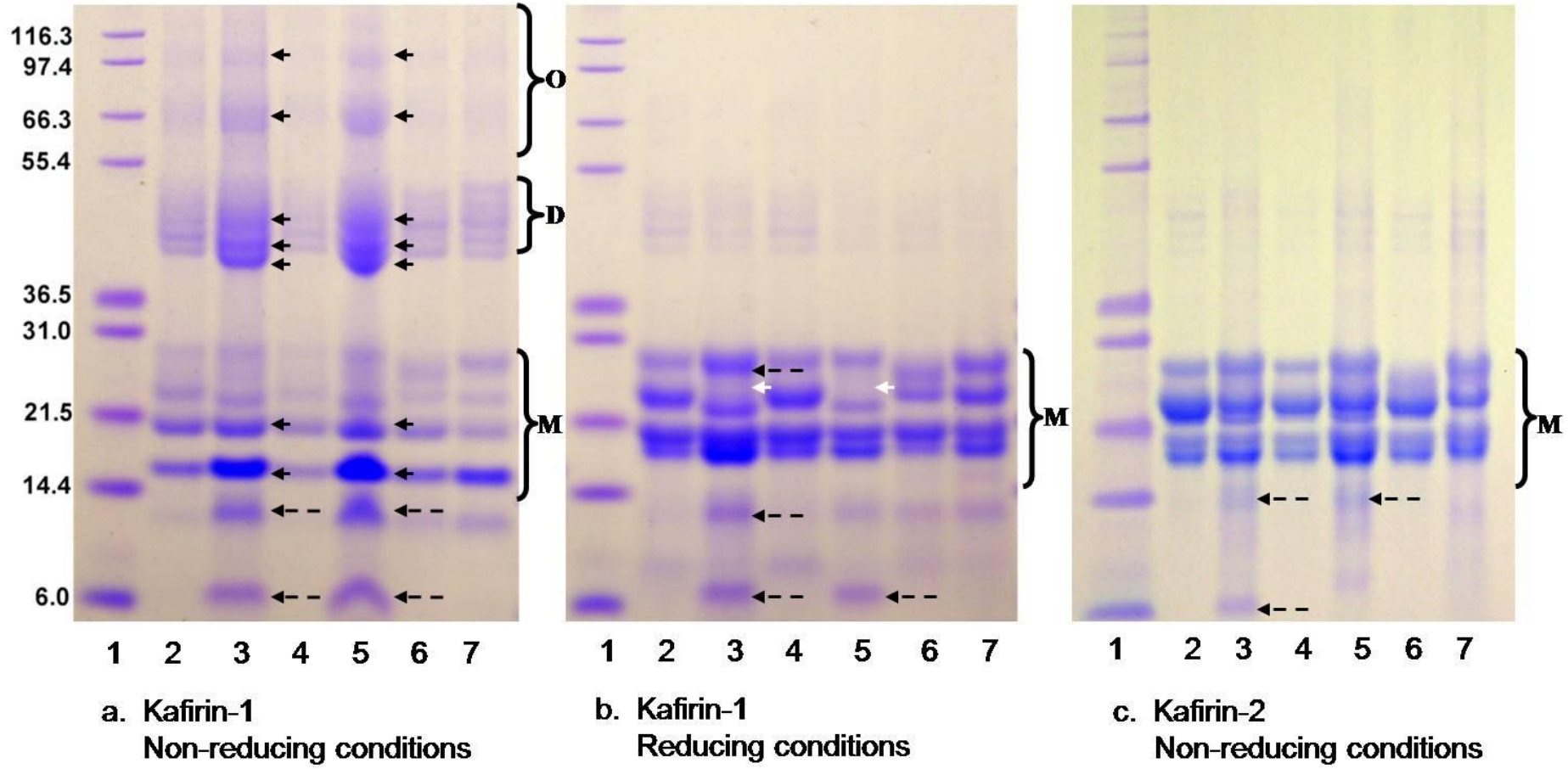

Figure 4.3.3 SDS-PAGE of extracted kafirin.
a-b) kafirin-1 extracted with 60% tert-butanol , c) kafirin-2 extracted with 60% tert-butanol plus 5% 2-mercaptoethanol.
1) Molecular weight markers (kDa); 2) NC1; 3) TG1; 4) NC2; 5) TG2; 6) HD; 7) Macia (in all cases). M = Monomers; D = Dimers; O = Oligomers. Black arrows show high band intensity. White arrows show reduced band intensity/or absence. Black dashed arrows show additional bands. Loading at constant protein (10 µg).

Additionally, there were significant differences between the kafirin monomer bands present in the TG lines and the other sorghums (Figure 4.3.3b), as in the PP by wet milling (Figure 4.3.2). At least one major band was absent in TG1 and TG2 at approximately 25 kDa (white arrows), which was present in their NCs, HD and Macia, showing that its synthesis was suppressed. Also, at least one new band was present in the TG lines at approximately 23 kDa (Figure 4.3.3b, top dashed black arrow), which was absent in the normal sorghums, indicating that compensatory synthesis of other kafirin proteins or possibly non-prolamin proteins had taken place.

Further, with SDS-PAGE under both non-reducing and reducing conditions additional high intensity low molecular weight bands (<14.4 and 6.0 kDa) were also present in kafirin-1 of the TG lines (Figure 4.3.3a,b lower black dashed arrows). These low molecular weight proteins are presumably kafirins as the extraction conditions used (60% tert-butanol extract) is specific for solubilizing prolamin proteins such as kafirin (Taylor et al., 1984; El Nour et al., 1998). The presence of these low molecular weight kafirins may be due to compensatory over expression of additional kafirin proteins, or kafirin fragments, or alcohol-soluble non-prolamin proteins (reviewed by Shewry and Pandya, 1999), as a result of modified gene expression.

The kafirin-2 proteins (Figure 4.3.3c), which were extracted under reducing conditions after extraction of kafirin-1, showed generally similar band patterns to the kafirin-1 proteins separated under reducing conditions (Figure 4.3.3b). The kafirin-2 proteins are presumably originally made up of the disulphide-bonded kafirin polymers, which were reduced into monomers. The low molecular weight kafirins were again present in the TG lines, although at a reduced intensity (Figure 4.3.3c, black dashed arrows). Of significance is that the pattern of TG1 and TG2 kafirin-2 proteins is more similar to that of the NCs, HD and Macia than was the case with the kafirin-1 proteins under reducing conditions (Figure 4.3.3b). This can be attributed to the kafirin-2 proteins originally being disulphide-bonded polymers, where the cysteine-rich ɣ- and β-kafirins, must be present in order for the kafirins to be polymerised (El Nour et al., 1998).

4.3.4.3 Pepsin digestion of the PP by starch digestion

Treating the cooked flours with amylases to remove all the gelatinized starch formed during cooking, which can embed the protein bodies and matrix, improved the cooked IVPD of all the sorghum samples by 14-36% compared with no pre-treatment (Table 4.3.2). The improved IVPD of the samples following starch removal is presumed to be due to the pepsin having greater access to the protein structures (Duodu et al., 2002). Importantly, however, after removal of the starch the TG lines still had substantially higher IVPD (25 to 32%) compared to their NCs. This shows that the protein in the TG lines was intrinsically more available to proteolysis. The fact that the IVPD of the TG lines was the same as that of Macia, a normal sorghum, and lower than that of HD, can be attributed to the presence of tannins in the TG lines, which would interfere with protein digestion (Butler, Riedl, Lebryk and Blyit, 1984; Taylor et al., 2007).

Table 4.3.2 Effect of altered kafirin synthesis on the *in vitro* protein digestibility of cooked sorghum flour pre-treated with amylase (PP by starch digestion) to digest the starch.

	IVPD no pre-treatment (%)	IVPD pre-treated (%)	Difference
NC-P898012-2 (NC1)	$37.7^{a}\pm1.4^{1,2}$	$52.7^{a}\pm2.7$	15.0
TG-P898012(ABS032)-2 (TG1)	$57.8^{bc1,2}\pm2.3$	$65.7^{b}\pm3.3$	7.9
NC-P898012-3 (NC2)	$38.8^{a}\pm2.0$	$51.7^{a}\pm1.9$	12.9
TG-P898012(ABS032)-3 (TG2)	$55.2^{b}\pm2.0$	$68.0^{b}\pm5.2$	12.8
HPDM8 (HD)	$55.0^{b}\pm2.0$	$74.8^{c}\pm4.6$	19.8
Macia	$60.0^{c}\pm2.0$	$69.2^{b}\pm0.8$	9.2
Overall mean	**50.7**	**63.7**	**13.0**

[1]Means and standard deviations, [2]Different letters in columns indicate significant difference ($p<0.05$).

The compositions of pepsin-indigestible proteins in the PP by starch digestion residues (Figure 4.3.4) were generally similar to the native proteins in the PP by wet milling samples (Figure 4.3.2). However, under non-reducing conditions it appeared that the relative ratio of oligomers and dimers to monomers was higher in the PP by starch digestion pepsin indigestible residues (Figure 4.3.4a) than in the PP by wet milling (Figure 4.3.2a). Also, of note is that higher numbers of oligomer and dimer bands occurred in the TG lines compared to their NCs, HD and Macia (Figure 4.3.4a, black arrows), as in the PP by wet milling, but the levels of monomers remaining in TG and their NCs were similar, unlike the situation in the PP by wet milling.

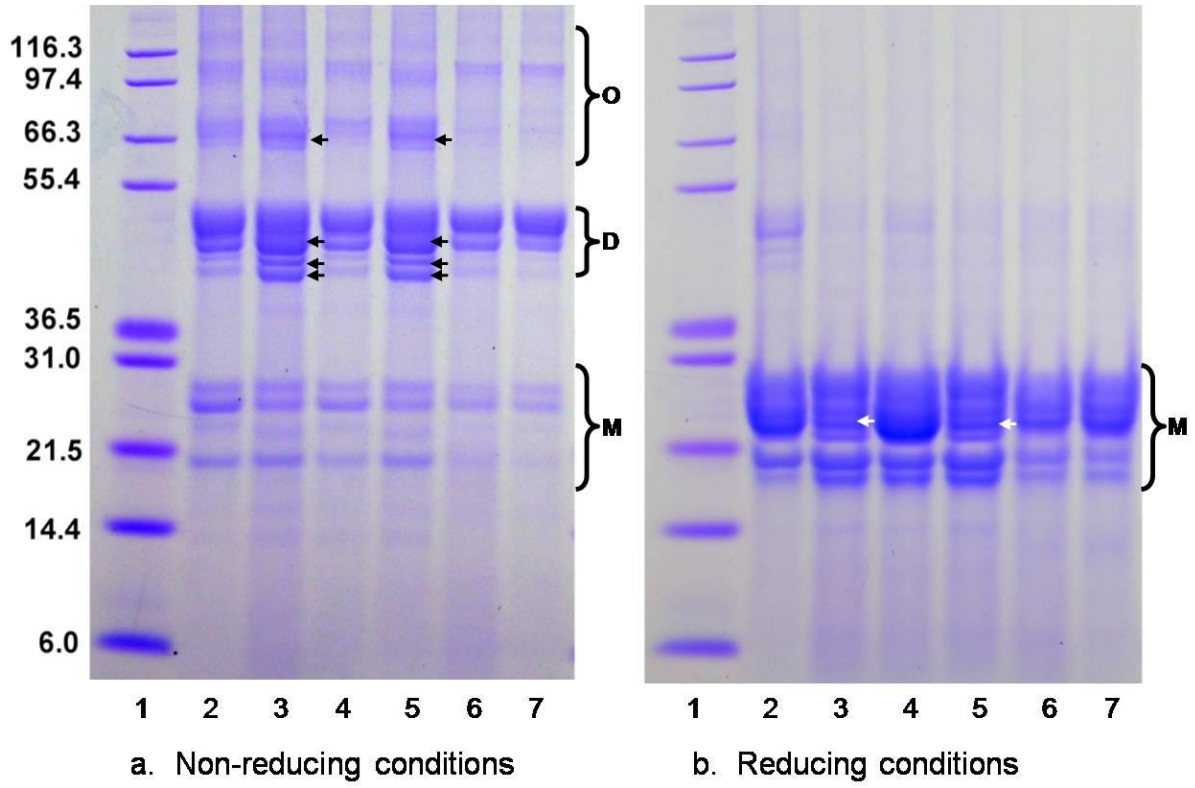

Figure 4.3.4 SDS-PAGE of pepsin-indigestible residue from cooked protein preparations by starch digestion.
1) Molecular weight markers; 2) C1; 3) T1; 4) C2; 5) T2; 6) HD; 7) Macia. M = Monomers; D = Dimers; O = Oligomers. Black arrows show high band intensity. White arrows show reduced band intensity/or absence. Loading proportional to the quantity of protein remaining after digestion.

The fact that substantial amounts of kafirin oligomers and dimers were present in the pepsin indigestible residues implies that even these relatively small disulphide-bonded kafirins are more resistant to hydrolysis than the monomers.

Upon reduction, the polymeric proteins were resolved into the different kafirin monomer sub-classes (Figure 4.3.4b). Significantly, the low molecular weight kafirins, which were present in TG1 and TG2 PP by wet milling, kafirin-1 and kafirin-2 preparations (Figures 4.3.2 and 4.3.3), were absent. This indicates that they were readily hydrolysed by pepsin. However, similar to the PP by wet milling (Figure 4.3.2b) and the kafirin-1 (Figure 4.3.3b), particular kafirin monomers, probably ɣ-kafirin, that were present in high concentration in the PP by starch digestion pepsin indigestible residues of the NCs were absent, or present at very reduced concentration in the TG lines (Figure 4.3.4b, white arrows). It should be noted that TG1 and TG2 were only 75% pure with regard to the ABS032 gene construct. Thus, the presence of the monomeric kafirins would be from the 25% normal sorghum fraction. Nevertheless, this suggests that the kafirins in PP by starch digestion pepsin indigestible residues of the TG lines were less polymerised than those of their NCs, due to the suppression of synthesis of ɣ-kafirin. This is consistent with the higher pepsin digestibility of the TG lines compared to their NCs.

TEM of the protein bodies in the PP by starch digestion of the TG lines, NCs and Macia after pepsin digestion, revealed disappearance of the matrix protein between the protein bodies and digestion of the kafirin ground material from the periphery of the protein bodies (Figure 4.3.5). This pattern of sorghum protein body digestion is normal (Taylor, Novellie and Liebenberg, 1985; Taylor and Evans, 1989; Ng'andwe, Hall and Taylor, 2008). In normal sorghum the ɣ- and β-kafirins are concentrated at the periphery of the protein bodies and appear as dark regions (Shull et al., 1992) (dashed black arrows in the NC (Figure 4.3.5a), and Macia (Figure 4.3.5c)) and are believed to inhibit access of proteases to the α-kafirin ground material (Oria et al., 1995a), thus majorly contributing to the poor protein digestibility of wet cooked sorghum. The absence of, or greatly reduced amount of dark staining regions (crosslinked ɣ- and β-kafirins) in the protein bodies of the TG (Figure 4.3.5b) presumably facilitates more rapid digestion of the protein bodies, but as observed does not affect the general pattern of protein body digestion.

With the HD, protein bodies could not be observed in the PP by starch digestion after pepsin digestion, presumably due to its higher IVPD (Table 4.3.2). However, in these HD mutants the protein bodies are very folded (invaginated) (Figure 4.3.1c), with the ɣ-kafirin located at the base of the invaginations (Oria et al., 2000), with the effect that proteases have easy accessibility to the α-kafirin. Thus, it appears that the reason for the high digestibility of the protein bodies in the TG lines is different from that in the HD types.

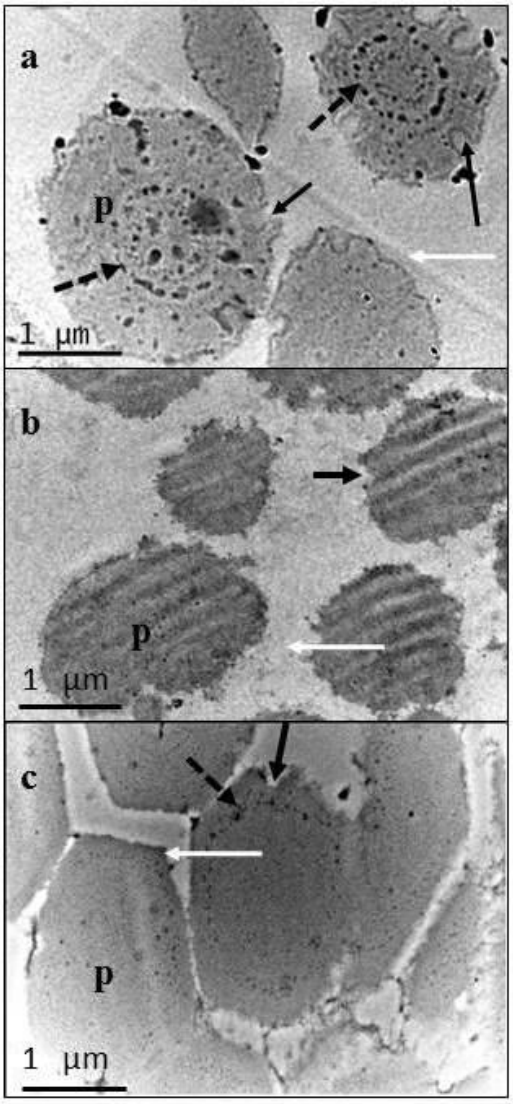

Figure 4.3.5 Representative images of TEM of pepsin-indigestible residue from cooked protein preparations by starch digestion.
a) NC1; b) TG1; c) Macia; p = protein body; Solid black arrows show proteolysis of protein body periphery; Dashed black arrows show dark staining regions, presumed primarily ɣ- and β-kafirin (Shull et al., 1992); White arrows show where matrix protein was.

4.3.5 Conclusions

Suppressed synthesis of the cysteine-rich ɣ-kafirin sub-class in these TG sorghum lines results in a substantially lower proportion of disulphide bonded kafirin polymers. Despite suppression of synthesis of these and other major kafirin sub-classes there is, however, compensatory synthesis of other kafirins. This results in a normal proportion of kafirins in the grain, which is probably related to their protein bodies being intermediate in appearance between the normal type and the characteristic invaginated HD type. The higher protein digestibility of these TG lines is most probably related to their lower levels of disulphide-bonded kafirin polymerisation, allowing better access of proteases. This work seems to confirm the theory of Hamaker et al. (1987) and Rom et al. (1992) that disulphide bond formation in kafirin is responsible for the reduced protein digestibility of cooked sorghum, where it was found that the disulphide bond-breaking reducing agents improved sorghum protein (Hamaker et al., 1987, Rom et al., 1992) and protein body digestion (Rom et al., 1992).

4.3.6 References

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