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Transgenic sorghum: Effects of altered kafirin synthesis on kafirin polymerisation, protein quality, protein body structure and endosperm texture

By

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DECLARATION

I hereby declare that the thesis submitted at the University of Pretoria for the award of PhD degree is my work and has not been submitted by me for a degree at any other university or institution of higher learning.

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Laura Suzanne da Silva

DEDICATION

I dedicate this thesis to my wonderful family. To my husband, José, for his continued support, understanding and patience over the last six years. To my sons, Daniel and Nathan, for ensuring that play time and walks at the Botanical Gardens were non-negotiable.

Perseverance is the hard work you do after you get tired of doing the hard work you already did.

Newt Gingrich

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ABSTRACT

Transgenic sorghum: Effects of altered kafirin synthesis on kafirin polymerisation, protein quality, protein body structure and endosperm texture

By

Laura Suzanne da Silva

Supervisor: Prof. J.R.N. Taylor
Co-supervisor: Dr J. Taylor

Transgenic (TG) sorghum genotypes with altered kafirin synthesis were developed by the Africa Biofortified Sorghum Project, employing recombinant DNA technology, with the aim of improving the protein nutritional quality of the grain. In this project, the effects of altered kafirin synthesis on kafirin polymerisation, protein quality, protein body structure and endosperm texture in different TG lines were investigated.

The first generation of TG lines were in a type II low-tannin sorghum background. Altered synthesis of different major kafirin sub-classes (α-, ɣ- and δ-kafirin) was targeted. Some TG lines had improved lysine content (3.17 g/100 g protein) and moderate (55%) to high (74%) cooked *in vitro* protein digestibility, compared to the parent (2.05 g/100 g protein; 47.4%, respectively). This is of significance as tannins reduce protein digestibility, by complexing with the proline-rich kafirins. Transmission electron microscopy revealed that the improved protein quality traits were associated with floury endosperm texture and irregular protein body structure. Irregular protein bodies were 2-3 µm in diameter, with few to numerous invaginations, compared to normal protein bodies. The high digestibility TG line also had a unique dense protein matrix, with occasional thick dark-staining inclusions. It appears that reduced kafirin synthesis, specifically ɣ-kafirin, has a major effect on the protein body structure, which in turn results in changes in protein digestibility and endosperm structure.

To further improve the protein quality and poor endosperm texture of the first generation of TG lines, improved non-tannin sorghums were transformed to suppress kafirin synthesis, or they were back-crossed into TG lines with improved protein quality. Co-suppression of the α-, ɣ- and δ-kafirin sub-classes and removal of the tannin trait, resulted in TG 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-TG null controls (±50%, 0.4 and 0.2, respectively). However, these high-protein quality lines still had a floury endosperm texture. They also had irregular shaped protein body structure, as described previously. When fewer kafirin sub-classes were suppressed (only ɣ- and δ-kafirin) the endosperm was corneous with normal protein body structure, but the improvement in cooked protein digestibility was less. Apparently, co-suppression of several kafirin sub-classes is required to obtain high-protein quality sorghum, but this seems to result in floury-type grain endosperm.

Further work conducted on the high digestible TG line revealed that the proportion of kafirin-1, extracted with 60% tert-butanol alone, was greatly increased. However, the total amount of kafirin remained unchanged. Also, the kafirin was much less polymerised by disulphide bonding, and there was evidence of compensatory synthesis of other kafirin proteins. Hence, the mechanism for the increased protein digestibility of TG lines is probably related to their lower levels of disulphide-bonded kafirins, 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.

Since grain hardness is an important grain quality attribute, playing a major agronomic role, in sorghum processing and in the end-use quality of sorghum-based foods, further research should focus on transforming sorghum to have both improved protein nutritional quality and good grain endosperm texture.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

Sorghum is a major subsistence cereal crop grown in Africa and Asia, due to its hardiness and drought-resistance, enabling its cultivation in semi-dry regions (FAO 1995, reviewed by Belton and Taylor, 2004). Hence, sorghum is a major food staple for the poorest people of Africa, and is the principal source of energy, protein, vitamins and minerals for these people (FAO, 2002; ICRISAT, 2009). Sorghum grain contains about 11% protein (ranging between 7 to 16%), which is similar to other major cereals (Serna-Saldivar and Rooney, 1995). However, the protein quality (specifically lysine protein content and protein digestibility) of sorghum is inferior to other cereals. The lysine content of its protein is substantially lower, by 35 to 90% compared to other cereals (reviewed by Henley, Taylor and Obukosia, 2010). The lower lysine content is due to the major proteins of sorghum, the kafirin prolamin storage proteins, being essentially lysine-free (reviewed by Belton, Delgadillo, Halford and Shewry, 2006). In addition, the digestibility of sorghum proteins is lower than that of maize, despite the fact that the proteins are very similar (reviewed by Duodu, Taylor, Belton and Hamaker, 2003). The low protein digestibility of sorghum is even further reduced after wet cooking, such as porridge making (Axtell, Kirleis, Hassen, D'Cros-Mason, Mertz, and Munck, 1981; Taylor and Taylor, 2002). The significance is that sorghum foods have much lower protein digestibility (protein quality) than foods made from other cereals (Mertz, Hassen, Cairns-Whittern, Kirleis, Tu and Axtell, 1984). These factors have a huge impact on communities that rely on sorghum as a major staple, where Protein Energy Malnutrition (PEM), insufficient food, lack of dietary diversity and disease outbreaks are common (Muller and Krawinkle, 2005).

Due to the above, efforts to improve the overall nutritional quality of sorghum are taking place. Nutrient fortification strategies for sorghum (and other cereals) include improved agronomic practices, breeding high nutrient yielding cultivars and nutrient targeted genetic modification (or biofortification) (reviewed by Poletti, Gruissem and Sautter, 2004). Biofortification can be defined as a process to increase the bioavailability and the concentration of nutrients in crops through both conventional plant breeding and recombinant DNA technology (genetic engineering) (Henley et

al., 2010). Effective biofortification of sorghum could contribute to improved nutritional quality of sorghum-based foods, which in turn could alleviate some of the macro- and micronutrient deficiencies of poorer communities. However, the accumulation of macro- and micronutrients in the grain is a limiting factor in health improvement, unless the bioavailability and retention of these nutrients in the processed food is improved.

Currently, nutritionally improved sorghum lines with reduced expression of different kafirin sub-classes, are being developed using genetic engineering techniques by the Africa Biofortified Sorghum (ABS) Project, under the Bill and Melinda Gates Foundation Grand Challenges (No. 9) in the Global Health Initiative (ABS Project, 2009). The Grand Challenge 9 (GC 9) intends to ‘‘Create a full range of optimal bioavailable nutrients in a single staple plant species’’ with the aim of improving nutrition to promote health. The specific aims of the ABS Project are to: 1) increase iron and zinc availability by 50%, 2) to increase provitamin A levels to up to 20 mg/kg, 3) to increase lysine content by 80–100%, 4) to increase tryptophan and threonine by 20%, 5) to concomitantly decrease leucine by 15%, and 6) to improve protein digestibility from its current value to approximately 60–80% (ABS Project, 2009; Henley et al., 2010). These improvements in sorghum nutrient content and availability are based on the average levels currently found in normal sorghum. In this work, only the improvements in lysine content and *in vitro* protein digestibility will be addressed.

2 LITERATURE REVIEW

In this review, focus is put on the development of high-lysine sorghum and maize mutants, with emphasis on protein nutritional quality (lysine content and protein digestibility) and grain endosperm quality. Research conducted on the sorghum protein composition (non-kafirins and kafirin proteins), the synthesis of sorghum and maize protein bodies, distribution of different prolamins in the protein bodies, and the role the different prolamins play in protein body structure and endosperm structure will be reviewed. Comparisons will be made between normal sorghum, normal maize, high-lysine and high-protein digestibility mutants, Quality Protein Maize and research conducted on transgenic maize. Biotechnology used to transform the protein nutritional quality of sorghum in the ABS Project will also be covered.

The nutritional quality of a protein source for humans (or animals) can be defined as the power of that protein source to cover the requirements for nitrogen and amino acids of these organisms (reviewed by Schaafsma, 2005). In this work the term '*protein nutritional quality'* will refer specifically to lysine content and protein digestibility.

2.1 Development of high-lysine maize and sorghum

Research to find cereals with improved protein quality has been ongoing since the early 1960's, beginning with reports of naturally occurring (spontaneous) high-lysine mutant maize genotypes, *opaque-2* (Mertz, Bates and Nelson, 1964) and *floury-2* (Nelson, Mertz and Bates, 1965). The lysine content of the high-lysine maize endosperm tissue was found to be 4 and 3 g lysine/100 g protein, respectively, compared to 2 g lysine/100 g protein of normal maize endosperm. The improved lysine content of these mutant grains was attributed to low levels of the lysine-poor seed storage proteins, the prolamins, which are called zeins in maize, with compensatory increases in the lysine- and tryptophan-rich non-zein seed proteins, as well as free lysine and tryptophan compared to normal maize (reviewed by Gibbon and Larkins, 2005). The improved protein nutritional value of *opaque*-2 maize was demonstrated in a series of growth experiments using rats, weaning pigs

and young children suffering from protein malnutrition (identified as kwashiorkor) (reviewed by Mertz, Axtell, Ejeta and Hamaker, 1993). Diets containing *opaque*-2 maize combined with minerals and vitamins were found to significantly increase the growth in rat and weaning pig feeding trials compared to normal maize, while in human feeding trials, diets based solely on *opaque*-2 flour was shown to cure children who suffered from kwashiorkor. The improved health in human children was attributed to the fact *opaque*-2 maize has 90% the protein nutritional value of milk protein (Mertz et al., 1993). Over the years, *opaque-2* has been used in a number of breeding trials to produce several high-lysine mutant maize genotypes with good corneous endosperm texture, referred to as Quality Protein Maize (QPM) (Gibbon and Larkins, 2005).

Success with *opaque*-2 maize encouraged extensive research to identify similar naturally occurring mutants in other cereals, including barley (Munck, Karlsson, Hagberg and Eggum, 1970) and sorghum (Singh and Axtell, 1973). The use of chemical mutagens to induce high-lysine mutants was also investigated (Mertz et al., 1993; Eggum, Brunsgaard and Jensen, 1995). In 1966, Purdue University, USA, initiated a comprehensive programme to systematically investigate the inheritance and improvement of protein content and protein quality in sorghum (reviewed by Wall and Paulis, 1978). Two naturally occurring high-lysine mutants (IS11167 and IS11758, of Ethiopian origin) were identified from 62 varieties, all with floury endosperm phenotype, obtained from the world sorghum collection (9 000 varieties) (Singh and Axtell, 1973). These high-lysine mutants were very high in protein (15.70% and 17.20%) and lysine content (3.33 and 3.13 g lysine /100 g protein), respectively, compared to the mean protein (12.7%) and lysine (2.05%) content of the remaining 60 normal varieties analysed.

Another approach used during this period was to induce high-lysine mutations in sorghums using the chemical mutagen, diethylsulphate (Mertz et al., 1993). The mutation resulted in at least a 60% increase in protein lysine content. High-lysine sorghum selection, P721 opaque (P721Q), contained similar protein content (12.9%) as the normal parent (P721N), but had 3.1 g lysine/100 g protein as opposed to 2.1 g lysine/100 g protein in the normal parent.

Inheritance studies on native high-lysine sorghums (Singh and Axtell, 1973) and the chemically induced high-lysine P721Q mutant, suggested that the increased lysine concentration is controlled by a single recessive gene that is simply inherited in both cases (Wall and Paulis, 1978). The high-lysine gene is therefore easily transferred by standard plant breeding procedures. Thus, both native high-lysine sorghums (Jambunathan, Mertz and Axtell, 1975) and the chemically induced high-lysine mutant P721Q (Weaver, Hamaker and Axtell, 1998; Tesso, Ejeta, Chandrashekar, Huang, Tandjung, Lewamy, Axtell and Hamaker, 2006; Nyannor, Adedokun, Hamaker, Ejeta and Adeola, 2007; Tesso, Hamaker and Ejeta, 2008) have been used in plant breeding programmes to develop high-lysine populations derived from crosses with normal commercially important sorghums.

2.2 Amino acid profile and protein composition of high-lysine mutants

In high-lysine sorghums, as is the case with other high-lysine cereals such as *opaque*-2 maize, the high-lysine gene alters the amino acid pattern in the floury endosperm tissue relative to normal vitreous endosperm tissue (Singh and Axtell, 1973). The major changes observed were increased lysine, arginine, aspartic acid, glycine and tryptophan concentration and decreased amounts of glutamic acid (glutamine), proline, alanine and leucine in the floury endosperm tissue. Differences in the distribution of soluble protein fractions between high-lysine and normal sorghums is considered to be the primary reason for the improved lysine content of high-lysine sorghums. Jambunathan et al. (1975) compared the distribution of soluble protein fractions in native high-lysine sorghum (IS11758), high-lysine hybrid (F_2) sorghums and normal sorghums with similar genetic background. It was shown by these authors that the native (IS11758) and hybrid high-lysine (F_2) kernels had a decrease in the concentration of lysine-poor kafirin proteins, with a compensatory increase in the lysine-rich non-kafirin proteins (albumins, globulins and glutelins, including free amino acids) compared to the normal sorghums. The reduced synthesis of kafirin proteins and compensatory increased synthesis in non-kafirin proteins no doubt accounts for the increased lysine content in high-lysine kernels. This was later confirmed in a study using high-lysine mutants (IS11167 and P721Q) and normal sorghums (Guiragossian, Chibber, Van Scoyoc, Jambunathan, Mertz

and Axtell, 1978). However, these authors showed that the modified distribution of soluble protein fractions and increased lysine content was endosperm specific. It was suggested that in high-lysine mutants it is necessary to look at the distribution of soluble proteins specifically in the endosperm tissue to fully understand the marked changes in amino acid patterns observed in the whole grain. This is due to the fact that lysine-rich albumin and globulin proteins are found primarily in the germ (and cells of the aleurone layer) (Wall and Paulis, 1978; Taylor and Schüssler, 1986). Sorghum kernels with a large germ and small endosperm portion, or floury endosperm can in fact show high lysine content. However, these grains generally have low protein content. Even so, differences in total protein soluble in 60% tertiary butanol with a reducing agent, 2-mercaptoethanol (2-ME) has been used to distinguish normal and high-lysine sorghums (Wall and Paulis, 1978).

In maize, a similar protein content was also found to occur between different high-lysine opaque mutants (ranging from 9.3 to 12.8%), QPM (ranging from 9.7 to 10.8%) and normal maize genotypes (ranging from 9.2 to 10.8%) (Gibbon and Larkins 2005). It would appear that the protein content of different sorghums (and maize) is relatively stable regardless of the genotype. Nitrogen fertilisation has been found to be a major factor in elevating the protein content in normal sorghum (Walls and Paulis, 1978). In these cases, a slight decline in lysine content is reported, in normal sorghum, which is thought to be due to increased synthesis of the lysine-poor storage proteins (kafirins) in the grain endosperm, thereby reducing the protein nutritional quality of the grain.

Nevertheless, the increased lysine content reported for high-lysine mutant sorghum and maize is still below that recommended by the World Health Organization (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). It is not known if genetic transformation can increase the lysine content of transformed cereals to meet the lysine requirements recommended by the WHO.

2.3 Classification of sorghum proteins

Sorghum proteins can be classified into five major classes namely: albumins (water-soluble), globulins (salt-soluble), prolamins (kafirins) (aqueous alcohol-soluble), crosslinked kafirins (aqueous alcohol + reducing agent-soluble) and glutelins (detergent + reducing agents + alkaline pH-soluble) (reviewed by Hamaker and Bugusu, 2003). This solubility-based classification scheme was proposed by Landry and Moureaux (1970) for the proteins of maize and was modified by Jambunathan et al. (1975) for sorghum. Later studies revealed that the kafirins and crosslinked kafirins, after reduction with a reducing agent like 2-ME, are essentially identical. Thus, sorghum proteins can be classified simply as kafirins and non-kafirins (Hamaker, Mohamed, Habben, Huang and Larkins, 1995).

2.3.1 Non-kafirin proteins

The albumin and globulin proteins are concentrated in the germ of the kernel, and are higher in nutritional value compared to the kafirin proteins, due to higher concentrations of lysine (Taylor and Schüssler, 1986). The glutelin proteins (also referred to as endosperm matrix protein) are the second major proteins of the endosperm (Serna-Saldivar and Rooney, 1995). They are higher in the basic amino acids, glutamic acid and lysine, compared to the kafirins. The glutelin proteins are thought to also function as a source of enzymes involved in the hydrolysis of starch and protein reserves, as opposed to being storage type proteins like the kafirins (Taylor, Novellie and Liebenberg, 1984). In maize (and possibly sorghum), the glutelin proteins making up the matrix protein are thought to be derived from cytoplasmic albumin and globulin proteins which form disulphide-linkages during the latter stages of seed development (Wall, Cooker and Bietz, 1988).

2.3.2 Kafirin proteins

The kafirin proteins are the most abundant proteins in whole grain sorghum, making up 50 to 60% of the total protein (Taylor and Schüssler, 1986; Shull, Watterson and Kirleis, 1991). However, values as high as 70% of whole grain protein and about

80% of decorticated grain protein have been reported (Hamaker et al., 1995). Kafirins are rich in glutamine, proline, alanine and leucine, but are essentially free of lysine (reviewed by Lásztity, 1984, reviewed by Shewry and Halford, 2002). It is due to the high content of kafirin prolamin storage proteins, which are deficient in essential amino acids, that sorghum (and other cereals) is rendered inadequate with regard to protein nutritional quality.

Kafirin proteins are known to be more hydrophobic than other prolamin proteins (such as zein) due to a higher proportion of non-polar amino acid residues (Duodu et al, 2003). Proline and glutamine constitute more than 30% of the kafirin proteins. Thus, the optimum solubility condition for kafirin is 60% aqueous tertiary butanol, due to the higher hydrophobicity of this solvent compared to other aqueous alcohol solvents, such as ethanol and propanol which are commonly used (Belton et al., 2006). Small quantities of cystine (sulphur-containing amino acid) contribute to intra- and inter-molecular disulphide bonding of kafirins. Disulphide bonding of kafirin proteins results in the formation of large polymeric kafirin proteins, which are insoluble in the aqueous alcohols and have low digestibility (Hamaker et al., 1995; El Nour, Peruffo and Curioni, 1998). Consequently a reducing agent such as 2-ME is required to break the disulphide bonds and improve extraction of the kafirin proteins.

2.3.2.1 Kafirin sub-classes

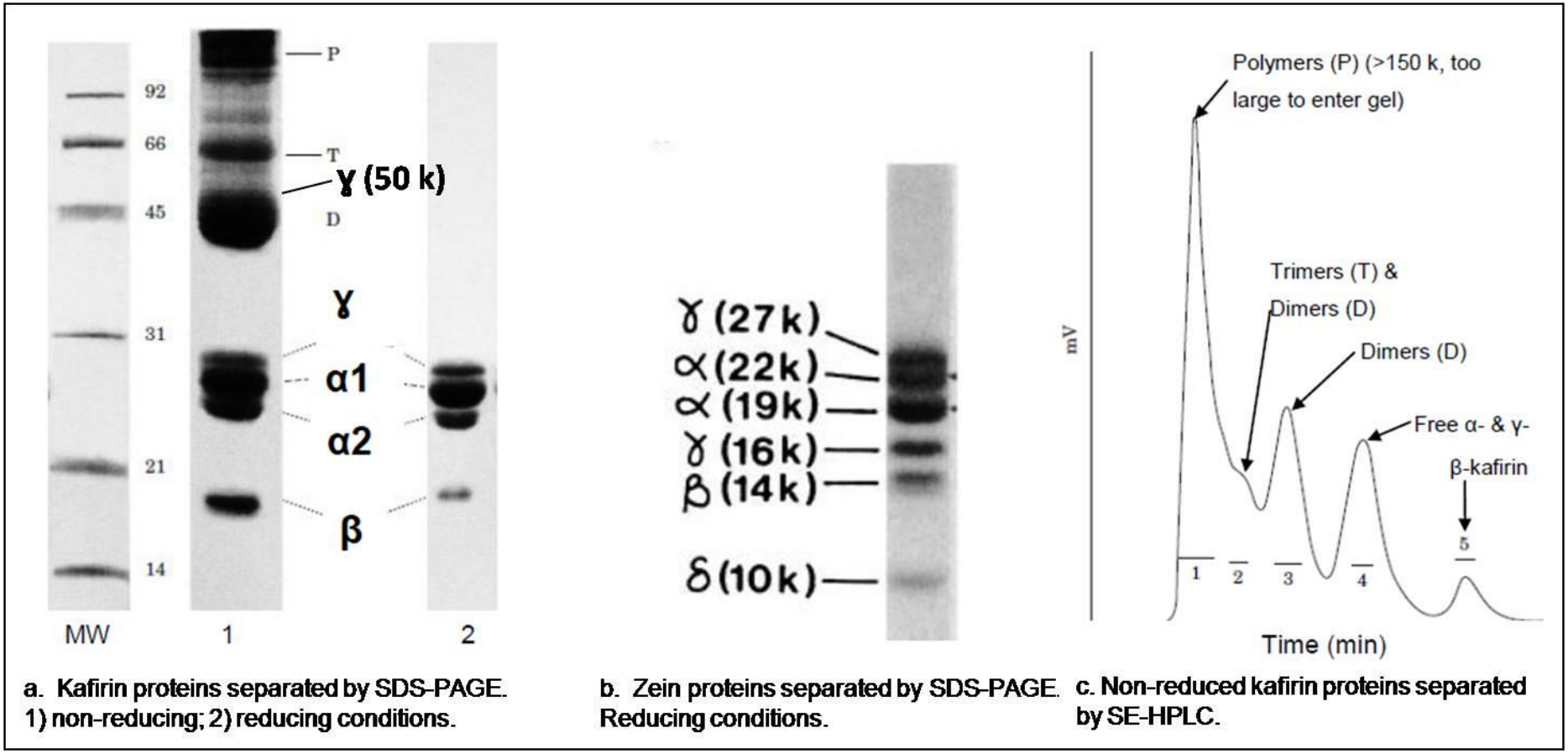
Kafirins are heterogeneous proteins separable into different sub-classes (α-, β- ɣ- and δ-kafirins) of different proportions (Belton et al., 2006). The sub-classes are characterised based on their solubility, amino acid composition, electrophoretic, chromatographic, and immunological property differences and DNA sequencing (Shull et al., 1991; Belton et al., 2006). Table 2.1 summarises the major properties of the different kafirin sub-classes with regard to percentage of total kafirin fractions in both corneous (vitreous) and opaque endosperm, number of resolving bands observed by SDS-PAGE, apparent molecular mass, amino acid composition and polymerisation (crosslinking) behaviour. Kafirin proteins are homologous to the zeins and many comparative studies regarding these two prolamin proteins have been done (Esen, 1987; Shull et al., 1991; Mazhar, Chandrashekar, and Shetty,

1993; El Nour et al., 1998; Nunes, Correia, Barros, and Delgadillo, 2005). Due to this homology a nomenclature for kafirin has been described, but is constantly being reviewed and modified as new and improved technologies are developed (reviewed by Bean and Lookhart, 2000). In this current research project, the different kafirin sub-classes targeted for suppressed synthesis are defined as, 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) (Dr R. Jung, Pioneer Hi-Bred, personal communication).

The crosslinking behaviour of the different monomeric kafirins to form dimers, oligomers and polymers is directly related to the number of cysteine residues per monomer. Figure 2.1 shows the separation of kafirin and zein proteins by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), and the separation of non-reduced kafirin proteins by SE-HPLC (size exclusion high performance liquid chromatography). According to El Nour et al. (1998) α-kafirins are present in whole grain flour as both monomeric and polymeric proteins, under non-reducing conditions (Figure 2.1a). The α-kafirin 1 (26.6 kDa, 2 cysteine residues) can link by disulphide bonds to form different sized oligomers, while α-kafirin 2 (24 kDa, one cysteine residue) forms dimers or small oligomers. Thus, α-kafirin 1 is considered to be a ‘chain extender’ and α-kafirin 2 a ‘chain terminator’. The β-kafirins contain 10 cysteine residues, which enables them to form either inter- or intra-molecular disulphide bonds, forming oligomers and polymers (Belton et al., 2006). According to El Nour et al. (1998), β-kafirin can act as a bridge between oligomers of α-kafirin 1 and ɣ-kafirins resulting in very large polymers, extractable only under reducing conditions. High molecular weight polymeric proteins (>150 kDa) are not observed by SDS-PAGE, as they are too large to enter the stacking gel (El Nour et al., 1998). However, other techniques such as size exclusion chromatography can be used to separate the large polymeric proteins (Figure 2.1c) as was demonstrated by El Nour et al. (1998) and later by Emmambux and Taylor (2009). The ɣ-kafirins contain substantially more cysteine residues (15 residues) and are present in their native state as polymers stabilised by disulphide bonds (Belton et al., 2006) and as disulphide crosslinked polymers with α-kafirin 1 (El Nour et al., 1998).

Table 2.1 Summary of the major properties of the four types of kafirin proteins and their crosslinking behaviour within the sorghum grain (modified from Belton et al. (2006)).

Kafirin type	Total kafirin fraction (%) (in vitreous (corneous) or opaque(floury) endosperm)	Number of resolving bands	Apparent molecular mass ranges reported (kDa)	Amino acid composition	Polymerisation behaviour
α-	80-84% (vitreous) 66-71% (opaque)	Two major bands. Can be resolved into more components which vary in number and properties between genotypes, and separation technique used.	19 to 27 (19, 22, 23, 24, 25)	240-250 amino acid residues. Rich in non-polar amino acids, no lys, 1 typ, 10 blocks of repeated amino acids	Monomers, oligomers and polymers α- 1, “Chain extender” α- 2, “Chain terminator”
β-	7-8% (vitreous) 10-13% (opaque)	Three major bands. Or a single band.	15, 17 and 18 or 18 only	172 amino acid residues. Rich in Met, and Cys, 2 Trp	Monomers with intra-chain disulphide bonds. Oligomers and polymers, stabilised by intra- and inter-chain disulphide bonds. “Chain extender”
ɣ-	9-12% (vitreous) 19-21% (opaque)	A number of resolving bands have been identified.	16 to 28 and 50 (minor band)	193 amino acid residues. Rich in Pro, Cys, His. No Lys, Asn, Asp, Trp. Four repeats (consensus PPPVHL)	Oligomers and polymers, stabilised by inter-chain disulphide bonds. “Chain extender’
δ-	Not known	Two sequences have been reported.	12.961 or 10 and 18	114 amino acid residues. Rich in Met, No Lys, 1 Trp	Not known



a. Kafirin proteins separated by SDS-PAGE. 1) non-reducing; 2) reducing conditions.

b. Zein proteins separated by SDS-PAGE. Reducing conditions.

c. Non-reduced kafirin proteins separated by SE-HPLC.

Figure 2.1 Separation of kafirin and zein proteins using SDS-PAGE, and kafirin proteins using SE-HPLC.
(a) Kafirin proteins separated by SDS-PAGE; MW M_r standard proteins; Lane 1, unreduced 60% (v/v) tertiary butanol extract; Lane 2, reduced 60% (v/v) tertiary butanol extract. P, T and D indicate protein polymers, trimers and dimers, respectively (El Nour et al., 1998). (b) Zein proteins separated by SDS-PAGE (Shewry and Halford, 2002). γ, α- 1, α- 2, and β indicate the zein/kafirin proteins. (c) Non-reduced kafirin proteins separated by SE-HPLC unreduced 60% (v/v) tertiary butanol sorghum flour extract. Numbers indicate collected peaks, and where identified by SDS-PAGE of the eluted peaks (El Nour et al., 1998).

2.4 Synthesis of prolamin storage protein bodies

Cereal seed storage proteins are synthesised in the rough endoplasmic reticulum (rough ER) and are deposited in discrete protein bodies within the grain endosperm (Shewry and Halford, 2002). The synthesis of maize (Gibbon and Larkins, 2005) and sorghum (Taylor, Schüssler and Liebenberg, 1985) protein bodies has been studied extensively, and found to be very similar. Hence the descriptive model for the development of zein protein bodies and the distribution of zein proteins in maize (Figure 2.2), proposed by Lending and Larkins (1989), is commonly used for sorghum. Figure 2.3 shows transmission electron micrographs of protein bodies in developing sorghum endosperm.

According to Lending and Larkins (1989), protein body formation in maize (and probably sorghum), begins with the initial accumulation of zein proteins within the rough endoplasmic reticulum, which consists of dark-staining deposits of both β- and ɣ-zeins, and contains little or no α-zein (Figure 2.2a). Subsequently, light-staining locules of α-zein begin to accumulate within the β- and ɣ-zeins (Figure 2.2b). As the interior of the protein body fills with α-zein, the locules of α-zein fuse and aggregate to form a central core. Some smaller locules of α-zein remain and are interspersed in the outer region of the protein body (Figure 2.2c). The dark-staining region that contains β- and ɣ-zein forms a continuous layer around the periphery of the protein body. In the final stages of protein body maturation, α-zein fills most of the protein body core and is surrounded by a thin layer of β- and ɣ-zeins (Figure 2.2d). Small, dark-staining patches of ɣ-zein, and to a lesser extent β-zein, may occur within the interior region.

According to Shull et al. (1992) slight differences may exist between the synthesis of sorghum and maize protein bodies. More β- and ɣ-protein crosslinking occurs in older more mature sorghum protein bodies compared to maize protein bodies, which may indicate that differences exist between the development of sorghum and maize protein bodies.

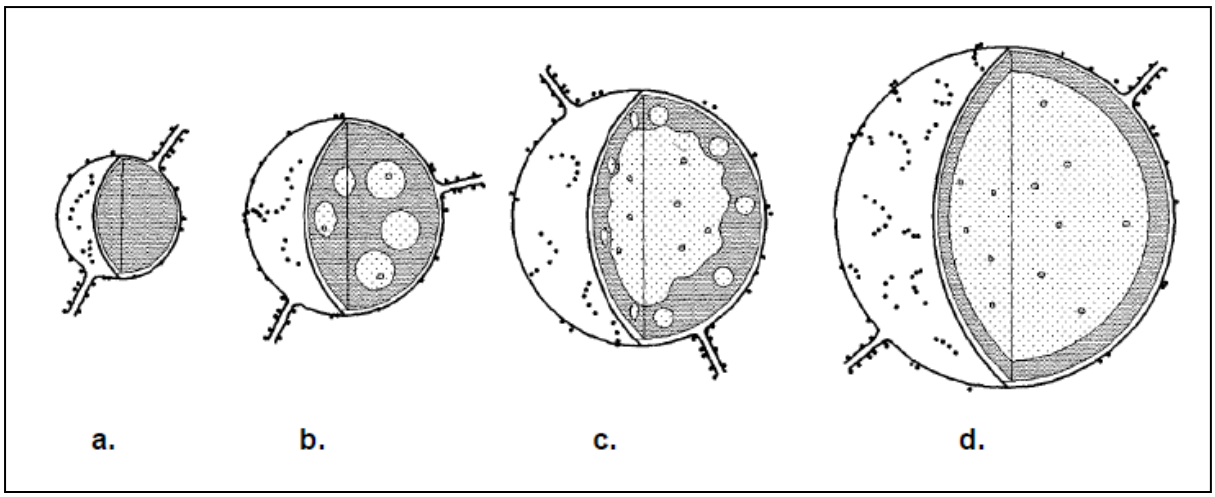


Figure 2.2 A diagrammatic representation of the development of protein bodies in maize endosperm.
The heavily stippled regions correspond to regions that are rich in β- and ɣ-zeins, and the lightly stippled regions correspond to regions rich in α-zein. The protein body is surrounded by rough endoplasmic reticulum (dark dots represent ribosomes). Some β- and ɣ-zeins are found within the regions that consist primarily of α-zein (heavily stippled inclusions). Localization of different zein proteins was done using immunolocalization techniques (taken from Lending and Larkins, 1989). Figure is not to scale.

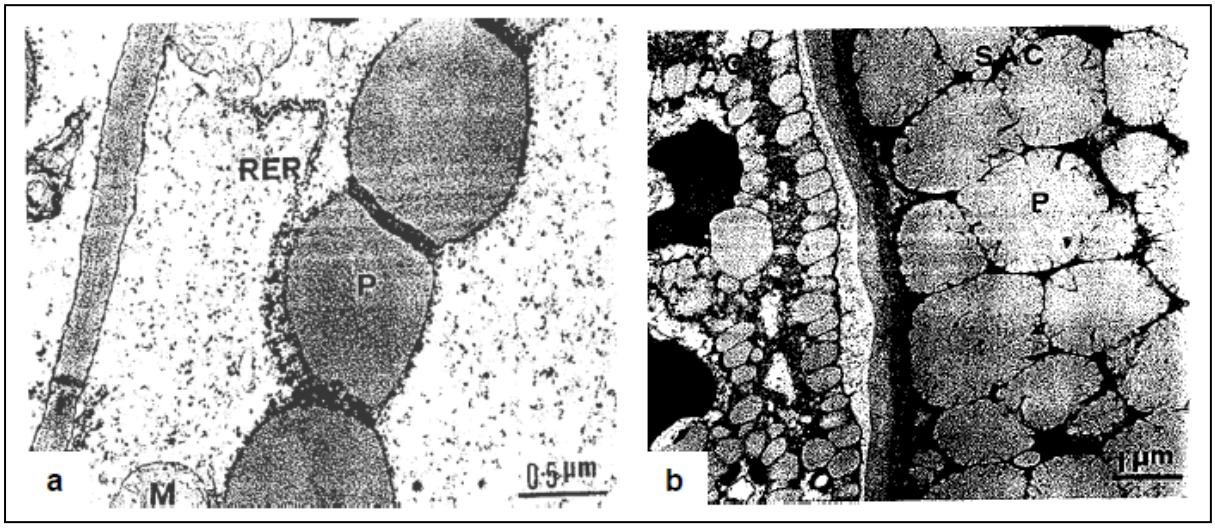


Figure 2.3 Transmission electron micrographs of developing sorghum endosperm.
a) Electron micrograph of sorghum endosperm at soft dough stage of seed development. M – mitochondria; P – protein body; RER – rough endoplasmic reticulum. b) Sorghum aleurone and sub-aleurone cells at the late hard dough stage of seed development; AC – aleurone cell; M- matrix protein; P – protein body; SAC – sub-aleurone cell (taken from Taylor, Schüssler and Liebenberg, 1985).

In addition, in a study conducted by Oria, Hamaker and Shull (1995a), the quantification of different kafirin sub-classes using solvent systems, showed α-kafirin was the first prolamin protein to accumulate in the developing kernel, followed by the accumulation of β- and ɣ-kafirin. It was also stated by these authors that the ratio of crosslinked kafirin fractions to uncrosslinked kafirin fractions increases significantly during grain maturity and desiccation. Kafirin crosslinking is due to the formation of disulphide bonds being formed due to oxidative processes or interchange involving sulfhydryl groups (Oria et al., 1995a). These differences may also be responsible for the lower protein digestibility of sorghum compared to maize (see section 2.6).

In mature sorghum, the amorphous matrix protein that encases the tightly packed protein bodies and starch granules, is composed of aggregated dense cytoplasm and disintegrated ER and mitochondria (Shull, Chandrashekar, Kirleis and Ejeta, 1990). In normal sorghum, mature protein bodies found in the sub-aleurone layer and vitreous endosperm are round in cross-section, with diameters ranging from 0.5 to 3.5 µm (Adams, Novellie and Liebenberg, 1976; Taylor et al., 1985). Crosslinked polymeric kafirins are commonly found within the protein bodies arranged in concentric rings (Seckinger and Wolf, 1973; Adams et al., 1976; Taylor et al., 1984; Krishnan, White and Pueppke, 1989).

2.4.1 Synthesis of protein bodies in mutant genotypes

In contrast to normal sorghum protein bodies, protein bodies in mutant sorghums (P721Q and derivatives) were found to be remarkably different and irregular shaped (Oria, Hamaker, Axtell and Huang, 2000) (Figure 2.4). These mutant sorghums are also characterised as having high protein digestibility, and are thus termed high-protein digestibility mutants (HPDM) (see section 2.7). The protein bodies of HPDM are characterised by deep invaginations or folds, and in many cases the folds reach the central area of the protein body forming irregular shaped lobes (Figure 2.4b, c) (Oria et al., 2000). The dark inclusions are also seen mainly at the base of the folds (Figure 2.4c), and are composed of crosslinked ɣ- and β-kafirins. Similarly, the protein bodies in developing endosperm of *floury-2* mutant maize (a mutation that results in reduced levels of all zein sub-classes) were also reported to differ in shape

from the discrete, spherical protein bodies formed in normal maize endosperm (Lending and Larkins, 1992). The protein bodies of *floury-2* were reported to be irregular in shape, with irregular clusters (or lobes) of zein proteins within the lumen of the rough ER (Figure 2.5b). This work is in agreement with earlier studies conducted by Wolf and co-workers who isolated small oddly shaped protein bodies in immature *floury-2* maize kernels (Wolf, Khoo and Seckinger, 1967). Of interest, these authors reported that there was an absence of protein bodies in mature *floury-2* maize. It was concluded that the protein bodies disintegrate during grain maturation.

In contrast to both *floury-2* maize mutant and the HPDM, the protein bodies in developing endosperm of *opaque-2* mutant maize (mutation results in considerable reduction in α-zein sub-class, M_r 22,000) are typically smaller than those of the normal genotype, but have a normal spherical shape (Geetha, Lending, Lopes, Wallace and Larkins, 1991) (Figure 2.5c). Decreased α-zein production in high-lysine maize genotypes is commonly associated with smaller and fewer protein bodies in the grain endosperm (Christianson, Khoo, Nielsen and Wall, 1974; Gibbon and Larkins, 2005).

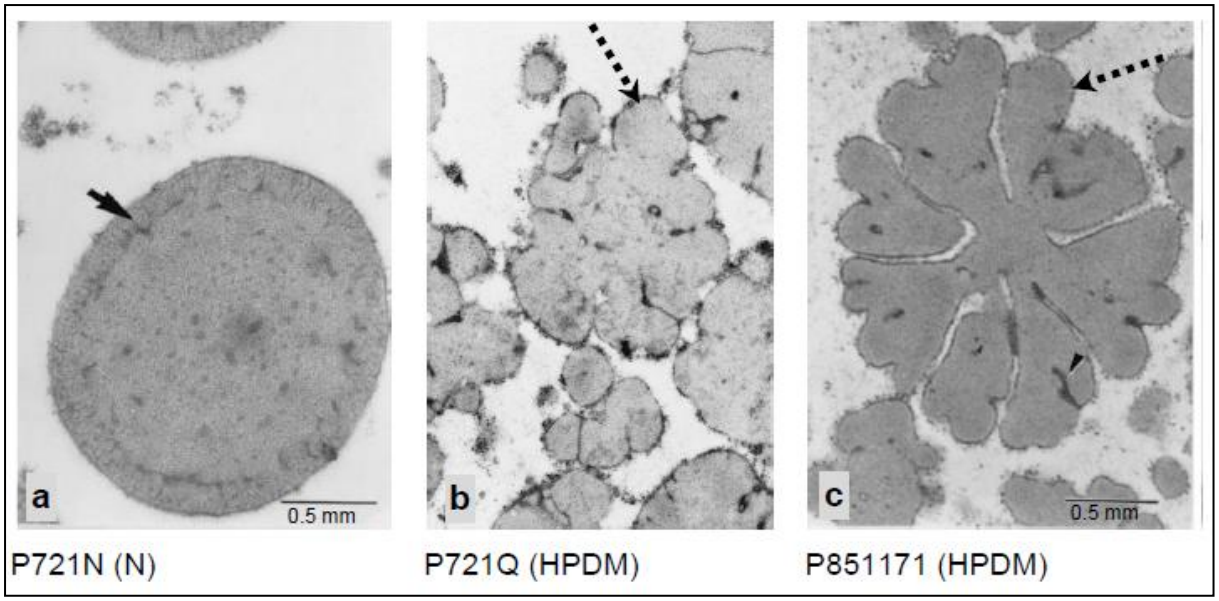


Figure 2.4 Transmission electron micrographs of protein bodies in the peripheral endosperm of normal (N) and high-protein digestibility mutant (HPDM) sorghum cultivars.
Black arrow points at dark-staining peripheral ring; black dashed arrow points at lobes of protein bodies, black arrow head points at dark-staining base folds. (From Oria et al., 2000).

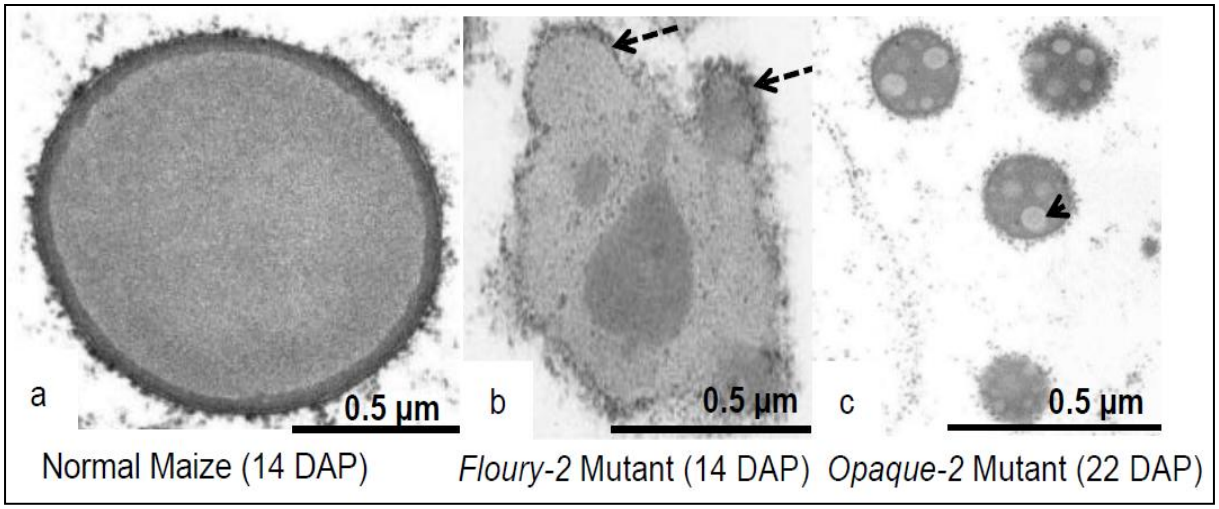


Figure 2.5 Transmission electron micrographs of representative protein bodies from developing endosperm of a) normal maize, b) *floury-2*, c) *Opaque-2* mutant.
Dashed arrows show irregular protein lobs (or clusters); arrow head shows light staining inclusions; DAP - days after pollination. (From: Lending and Larkins (1989); Lending and Larkins (1992) and Geetha et al. (1991), respectively).

2.5 Organisation and role of prolamin proteins in storage protein bodies

In normal sorghum, the distribution of the different kafirin sub-classes within storage protein bodies is non-homogeneous. Immunocytochemical studies show that the α-kafirins are located mainly in the interior of the protein body, as light staining areas, while the β- and ɣ-kafirins are found inside and at the periphery of the protein bodies, as dark-staining areas (Shull et al., 1992). At the protein body periphery, ɣ-kafirins and to a lesser degree β-kafirins form a disulphide-bound polymeric network which encapsulates the α-kafirins (Figure 2.6a).

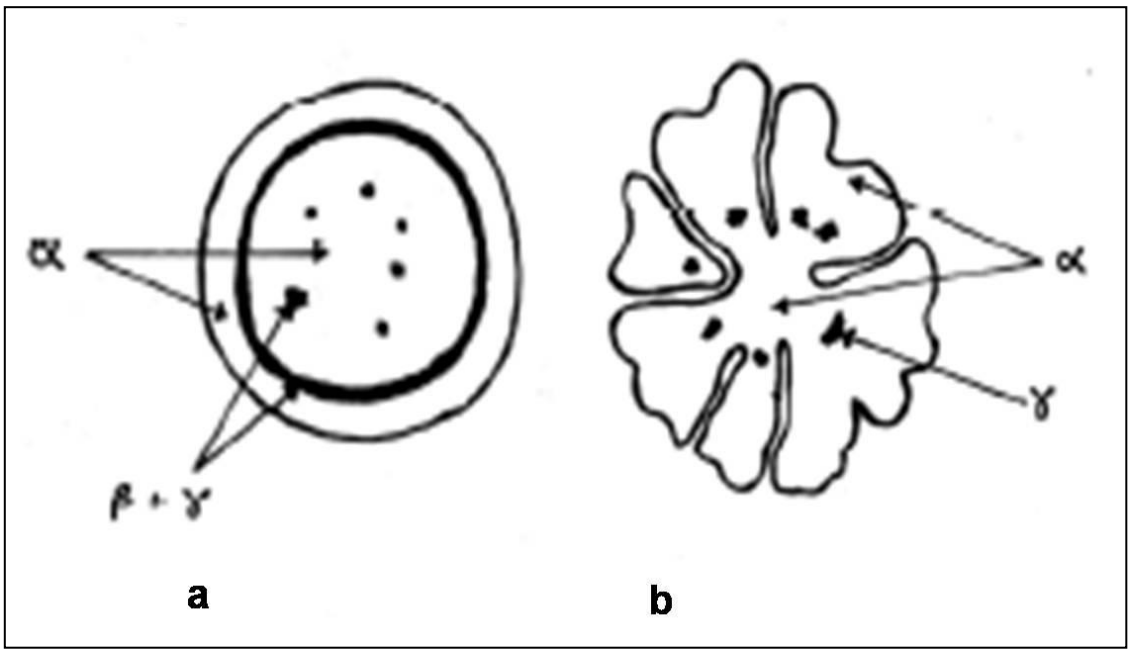


Figure 2.6 A diagrammatic representation of a section through a) a normal sorghum protein body and b) a high-protein digestibility mutant protein body showing differential location of α-, β- and ɣ-kafirins (from Duodu et al., 2003).

In HPDM sorghum protein bodies, the distribution of kafirin proteins is modified compared to that of normal sorghum protein bodies. Immunolocalization studies, conducted by Oria et al. (2000) on protein bodies from HPDM, revealed that α-kafirin is localized homogeneously throughout the light-staining regions of the interior of the protein bodies, β-kafirin is found predominantly as dark staining areas localised at the periphery of the protein body lobes, and ɣ-kafirin is also found as dark staining areas localised at the base of the invaginations and to a lesser extent at the

peripheral regions (Figure 2.6b). Very small amounts of β- and ɣ-kafirins also occur in the light staining area of the protein body.

In a study to characterize the associations and distribution of different zein sub-classes in developing endosperm of the *floury-2* maize mutant, Lending and Larkins (1992) found that the protein bodies were irregular in shape (Figure 2.5b). Also, the zein proteins were also disorganised compared to those observed in normal maize genotypes (Lending, Kriz, Larkins and Bracker, 1988; Lending and Larkins, 1992). In the initial stages of endosperm development, protein body formation followed the same pattern observed in normal maize, namely the initial aggregates within the rough ER consist predominantly of β- and ɣ-zeins, as described by Lending and Larkins (1989), (Figure 2.2). However, as the α-zeins accumulated, the morphology of the protein bodies began to change from that in normal maize. As the protein bodies in *floury-2* endosperm became enlarged, they started to develop irregular lobes. Immunocytochemical staining revealed that the distribution of the different zein sub-classes was disorganised in *floury-2* maize, with patches of β- and ɣ-zein occurring within irregular lobed clusters of α-zeins (Lending and Larkins, 1992). In addition, within the central regions of endosperm cells, clusters of protein deposits were observed to form within the rough ER aggregates and were dispersed throughout the cytoplasm. This was in direct contrast to the formation of protein bodies in normal maize genotypes. Additionally, granular, electron-dense deposits, unique to *floury-2* maize, were also observed between the rough ER and the protein aggregates during all stages of protein body formation.

In the endosperm of *opaque-2* mutant maize, the level of one zein sub-class, M_r 22,000 α-zein, is greatly reduced (Jones, Larkins and Tsai, 1977; Burr and Burr 1982; Kodrzycki, Boston, and Larkins, 1989). The protein bodies in *opaque-2* maize are typically smaller than those of the normal maize, due to the great decrease in the amount of the M_r 22,000 α-zein (Geetha et al., 1991).

Although the mechanism of protein body assembly is not fully understood, it has been hypothesized that specific interactions must occur between the various prolamin sub-classes. In maize, it is thought that the sulphur-poor α-zein must pass

into the sulphur-rich β- and ɣ-zeins that initially aggregate within the rough ER (Lending and Larkins, 1992). Research into understanding the mechanisms involved in protein body assembly in cereals is a major focus area (Geli, Torrent, and Ludevid., 1994; Bagga, Adams, Kemp, and Sengupta-Gopalan, 1995; Coleman, Herman, Takasaki and Larkins, 1996; Bagga, Adams, Rodriguez, Kemp and Sengupta-Gopalan, 1997; Kim, Woo, Clore, Burnett, Carneiro and Larkins, 2002; Coleman, Yoho, Escobar and Ogawa, 2004). These authors used *in planta* transgenic tobacco seed or yeast two-hybrid systems, to study the interactions between different zein proteins and their role in the formation of a maize protein body. There is evidence that specific protein–protein interactions play an important role in storage protein body formation. Geli et al. (1994) and Bagga et al. (1995) showed that β-zein and ɣ-zein, when expressed separately, accumulate as protein accretions within tissues of transgenic tobacco seed. Delta-zein was also found to accumulate in transgenic tobacco seed, but the amount of δ-zein was enhanced when it was co-expressed with β-zein (Bagga et al. 1997). However, the δ-zein/β-zein accretions had an irregular structure compared to the spherical protein bodies of normal maize endosperm. This result suggests that formation of a spherical protein body requires the inclusion of both ɣ- and β-zein proteins.

Other studies have shown that α-zein accumulation in transgenic tobacco seed is only possible when either ɣ-zein (Coleman et al., 1996), or β-zein (Coleman et al., 2004) is co-expressed in the transgenic seed. The resulting α-zein/ɣ-zein or α-zein/β-zein accretions formed were similar in appearance to those of maize endosperm protein bodies. These results indicate that either β- or ɣ-zein is able to stabilise the accumulation of α-zein in transgenic tobacco seed endosperm. These also demonstrate the importance of the sulphur-rich prolamin proteins in initiating and maintaining protein body structure.

Considering that β- and ɣ-zein are structurally related and appear to have similar roles in the initiation of protein bodies in developing maize endosperm (Lending and Larkins, 1989; Woo, Hu, Larkins and Jung, 2001; Kim et al., 2002), the findings of Coleman and co-authors (1996 and 2004) are not surprising. The mechanism involved in the initiation and development of zein protein bodies is described as the

aggregation of cysteine-rich β- and ɣ-zein proteins via the formation of inter-molecular disulphide bonds, forming an insoluble protein body core, required for integration of α-zein (Coleman et al., 2004). Both β- and ɣ-zein proteins may possess intrinsic properties that allow them, when assembled as aggregates, to interact with α-zein. Such interactions may involve the hydrophobicity of the α-zein protein or disulphide bonding between α-zein and β- or ɣ-zein (Coleman et al., 2004). A study by Kim et al. (2002) using the yeast two-hybrid system, also confirmed that the various zein proteins either interact with themselves or with other zein proteins during protein body synthesis, but the role of these interactions in initiating or maintaining zein protein body structure was unclear. The model described by Lending and Larkins (1989) shows great similarity to that described above.

2.6 Grain endosperm quality of high-lysine maize and sorghum

The discovery of high-lysine genotypes in maize and sorghum stimulated a great deal of excitement due to the potential of these genotypes for enhancing the nutritional value of these grains (Mertz et al., 1964). However, high-lysine mutant cereals are renowned for having soft, floury endosperm, resulting in lower kernel density and increased susceptibility to insects, pathogens, mechanical damage and reduced yields compared to their normal vitreous counterparts (Mertz et al., 1964; Ortega and Bates, 1983, Lending and Larkins, 1992; Weaver et al., 1998; Tesso et al., 2006; Tesso et al., 2008). These agronomic problems have inhibited the widespread cultivation of high-lysine maize and sorghum.

Grain hardness or endosperm texture, is a key trait in maize and sorghum as it provides resistance to damage during harvesting and marketing, as well as to insect and fungal damage (Maxson, Fryar, Rooney, and Krishnaprasad, 1971). Also grain hardness is the main characteristic that determines milling performance and end-use quality of the decorticated flour (Maxson et al., 1971; Awika, Suhendro and Rooney, 2002). During milling, sorghum and maize with corneous endosperm yield more complete endosperms and fewer broken particles of endosperm than grains with floury endosperm when decorticated (Maxson et al., 1971; Awika et al., 2002). The

poor milling properties of grains with soft, floury endosperm texture is due to the weak protein-protein and protein-starch association in the grain endosperm, and results in shattering of the endosperm during milling (Maxson et al., 1971; reviewed by Chandrashekar and Mazhar, 1999; Awika et al., 2002). In addition, grain endosperm texture also has a profound effect on the food making quality and sensory properties of sorghum and maize foods (Murty and Kumar, 1995; Chandrashekar and Mazhar, 1999).

Endosperm texture is determined in maize and sorghum by the relative amounts of hard (corneous) and soft (floury) endosperm in the grain. There is some correlation between prolamin storage proteins and corneous kernel (Maxson et al., 1971). A 3-point rating system for evaluating sorghum endosperm texture was developed by Taylor and Taylor (2008) (Figure 2.7), and has been adopted by the International Association for Cereal Science and Technology (ICC) as a draft ICC standard method for determining sorghum grain endosperm texture (ICC, 2008). This rating system distinguishes sorghum endosperm texture into corneous, intermediate or floury phenotype.

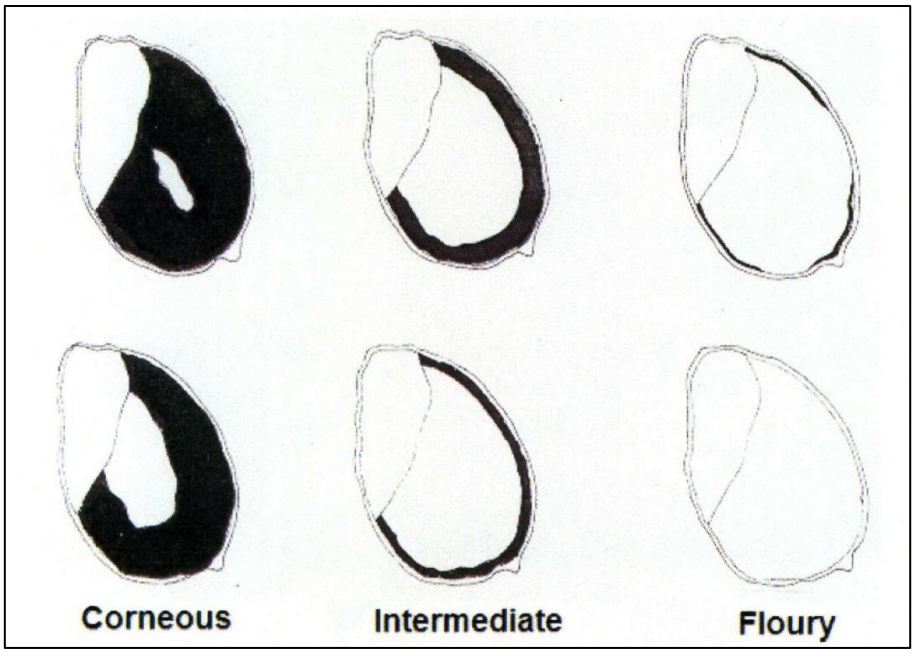


Figure 2.7 Illustration of the 3-point rating system for evaluation sorghum endosperm texture (ICC, 2008).

According to a review of the major causes of grain hardness in sorghum and maize, it was concluded by Chandrashekar and Mazhar (1999) that grain hardness is multifactorial, which includes: Cell wall structure and the types and concentrations of prolamins present in the endosperm. In hard grains the cell wall polymers are more rigid, with more protein bodies which are evenly distributed in the peripheral endosperm tissue than the protein bodies found in floury grains. In addition, the protein bodies in hard endosperm generally contain more ɣ-prolamins which seem to be crosslinked by disulphide bonds (Chandrashekar and Mazhar, 1999).

2.6.1 Quality Protein Maize (QPM)

Research to improve the endosperm texture of high-lysine maize has resulted in the development of high-lysine phenotypes with corneous endosperm texture for maize, known as Quality Protein Maize (QPM) (Gibbon and Larkins, 2005). QPM was developed by plant breeders in South Africa and the International Maize and Wheat Improvement Centre (CIMMYT) in Mexico. Researchers identified a number of modifier genes that alter the soft, floury texture of the endosperm, giving the grain a normal appearance while maintaining the increased essential amino acid content (due to low α-zein content) of *opaque-2* (Gibbon and Larkins, 2005). The mechanism by which the opaque phenotype of *opaque-2* endosperm is modified in QPM is still under investigation (Wu, Holding and Messing, 2010). Genetic mapping of *opaque-2* modifiers has indicated that there is a link between the locus encoding the 27 kDa ɣ-zein storage protein and a region near the end of the long arm of chromosome 7 (Lopes, Takasaki, Bostwick, Helentjaris, and Larkins, 1995). Other studies on QPM have shown that, compared to *opaque-2*, the 27 kDa ɣ-zein is increased two- to three-fold (Wallace, Lopes, Paiva, and Larkins, 1990; Wu et al., 2010). The increase in ɣ-zein is dependent on the dosage of *opaque-2* modifiers, which appear to act in a semi-dominant manner (Lopes and Larkins, 1991). In addition, numbers of zein protein bodies are also increased, forming a compacted protein mass between the starch granules in the endosperm (Dannenhoffer, Bostwick, Or and Larkins, 1995). According to Wu et al. (2010) ɣ-zeins are essential for ensuring protein body density and starch grain interaction, thus giving QPM a corneous endosperm texture.

A model depicting the corneous or floury starchy endosperm cells for wild type, QPM and *opaque-2* maize, at the mid-maturation stage (18 DAP) is shown in Figure 2.8 (Wu et al., 2010). In the wild-type maize and QPM, compact stable matrices are visible, giving rise to glass-like, vitreous endosperm at maturity. Also of note, the protein bodies in QPM endosperm are generally smaller than that of the wild-type. In the opaque mutant (low-zein line), small, sparsely distributed protein bodies are formed, producing loose, unstable matrices, which shatter during desiccation, resulting in an opaque texture at maturity.

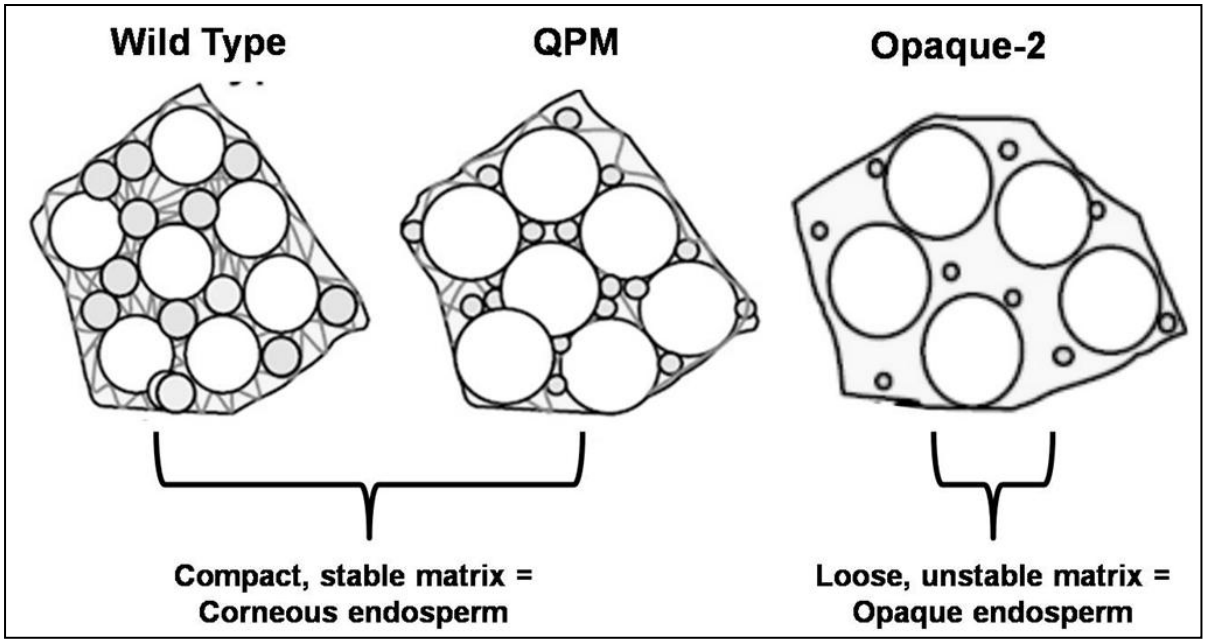


Figure 2.8 Model for corneous endosperm formation in which mid-maturation stage (18 DAP) starchy endosperm cells are depicted for wild type, QPM (Quality Protein Maize) and *opaque-2* mutant.
The protein bodies are represented by small grey spheres, starch grains with white spheres, and proteinaceous matrix with grey lines (from Wu et al., 2010).

In the case of sorghum, Tesso et al. (2006) identified a novel sorghum mutant with both high-protein digestibility and high-lysine (HDP/*hl*) trait and relatively hard endosperm texture. The HPD/hl mutant was an F6 generation of crosses between P721Q mutant and elite hard endosperm sorghum lines. Improved protein digestibility and lysine content were 20% and 44% higher than normal sorghum, respectively. The relatively hard endosperm texture was attributed to areas of densely packed starch granules, but without the typically associated continuous

protein matrix of normal sorghum. In addition, the protein bodies of HDP/*hl* had irregular, invaginated shapes, as previously described for P721Q derived lines (Oria et al., 2000).

2.7 Factors affecting *in vitro* protein digestibility of sorghum

The protein digestibility of normal sorghum is particularly low, especially after wet cooking. According to MacLean, de Romańa, Placko and Graham (1981), the apparent protein digestibility of cooked sorghum porridge can be as low as 46%, compared to values for cooked wheat (81%), rice (66%) and even maize (73%), despite the fact the proteins of sorghum and maize are very similar. Factors affecting the digestibility of sorghum proteins have been studied by various authors and can be divided into two broad categories: endogenous and exogenous factors, and have been reviewed extensively by Duodu et al. (2003). The endogenous factors include factors that arise out of changes within the sorghum proteins themselves, namely disulphide and non-disulphide crosslinking, kafirin hydrophobicity and changes in protein secondary structure. The exogenous factors include grain organisational structure, and the interaction of sorghum proteins with non-protein compounds inherent in the whole grain, namely polyphenols, non-starch polysaccharides, starch, phytate and lipids. Reduced protein digestion is due to either the formation of chemically indigestible products or the formation of a physical barrier preventing access of proteases to the proteins during processing. All the above factors have been shown to influence sorghum protein digestibility to different degrees and more than one factor can be involved at any time. The nature or state of the sorghum grain (whole grain or endosperm, protein body preparation, and tannin content) has also been reported to influence the degree of protein digestibility.

Of the factors listed above, it is the formation of disulphide crosslinked kafirins, specifically between the cysteine-rich ɣ- and β-kafirins, during grain development, desiccation and maturity (Hamaker, Kirleis, Mertz and Axtell, 1986; Oria et al., 2000), as well as those that are formed during cooking (Axtell, et al., 1981; Hamaker et al., 1986; Rom, Shull, Chandrashekar and Kirleis, 1992; Oria, Hamaker and Shull, 1995b; Ezeogu, Duodu and Taylor, 2005), that are considered to play major roles in

lowering the protein digestibility of sorghum. Hamaker, Kirleis, Butler, Axtell and Mertz (1987) demonstrated that kafirins are the last proteins to be digested and require the addition of a reducing agent such as 2-ME to break the disulphide crosslinks between the kafirin proteins to improve their uncooked and cooked digestibilities. According to Ezeogu, Duodu, Emmambux and Taylor (2008), cooking in the presence of 2-ME also improves the starch digestibility of wet cooked sorghum and maize. This is due to 2-ME breaking the disulphide bonds in the prolamin-protein matrix, causing an expansion of the corneous endosperm matrix protein mesh and resulting in improved access of α-amylase to the gelatinised starch.

In addition, isolated native (unreduced) α-kafirins are in fact highly digestible and retain their high digestibility after cooking (Hamaker et al., 1986; 1987). These results, and a scanning electron microscopy study, indicate that the breakdown of kafirin protein bodies starts at the protein body periphery and progresses inward (Rom et al., 1992). Hence, the poor digestibility of α-kafirin is caused by its location within the interior of the protein body, as it is surrounded by an enzyme-resistant structure (disulphide crosslinked ɣ- and β-kafirins) that retards digestion of the α-kafirin (Figure 2.4a) (Oria et al., 2000).

Mutant sorghum lines, developed by crossing P721Q with normal hard endosperm lines, have been found to have substantially improved *in vitro* protein digestibility, 10-15% higher in uncooked flour and 25% higher in cooked flour, compared to normal sorghum (Weaver et al., 1998). The improved protein digestibility is reported to be due to change in the shape of the kafirin protein bodies from spherical to invaginated (Figure 2.4b,c) where the ɣ-kafirin is concentrated at the base of folds, exposing the more digestible α-kafirins (Oria et al., 2000). As a result, the α-kafirins are more exposed to digestive enzymes compared to that of normal sorghum protein bodies.

2.8 Africa Biofortified Sorghum (ABS) Project

Due to the fact that sorghum is a major staple food in developing countries and has major limitations regarding protein nutritional quality, it has been identified as one of the crops for biofortification, namely the Africa Biofortified Sorghum (ABS) project

(reviewed by O'Kennedy, Grootboom and Shewry, 2006; Henley et al., 2010). The ABS project is employing recombinant DNA technology with the aim of improving both sorghum lysine content and wet-cooked protein digestibility. Sorghum transformation is brought about using a ''super-binary'' *Agrobacterium*-mediated vector (Zhao, Cai, Tagliani, Miller, Wang, Pang, Rudert, Schroeder, Hondred, Seltzer and Pierce, 2000; Zhao, Glassman, Sewalt, Wang, Miller, Chang, Thompson, Catron, Wu, Bidney, Kedebe and Jung, 2003), or particle bombardment (Grootboom, Mkhonza, O'Kennedy, Chakauka, Kunert and Chikwamba, 2010) of immature zygotic embryos as explant.

Approaches for improving the protein nutritional quality traits of sorghum include:
1) Suppressing the synthesis of specific kafirin sub-classes that are very low in lysine and that are responsible for poor protein digestibility (i.e., suppression of the synthesis of various combinations of α-, ɣ-, and δ-kafirins) (O'Kennedy et al., 2006; Henley et al., 2010). Decreased zein and kafirin synthesis commonly occurs in high-lysine mutants, however, this results in floury endosperm texture. Hence, genetic engineering approaches have been used in maize to specifically down-regulate zein synthesis, with the aim of avoiding the deleterious effects associated with the high-lysine mutations (reviewed by Shewry, 2007).
2) Increasing the lysine content of sorghum by the synthesis of lysine-rich proteins (such as HT12, an analogue of barley hordothionin), expressed in the grains endosperm, and reduced catabolism of free lysine in the grain endosperm by suppressed synthesis of the enzyme lysine ketoglutarate reductase (LKR) (Zhao et al., 2003).

2.9 Conclusions

Sorghum contributes significantly to the protein content of many people's diets, even though it has low protein nutritional quality with regard to low lysine content and poor cooked protein digestibility. The search for high-lysine and high-protein digestibility mutant lines has been somewhat successful but very slow. It would appear that the mutants discovered so far all have floury endosperm texture, requiring considerable effort through breeding programmes to slightly improve the endosperm texture.

Recombinant DNA technology is now being used to co-suppress the synthesis of target kafirin proteins with the aim of developing biofortified sorghum with improved protein nutritional quality. These transgenic sorghums are the first of their kind. Thus, little is known about the effects the different kafirin protein co-suppressions will have on the physical (endosperm texture, protein body structures) and chemical (protein composition, amino acid composition, cooked IVPD and kafirin polymerisation behaviour) characteristics of the transformed grain. Research is thus needed to understand the effect the target kafirin co-suppressions will have on the physical and chemical characteristics of the transformed grains before these biofortified sorghums can be considered for commercial production and processed into foods.

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

This chapter has been submitted in part for publication in the journal Transgenic Research.

Grootboom, A.W., Mkhonza, N.L., O'Kennedy, M.M., da Silva, L.S., Taylor, J., Chikwamba, R., Taylor, J.R.N., Mehlo, L. Effect of different kafirin species suppression on endosperm structure and protein digestibility of transgenic sorghum generated via particle bombardment. *Transgenic Research* (submitted August 2011).

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).

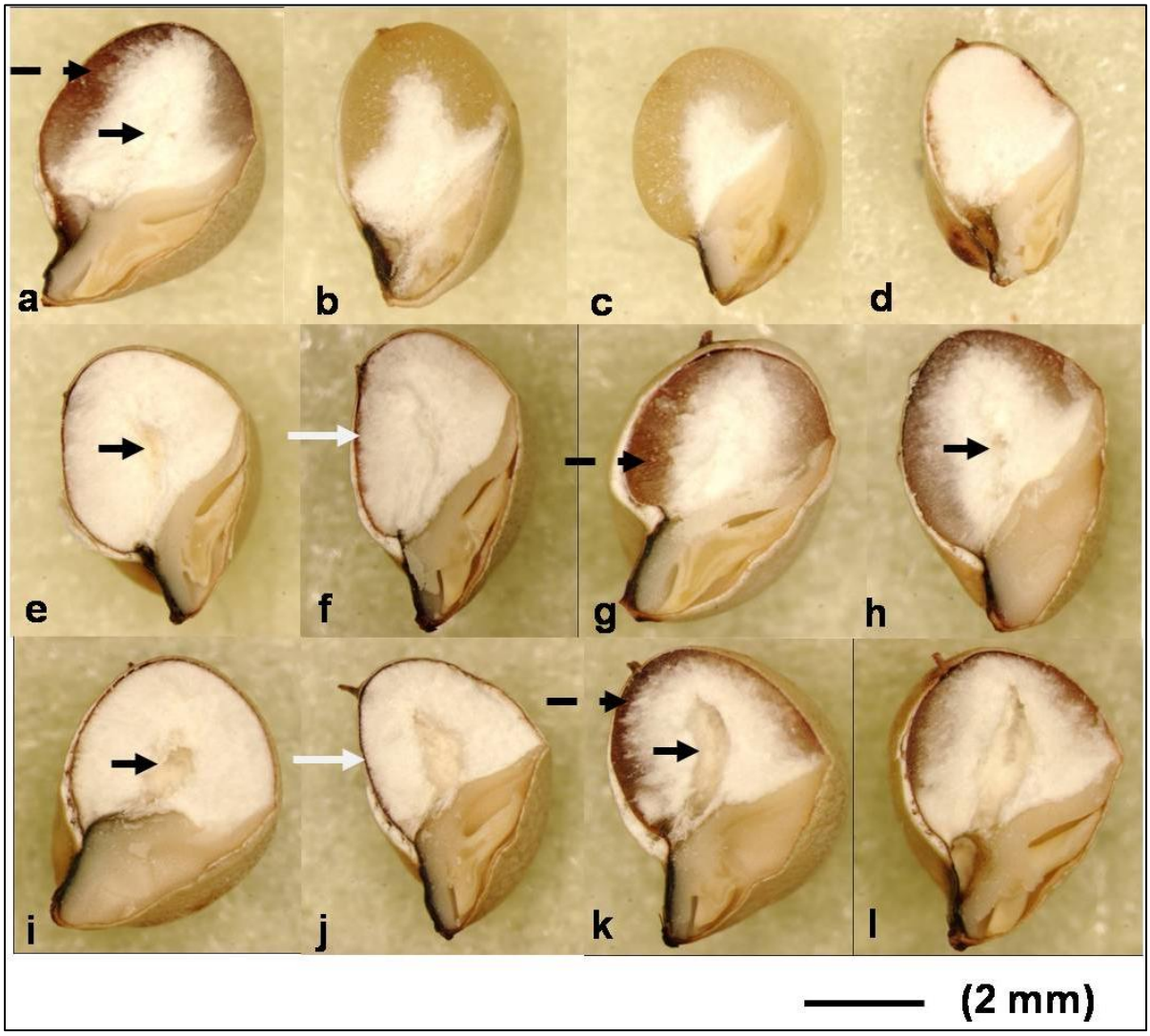


Figure 4.1.1 Longitudinal cross-sections of whole sorghum grains.
a) P898012; b) Macia; c) NPD-2; d) HPDM-1, e-f) TG-ABS042, g-h) Null controls - ABS042; i-j) TG-ABS044; k-l) Null controls - ABS044. Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining; white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

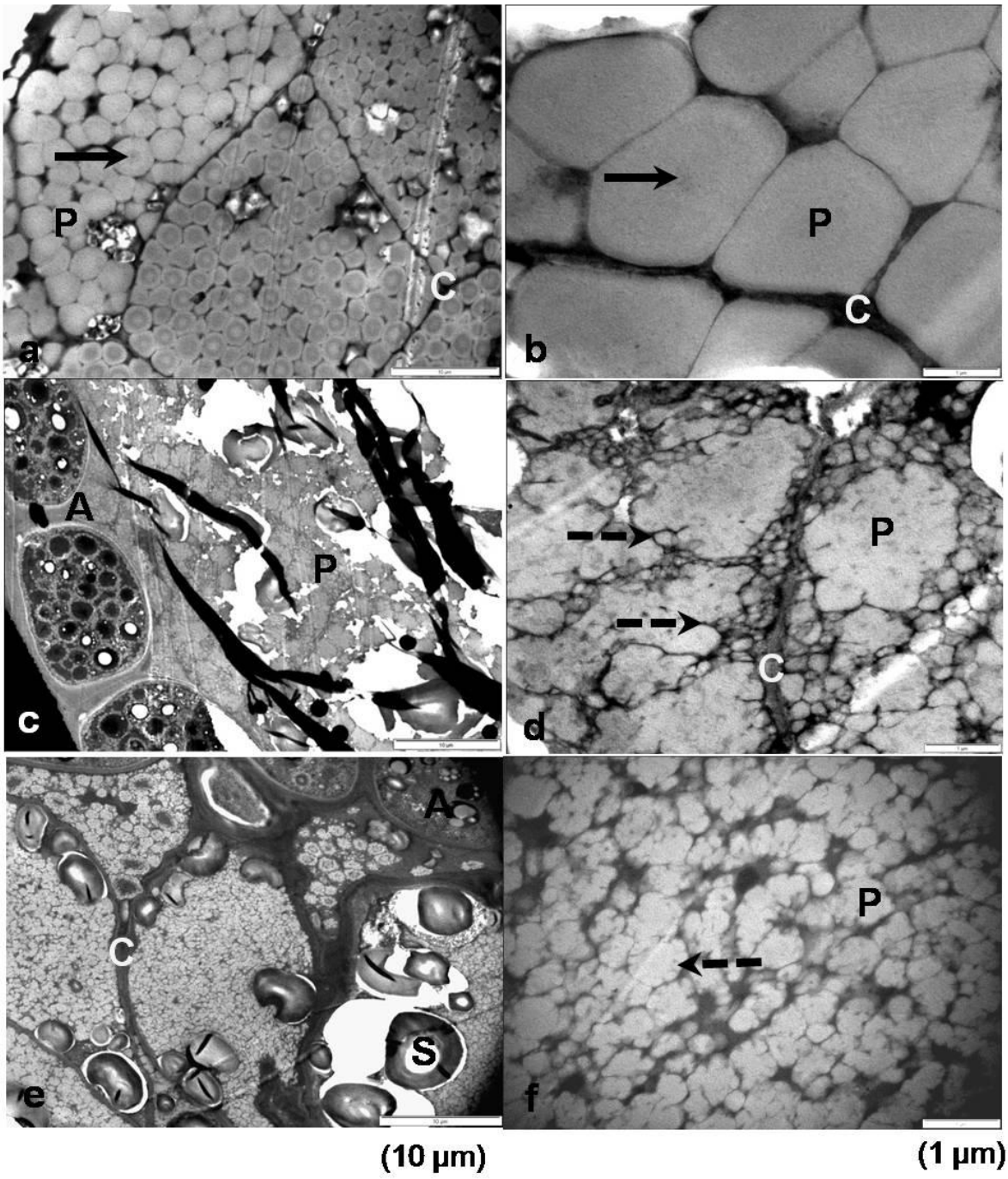


Figure 4.1.2 Transmission electron micrographs of protein bodies in the peripheral endosperm of transgenic and HPDM lines.
a-b) TG-ABS042; c-d) TG-ABS044; e-f) HPDM-1. Where indicated: A, aleurone; C, cell wall; P, protein body; S, starch granule; black arrows indicate dark-staining inclusions within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

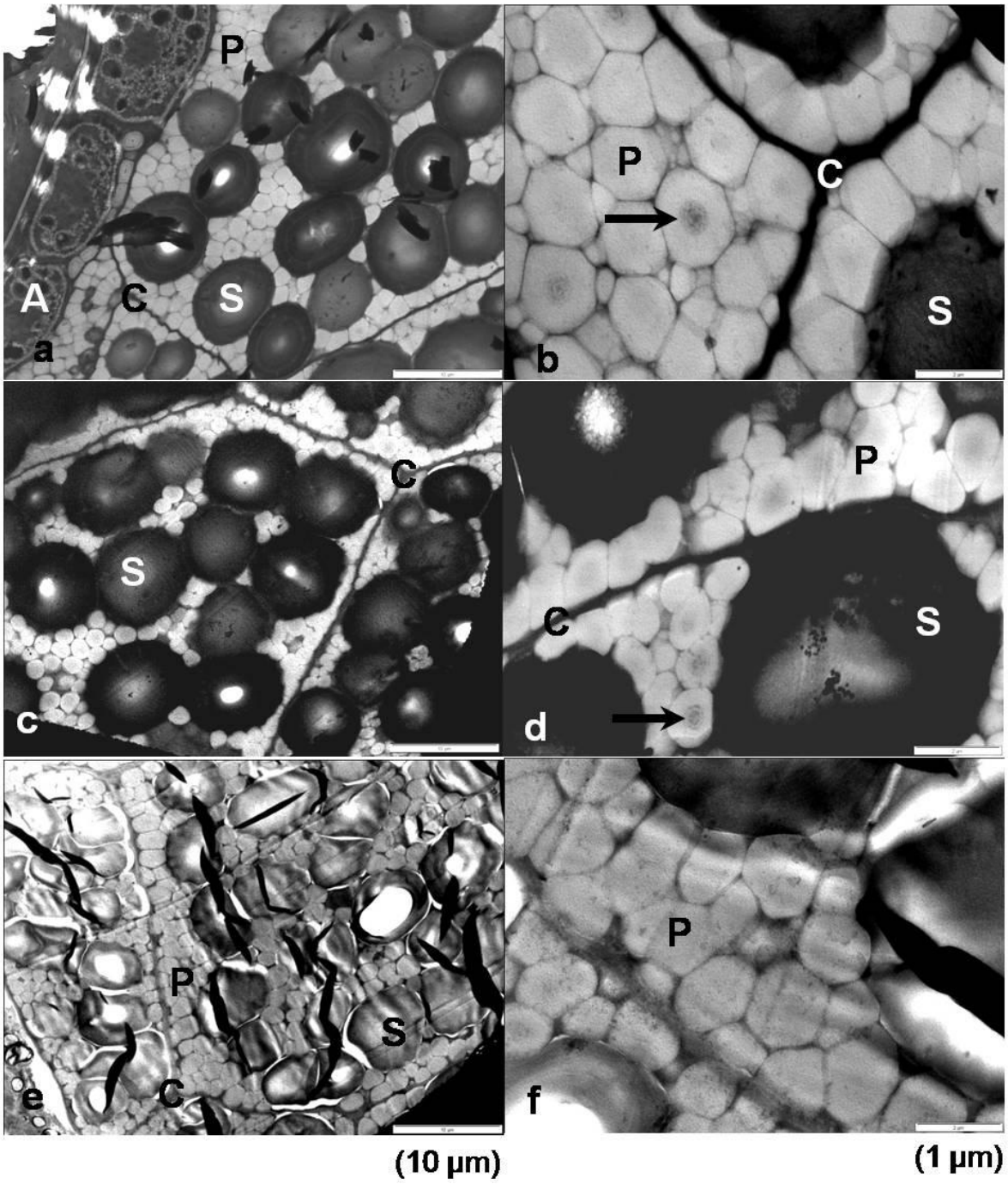


Figure 4.1.3 Transmission electron micrographs of protein bodies in the peripheral endosperm of normal sorghum lines.
a-b) P898012; c-d) NPD-2; e-f) Macia. Where indicated: A, aleurone; C, cell wall; P, protein body; S, starch granule; black arrows indicate dark-staining inclusions within protein bodies.

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

AACC International, 2000. Moisture content, Standard Method 44-15A. Crude protein-combustion, Standard Method 46-30. Test weight per bushel, Standard Method 55-10. *Approved Methods of the AACC, 10th ed*. American Association of Cereal Chemists: St Paul, MN.

ABS (Africa Biofortified Sorghum Project). 2009. http://biosorghum.org/, accessed March 2009.

Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography* 366, 93-104.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. and Hamaker, B.R. 2003. Factors affecting protein digestibility. *Journal of Cereal Science* 38, 117-131.

FAO (Food and Agriculture Organization). 2002. FAO Animal Production and Health, *Proceedings:* Protein Sources for the Animal feed Industry. Expert Consultation and Workshop, Bangkok, 29 April – 3 May.

Gomez, M.I., Obilana, A.B., Martin, D.F., Madzvamuse, M., Monyo, E.S. 1997. Manual of Procedures for Quality Evaluation of Sorghum and Pearl Millet. ICRISAT, Patancheru, India.

Grootboom, A.W., Mkhonza, N.L., O'Kennedy, M.M., Chakauya, E., Kunert, K., Chikwamba, R.K. 2010. Biolistic mediated sorghum (*Sorghum bicolor* L. Moench) transformation via mannose and bialaphos based selection systems. *International Journal of Botany* 6, 89-94.

Guiragossian, V., Chibber, B.A.K., Van Scoyoc, S., Jambunathan, R., Mertz, E.T., Axtell, J.D. 1978. Characteristics of proteins from normal, high lysine, and high tannin sorghums. *Journal of Agricultural and Food Chemistry* 26, 219-223.

Hamaker, B.R., Kirleis, A.W., Mertz, E.T., Axtell, J.D. 1986. Effect of cooking on the protein profiles and *in vitro* digestibility of sorghum and maize. *Journal of Agricultural and Food Chemistry* 34, 647-649.

Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D., Mertz, E.T. 1987. Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceedings of the National Academy of Sciences of the United States of America* 84, 626-628.

ICC (International Association for Cereal Science and Technology). 2008. Estimation of sorghum grain endosperm texture. ICC Standard 176. ICC, Vienna. http://www.icc.or.at/standard_methods/176, accessed April 2009

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 2009. Sorghum. www.icrisat.org/newsite/crop-sorghum.htm, accessed November 2009.

Maxson, E.D., Rooney, L.W. 1972. Evaluation of methods for tannin analysis in sorghum grain. *Cereal Chemistry* 49, 719-729.

Mazhar, H., Chandrashekar, A. 1993. Differences in kafirin composition during endosperm development and germination in sorghum cultivars of varying hardness. *Cereal Chemistry* 70, 667-671.

O'Kennedy, M.M., Grootboom, A., Shewry, P.R. 2006. Harnessing sorghum and millet biotechnology for food and health. *Journal of Cereal Science* 44, 224-235.

Oria, M.P., Hamaker, B.R., Shull, J.M. 1995a. *In vitro* protein digestibility of developing and mature sorghum grain in relations to α-, β- and γ-kafirin disulphide crosslinking. *Journal of Cereal Science* 22, 85-93.

Oria. M.P., Hamaker., B.R., Axtell, J.D., Huang, C.P. 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm

protein bodies. *Proceedings of the National Academy of Sciences of the United States of America* 97, 5065-5070.

Rooney, L.W., Miller, F.R. 1982. Variation in the structure and kernel characteristics of sorghum. In: Mertin, J.V. (Ed), International Symposium on Sorghum Grain Quality, ICRISAT, Patancheru, India, pp. 143-162.

Rooney, L.W., Serna-Saldivar, S.O. 1990. Sorghum. In: Lorenz, K.J. and Kulp, K., (Eds.), Handbook of Cereal Science and Technology, Markel Dekker, New York, pp. 233-270.

Shull, J.M., Watterson, J.J., Kirleis, A.W. 1992. Purification and immunocytochemical localization of kafirins in *Sorghum bicolor* (L. Moench) endosperm. *Protoplasma* 171, 64-74.

Singh, R., Axtell, J.D. 1973. High-lysine mutant gene (hl) that improves protein quality and biological value of grain sorghum. *Crop Science* 13, 535.

Taylor, J., Taylor, J.R.N. 2011. Protein biofortified sorghum: Effect of processing into traditional African foods on their protein quality. *Journal of Agricultural and Food Chemistry* 59, 2386-2392.

Taylor, J.R.N., Schüssler, L. 1986. The protein compositions of the different anatomical parts of sorghum grain. *Journal of Cereal Science* 4, 361-369.

Taylor, J.R.N., Taylor, J. 2008. Five simple methods for the determination of sorghum grain end-use quality (with adaptations for those without laboratory facilities). University of Pretoria, South Africa. http://www.intsormil.org, accessed August 2008.

Taylor, J.R.N., Schüssler, L., Liebenberg, N. VdW. 1985. Protein body formation in the starchy endosperm of developing *Sorghum bicolor* (L.) Moench seeds. *South African Journal of Botany* 51, 35-40.

Tesso, T., Ejeta, G., Chandrashekar, A., Huang, C.P., Tandjung, A., Lewamy, M., Axtell, J.D., Hamaker, B.R. 2006. A novel modified endosperm texture in a mutant high-protein digestibility/high-lysine grain sorghum (*Sorghum bicolor* (L.) Moench). *Cereal Chemistry* 83, 194-201.

Weaver, C.A., Hamaker, B.R., Axtell, J.D. 1998. Discovery of grain sorghum germ plasm with high uncooked and cooked *in vitro* protein digestibilities. *Cereal Chemistry* 75, 665-670.

WHO/FAO/UNU Expert Consultation. 2007. Protein and Amino Acid Requirements in Human Nutrition, Report of a Joint WHO/FAO/UNU Expert Consultation.

World Health Organization Technical Report No. 935. World Health Organization, Geneva.

Zhao, Z-Y., Glassman, K., Sewalt, V., Wang, N., Miller, M., Chang, S., Thompson, T., Catron, S., Wu, E., Bidney, D., Kedebe, Y., Jung, R. 2003. Nutritionally improved transgenic sorghum. In: Vasil, I.K., (Ed), Plant Biotechnology 2002 and Beyond, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 413-416.

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.

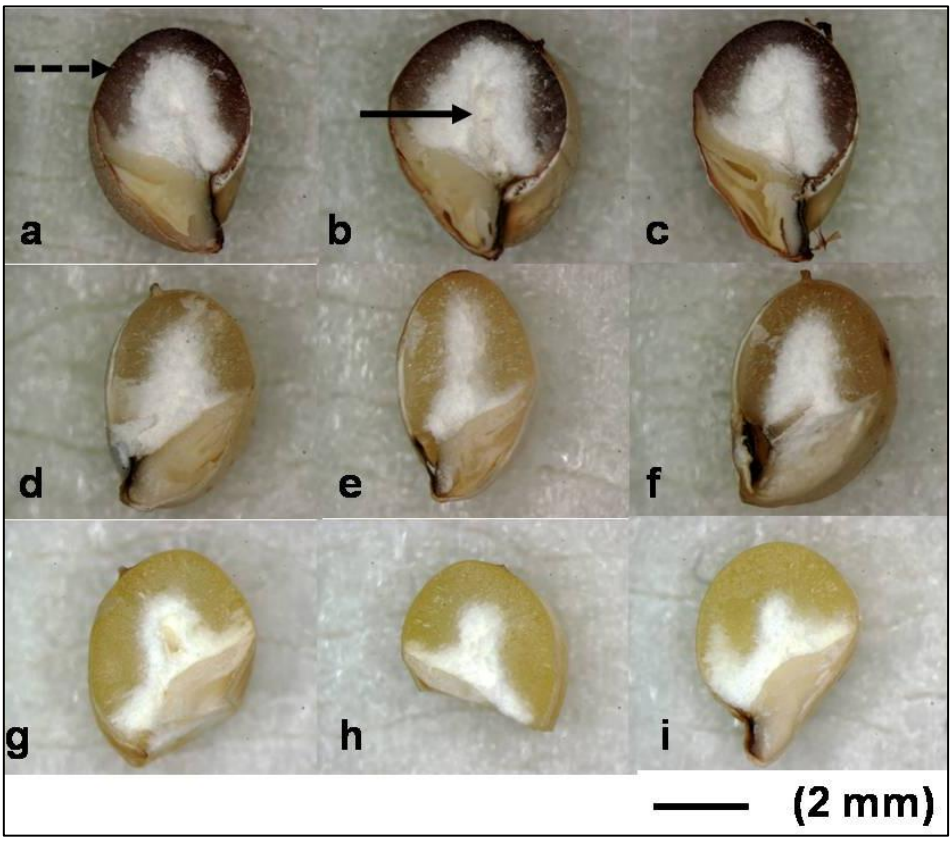

Figure 4.2.1 Sectioned kernels (with or without germ) of the normal sorghum parent lines used for transformation or backcrossing.
a-c) P898012; d-f) Macia; g-i) Tx430. Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining.

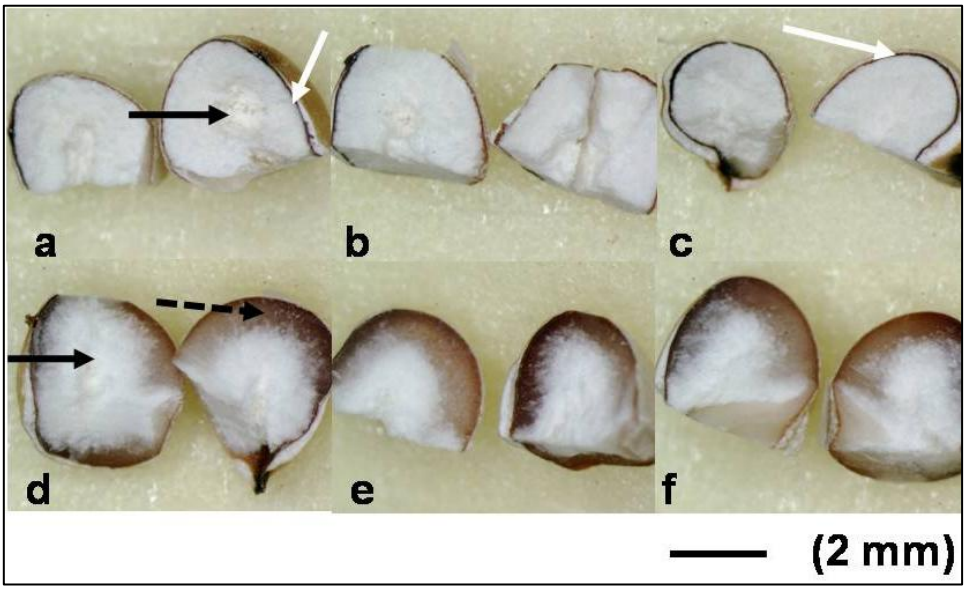

Figure 4.2.2 Sectioned kernels (without germ) of trangenic line (ABS032 gene construct) and its null control.
a-c) TG-P898012 (ABS032); d-f) NC-P898012. Black arrow indicates lumen in centre of endosperm; black dashed arrow indicates tannin staining; white arrow indicates pigmented testa layer.

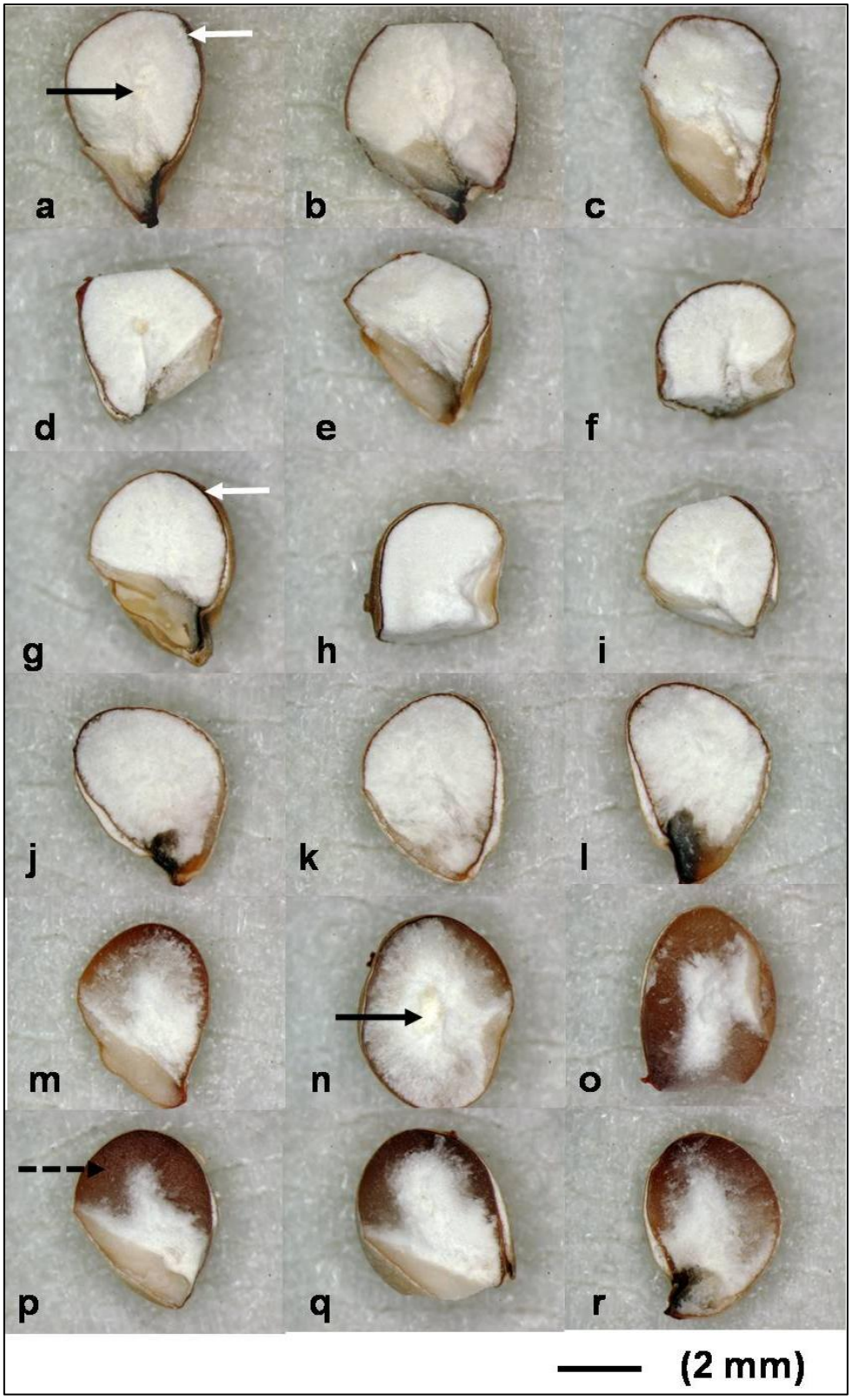

Figure 4.2.3 Sectioned kernels (with or without germ) of transgenic line (ABS032 gene construct backcrossed into Macia, type II tannin) and its null control.

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.

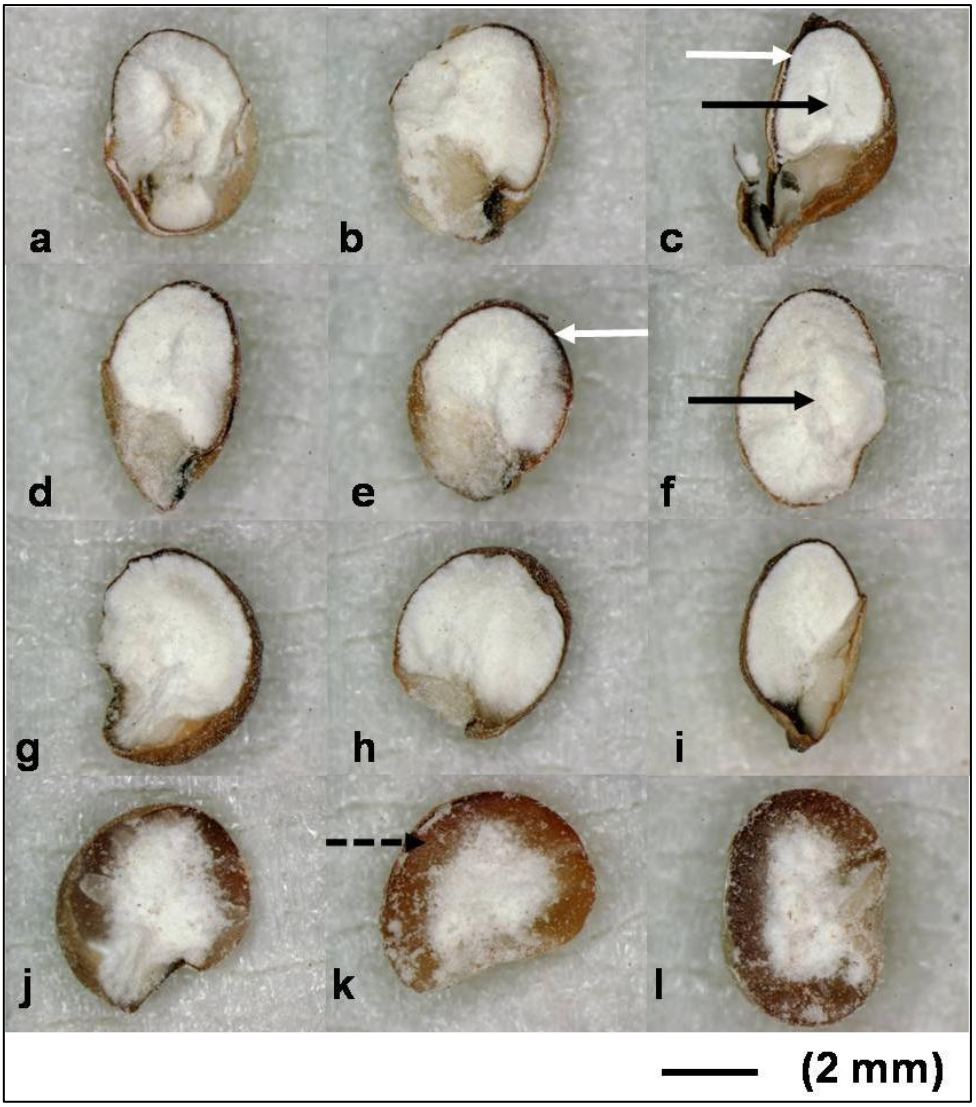

Figure 4.2.4 Sectioned kernels (with or without germ) of transgenic line (ABS032 gene construct backcrossed into Macia, type II tannin) and its null control.

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.

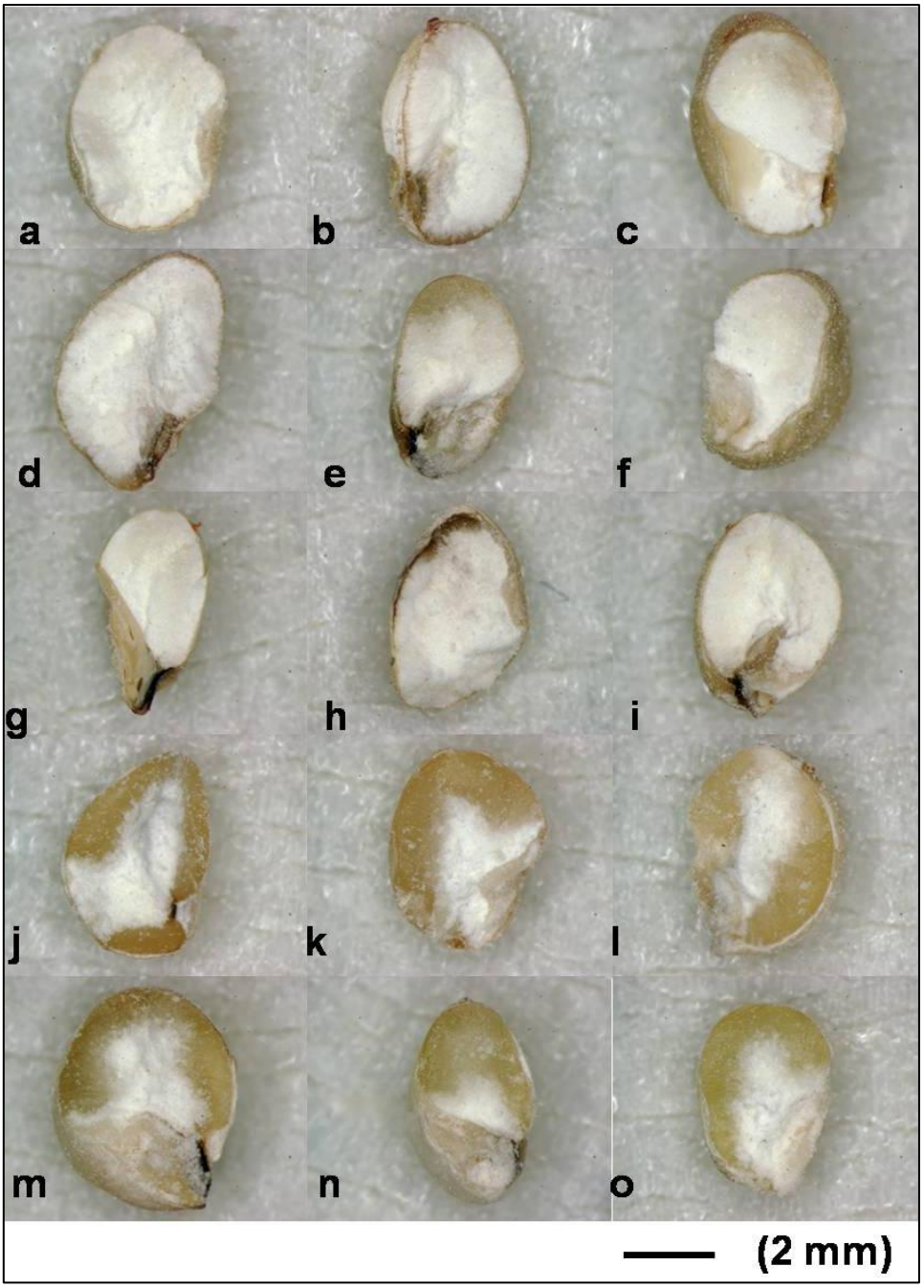

Figure 4.2.5 Sectioned kernels (with or without germ) of transgenic line (ABS032 gene construct backcrossed into Macia, non-tannin) and its null control.
a-i) TG-P898012xMacia (ABS032)-3; j-o) NC-P898012xMacia-3.

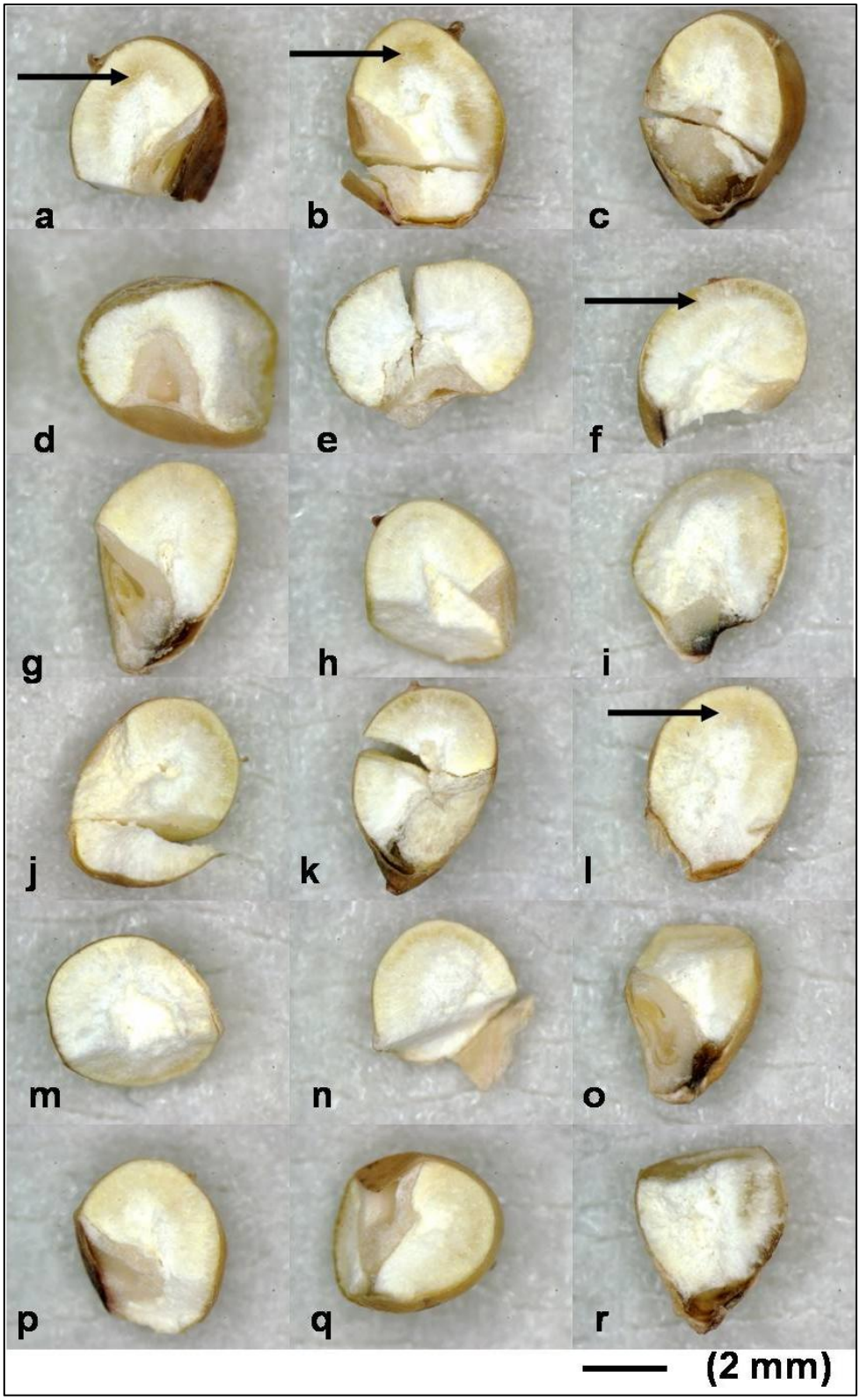

Figure 4.2.6 Sectioned kernels (with or without germ) of transgenic line (ABS166 gene construct) and its null control.
a-r) TG-Tx430 (ABS166); s-aj) NC-Tx430-1. Black arrow indicates patches of corneous endosperm texture.

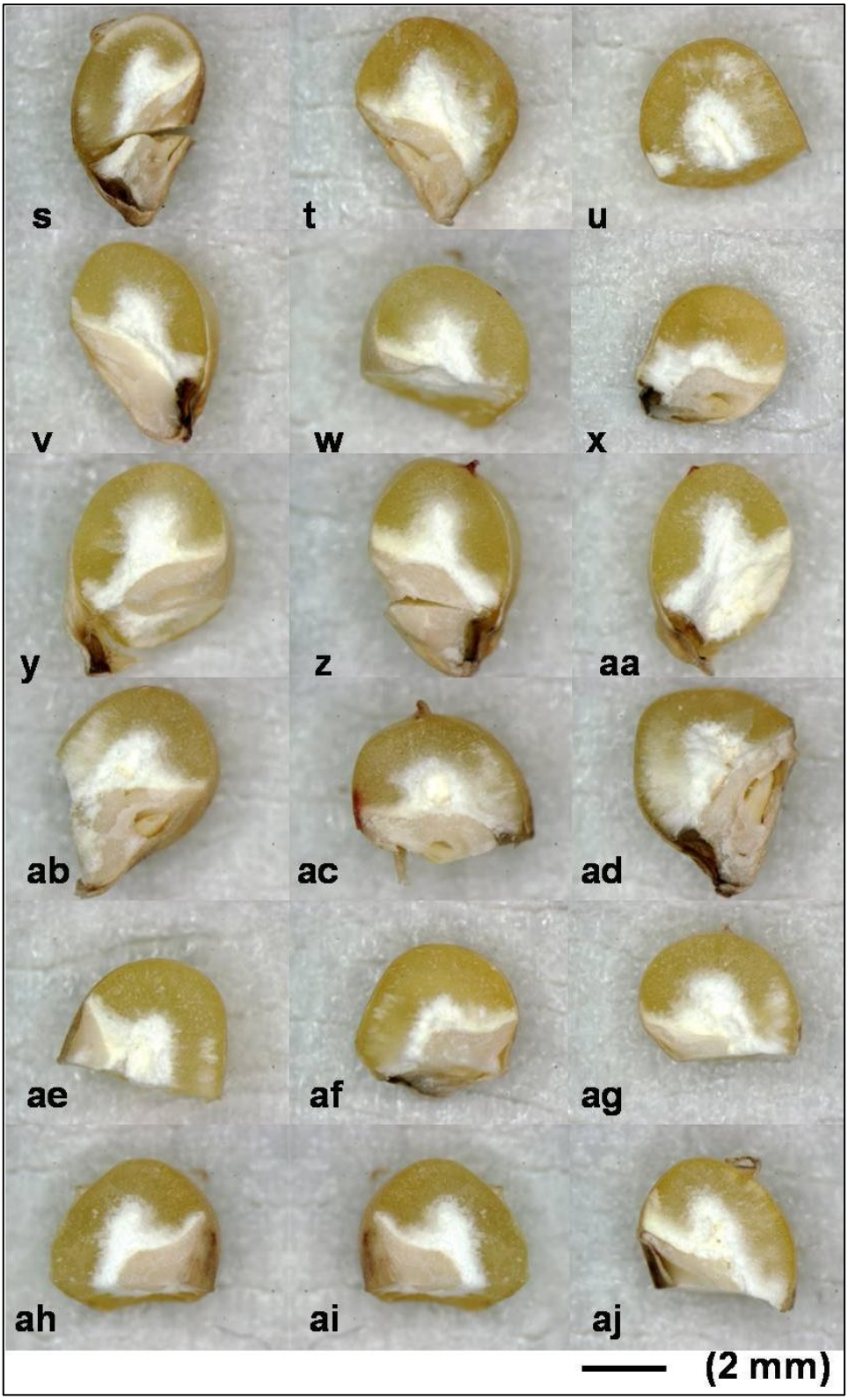

Figure 4.2.6. Continued.

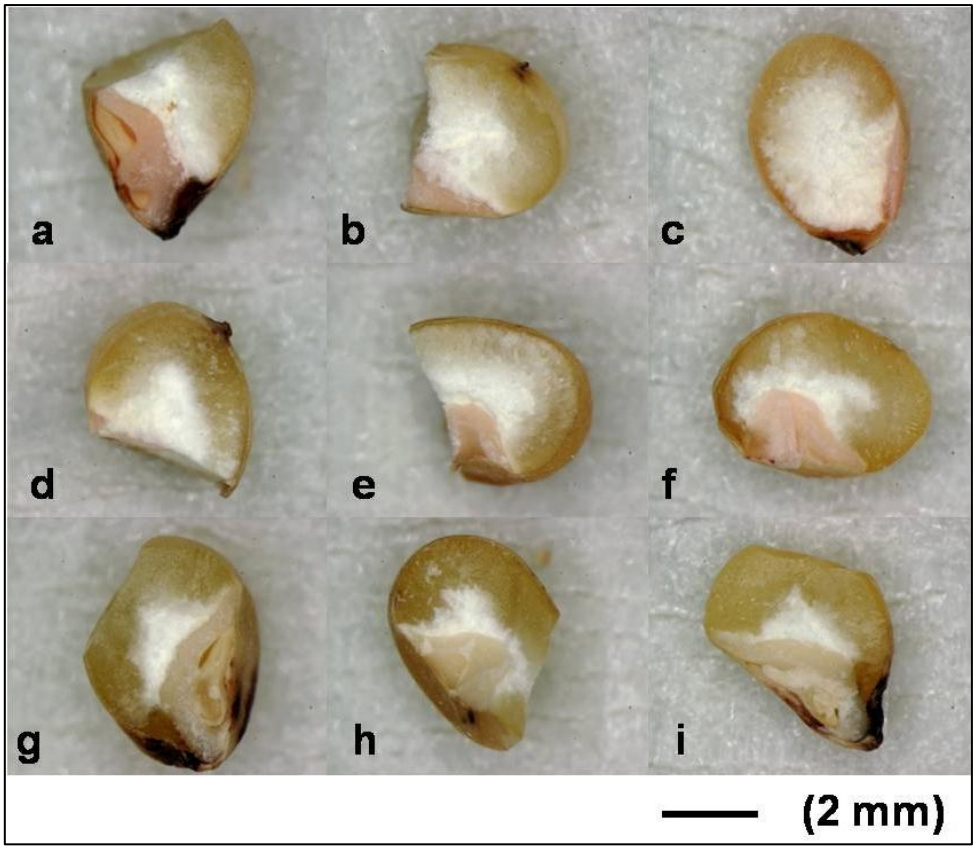

Figure 4.2.7 Sectioned kernels (with or without germ) of transgenic line (ABS0149 gene construct) and its null control.
a-f) TG-Tx430 (ABS149); g-i) NC-Tx430-2.

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.

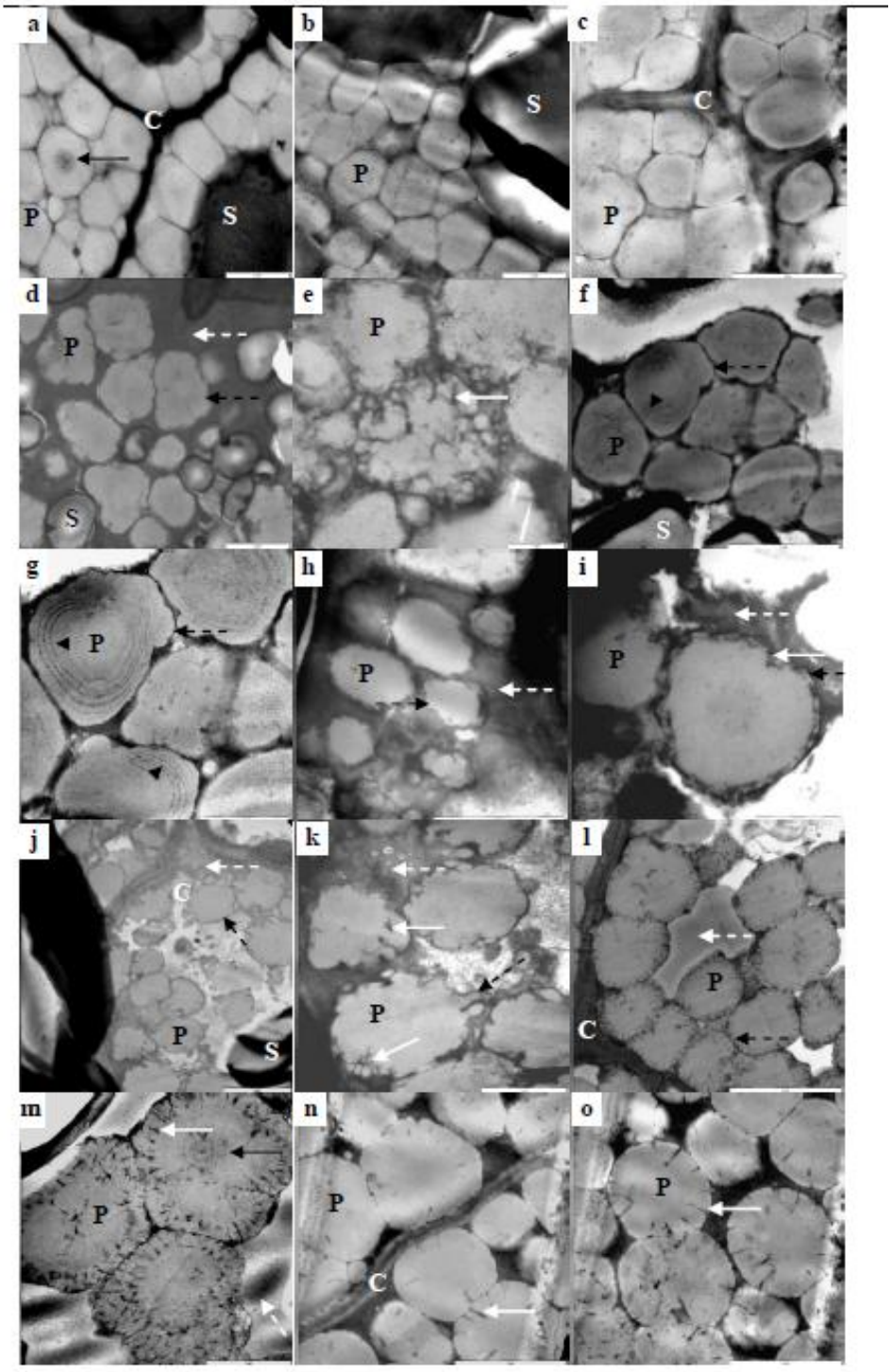

Figure 4.2.8 TEM of protein bodies in the peripheral endosperm of different sorghum lines.

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.

4.2.6 References

AACC International, 2000. Crude protein-combustion, Standard Method 46-30, *Approved Methods of the AACC, 10th ed*. American Association of Cereal Chemists: St Paul, MN.

ABS (Africa Biofortified Sorghum Project). 2009. http://biosorghum.org/, accessed March 2009.

Adams, C.A., Novellie, L., Liebenberg, N. VdW. 1976. Biochemical properties and ultrastructure of protein bodies isolated from selected cereals. *Cereal Chemistry* 53, 1-12.

Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography* 366, 93-104.

Coleman, C.E., Herman, E.M., Takasaki, K., Larkins, B.A. 1996. The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. *The Plant Cell* 8, 2335-2345.

Coleman, C.E., Yoho, P.R., Escobar, S., Ogawa, M. 2004. The accumulation of α-zein in transgenic tobacco endosperm is stabilized by co-expression of β-zein. *Plant Cell Physiology* 45, 864-871.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. and Hamaker, B.R. 2003. Factors affecting protein digestibility. *Journal of Cereal Science* 38, 117-131.

Dykes, L., Rooney, L.W. 2006. Sorghum and millets phenols and antioxidants. *Journal of Cereal Science* 44, 236-251.

FAO (Food and Agriculture Organization). 2002. FAO Animal Production and Health, *Proceedings:* Protein Sources for the Animal feed Industry. Expert Consultation and Workshop, Bangkok, 29 April – 3 May.

Gibbon, B.C., Larkins, B.A. 2005. Molecular genetic approaches to developing quality protein maize. *Trends in Genetics* 21, 227-233.

Guiragossian, V., Chibber, B.A.K., Van Scoyoc, S., Jambunathan, R., Mertz, E.T., Axtell, J.D. 1978. Characteristics of proteins from normal, high lysine, and high tannin sorghums. *Journal of Agricultural and Food Chemistry* 26, 219-223.

Habben, J.E., Moro, G.L., Hamaker, B.R., Larkins, B.A. 1995. Elongation factor 1α concentration is highly correlated with the lysine content of maize endosperm. *Proceedings of the National Academy of Sciences of the United States of America* 92, 8640-8644.

ICC (International Association for Cereal Science and Technology). 2008. Estimation of sorghum grain endosperm texture. ICC Standard 176. ICC, Vienna. http://www.icc.or.at/standard_methods/176, accessed April 2009

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 2009. Sorghum. www.icrisat.org/newsite/crop-sorghum.htm, accessed November 2009.

Kim, C.S., Woo, Y.-M., Clore, A.M., Burnett, R.J., Carneiro, N.P., Larkins, B.A. 2002. Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. *The Plant Cell* 14, 655-672.

Lending C.R., Larkins, B.A. 1992. Effect of the *floury-2* locus on protein body formation during maize endosperm development. *Protoplasma* 171, 123-133.

Maxson, E.D., Rooney, L.W. 1972. Evaluation of methods for tannin analysis in sorghum grain. *Cereal Chemistry* 49, 719-729.

Mertz, E.T., Axtell, J.D., Ejeta, G., Hamaker, B.R. 1993. Development and recent impact of quality protein maize and sorghum. In: Taylor, J.R.N., Randall, P.G. and Viljoen, J.H., (Eds), Cereal Science and Technology. Impact on a

Changing Africa. Selected Papers from the ICC International Symposium. The CSIR, Pretoria, South Africa, pp. 115-131.

Oria, M.P., Hamaker, B.R., Shull, J.M. 1995a. *In vitro* protein digestibility of developing and mature sorghum grain in relations to α-, β- and γ-kafirin disulphide crosslinking. *Journal of Cereal Science* 22, 85-93.

Oria. M.P., Hamaker., B.R., Axtell, J.D., Huang, C.P. 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. *Proceedings of the National Academy of Sciences of the United States of America* 97, 5065-5070.

Rooney, L.W., Serna-Saldivar, S.O. 1990. Sorghum. In: Lorenz, K.J. and Kulp, K., (Eds.), Handbook of Cereal Science and Technology, Markel Dekker, New York, pp. 233-270.

Shewry, P.R. 2007. Improving the protein content and composition of cereal grain. *Journal of Cereal Science* 46, 239-250.

Shull, J.M., Watterson, J.J., Kirleis, A.W. 1992. Purification and immunocytochemical localization of kafirins in *Sorghum bicolor* (L. Moench) endosperm. *Protoplasma* 171, 64-74.

Singh, R., Axtell, J.D. 1973. High-lysine mutant gene (hl) that improves protein quality and biological value of grain sorghum. *Crop Science* 13, 535.

Tesso, T., Ejeta, G., Chandrashekar, A., Huang, C.P., Tandjung, A., Lewamy, M., Axtell, J.D., Hamaker, B.R. 2006. A novel modified endosperm texture in a mutant high-protein digestibility/high-lysine grain sorghum (*Sorghum bicolor* (L.) Moench). *Cereal Chemistry* 83, 194-201.

Taylor, J., Bean, S.B., Ioerger, B.P., Taylor, J.R.N. 2007. Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. *Journal of Cereal Science* 46, 22-31.

Vendemiatti, A., Ferreira, R.R., Gomes, L.H., Medici, L.O., Azevedo, R.A. 2008. Nutritional quality of sorghum seeds: storage proteins and amino acids. *Food Biotechnology* 22, 377-397.

Weaver, C.A., Hamaker, B.R., Axtell, J.D. 1998. Discovery of grain sorghum germ plasm with high uncooked and cooked *in vitro* protein digestibilities. *Cereal Chemistry* 75, 665-670.

WHO/FAO/UNU Expert Consultation. 2007. Protein and Amino Acid Requirements in Human Nutrition, Report of a Joint WHO/FAO/UNU Expert Consultation.

World Health Organization Technical Report No. 935. World Health Organization, Geneva.

Zarkadas, C.G., Hamilton, R.I., Yu, Z.R., Choi, V.K., Khanizadeh, S., Rose, N.G.W., Pattison, P.L. 2000. Assessment of protein quality of 15 new northern adapted cultivars of quality protein maize using amino acid analysis. *Journal of Agricultural and Food Chemistry* 48, 5351-5361.

Zhao, Z-Y., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J., Pierce, D. 2000. *Agrobacterium*-mediated sorghum transformation. *Plant Molecular Biology* 44, 798-798.

Zhao, Z-Y., Glassman, K., Sewalt, V., Wang, N., Miller, M., Chang, S., Thompson, T., Catron, S., Wu, E., Bidney, D., Kedebe, Y., Jung, R. 2003. Nutritionally improved transgenic sorghum. In: Vasil, I.K., (Ed), Plant Biotechnology 2002 and Beyond, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 413-416.

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).

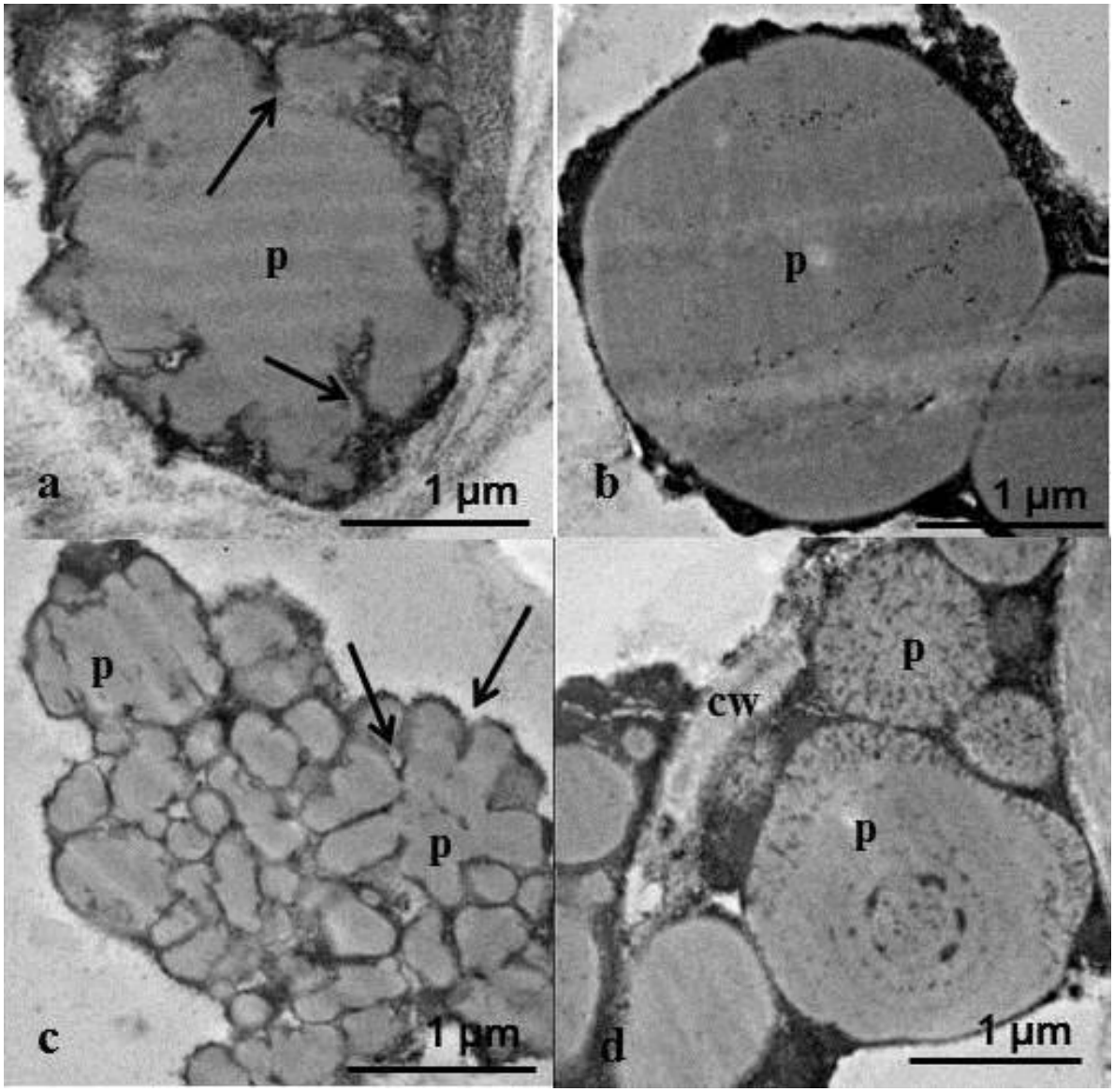

Figure 4.3.1 Representative images of TEM of sorghum protein body preparations by wet milling.
a) TG1; b) NC1; c) HD; d) Macia. cw = cell wall; p = protein body, arrows show invaginations

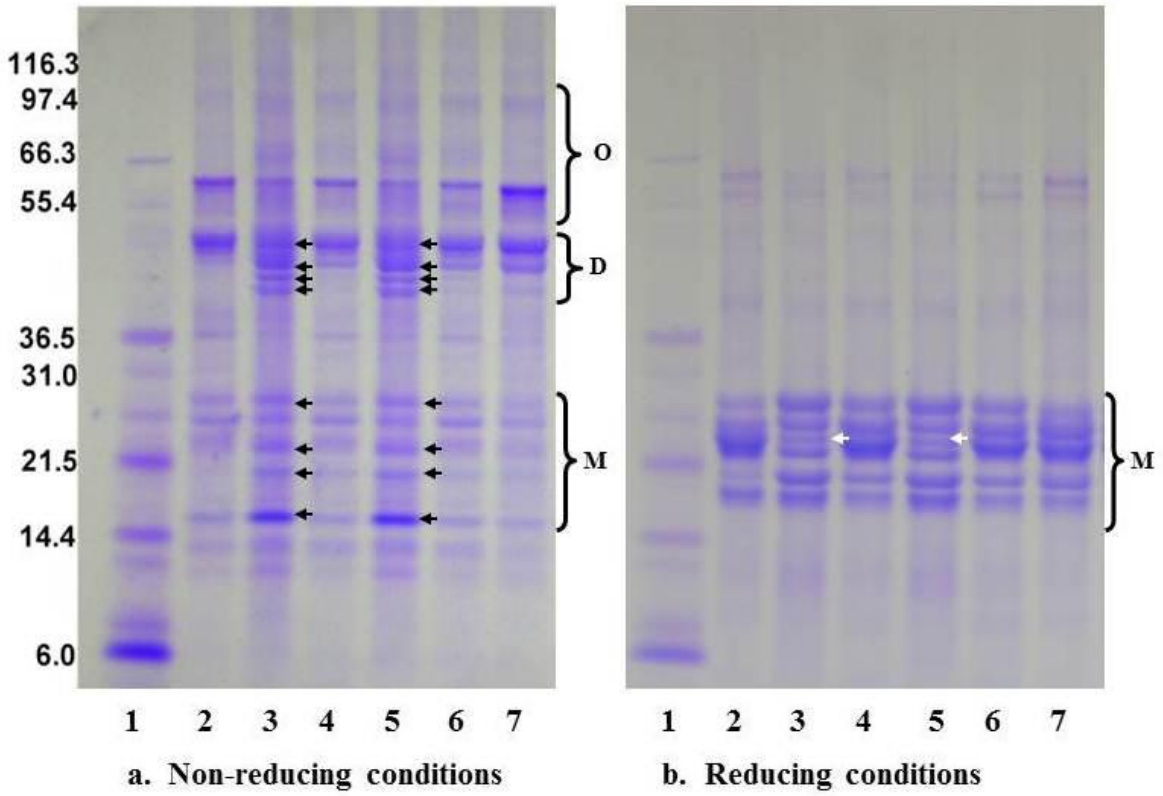

Figure 4.3.2 SDS-PAGE of sorghum protein preparations by wet milling.
1) Molecular weight markers (kDa); 2) C1; 3) T1; 4) C2; 5) T2; 6) HD; 7) Macia. M = Monomers; D = Dimers; O = Oligomers. Black arrows show high band intensity. White arrows show reduced band intensity. Loading at constant protein (10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

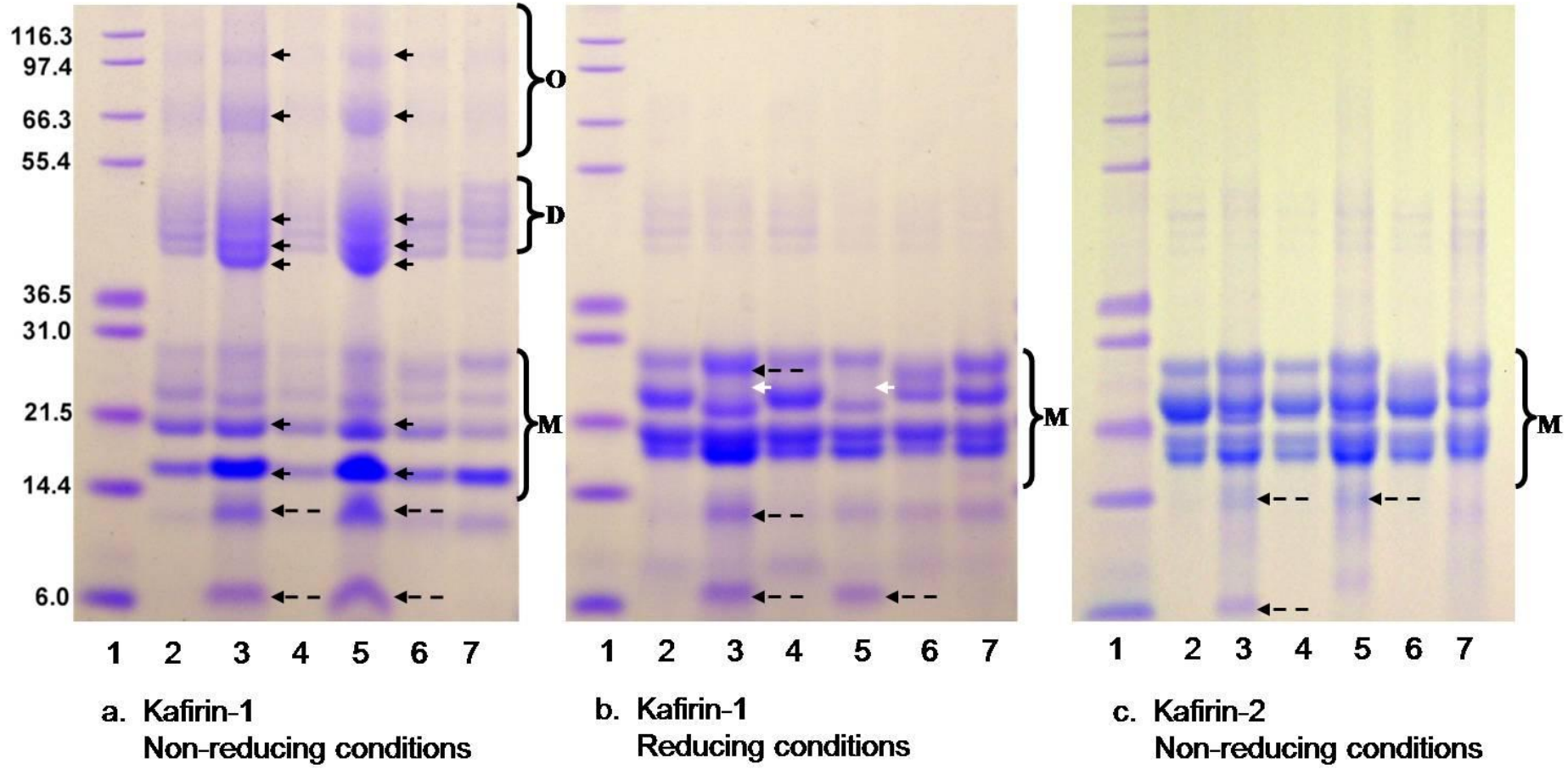

Figure 4.3.3 SDS-PAGE of extracted kafirin.
a-b) kafirin-1 extracted with 60% tert-butanol , c) kafirin-2 extracted with 60% tert-butanol plus 5% 2-mercaptoethanol.
1) Molecular weight markers (kDa); 2) NC1; 3) TG1; 4) NC2; 5) TG2; 6) HD; 7) Macia (in all cases). M = Monomers; D = Dimers; O = Oligomers. 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}±1.4[1, 2]	52.7^{a}±2.7	15.0
TG-P898012(ABS032)-2 (TG1)	$57.8^{bc1,2}$±2.3	65.7^{b}±3.3	7.9
NC-P898012-3 (NC2)	38.8^{a}±2.0	51.7^{a}±1.9	12.9
TG-P898012(ABS032)-3 (TG2)	55.2^{b}±2.0	68.0^{b}±5.2	12.8
HPDM8 (HD)	55.0^{b}±2.0	74.8^{c}±4.6	19.8
Macia	60.0^{c}±2.0	69.2^{b}±0.8	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.

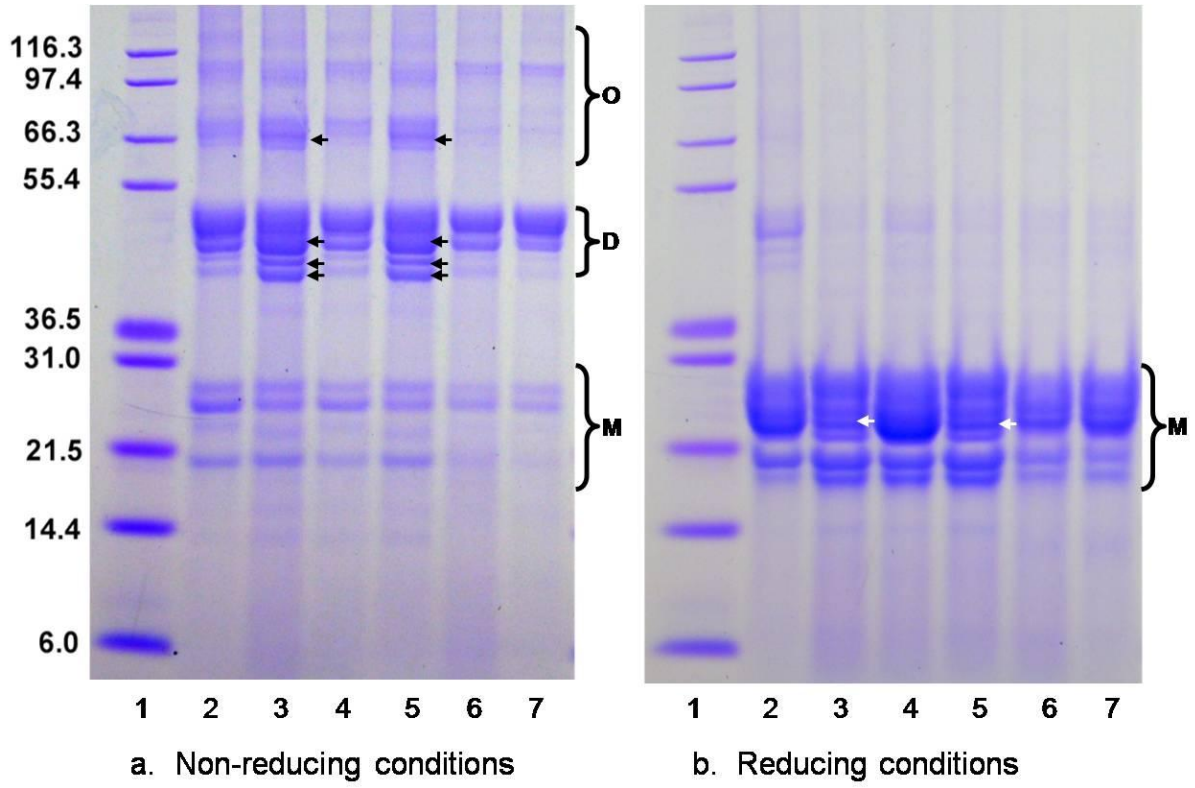

Figure 4.3.4 SDS-PAGE of pepsin-indigestible residue from cooked protein preparations by starch digestion.
1) Molecular weight markers; 2) C1; 3) T1; 4) C2; 5) T2; 6) HD; 7) Macia. M = Monomers; D = Dimers; O = Oligomers. Black arrows show high band intensity. White arrows show reduced band intensity/or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

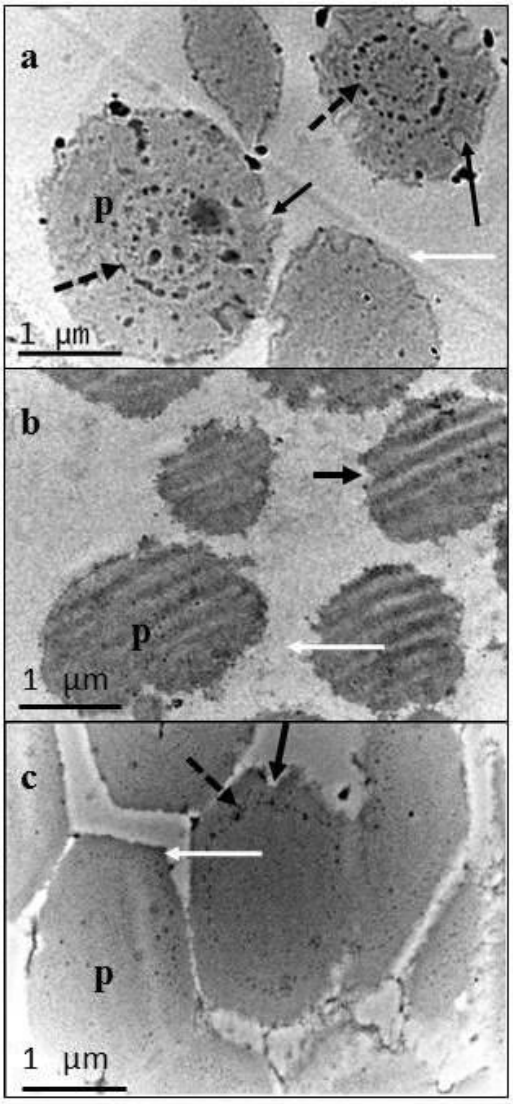

Figure 4.3.5 Representative images of TEM of pepsin-indigestible residue from cooked protein preparations by starch digestion.
a) NC1; b) TG1; c) Macia; p = protein body; Solid black arrows show proteolysis of protein body periphery; Dashed black arrows show dark staining regions, presumed primarily 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

AACC International, 2000. Crude protein-combustion, Standard Method 46-30. *Approved Methods of the AACC, 10th ed*. American Association of Cereal Chemists: St Paul, MN.

ABS (Africa Biofortified Sorghum Project). 2009. http://biosorghum.org/, accessed March 2009.

Belton, P.S., Delgadillo, I., Halford, N.G., Shewry, P.R. 2006. Kafirin structure and functionality. *Journal of Cereal Science* 44, 272-286.

Butler, L.G., Riedl, D.J., Lebryk, D.G., Blyit, H.J. 1984. Interaction of proteins with sorghum tannin: Mechanism, specificity and significance. *Journal of the American Oil Chemists' Society* 61, 916-920.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. and Hamaker, B.R. 2003. Factors affecting protein digestibility. *Journal of Cereal Science* 38, 117-131.

Duodu K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., Taylor, J.R.N. 2002. Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *Journal of Cereal Science* 35, 161-174.

El Nour I.N., Peruffo, A.D.B., Curioni, A. 1998. Characterisation of sorghum kafirins in relation to their cross-linking behaviour. *Journal of Cereal Science* 28, 197-207.

Emmambux, M.N., Taylor J.R.N. 2009. Properties of heat-treated sorghum and maize meal and there prolamin proteins. *Journal of Agricultural and Food Chemistry* 57, 1045-1050.

Ezeogu, L.I., Duodu, K.G., Taylor, J.R.N. 2005. Effects of endosperm texture and cooking conditions on the *in vitro* starch digestibility of sorghum and maize flours. *Journal of Cereal Science* 42, 33-44.

Gallagher, S.R. 1999. Electrophoretic separation of proteins. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A., and Strule, K. (Eds), Current Protocols in Molecular Biology, John Wiley and Sons, New York, pp. 10.2, A1-9.

Hamaker, B.R., Kirleis, A.W., Mertz, E.T., Axtell, J.D. 1986. Effect of cooking on the protein profiles and *in vitro* digestibility of sorghum and maize. *Journal of Agricultural and Food Chemistry* 34, 647-649.

Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D., Mertz, E.T. 1987. Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceedings of the National Academy of Sciences of the United States of America* 84, 626-628.

Henley, E.C., Taylor, J.R.N., Obukosia, S.D. 2010. The importance of dietary protein in human health: Combating protein deficiency in sub-Saharan Africa through transgenic biofortified sorghum. *Advances in Food and Nutrition Research* 60, 21-52.

Jackson, D.S., Shandera, D.L. 1995. Corn wet milling: separation chemistry and technology. *Advances in Food and Nutrition Research* 38, 271-300.

Ng'andwe, C.C., Hall, A.N., Taylor, J.R.N. 2008. Proteolysis of sorghum endosperm proteins when mashing with raw grain plus exogenous protease and potassium metabisulphite. *Journal of the Institute of Brewing* 114, 343-348.

Oria, M.P., Hamaker, B.R., Shull, J.M. 1995a. *In vitro* protein digestibility of developing and mature sorghum grain in relations to α-, β- and γ-kafirin disulphide crosslinking. *Journal of Cereal Science* 22, 85-93.

Oria. M.P., Hamaker., B.R., Axtell, J.D., Huang, C.P. 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. *Proceedings of the National Academy of Sciences of the United States of America* 97, 5065-5070.

Rom, D.L., Shull, J.M., Chandrashekar, A., Kirleis, A.W. 1992. Effects of cooking and treatment with sodium disulfite on *in vitro* protein digestibility and microstructure of sorghum flour. *Cereal Chemistry* 69, 178-181.

Shewry, P.R. 2007. Improving the protein content and composition of cereal grain. *Journal of Cereal Science* 46, 239-250.

Shewry, P.R., Pandya, M.J. 1999. The 2S albumin storage proteins. In: Shewry, P.R. and Casey, R., (Eds), Seed Proteins, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 563-86.

Shull, J.M., Watterson, J.J., Kirleis, A.W. 1992. Purification and immunocytochemical localization of kafirins in *Sorghum bicolor* (L. Moench) endosperm. *Protoplasma* 171, 64-74.

Taylor, J., Taylor, J.R.N. 2011. Protein biofortified sorghum: Effect of processing into traditional African foods on their protein quality. *Journal of Agricultural and Food Chemistry* 59, 2386-2392.

Taylor, J.R.N., Evans, D.J. 1989. Action of sorghum proteinase on the protein bodies of sorghum starchy endosperm. *Journal of Experimental Botany* 40, 763-768.

Taylor, J.R.N., Novellie, L., Liebenberg, N. VdW. 1984. Sorghum protein body composition and ultrastructure. *Cereal Chemistry* 61, 69-73.

Taylor, J.R.N., Novellie, L., Liebenberg, N. VdW. 1985. Protein body degradation in the starchy endosperm of germinating sorghum. *Journal of Experimental Botany* 36, 1287-1295.

Xie, X.J., Seib, P.A. 2000. Laboratory procedure to wet-mill 100 g of grain sorghum into six fractions. *Cereal Chemistry* 77, 392-395.

Zhao, Z-Y., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J., Pierce, D. 2000. *Agrobacterium*-mediated sorghum transformation. *Plant Molecular Biology* 44, 798-798.

5 GENERAL DISCUSSION

The general discussion will firstly critically examine the experimental methodologies as applied in this research. Secondly, the key mechanisms responsible for the improved protein quality in terms of lysine content and protein digestibility of transgenic genotypes with altered synthesis of major kafirin sub-classes will be considered based on the findings in the research. Then the effects of kafirin synthesis suppression on endosperm structure will be discussed. Lastly, a short discussion on the potential for continued development of transgenic sorghum with both improved protein quality and good grain endosperm quality will be given.

5.1 Methodological considerations

A major objective of this study was to determine the effect that altered kafirin synthesis has on the protein quality of different transgenic sorghums compared to normal sorghum and high protein digestibility mutant sorghum. Protein digestibility is commonly used as an indicator of protein quality as it is a measure of the availability of a protein to proteolysis during digestion (Poletti et al., 2004). Hence, food prepared from sorghum with high protein digestibility is considered to have better nutritional value compared to one with low protein digestibility, as it would provide the body with more amino acids after digestion. In this study, protein digestibility was determined *in vitro* using the pepsin digestibility assay of Hamaker et al. (1986). This method is considered reasonably useful, as it provides results that correlate with human child protein digestibility studies (MacLean et al., 1981; Mertz et al., 1984). However, a relatively large sample size (200 mg flour) is normally used, but in this project, transgenic material received was as little as a few grains. This limited the amount of material available for analysis. Hence, the digestibility method of Hamaker et al. (1986), suitably modified for small-scale assay (20 mg) was used. The small scale method (20 mg versus 200 mg) developed was shown to have high correlation with that of large sample size (Taylor and Taylor, 2007). A limitation when working with such small sample sizes is the fact that the margin for error is increased significantly. To reduce the error, at least two analyses with replicates were performed. In addition, sorghum condensed tannins present in P898012

parent line and certain transgenic lines could interfere with the IVPD assay. Sorghum condensed tannins are reported to reduce sorghum protein digestibility values (Butler et al., 1984). This is due to the condensed tannins binding to proteins forming indigestible protein-tannin complexes. There is also the chance that condensed tannins could inhibit the pepsin enzyme during the assay (Nguz, Van Gaver and Huyghebaert, 1998). Hence the low protein digestibility of certain tannin containing transgenic lines, does not indicate the true potential of different target kafirin suppressions with regard to protein digestibility.

Transmission electron microscopy (TEM) was used to visualise the effect of altered kafirin synthesis on the protein body structure of transgenic sorghum genotypes. The fact that only mature grains were obtained for this present study means a number of TEM sample preparation steps could have interfered with the structure of kafirin protein bodies: 1) In mature grains the protein bodies found in the peripherial endosperm below the aleurone layer are considered to best represent the actual size and shape of mature sorghum protein bodies (Krishnan et al., 1989). Since mature grains are typically very hard, it was difficult to cut thin sections of peripheral endosperm. Due to the thickness of the endosperm sections, extended time was needed for the dehydration and resin infiltration steps, and obtaining ultra thin specimens to view tended to be problematic; and 2) For dehydration, samples for TEM are commonly washed sequentially with increasing concentrations of aqueous ethanol (reviewed by Egerton, 2005). Since kafirin proteins are soluble in 70% aqueous ethanol, increasing concentrations of aqueous acetone was used to prevent solubilisation of the kafirins. Acetone has been used previously to successfully prepare kafirin microparticles (made from pure kafirin) for TEM (Taylor, Taylor, Belton and Minnaar, 2009), without them dissolving. This indicates acetone does not dissolve kafirin, and is thus a suitable dehydrating agent for kafirin protein bodies. The fact that the TEM methodology applied revealed that the protein bodies from normal sorghum were typical in size, shape and structure, as described by many workers (Adams et al., 1976; Taylor, Schüssler and Liebenberg, 1985; Krishnan et al., 1989), shows that it was effective. Hence, it can be concluded that TEM of transgenic protein bodies, which showed irregular shape and size, revealed their true morphology, and that the modified structures were not due to artefacts resulting from sample preparation.

The value of using TEM to study sorghum endosperm is that it gives detail with regard to protein body size, shape, packing density and associations between the protein bodies, matrix protein and the surrounding starch granules. However, TEM alone does not indicate the location and/or distribution of the different kafirin sub-classes within and at the periphery of the protein bodies. TEM also did not reveal whether the dark staining matrix protein commonly surrounding the modified transgenic protein bodies was kafirin or non-kafirin proteins. The use of TEM combined with immunolabeling would have been of great value in this work. Immunolabeling methods are commonly used to localise a compound of interest, using an antibody raised against an antigenic determinant (epitope) from that compound (reviewed by Stirling, 1990). The basic steps involved in this process are the initial stabilization of the tissue in a manner that preserves both morphology and antigenicity, followed by application of the antibody and visualization of the antibody-antigen complex in situ. Immunolabeling techniques have been used to localise and show the distribution of different kafirin and zein sub-classes in protein bodies of different sorghum and maize genotypes (Krishnan et al. 1989, Lending and Larkins, 1989; Geetha et al., 1991; Shull et al., 1992; Oria et al., 2000; Lending and Larkins, 1992). In addition, immunolabeling techniques have been used extensively to study the interactions between different zein proteins and their role in the formation of a maize protein body (Geli et al. 1994, Bagga et al. 1995, Coleman et al., 1996; Kim et al., 2002; Coleman et al., 2004). In this current work immunolabeling studies were not carried out due to the unavailability of antibodies.

Another significant limitation in this study was that protein body synthesis was not studied in the developing transgenic endosperm. In this research, only mature transgenic grains could be obtained. Such a study, linked with immunolabeling would give valuable insight into the effect altered kafirin synthesis has on the synthesis of protein bodies and matrix proteins. Studies conducted on developing and mature *floury-2* maize showed that protein bodies can in fact disintegrate during grain maturation (Christianson et al., 1974). Thus, loss of valuable information regarding protein body synthesis can occur when working with mature grains.

Another limitation was that SDS-PAGE alone was used to study the proteins. Thus, the identity of the different kafirin monomers could not be confirmed, except by

comparison with published data. During the development of the different transgenic sorghum genotypes, Western blotting using kafirin specific antibodies was used to identify different kafirin proteins and confirm target protein suppressions (Getu Beyene, Pioneer Hi-Bred, personal communication). In this current project, the antibodies were not available to us, thus Western blotting was not done. However, since cysteine-rich ɣ-kafirins were major kafirins targeted for suppression, it can be assumed that the missing band observed by SDS-PAGE is that of ɣ-kafirin. In addition, size exclusion chromatography (SEC) in conjunction with SDS-PAGE, would have been useful to clarify the effects of cooking on the polymerisation behavior of sorghum proteins as was done by Emmambux and Taylor (2009). SEC was able to confirm highly polymerised kafirins occur in cooked kafirin, which were not observed by SDS-PAGE, as the proteins were too large to enter the stacking gel. In this present study, if time had permitted, SEC could have been employed to quantify the highly polymerised kafirins (>200 kDa) in the transgenic and normal sorghum lines.

5.2 Potential mechanisms responsible for improved protein quality

What was most apparent in this study is that co-suppression of synthesis of major kafirin sub-classes, namely α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2 (ABS032 gene constructs), resulted in TG lines with considerably improved protein quality with regard to cooked protein digestibility (maximum 73.7%, type II tannin lines, and maximum 80.8%, non-tannin line), and lysine content (maximum 4.1 g/100 g protein) compared to that of their null controls and other TG lines (Table 5.1). When fewer kafirin sub-classes were suppressed the improvement in protein quality was considerably lower, such was in the case of TG-ABS044 and TG-ABS166 lines (with α-kafirin-1, ɣ-kafirin-1 and ɣ-kafirin-2 suppression), and TG-ABS042 (with ɣ-kafirin-1 and -2 suppression) and TG-ABS142 (with ɣ-kafirin-1 and -2 and δ-kafirin-2 suppression). The mechanisms involved in the TG lines responsible for the high-protein digestibility and high-lysine traits can therefore be surmised.

Table 5.1 Summary of results: Protein and lysine content, *in vitro* protein digestibility (IVPD), protein body structure, tannin type and endosperm texture for different transgenic sorghum lines, non-transgenic null controls and normal lines studied.

Line and Gene Construct	Protein (g/100 g flour dry basis) (N x 6.25)	Lysine content (g/100 g protein)	IVPD (%) wet cooked	Protein body structure	Tannin type, Endosperm texture
TG P898012 (ABS042 gene construct) Greenhouse trial, T1 seed, ɣ-kafirin 1 and 2; suppression	9.07-10.6	2.04-2.36	27.6-39.9	Spherical (±2-3 µm), densely packed showing concentric rings	Type II Tannin Floury endosperm
TG P898012 (ABS044 gene construct) Greenhouse3 trial, T1 seed, α-kafirin A1, ɣ-kafirin 1 and 2	12.1-16.0	3.06	48.9-56.2	Deeply folded (invaginated) (±2-3 µm), densely packed with dark-staining inclusions, concentric rings absent	Type II Tannin Floury endosperm
TG P898012 (ABS032 gene construct), greenhouse trial, T1 seed, α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2 suppression.	12.8	3.28	73.7	Slightly folded (invaginated) (±2 µm), occasional dark-staining inclusions, sparsely packed, dense continuous dark protein matrix, concentric rings absent	Type II Tannin Floury endosperm
TG P898012 (ABS032 gene construct) backcrossed into Macia, greenhouse trial, F3 seed, α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2 suppression.	8.8-12.4	3.65	43.0-59.2	Slightly folded (invaginated) (±2 µm), occasional dark-staining inclusions, sparsely packed, dense continuous dark protein matrix, irregular concentric rings	Type II Tannin Floury endosperm
TG P898012 (ABS032 gene construct) backcrossed into Macia, summer confined field trial, F3 seed, α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2 suppression.	11.3-12.8	4.1	54.1-61.0	Slightly folded (invaginated) (±2 µm), occasional dark-staining inclusions, sparsely packed, dense continuous dark protein matrix, concentric rings absent	Type II Tannin Floury endosperm
Transgenic P898012 (ABS032 gene construct) backcrossed into Macia, summer confined field trial, F3 seed, α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2 suppression.	12.1-12.8	4.1	78.4	Slightly folded (invaginated) (±2 µm), occasional dark-staining inclusions, sparsely packed, dense continuous dark protein matrix	Non-tannin Floury endosperm

Table 5.1 Continued

Line and Gene Construct	Protein (g/100 g flour dry basis) (N x 6.25)	Lysine content (g/100 g protein)	IVPD (%) wet cooked	Protein body structure	Tannin type, Endosperm texture
TG P898012 (ABS032 gene construct) T2 selfed seeds, confined field trail, 75% pure with respect to ABS032, α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2 suppression.	11.6-12.1	2.38-2.69	55.2-57.8	Slightly folded (invaginated) (±2 µm), occasional dark-staining inclusions, sparsely packed, dense continuous dark protein matrix, concentric rings absent	Type II Tannin Floury endosperm
Transgenic Tx430 (ABS166 gene construct), greenhouse trial, T1 seed, α-kafirin A1 and ɣ-kafirin 1 and 2 suppression.	8.9-15.5	2.1	50.9-71.6	Spherical (±2 µm), dark staining inclusions and presence of concentric rings, less dense packing density with patches of dark protein matrix	Non-tannin Intermediate endosperm (modified with a large central floury portion with faint bands or patches of corneous-like endosperm)
Transgenic Tx430 (ABS149 gene construct), greenhouse trial, T1 seed, δ-kafirin 2 and ɣ-kafirin 1 and 2 suppression.	11.7-15.6	2.43	40.9-41.2	Spherical (±2 µm) with normal packing density. Presence of dark staining inclusions.	Non-tannin Corneous endosperm
High protein digestibility muntant (HPDM)	10.4-15.0	1.87–2.41	44.0-64.6	Invaginated (±1 µm), densely packed, concentric ring absent	Non-tannin Floury endosperm
Macia	10.1	1.95	59.2	Spherical (±2 µm), densely packed showing concentric rings	Non-tannin Intermediate endosperm
P898012	10.2-13.9	2.10	19.4-41.1	Spherical (±2 µm), densely packed showing concentric rings	Type II Tannin Intermediate endosperm
Tx430	10.2	2.18	48.2	Spherical (±2 µm), densely packed showing concentric rings	Non-Tannin Corneous endosperm
Normal Protein Digestibility	10.6-15.1	1.20-2.05	30.3-39.8	Spherical (±2-3 µm), densely packed showing concentric rings	Non-tannin Corneous endosperm

The major factors responsible for the high-protein digestibility of TG-ABS032 lines seem to be: 1) Significant reduction in the formation of highly crosslinked polymeric kafirins (>200 kDa) during grain development; 2) The synthesis of modified (irregular shaped) protein body structure, and 3) The presence of a dense continuous protein matrix around the protein bodies.

Significant reduction in the formation of highly crosslinked polymeric kafirins (>200 kDa) during grain development resulted in proportionally more dimers, oligomers, and certain monomers present in the TG-ABS032 grain endosperm (Figures 4.3.2 - 4.3.4). Reduced kafirin crosslinking was no doubt also responsible for the improved extractability of kafirin-1 (approximately 69% of total kafirin extracted) from TG-ABS032 lines, compared to the null controls (40-42%). According to Oria et al. (1995a), some 89% of the total kafirin in mature sorghum grains is crosslinked. Considering the fact that both the TG-ABS032 lines and their null controls had similar total kafirin content (ranging from 47.2-49.9%), it can be assumed 27-29% less crosslinking occurred in the TG-ABS032 lines compared to that of their null controls. Reduced crosslinking of TG-ABS032 kafirin proteins can be attributed to the targeted suppression of ɣ-kafirin synthesis. The ɣ-kafirins are required for kafirin polymerisation through disulphide bonding (El Nour et al., 1998).

In addition, kafirin oligomers have been found to comprise α- and ɣ-kafirin sub-classes with only trace amounts of β-kafirin, and only highly polymeric kafirins comprise α-, ɣ- and β-kafirins (El Nour et al., 1998; Nunes et al., 2005). The pattern of crosslinking behaviour of kafirin sub-classes is reported to be as follows: ɣ-kafirin (15 cysteine residues) can link with α-kafirin A1 (2 cysteine residues) to form different sized oligomers and high molecular weight polymers, or link with α-kafirin A2 (one cysteine residue) to forms dimers and small oligomers (El Nour et al., 1998). Dimers of α-kafirin A2 also occur and form part of small oligomers. Beta-kafirin (10 cysteine resudues) acts as a bridge linking oligomers of ɣ-kafirin and α-kafirin A1 forming very large polymers, extractable only under reducing conditions. The role of δ-kafirin (4 cysteine residues) in kafirin crosslinking is not clear (Belton et al., 2006).

The crosslinking behaviour of the kafirin sub-classes is believed to be directly related to the number of cysteine residues. Since α-kafirin A2 has only one free sulphydryl

group (-SH), it is not possible for this kafirin to form multiple disulphide bonds, thus acting as a 'chain terminator' (El Nour et al., 1998). Kafirins (α-kafirin A1, ɣ-, β- and possibly δ-kafirin) with more than one free -SH group will contribute to increasing the polymer size, thus acting as a 'chain extender'. In consideration of the work of El Nour et al. (1998), it is most probable that any changes in the proportions and quantities of the different kafirin sub-classes during grain development will have a dramatic effect on the kafirin polymerisation behaviour of TG sorghum with altered kafirin synthesis. Hence, it can be assumed that the composition of kafirin oligomers in the TG-ABS032 lines is altered and comprise more crosslinked β- and α-kafirin sub-classes and less or no crosslinked ɣ- and α-kafirin sub-classes, in comparison with normal sorghum. Additionally, the fact that kafirins in the TG-ABS032 lines were more readily extractable in aqueous tert-butanol without the aid of a reducing agent indicates that the kafirins are also more available to pepsin digestion. Hence, this would in part result in the high-protein digestibility of the TG-ABS032 line.

TG-ABS032 lines had modified protein body structure, being irregular in shape with slightly folded (invaginated) periphery in comparison with the spherical protein bodies of normal sorghums (Figure 4.2.8). Irregular shaped, highly invaginated protein bodies have been reported to be a major factor in the improved protein digestibility of HPDM lines (Oria et al., 2000). The improved protein digestibility of HPDM sorghums is believed to be due to increased protein body surface area and easy accessibility of digestive enzymes to the more digestible α-kafirins. The same could be assumed for those highly digestible TG lines exhibiting irregular protein body structure.

However, the protein bodies of the TG-ABS044 line, as well as the HPDM-1 lines used in this study, also had irregular protein body shape (Figure 4.1.2). But, their protein digestibility was, surprisingly, considerably lower (maximum 56.2%, type II tannin for TG-ABS044 and maximum 64.6%, non-tannin for HPDM-1) than that of TG-ABS032 (maximum 73.7%, type II tannin, and maximum 80.8%, non-tannin) (Table 5.1), as well as mutant sorghums reported in the literature (Weaver et al., 1998). The fact that the HPDM-1 line used in this study had a lower proportion of kafirin-1 (42.8%) and had low levels of dimers, oligomers and monomers (by SDS-PAGE, Figure 4.3.2-4.3.4), compared to TG-ABS032, indicates that the kafirins in

the HPDM-1 line were more crosslinked. Although the proportion of kafirin-1 to kafirin-2 was not determined for TG-ABS044 (due to small sample size), it can be assumed that kafirins in the TG-ABS044 line were also more crosslinked than those of TG-ABS032. Nevertheless, the fact that TG-ABS044 had modified protein body structure still indicates that the co-suppression of α- and ɣ-kafirins was sufficient to interfere with normal protein body synthesis. Thus, it would appear that the relative proportion of highly crosslinked kafirins versus less crosslinked kafirins in TG lines plays a more important role in the digestibility of kafirins, than the modified protein body structure alone.

The development of modified protein body structures in TG-ABS032 is also probably related to the fact that the kafirins were considerably less crosslinked than those of normal sorghum. The mechanism involved in the initiation and development of zein protein bodies in maize is described as the aggregation of cysteine-rich β- and ɣ-zein proteins via the formation of inter-molecular disulphide bonds, forming an insoluble protein body core, required for integration of α-zein (Lending and Larkins, 1998; Coleman et al., 2004). Hence, in the case of TG-ABS032, the absence of ɣ-kafirin would interfere with the aggregation of β- and ɣ-kafirins, resulting in less crosslinked kafirins forming via inter-molecular disulphide bonds. This in turn would prevent the formation of an insoluble protein body core, which is presumed to be required for integration of accumulating α-kafirin (Lending and Larkins, 1998). Further, aggregates of both β- and ɣ-zein proteins are believed to interact with α-zein via hydrophobic and/or disulphide bonding (Coleman et al., 2004). Also, the various zein sub-classes are believed to interact with themselves during protein body synthesis (Kim et al., 2002). Hence, it can be assumed that protein-protein interactions between β- and α-kafirins, as well as between the α-kafirins themselves still occurred in TG-ABS032 lines, but to a less extent than in normal sorghums, resulting in the formation of various kafirin dimers and oligomers. In addition, it can be assumed that the organisation and distribution of the different kafirin sub-classes are disorganised in TG-ABS032 and TG-ABS044, as described for irregular protein bodies of *floury-2* mutant (Lending and Larkins, 1992) and HPDM (Oria et al., 2000) lines.

The dense protein matrix observed in the peripheral endosperm of TG-ABS032 (Figure 4.2.8) most probably played a role in its improved protein digestibility. Although, the protein composition of the dense protein matrix is not known, it can be assumed that the dense protein matrix is composed in part of digestible non-prolamin proteins. According to a review by Shewry (2007), in high-lysine cereal genotypes, reduced synthesis of prolamin proteins typically results in compensatory synthesis of highly-digestible non-prolamin proteins in the grain endosperm. However, for TG-ABS032 this does not appear to be the case, since it did not have reduced synthesis of prolamin proteins. Thus, it is not likely that the dense protein matrix is composed solely of non-prolamin proteins, and may actually contain low molecular weight kafirin proteins (monomers and dimers). The irregular shaped protein body structures, composed of less crosslinked kafirins is a good indication that modified protein body synthesis occurred. Thus, it is possible that during protein body synthesis, low molecular weight kafirins may have escaped through the endoplasmic reticulum into the surrounding endosperm tissue. Kafirins in the endosperm matrix would presumably be more digestible than kafirins contained within protein bodies. According to Oria, Hamaker and Shull (1995b) native, unreduced, α-kafirins are highly digestible under both uncooked and cooked conditions. However, in mature grains, the poor digestibility and low extractability of α-kafirins is due to the location of the α-kafirins surrounded by layers of crosslinked β- and ɣ-kafirins at the periphery of the protein body preventing α-kafirin from being readily extracted or digested.

TG-ABS032 lines, where α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2 were co-suppressed, resulted in the greatest increase in lysine content (up to 4.1 g/100 g protein) (Table 5.1). Nevertheless, the increased lysine content is somewhat surprising considering that the TG lines had similar kafirin content as their respective null controls. This may be due in part to the fact that the TG-ABS032 line used for kafirin extraction studies was only 75% pure. In pure lines it may be possible that reduced total kafirin content could occur. In high-lysine, non-transgenic cereal genotypes improved lysine content is attributed to significant reduction in lysine-poor prolamin proteins, with compensatory synthesis of lysine-rich non-prolamin proteins (Shewry, 2007). The high lysine content of *opaque-2* mutant maize has been attributed to significantly more (almost double) non-zein cytoskeletal proteins

attached to polysomes surrounding the zein proteins bodies, compared to normal maize (Azama, Abe, Sugimoto and Davies, 2003). The cytoskeletal proteins identified include: actin, tubulin, elongation factor 1-a (eEF1a), UDP-glucose starch glucosyltransferase (UDP-GSGT), sucrose synthase 1 (SuSy-1) and fructose-1,6 bisphosphate aldolase (FBA), which are rather high in lysine (5–11%). It is possible similar lysine-rich cytoskeletal proteins were in the dense protein matrix around the protein bodies in TG-ABS032. In addition, SDS-PAGE of the kafirin-1 and kafirin-2 extracts showed the presence of considerable levels of low-molecular weight proteins (<14 kDa) not previously observed in normal sorghum. Since these proteins were soluble in aqueous tert-butanol solvent, it is possible these low molecular proteins could be alcohol-soluble, non-prolamin proteins (Shewry and Pandya, 1999), and may also have contributed to increasing the lysine content of the TG-ABS032. Further investigation to identify possible lysine-rich non-kafirin proteins in TG-ABS032 lines is required.

Lastly, the overall increased lysine content in TG-ABS032, may also be due in part to the reduced expression of the lysine catabolic enzyme lysine ketoglutarate reductase (LKR). As stated, reduced expression of LKR in high-lysine mutant cereals results in increased levels of free (non-protein) lysine in the grain endosperm (Shewry, 2007). According to Brochetto-Braga, Leite and Arruda (1992) the LKR activity in *opaque-2* mutant maize endosperm is 3-5 fold lower than in normal maize, contributing to reduced lysine catabolism during gain development, resulting in increased lysine content. In addition, in *opaque-2* lines low LKR activity appears to be associated with the low rate of zein accumulation, suggesting that LKR is under the control of the mutant gene that transactivates the expression of the 22 kDa α-zein genes in maize endosperm. Although target LKR suppression also occurred in TG-ABS149 lines, the lysine content appeared normal (2.43 g/100 g protein) (Table 5.1). Thus, it can be assumed that the reasons for increased lysine content of TG-ABS032 lines is multifactorial.

5.3 Floury endosperm texture

The floury endosperm texture of TG lines with high protein quality seems to be a direct consequence of suppression of ɣ-kafirin synthesis, leading to somewhat irregular shaped protein bodies with low packing density. This is due primarily to the fact that ɣ-kafirin suppression altered kafirin polymerisation behaviour by forming less highly crosslinked kafirin polymers, and higher concentrations of dimers and oligomers. Significant reduction in disulphide crosslinking of endosperm proteins appears to, in turn, disrupt the grain endosperm structure. This would cause a loose association of endosperm components, where irregular protein bodies are not tightly packed and the protein matrix is discontinuous. It is most likely that such loose, unstable matrices will shatter during grain desiccation, producing the floury texture at maturity. These findings reinforce the fact that ɣ-kafirins confer rigidity by crosslinking with β-kafirins at the periphery of protein bodies, and forming crosslinks with matrix proteins (Wall et al., 1988; Shull et al., 1990; Paiva, Kris, Peixoto, Wallace and Larkins, 1991; Robutti, Borras and Eyherabide, 1997; Mazhar and Chandrashekar, 1995; Chandrashekar and Mazhar, 1999). These crosslinks are thus essential for forming a compact, continuous structure commonly associated with corneous endosperm.

In addition, what was apparent in this present study is that when several kafirin sub-classes were co-suppressed (α-, ɣ- and δ-kafirin), complete floury endosperm texture occurred. However, when fewer kafirin sub-classes were co-suppressed (only α- and ɣ-kafirin, or only δ- and ɣ-kafirin) the endosperm texture was slightly or completely restored to that of their respective null controls, especially in TG lines (TG-ABS166 and TG-ABS149) where parent line Tx430 was used for transformation. This indicates that synthesis of the correct proportions of the different kafirin sub-classes may also be necessary to ensure adequate kafirin and matrix protein crosslinking, to maintain endosperm integrity. Again, these findings suggest that the different kafirin sub-classes have evolved to play distinct roles in the development of sorghum endosperm. In the case of QPM, modifier genes are used to elevate the accumulation of 27 kDa ɣ-zein in the endosperm (Wu et al., 2010). Increased 27 kDa ɣ-zein allows the accumulation of small but numerous protein bodies, which

have a strong association with the proteinaceous matrix and starch granules, resulting in the formation of corneous endosperm of QPM.

The use of modifier genes to increase ɣ-kafirin synthesis in sorghum to improve endosperm texture would have negative consequences on protein digestibility. Nevertheless, development of high-lysine high-protein digestibility sorghum mutant lines with relatively hard endosperm phenotype is possible through breeding programmes. Tesso et al. (2006) reported the identification of a novel high-lysine high-protein digestibility (HPD/*hl*) sorghum genotype with a unique modified relatively hard endosperm texture. The microstructure of the corneous endosperm fraction was dramatically different from that of normal corneous kernels, in that polygonal starch granules were densely packed but without the typically associated continuous protein matrix.

What appears to be highly relevant to the issue of sorghum floury endosperm structure is that normal tannin sorghums such as the P898012 parent line used in this present study commonly have a floury endosperm. According to Waniska, Poe and Bandyopadhyay (1989), tannin sorghums (with a pigmented testa) are more resistant to grain moulding, contain higher levels of free phenolic compounds, and have a softer endosperm texture than non-tannin sorghum. These authors suggested phenolic compounds are involved in the plant's defensive mechanism against fungi. However, the cause of floury endosperm does not appear to be known. Since sorghums with hard endosperm texture are generally more mould resistant (Glueck and Rooney, 1980), the presence of phenolic compounds in tannin sorghums may alter the biochemical mechanisms in these sorghums, allowing altered synthesis of kafirin proteins and resulting in more floury endosperm texture. Alternatively, tannins are well known to complex with proline-rich proteins, such as the kafirins (reviewed by Spencer, Cai, Gaffney, Goulding, Magnolato, Lilley, and Haslam, 1988). Compared to other kafirins, ɣ-kafirin has been reported to bind more tannins as it contains far more proline than total kafirin (Taylor et al., 2007). Thus, tannins can possibly interfere with ɣ-kafirin crosslinking, resulting in floury endosperm texture.

5.4 Future work

TG lines with the ABS032 gene construct (α-kafirin A1, B1 and B2; ɣ-kafirin 1 and 2, and δ-kafirin 2) showed the greatest potential for improving the protein nutritional quality of sorghum, having high-lysine and high-protein digestibility traits (Table 5.1), but TG-ABS032 resulted in floury endosperm texture. Since grain hardness is an important grain quality attribute that plays a major role in sorghum agronomic properties, processing and the end-use quality of sorghum based foods (reviewed by Rooney, Kirleis and Murty, 1986), improving the grain hardness in TG-ABS032 needs to be the focus of future work. Options for consideration include: 1) Using elite hard endosperm normal sorghum lines for transformation and backcrossing, such as Tx430 used in this present study. According to Tesso et al. (2006) elite hard endosperm sorghum can be used successfully to improve the endosperm texture of high-protein quality opaque mutants. However, several breeding cycles (F6) of phenotypic agronomic selections may be needed to obtain grain with improved endosperm texture; 2) The use of the mutant germplasm (HPD/*hl*) with both high protein quality and improved endosperm texture (Tesso et al., 2006) should be considered for transformation work, or backcrossed with non-tannin TG-ABS032 lines. Since the translucent, vitreous endosperm of HPD/*hl* is due solely to the dense packing of its starch granules and not protein matrix, the hard endosperm trait might be retained; and 3) Due to the fact that ɣ-kafirin plays a major role in grain endosperm texture, transgenes should be designed to partially suppress the synthesis of ɣ-kafirin. Partial ɣ-kafirin suppression could restore in part the endosperm texture, but protein digestibility might be reduced.

An alternative approach may be to use TG lines with the ABS166 gene construct (α-kafirin A1 and ɣ-kafirin 1), which also showed potential for improving the cooked protein digestibility as well as retaining some hard endosperm texture trait. However, TG-ABS166 lines had normal lysine content (2.1 g/100g protein). Since reducing the expression of additional kafirins may have a negative effect on endosperm texture, it may be necessary to use alternative methods to improve the lysine content of TG-ABS166. For instance, in early transformation studies on nutritionally improved sorghum, Hordothionin (*HT12* gene), a small lysine-rich (12 lysine residues) protein from barley was expressed in the grain, resulting in a 50%

increase in total grain lysine (Zhao et al., 2003). Additionally, reduced expression of LKR, as used in TG-ABS032, could also be included to reduce lysine catabolism and increase free lysine content in the grain.

Lastly, little is known as to the cause of floury endosperm texture common to tannin containing sorghums. A study of the different kafirin sub-classes present in these sorghum genotypes could help in understanding why tannin sorghums have soft endosperm.

6 CONCLUSIONS AND RECOMMENDATIONS

Co-suppression of the major kafirin sub-classes, α-, ɣ- and δ-kafirin, substantially improves the protein nutritional quality of TG sorghum. The improved protein lysine content is presumably due, in part, to compensatory synthesis of lysine-rich cytoskeletal proteins, as reported for high-lysine mutants. Improved cooked protein digestibility is primarily due to reduced kafirin disulphide crosslinking behaviour. This is as a result of suppressed synthesis of the cysteine-rich ɣ-kafirins, resulting in a lower proportion of highly crosslinked kafirin polymers being formed. Lower levels of indigestible crosslinked kafirin polymers would presumably allow better access of proteases to the highly digestible α-kafirins, improving protein digestibility of the cooked flour. The significance of involving ɣ-kafirin disulphide crosslinking as a major factor for the poor protein digestibility of cooked sorghum has been confirmed. The presence of more digestible non-kafirin proteins, upregulated in the grain endosperm, may also play a role in improving the protein digestibility of TG sorghum.

The reduction in crosslinked kafirin polymers is, no doubt, related to the occurrence of modified protein body structure and floury endosperm texture of the TG lines with improved protein quality traits. This appears to be due to the fact ɣ-kafirins confer rigidity by crosslinking with β-kafirin at the outer edges of normal spherical protein bodies, as well as crosslinking with matrix proteins necessary for corneous endosperm development. Moreover, it appears that irregular shaped protein bodies, in the TG sorghums, are associated with improved cooked protein digestibility and floury endosperm texture.

What was apparent in this present study is the fact that target kafirin suppression is directly related to the degree of structural change observed, and is also directly related to the improvements in protein nutritional quality. This was clearly demonstrated when fewer kafirin sub-classes are co-suppressed (only ɣ- and δ-kafirin). In these grains, the protein bodies appear normal and endosperm texture is somewhat restored. However, this results in reduced improvement in protein nutritional quality.

The future of biofortified sorghum will depend on the ability to produce transformed sorghum with both improved protein nutritional quality, agronomic and good grain processing quality. Due to the fact that major kafirin target co-suppression has a dramatic effect on both protein quality (positive) and endosperm texture (negative), it may be necessary to make certain compromises to accomplish this task. For instance, partial γ-kafirin suppression may be necessary to maintain some corneous endosperm phenotype, but this could be at the expense of high protein digestibility. Additional recombinant DNA technology may be necessary to express additional highly-digestible, nutritionally enhanced proteins such as high-lysine HT12, to increase the digestibility and grain lysine content beyond that possible by kafirin suppression alone. Further research into the use of novel mutant sorghum germplasm with hard endosperm, such as recently developed (HPD/hl), may also be considered for backcrossing.

7 REFERENCES

AACC International, 2000. Moisture content, Standard Method 44-15A. Crude protein-combustion, Standard Method 46-30. Test weight per bushel, Standard Method 55-10. . *Approved Methods of the AACC, 10th ed*. American Association of Cereal Chemists: St Paul, MN.

ABS (Africa Biofortified Sorghum Project). 2009. http://biosorghum.org/, accessed March 2009.

Adams, C.A., Novellie, L., Liebenberg, N. VdW. 1976. Biochemical properties and ultrastructure of protein bodies isolated from selected cereals. *Cereal Chemistry* 53, 1-12.

Awika, J.M., Suhendro, E.L., Rooney, L.W. 2002. Milling value of sorghums compared by adjusting yields to a constant product color. *Cereal Chemistry* 79, 249-251.

Axtell, J.D., Kirleis, A., Hassen, M.M., D'Croz-Mason, N., Mertz, E.T., Munck, L. 1981. Digestibility of sorghum proteins. *Proceedings of the National Academy of Sciences of the United Sates of America* 78, 1333-1335.

Azama, K., Abe, S., Sugimoto, H., Davies, E. 2003. Lysine-containing proteins in maize endosperm: a major contribution from cytoskeleton-associated carbohydrate-metabolizing enzymes. *Planta* 217, 628-638.

Bagga, S., Adams, H., Kemp, J.D., Sengupta-Gopalan, C. 1995. Accumulation of 15-kilodalton zein in novel protein bodies in transgenic tobacco. *Plant Physiology* 107, 13-23.

Bagga, S., Adams, H.P., Rodriguez, F.D., Kemp, J.D., Sengupta-Gopalan, C. 1997. Coexpression of the maize δ-zein and β-zein genes results in stable accumulation of δ-zein in endoplasmic reticulum-derived protein bodies formed by β-zein. *The Plant Cell* 9, 1683-1696.

Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography* 366, 93-104.

Bean, S.R., Lookhart, G.L. 2000. Electrophoresis of cereal storage proteins. *Journal of Chromatography* A, 881, 23–36.

Belton, P.S., Taylor, J.R.N. 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science and Technology* 15, 94-98.

Belton, P.S., Delgadillo, I., Halford, N.G., Shewry, P.R. 2006. Kafirin structure and functionality. *Journal of Cereal Science* 44, 272-286.

Brochetto-Braga, M.R., Leite, A., Arruda, P. 1992. Partial purification and characterization of lysine-ketoglutarate reductase in normal and opaque-2 maize endosperms. *Plant Physiology* 98, 1139-1147

Burr, F.A., Burr, B. 1982. Three mutations in Zea mays affecting zein accumulation: a comparison of zein polypeptides, *in vitro* synthesis and processing, mRNA levels, and genomic organization. *Journal of Cell Biology* 194, 201-206.

Butler, L.G., Riedl, D.J., Lebryk, D.G., Blyit, H.J. 1984. Interaction of proteins with sorghum tannin: Mechanism, specificity and significance. *Journal of the American Oil Chemists' Society* 61, 916-920.

Chandrashekar, A., Mazhar, H. 1999. The biochemical basis and implications of grain strength in sorghum and maize. *Journal of Cereal Science* 30, 193-207.

Christianson, D.D., Khoo, U., Nielsen, H.C., Wall, J.S. 1974. Influence of opaque-2 and floury-2 genes on formation of proteins in particulates of corn endosperm. *Plant Physiology* 53, 851-855.

Coleman, C.E., Herman, E.M., Takasaki, K., Larkins, B.A. 1996. The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. *The Plant Cell* 8, 2335-2345.

Coleman, C.E., Yoho, P.R., Escobar, S., Ogawa, M. 2004. The accumulation of α-zein in transgenic tobacco endosperm is stabilized by co-expression of β-zein. *Plant Cell Physiology* 45, 864-871.

Dannenhoffer, J.M., Bostwick, D.E., Or, E., Larkins, B.A. 1995. Opaque-15, a maize mutation with properties of a defective opaque-2 modifier. *Proceedings of the National Academy of Sciences of the United States of America* 92, 1931-1935.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. and Hamaker, B.R. 2003. Factors affecting protein digestibility. *Journal of Cereal Science* 38, 117-131.

Duodu K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., Taylor, J.R.N. 2002. Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *Journal of Cereal Science* 35, 161-174.

Dykes, L., Rooney, L.W. 2006. Sorghum and millets phenols and antioxidants. *Journal of Cereal Science* 44, 236-251.

Egerton, R.F. 2005. Physical Principles of Electron Microscopy. An Introduction to TEM, SEM, and AEM. Springer, New York, pp. 120-125.

Eggum, B.O., Brunsgaard, G., Jensen, J. 1995. The nutritive value of new high-lysine barley mutants. *Journal of Cereal Science* 22, 171-176.

El Nour I.N., Peruffo, A.D.B., Curioni, A. 1998. Characterisation of sorghum kafirins in relation to their cross-linking behaviour. *Journal of Cereal Science* 28, 197-207.

Emmambux, M.N., Taylor J.R.N. 2009. Properties of heat-treated sorghum and maize meal and there prolamin proteins. *Journal of Agricultural and Food Chemistry* 57, 1045-1050.

Esen, A. 1987. A proposed nomenclature for the alcohol-soluble proteins (zein) of maize (*Zea mays L*.). *Journal of Cereal Science* 5, 117.

Ezeogu, L.I., Duodu, K.G., Taylor, J.R.N. 2005. Effects of endosperm texture and cooking conditions on the *in vitro* starch digestibility of sorghum and maize flours. *Journal of Cereal Science* 42, 33-44.

Ezeogu, L.I., Duodu, K.G., Emmambux, M.N., Taylor, J.R.N. 2008. Influence of cooking conditions on the protein matrix of sorghum and maize endosperm flours. *Cereal Chemistry* 85, 397-402.

FAO (Food and Agriculture Organization). 1995. Sorghum and Millets in Human Nutrition. *FAO Food and Nutrition Series* No. 27. Food and Agriculture Organization of the United Nations, Rome.

FAO (Food and Agriculture Organization). 2002. FAO Animal Production and Health, *Proceedings:* Protein Sources for the Animal feed Industry. Expert Consultation and Workshop, Bangkok, 29 April – 3 May.

Gallagher, S.R. 1999. Electrophoretic separation of proteins. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A., and Strule, K. (Eds), Current Protocols in Molecular Biology, John Wiley and Sons, New York, pp. 10.2, A1-9.

Geetha, K.B., Lending, C.R., Lopes, M.A., Wallace, J.C., Larkins, B.A. 1991. Opaque-2 modifiers increase gamma-zein synthesis and alter its spatial distribution in maize endosperm. *The Plant Cell* 3:1207-1219.

Geli, M.I., Torrent, M., Ludevid, D. 1994. Two structural domains mediate two sequential events in y-zein targeting: Protein endoplasmic reticulum retention and protein body formation. *The Plant Cell* 6, 1911-1922.

Gibbon, B.C., Larkins, B.A. 2005. Molecular genetic approaches to developing quality protein maize. *Trends in Genetics* 21, 227-233.

Glueck, J.A., Rooney, L.W. 1980. Sorghum diseases, A world review. In: Bengston G.D. (Ed), Proceedings of the International Workshop on Sorghum Diseases, ICRISAT, Patancheru, India, pp. 119-140.

Gomez, M.I., Obilana, A.B., Martin, D.F., Madzvamuse, M., Monyo, E.S. 1997. Manual of Procedures for Quality Evaluation of Sorghum and Pearl Millet. ICRISAT, Patancheru, India.

Grootboom, A.W., Mkhonza, N.L., O'Kennedy, M.M., Chakauya, E., Kunert, K., Chikwamba, R.K. 2010. Biolistic mediated sorghum (*Sorghum bicolor* L. Moench) transformation via mannose and bialaphos based selection systems. *International Journal of Botany* 6, 89-94.

Guiragossian, V., Chibber, B.A.K., Van Scoyoc, S., Jambunathan, R., Mertz, E.T., Axtell, J.D. 1978. Characteristics of proteins from normal, high lysine, and high tannin sorghums. *Journal of Agricultural and Food Chemistry* 26, 219-223.

Habben, J.E., Moro, G.L., Hamaker, B.R., Larkins, B.A. 1995. Elongation factor 1α concentration is highly correlated with the lysine content of maize endosperm. *Proceedings of the National Academy of Sciences of the United States of America* 92, 8640-8644.

Hamaker, B.R., Bugusu, B.A. 2003. Overview: Sorghum proteins and food quality. http://www.afripro.org.uk/papers/Paper08Hamaker.pdf cited 04/04/2006, accessed March 2008.

Hamaker, B.R., Kirleis, A.W., Mertz, E.T., Axtell, J.D. 1986. Effect of cooking on the protein profiles and *in vitro* digestibility of sorghum and maize. *Journal of Agricultural and Food Chemistry* 34, 647-649.

Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D., Mertz, E.T. 1987. Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceedings of the National Academy of Sciences of the United States of America* 84, 626-628.

Hamaker, B.R., Mohamed, A.A., Habben, J.E., Huang, C.P., Larkins, B.A. 1995. An efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamin contents than the conventional method. *Cereal Chemistry* 72, 583-588.

Henley, E.C., Taylor, J.R.N., Obukosia, S.D. 2010. The importance of dietary protein in human health: Combating protein deficiency in sub-Saharan Africa through transgenic biofortified sorghum. *Advances in Food and Nutrition Research* 60, 21-52.

ICC (International Association for Cereal Science and Technology). 2008. Estimation of sorghum grain endosperm texture. ICC Standard 176. ICC, Vienna. http://www.icc.or.at/standard_methods/176, accessed April 2009

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 2009. Sorghum. www.icrisat.org/newsite/crop-sorghum.htm, accessed November 2009.

Jackson, D.S., Shandera, D.L. 1995. Corn wet milling: separation chemistry and technology. *Advances in Food and Nutrition Research* 38, 271-300.

Jambunathan, R., Mertz, E.T., Axtell, J.D. 1975. Fractionation of soluble proteins of high-lysine and normal sorghum grain. *Cereal Chemistry* 52,119-121.

Jones, R.A., Larkins, B.A., Tsai, C.Y. 1977. Storage protein synthesis in maize. II. Reduced synthesis of a major zein component by the opaque-2 mutant of maize. *Plant Physiology* 59, 525-529.

Kim, C.S., Woo, Y.-M., Clore, A.M., Burnett, R.J., Carneiro, N.P., Larkins, B.A. 2002. Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. *The Plant Cell* 14, 655-672.

Kodrzycki, R., Boston, R.S., Larkins, B.A. 1989. The opaque-2 mutation of maize differentially reduces zein gene transcription. *The Plant Cell* 1, 105-114.

Krishnan, H.B., White, J.A., Pueppke, S.G. 1989. Immunocytochemical analysis of protein body formation in seeds of Sorghum bicolor. *Canadian Journal of Botany* 67, 2850-2856.

Landry, J., Moureaux, T. 1970. Heterogeneite des glutelines du grain de mais: Extraction selective et composition en acides amines des trios fractions isolees. *Bulletin de la Société de Chimie Biologique* 52, 1021-1031.

Lásztity, R. 1984. The Chemistry of Cereal Proteins. CRC Press, Boca Raton, FL, pp. 177-184.

Lending, C.R., Larkins, B.A. 1989. Changes in the zein composition of protein bodies during maize endosperm development. *The Plant Cell* 1, 1011-1023.

Lending C.R., Larkins, B.A. 1992. Effect of the *floury-2* locus on protein body formation during maize endosperm development. *Protoplasma* 171, 123-133.

Lending, C.R., Kriz, A.L., Larkins, B.A., Bracker, C.E. 1988. Structure of maize protein bodies and immunocytochemical localization of zeins. *Protoplasma* 143, 51-62.

Lopes, M.A., Larkins, B.A. 1991. Gamma-zein content is related to endosperm modification in Quality Protein Maize (QPM). *Crop Science* 31, 1655 1662.

Lopes, M.A., Takasaki, K., Bostwick, D.E., Helentjaris, T., Larkins, B.A. 1995. Identification of two opaque2 modifier loci in Quality Protein Maize. *Molecular and General Genetics* 247, 603-613

MacLean, W.C., de Romańa, G.L., Placko, R.P., Graham, G.G. 1981. Protein quality and digestibility of sorghum in preschool children: balance studies and plasma free amino acids. *Journal of Nutrition* 111, 128-136.

Maxson, E.D., Rooney, L.W. 1972. Evaluation of methods for tannin analysis in sorghum grain. *Cereal Chemistry* 49, 719-729.

Maxson, E.D., Fryar, W.B., Rooney, L.W., Krishnaprasad, M.N. 1971. Milling properties of sorghum grain with different propositions of corneous to flour endosperm. *Cereal Chemistry* 48, 478-490.

Mazhar, H., Chandrashekar, A. 1993. Differences in kafirin composition during endosperm development and germination in sorghum cultivars of varying hardness. *Cereal Chemistry* 70, 667-671.

Mazhar, H., Chandrashekar, A. 1995. Quantification and distribution of kafirins in the kernels of sorghum cultivars varying in endosperm hardness. *Journal of Cereal Science* 21, 155-162.

Mazhar, H., Chandrashekar, A., Shetty, H.S., 1993. Isolation and immunochemical characterization of the alcohol-extractable proteins (kafirins) of *Sorghum bicolor* (L.) Moench. *Journal of Cereal Science* 17, 83–93.

Mertz, E.T., Bates, L.S., Nelson, O.E. 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science* 145, 279-280.

Mertz, E.T., Axtell, J.D., Ejeta, G., Hamaker, B.R. 1993. Development and recent impact of quality protein maize and sorghum. In: Taylor, J.R.N., Randall, P.G. and Viljoen, J.H., (Eds), Cereal Science and Technology. Impact on a

Changing Africa. Selected Papers from the ICC International Symposium. The CSIR, Pretoria, South Africa, pp. 115-131.

Mertz, E.T., Hassen, M.M., Cairns-Whittern, C., Kirleis, A.W., Tu, L., Axtell, J.D. 1984. Pepsin digestibility of proteins in sorghum and other major cereals. *Proceedings of the National Academy of Sciences of the United States of America* 81, 1-2.

Muller, O., Krawinkle, M. 2005. Malnutrition and health in developing countries. *Canadian Medical Association Journal* 173, 279-286.

Munck, L., Karlsson, K.E., Hagberg, A., Eggum, B.O. 1970. Gene for improved nutritional value in barley seed protein. *Science* 168, 985-987.

Murty, D.S., Kumar, K.A. 1995. Traditional uses of sorghum and millets. In: Dendy, D.A.V., (Ed), Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN, pp. 223-281.

Nelson, O.E., Mertz, E.T., Bates, L.S. 1965. Second mutant gene affecting the amino acid pattern of maize endosperm proteins. *Science* 150, 1469-1470.

Ng'andwe, C.C., Hall, A.N., Taylor, J.R.N. 2008. Proteolysis of sorghum endosperm proteins when mashing with raw grain plus exogenous protease and potassium metabisulphite. *Journal of the Institute of Brewing* 114, 343-348.

Nguz, K., Van Gaver, D., Huyghebaert, A. 1998. *In vitro* inhibition of digestive enzymes by sorghum condensed tannins (*Sorghum bicolor* (L Moench)). *Science Alimentaris* 18, 507-514.

Nunes, A., Correia, I., Barros, A., Delgadillo, I. 2005. Characterization of kafirin and zein oligomers by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Agricultural and Food Chemistry* 53, 639–643.

Nyannor, E. K. D., Adedokun, S. A., Hamaker, B. R., Ejeta, G., Adeola, O. 2007. Nutritional evaluation of high-digestible sorghum for pigs and broiler chicks. *Journal of Animal Science* 85, 196-203.

O'Kennedy, M.M., Grootboom, A., Shewry, P.R. 2006. Harnessing sorghum and millet biotechnology for food and health. *Journal of Cereal Science* 44, 224-235.

Oria, M.P., Hamaker, B.R., Shull, J.M. 1995a. *In vitro* protein digestibility of developing and mature sorghum grain in relations to α-, β- and γ-kafirin disulphide crosslinking. *Journal of Cereal Science* 22, 85-93.

Oria, M.P., Hamaker, B.R., Shull, J.M. 1995b. Resistance of sorghum α-, β- and γ-kafirins to pepsin digestion. *Journal of Agricultural and Food Chemistry* 43, 2148-2152.

Oria. M.P., Hamaker., B.R., Axtell, J.D., Huang, C.P. 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. *Proceedings of the National Academy of Sciences of the United States of America* 97, 5065-5070.

Ortega, E.I., Bates, L.S. 1983. Biochemical and agronomical studies of 2 modified hard-endosperm *opaque-2* maize (*Zea mays* L.) populations. *Cereal Chemistry* 60, 107-111.

Paiva, E., Kris, A.L., Peixoto, M.J.V.V.D., Wallace, J.C., Larkins, B.A. 1991. Quantitation and distribution of ɣ-zein in the endosperm of maize kernels. *Cereal Chemistry* 68, 176-279.

Poletti, S., Gruissem, W., Sautter, C. 2004. The nutritional fortification of cereals. *Current Opinion in Biotechnology* 15,162-165.

Robutti, J.L., Borras, F.S., Eyherabide, G.H. 1997. Zein compositions of mechanically separated coarse and fine portions of maize kernels. *Cereal Chemistry* 74, 75-78.

Rom, D.L., Shull, J.M., Chandrashekar, A., Kirleis, A.W. 1992. Effects of cooking and treatment with sodium disulfite on *in vitro* protein digestibility and microstructure of sorghum flour. *Cereal Chemistry* 69, 178-181.

Rooney, L.W., Miller, F.R. 1982. Variation in the structure and kernel characteristics of sorghum. In: Mertin, J.V. (Ed), International Symposium on Sorghum Grain Quality, ICRISAT, Patancheru, India, pp. 143-162.

Rooney, L.W., Serna-Saldivar, S.O. 1990. Sorghum. In: Lorenz, K.J. and Kulp, K., (Eds.), Handbook of Cereal Science and Technology, Markel Dekker, New York, pp. 233-270.

Rooney, L.W., Kirleis, A.W., Murty, D.S. 1986. Traditional foods from sorghum: their production evaluation and nutritional value. In: Pomeranz, Y., (Ed), Advances in Cereal Science and Technology, Vol. VIII, American Association of Cereal Chemists, St. Paul, MN, pp. 317-353.

Schaafsma, G. 2005. The protein digestibility-corrected amino acid score (PDCAAS) - A concept for describing protein quality in foods and food

ingredients: A critical Review. *Journal of the American Association of Analytical Chemists International* 88, 988-994.

Seckinger, H.L., Wolf, M.L. 1973. Sorghum protein ultrastructure as it relates to composition. *Cereal Chemistry* 50, 455-465.

Serna-Saldivar, S., Rooney, L. W. 1995. Structure and chemistry of sorghum and millets. In: Dendy, D.A.V., (Ed), Sorghum and Millets: Chemistry and Technology, St. Paul, American Association of Cereal Chemists, pp. 69-124.

Shewry, P.R. 2007. Improving the protein content and composition of cereal grain. *Journal of Cereal Science* 46, 239-250.

Shewry, P.R., Halford, N.G. 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany* 53, 947–958.

Shewry, P.R., Pandya, M.J. 1999. The 2S albumin storage proteins. In: Shewry, P.R. and Casey, R., (Eds), Seed Proteins, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 563-86.

Shull, J.M., Watterson, J.J., Kirleis, A.W. 1991. Proposed nomenclature for the alcohol-soluble proteins (kafirins) of *Sorghum bicolor (*L. Moench*)* based on molecular weight, solubility, and structure. *Journal of Agricultural and Food Chemistry* 39, 83-87.

Shull, J.M., Watterson, J.J., Kirleis, A.W. 1992. Purification and immunocytochemical localization of kafirins in *Sorghum bicolor* (L. Moench) endosperm. *Protoplasma* 171, 64-74.

Shull, J.M., Chandrashekar, A., Kirleis, W., Ejeta, G. 1990. Development of sorghum endosperm in varieties of varying hardness. *Food Structure* 9, 253-267.

Singh, R., Axtell, J.D. 1973. High-lysine mutant gene (hl) that improves protein quality and biological value of grain sorghum. *Crop Science* 13, 535.

Spencer, C.A., Cai, Y., Gaffney, R.M.S.H., Goulding, P.N., Magnolato, D., Lilley, T.H., Haslam, E. 1988. Polyphenol complexation - Some thoughts and observations. *Phytochemistry* 27, 2397-2409.

Stirling, J.W. 1990. Immuno- and affinity probes for electron microscopy: A review of labelling and preparation techniques. *The Journal of Histochemistry and Cytochemistry* 38, 145-157.

Taylor, J., Taylor, J.R.N. 2002. Alleviation of the adverse effects of cooking on protein digestibility in sorghum through fermentation in traditional African porridges. *International Journal of Food Science and Technology* 37, 129-138.

Taylor J., Taylor, J.R.N. 2007. An *in vitro* protein digestibility method for cereal grains using small sample sizes. Proceedings of the 1st Latin American Cereal Science and Technology Conference. Rosario, Argentina. International Association for Cereal Science and Technology (ICC), Vienna.

Taylor, J., Taylor, J.R.N. 2011. Protein biofortified sorghum: Effect of processing into traditional African foods on their protein quality. *Journal of Agricultural and Food Chemistry* 59, 2386-2392.

Taylor, J., Bean, S.B., Ioerger, B.P., Taylor, J.R.N. 2007. Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. *Journal of Cereal Science* 46, 22-31.

Taylor, J., Taylor, J.R.N., Belton, P.S., Minnaar, A. 2009. Preparation of free-standing films from kafirin protein microparticles: Mechanism of formation and functional properties. *Journal of Agricultural and Food Chemistry* 57, 6729-6735.

Taylor, J.R.N., Evans, D.J. 1989. Action of sorghum proteinase on the protein bodies of sorghum starchy endosperm. *Journal of Experimental Botany* 40, 763-768.

Taylor, J.R.N., Schüssler, L. 1986. The protein compositions of the different anatomical parts of sorghum grain. *Journal of Cereal Science* 4, 361-369.

Taylor, J.R.N., Taylor, J. 2008. Five simple methods for the determination of sorghum grain end-use quality (with adaptations for those without laboratory facilities). University of Pretoria, South Africa. http://www.intsormil.org, accessed August 2008.

Taylor, J.R.N., Novellie, L., Liebenberg, N. VdW. 1984. Sorghum protein body composition and ultrastructure. *Cereal Chemistry* 61, 69-73.

Taylor, J.R.N., Novellie, L., Liebenberg, N. VdW. 1985. Protein body degradation in the starchy endosperm of germinating sorghum. *Journal of Experimental Botany* 36, 1287-1295.

Taylor, J.R.N., Schüssler, L., Liebenberg, N. VdW. 1985. Protein body formation in the starchy endosperm of developing *Sorghum bicolor* (L.) Moench seeds. *South African Journal of Botany* 51, 35-40.

Tesso, T., Hamaker, B.R., Ejeta, G. 2008. Sorghum protein digestibility is affected by dosage of mutant alleles in endosperm cells. *Plant Breeding* 127, 579-586.

Tesso, T., Ejeta, G., Chandrashekar, A., Huang, C.P., Tandjung, A., Lewamy, M., Axtell, J.D., Hamaker, B.R. 2006. A novel modified endosperm texture in a mutant high-protein digestibility/high-lysine grain sorghum (*Sorghum bicolor* (L.) Moench). *Cereal Chemistry* 83, 194-201.

Vendemiatti, A., Ferreira, R.R., Gomes, L.H., Medici, L.O., Azevedo, R.A. 2008. Nutritional quality of sorghum seeds: storage proteins and amino acids. *Food Biotechnology* 22, 377-397.

Wall, J.S., Paulis, J.W. 1978. Corn and sorghum grain proteins. In: Pomeranz, Y. (Ed), Advances in Cereal Science and Technology, Vol. II, American Association of Cereal Chemists, St. Paul, MN, pp. 135-219.

Wall, J.S., Cooker, L.A., Bietz, J.A. 1988. Structure and origin of maize endosperm alcohol-insoluble glutelin. *Journal of Agricultural and Food Chemistry* 36, 722-728.

Wallace, J.C., Lopes, M.A., Paiva, E, Larkins, B.A. 1990. New methods for extraction and quantitation of zeins reveal a high content of gamma-zein in modified opaque-2 maize. *Plant Physiolology* 92, 191-196

Waniska, R.D., Poe, J.H., Bandyopadhyay, R. 1989. Effects of growth conditions on grain molding and phenols in sorghum caryopsis. *Journal of Cereal Science* 10, 217-225.

Weaver, C.A., Hamaker, B.R., Axtell, J.D. 1998. Discovery of grain sorghum germ plasm with high uncooked and cooked *in vitro* protein digestibilities. *Cereal Chemistry* 75, 665-670.

WHO/FAO/UNU Expert Consultation. 2007. Protein and Amino Acid Requirements in Human Nutrition, Report of a Joint WHO/FAO/UNU Expert Consultation. World Health Organization Technical Report No. 935. World Health Organization, Geneva.

Wolf, M.J., Khoo, U., Seckinger, H.S. 1967. Subcellular structure of endosperm protein in high-lysine and normal corn. *Science* 157, 556-557.

Woo, Y.-M., Hu, D.W.-N., Larkins, B.A., Jung, R. 2001. Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. *The Plant Cell* 13, 2297-2317.

Wu, Y., Holding, D.R., Messing, J. 2010. ɣ-Zeins are essential for endosperm modification in quality protein maize. *Proceedings of the National Academy of Sciences of the United States of America* 107, 12810-12815.

Xie, X.J., Seib, P.A. 2000. Laboratory procedure to wet-mill 100 g of grain sorghum into six fractions. *Cereal Chemistry* 77, 392-395.

Zarkadas, C.G., Hamilton, R.I., Yu, Z.R., Choi, V.K., Khanizadeh, S., Rose, N.G.W., Pattison, P.L. 2000. Assessment of protein quality of 15 new northern adapted cultivars of quality protein maize using amino acid analysis. *Journal of Agricultural and Food Chemistry* 48, 5351-5361.

Zhao, Z-Y., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J., Pierce, D. 2000. *Agrobacterium*-mediated sorghum transformation. *Plant Molecular Biology* 44, 798-798.

Zhao, Z-Y., Glassman, K., Sewalt, V., Wang, N., Miller, M., Chang, S., Thompson, T., Catron, S., Wu, E., Bidney, D., Kedebe, Y., Jung, R. 2003. Nutritionally improved transgenic sorghum. In: Vasil, I.K., (Ed), Plant Biotechnology 2002 and Beyond, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 413-416.

8 APPENDIX

Publications and presentations from this work

Scientific Papers

Da Silva, L.S., Jung, R., Zhao, Z., Glassman, K., Taylor, J., Taylor, J.R.N. 2011. 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. *Journal of Cereal Science* 54, 160-167.

Da Silva, L.S., Taylor, J., Taylor, J.R.N. 2011. Transgenic sorghum with altered kafirin synthesis: Kafirin solubility, polymerization, and protein digestion. *Journal of Agricultural and Food Chemistry* 59, 9265-9270.

Conference Posters

Da Silva, L.S., Taylor, J.R.N. 2010. Protein digestibility and endosperm matrix protein structure of transgenic sorghums with reduced storage protein synthesis. Cereal Science and Technology - South Africa (CST-SA) - International Association for Cereal Science and Technology (ICC) International Grains Symposium on Quality and Safety of Grain Crops and Foods. 3-5 February 2010, Pretoria, South Africa.

Da Silva, L.S., Taylor, J.R.N. 2010. Protein digestibility and endosperm matrix protein structure of transgenic sorghums with reduced storage protein synthesis. 15th IUFOST World Congress of Food Science and Technology. 22-26th August 2010, Cape Town, South Africa.

Oral Presentation

Da Silva, L.S., Taylor, J., Taylor, J.R.N. 2008. Protein body structure of experimental sorghum lines with improved protein quality. Research Day, 26 August, Faculty of Science, Tshwane University of Technology, South Africa.