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.