

Transgenic Sorghum with altered Kafirin Synthesis: Kafirin Solubility, Polymerization and Protein Digestion

Laura S. da Silva^{a,b}; Janet Taylor^a and John R. N. Taylor^{a,*}

^aUniversity of Pretoria, Department of Food Science, Private Bag X20, Hatfield 0028, South Africa

^bTshwane University of Technology, Department of Biotechnology and Food Technology, Pretoria 0001, South Africa

*Corresponding author. Tel.: +27 12 420 4296; Fax: +27 12 420 2839

e-mail address: john.taylor@up.ac.za

ABSTRACT

Transgenic sorghum (TG) lines with altered kafirin synthesis, particularly suppression of gamma-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 polymerized by disulfide 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 disulfide-bonded kafirin polymerization, allowing better access of proteases. This work appears to confirm that disulfide bond formation in kafirin is responsible for the reduced protein digestibility of cooked sorghum.

KEYWORDS: Sorghum, transgenic, kafirin; protein digestibility, disulfide crosslinking, polymerization.

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 (1). Numerous factors contribute to the low protein digestibility of sorghum. However, crosslinking of the kafirin proteins through disulfide bonding and the organization of the various kafirin sub-classes within protein bodies are major factors (2,3).

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 (4). We have shown that transgenic sorghum lines (TG) with co-suppression of synthesis of alpha-, gamma- and delta-kafirin sub-classes and removal of the tannin trait have high cooked in vitro protein digestibility (IVPD) (approx. 60%), improved Lysine Score (0.5) and protein digestibility corrected amino acid score (0.4) (5). The improved protein quality traits are maintained when the TG sorghum is used to produce porridge, a major African staple food (6) and a wide range of other sorghum food products (5).

A problem, however, with these high protein quality sorghum TG lines is that they have a floury endosperm phenotype and a modified endosperm structure, with the protein bodies irregular shaped and surrounded by a dense protein matrix (7). Therefore this study investigated the effect of reduced synthesis of the major kafirin sub-classes on kafirin solubility, polymerization and protein digestibility, with the aim understanding of the mechanisms responsible for the TG sorghum lines having improved protein digestibility but modified endosperm structure.

MATERIALS AND METHODS

Materials. The sorghum samples used were two independent transgenic events (T1 event 5/15028 and T2 event 6/15032) and their respective non-transgenic null controls (C1 and C2) (parent variety P898012, a purple plant, Type II tannin type, 3.26 mg catechin equiv./100 mg flour). The transgenic samples were T2 selfed seeds, 75% pure with respect to the ABS032 gene construct, that suppresses synthesis of alpha-kafirin A1 and alpha-kafirin B1 and B2 (which correspond to 19 kDa and 22 kDa alpha-kafirin classes respectively, (3)); gamma-kafirin 1 and 2, and delta-kafirin 2. The transgenics and their null controls were developed using *Agrobacterium*-mediated transformation, as described by Zhao et al. (8), 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 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.

Methods. 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 content, protein, lysine, and in vitro protein digestibility of raw and wet cooked flour (5).

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 temp., 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 (X). Extraction 2 – The residue from extraction 1 was re-suspended in 40 ml 60% (v/v) aqueous tertiary butanol plus 5% (v/v) 2-mercaptoethanol and agitated for 3 h at room temp., 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 (X). The supernatants and residues were freeze dried, and the weights recorded and the protein contents determined.

Isolation of Protein Preparations (PP) by Wet Milling. To study the protein body proteins, PP by wet milling were prepared (8). 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.

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)propanesulfonic acid (MOPS) buffer (6 ml) was added to flour (200 mg) and the samples incubated at boiling temp. (95°C) for 15 min. Sodium acetate (8 ml) and amyloglucosidase (0.2 ml) was 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.

Analyses.

Protein. Protein (N x 6.25) was determined by a Dumas combustion method (AACC Standard Method 46-30) (10).

Pepsin Digestion: The in vitro pepsin digestibility (IVPD) method of Hamaker et al. was used, modified for small-scale assay (200 mg) (5).

Transmission Electron Microscopy (TEM). 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 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).

SDS-PAGE: This was carried out under both non-reducing and under reducing conditions using a 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 k. For reducing conditions 10% 2-mercaptoethanol was added to the sample buffer. Procedures were according to Gallagher (11) 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.

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.

RESULTS AND DISCUSSION

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 C 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 C had some peripheral corneous endosperm and Macia had intermediate endosperm texture (7). 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 (12). The relatively low protein contents of the PP by wet milling were due to that fact that a reducing agent, such as sodium metabisulfite, was not used during wet milling process, unlike in commercial wet milling (13). The reducing agent was not included so as 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 (X). Significantly, the TG had somewhat more irregular shaped and invaginated protein bodies (Figure 1a), as compared to those of C (Figure 1b) and Macia (Figure 1d). The TG protein bodies were, however, less irregular and invaginated than the characteristic HD protein bodies (14) (Figure 1c). The TG protein bodies could be considered intermediate in form between those of C and 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 (7).

The protein composition of the PP by wet milling was heterogenous (Figure 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 1). Under both non-reducing and reducing conditions at least six

monomeric kafirin bands with apparent molecular weights ranging from approx. 18 to 27 kDa were present, as was described by El Nour et al. (15). The individual kafirin monomers are not identified as unpublished work (Dr R. Jung, Pioneer Hi-Bred, personal communication) showed that the important 27 kDa gamma-kafirin was not separated from the alpha-kafirins by SDS-PAGE in these sorghum lines.

Under non-reducing conditions, kafirin dimers and oligomers were also present in relatively high concentration (Figure 2a). The latter have been identified as disulfide-bonded kafirins, pre-existing in the endosperm tissue (8, 15, 16). 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 Cs, 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) (16, 17) was formed in the TG lines during protein body synthesis.

Under reducing conditions, essentially only kafirin monomers were present (Figure 2b). This suggests that the kafirin polymers, oligomers and dimers were essentially all reduced by disulfide bond reduction into the various kafirin monomer sub-classes. In the TG lines, a major monomeric kafirin band was absent or present, at much lower concentration, (white arrow) compared to their Cs, HD and Macia, presumably as result of suppressed synthesis of that particular kafirin protein.

Kafirin Solubility and Composition. Total kafirin extracted from whole grain flour of the TG lines and their Cs was in the range 47 to 50 g/100 g protein (Table 1), showing that they both had similar total kafirin content. The total kafirin content of the TG lines falls within normal range for whole grain flour, approx. 50-60% of the total protein in whole grain flour (18). The high kafirin content of the TG lines is somewhat surprising as the ABS032 gene construct causes suppressed synthesis of alpha-kafirin A1, B1 and B2;

gamma-kafirin 1 and 2, and delta-kafirin 2 through RNA interference technology (6). Also, these TG lines were shown to contain considerably more total lysine, mean 2.65 g/100 g protein, than their Cs, mean 1.84 g/100 g protein. Also, the total protein content of the TG lines fell within the normal range for sorghum (5). 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 prolamins content, with a compensatory increase in the lysine-rich non-prolamins proteins (1), as can be seen in the low percentage of total kafirin in the HD mutant, 33 g/100 protein. 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 (8, 15, 16) from the TG lines (approx. 69% of total kafirin extracted) compared to their Cs (40-42%), HD (43%) and Macia (56%) (Table 1). Since the total amount of kafirin extracted (kafirin-1 plus kafirin-2) from the TG lines was the same as their Cs and more than from HD and Macia, this indicates that the kafirin proteins in the TG lines were less crosslinked by disulfide bonding than the kafirins in normal sorghums, as was suggested by SDS-PAGE (Figure 2).

SDS-PAGE of kafirin-1 under non-reducing conditions (Figure 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 Cs, 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 3b) all the sorghum types essentially only exhibited kafirin monomers, which were at much higher band intensity than under non-reducing conditions (Figure 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 polymerized than that of their Cs, HD and Macia (Figure 3a). This can be attributed to suppression of synthesis of the cysteine-rich gamma-kafirins, which are required for polymerization through disulfide bonding (15).

Additionally, there were significant differences between the kafirin monomer bands present in the TG lines and the other sorghums (Figure 3b), as in the PP by wet milling (Figure 2). At least one major band was absent in T1 and T2 at approx. 25 kDa (white arrows), which was present in their Cs, HD and Macia, showing that its synthesis was suppressed. Also, at least one new band was present in the TG lines at approx. 23 kDa (top dotted black arrow), which was absent in the normal sorghums, indicating that compensatory synthesis of other kafirin proteins or possibly non-prolamins 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 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 (15, 18). 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-prolamins (X), as a result of modified gene expression.

The kafirin-2 proteins (Figure 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 3b). The kafirin-2 proteins are presumably originally made up of the disulfide-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 3c, black dashed arrows). Of significance is that the pattern of T1 and T2 kafirin-2 proteins is more similar to that of the Cs, HD and Macia than was the case with the kafirin-1 proteins under reducing conditions (Figure 3b). This can be attributed to the kafirin-2 proteins originally being disulfide-bonded polymers where the cysteine-rich gamma- and beta-kafirins must be present in order for the kafirins to be polymerized (15).

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 2). The improved IVPD of the samples following starch removal, is presumed to be due to the pepsin have greater access to the protein structures (8). Importantly, however, after removal of the starch the TG lines still had substantially higher IVPD (25 to 32%) compared to their Cs. This shows that the protein in the TG lines was intrinsically more available to proteolysis. The fact 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 (19, 20).

The compositions of pepsin-indigestible proteins in the PP by starch digestion residues (Figure 4) were generally similar to the native proteins in the PP by wet milling samples (Figure 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 4a) than in the PP by wet milling (Figure 2a). Also, of note is

that higher numbers of oligomer and dimer bands occurred in the TG lines compared to their Cs, HD and Macia (Figure 4a, black arrows), as in the PP by wet milling, but the levels of monomers remaining in TG and their C' were similar, unlike situation in the PP by wet milling. The fact that substantial amounts of kafirin oligomers and dimers were present in the pepsin indigestible residues implies that even these relatively small disulfide-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 4b). Significantly, the low molecular weight kafirins, which were present in T1 and T2 PP by wet milling, kafirin-1 and kafirin-2 preparations (Figures 2 and 3), were absent. This indicates that they were readily hydrolysed by pepsin. However, similar to the PP by wet milling (Figure 2b) and the kafirin-1 (Figure 3b), particular kafirin monomers, probably gamma-kafirin, that was present in high concentration the PP by starch digestion pepsin indigestible residues of the Cs were absent, or present at very reduced concentration, in the TG lines (Figure 4b, white arrows). This suggests that the kafirins in PP by starch digestion pepsin indigestible residues of the TG lines were less polymerized than those of their Cs, due to the suppression of synthesis of gamma-kafirin. This is consistent with the higher pepsin digestibility of the TG lines compared to their Cs.

TEM of the protein bodies in the PP by starch digestion of the TG lines, Cs 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 5). This pattern of sorghum protein body digestion is normal (21-23). In normal sorghum the gamma- and beta-kafirins are concentrated at the periphery of the protein bodies and appear as dark regions (24) (dashed black arrows in null control (Figure 5a) and Macia (Figure 5c) and are believed to inhibit access of proteases to the

alpha-kafirin ground material (25), thus majorly contributing to the poor protein digestibility of wet cooked sorghum. The absence of, or greatly reduced amount of dark staining regions (cross-linked gamma- and beta-kafirins) in the protein bodies of the TG (Figure 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 2). However, in these HD mutants the protein bodies are very folded (invaginated) (Figure 1c), with the gamma-kafirin located at the base of the invaginations (14), with the effect that proteases have easy accessibility to the alpha-kafirin. Thus, it appears that reason for the high digestibility of the protein bodies the TG lines is different from that in the HD types.

Suppressed synthesis of the cysteine-rich gamma-kafirin sub-class in these TG sorghum lines results in a substantially lower proportion of disulfide 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 disulfide-bonded kafirin polymerization, allowing better access of proteases. This work seems to confirm the theory of Hamaker (26) and Rom et al. (27) that disulfide bond formation in kafirin is responsible for the reduced protein digestibility of cooked sorghum, where it was found that the disulfide bond-breaking reducing agents improved sorghum protein (26, 27) and protein body digestion (27).

ABBREVIATIONS USED

ABS, Africa Biofortified Sorghum

TG, transgenic sorghum

HD, high protein digestibility mutant

C, non-transgenic null control

IVPD, in vitro prot

ein digestibility

SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PP, protein preparations

MOPS, 3-(N-morpholine)propanesulfonic acid

LITERATURE CITED

- (1) Shewry, P.R. . Improving the protein content and composition of cereal grain. *J. Cereal Sci.* **2007**, 46, 239-250.
- (2) Duodu, K.G.; Taylor, J.R.N.; Belton, P.S.; Hamaker, B.R. Factors affecting sorghum protein digestibility. *J. Cereal Sci.* **2003**, 38, 117-131.
- (3) Belton, P.S.; Delgadillo, I.; Halford, N.G.; Shewry, P.R. Kafirin structure and functionality. *J. Cereal Sci.* **2006**, 44, 272-286.
- (4) African Biofortified Sorghum Project (ABS). **2009**, <http://biosorghum.org/> (accessed 10 March 2010).
- (5) Taylor, J.; Taylor, J.R.N. Protein biofortified sorghum: Effect of processing into traditional African foods on their protein quality. *J. Agric. Food Chem.* **2011**, 59, 2386-2392.
- (6) Henley, E.C.; Taylor, J.R.N.; Obukosia, S.D. The importance of dietary protein in human health: Combating protein deficiency in sub-Saharan Africa through transgenic biofortified sorghum. *Adv. Food Nutr. Res.* **2010**, 60, 21-52.
- (7) Da Silva, L.S.; Jung, R.; Zhao, Z.; Glassman, K.; Taylor, J.; Taylor, J.R.N. 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. *J. Cereal Sci.* **2011**, (in press).

- (8) Zhao, Z-Y., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J., Pierce, D. *Agrobacterium*-mediated sorghum transformation. *Plant Mol. Biol.* **2000**, 44, 798-798.
- (8) Duodu K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., Taylor, J.R.N. Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *J. Cereal Sci.* **2002**, 35, 161-174.
- (9) AACC. Crude protein-combustion, Standard Method 46-30. *Approved Methods of the AACC 10th ed.*; American Association of Cereal Chemists: St Paul, MN. 2000.
- (10) Hamaker, B.R.; Kirleis, A.W.; Mertz, E.T.; Axtell, J.D. Effect of cooking on the protein profiles and *in vitro* digestibility of sorghum and maize. *J. Agric. Food Chem.* **1986**, 34, 647-649.
- (11) Gallagher, S.R., Electrophoretic separation of proteins. In: *Current Protocols in Molecular Biology*, Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A., Strule, K. Eds., John Wiley and Sons, New York, 1999; pp. 10.2A1-9.
- (12) Xie, X.J.; Seib, P.A. Laboratory procedure to wet-mill 100 g of grain sorghum into six fractions. *Cereal Chem.* **2000**, 77, 392-395.
- (13) Jackson, D.S.; Shandera, D.L. Corn wet milling: separation chemistry and technology. *Adv. Food Nutr. Res.* **1995**, 38, 271-300.

(14) Oria. M.P.; Hamaker., B.R.; Axtell, J.D.; Huang, C.P. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. *PNAS*, **2000**, *97*, 5065-5070.

(15) El Nour I.N.; Peruffo, A.D.B.; Curioni, A. Characterisation of sorghum kafirins in relation to their cross-linking behaviour. *J. Cereal Sci.* **1998**, *28*, 197-207.

(16) Emmambux M.N., Taylor J.R.N. Properties of heat-treated sorghum and maize meal and there prolamin proteins. *J. Agric. Food Chem.* **2009**, *57*, 1045-1050.

(17) Ezeogu, L.I.; Duodu, K.D.; Taylor, J.R.N. Effects of endosperm texture and cooking conditions on the in vitro starch digestibility of sorghum and maize flours. *J. Cer. Sci.* **2005**, *42*, 33-44.

(18) Taylor, J.R.N.; Novellie, L.; Liebenberg, N., VdW. Sorghum protein body composition and ultrastructure. *Cereal Chem.* **1984**, *61*, 69-73.

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

(19) Butler, L.G.; Riedl, D.J.; Lebryk, D.G.; Blyit, H.J. Interaction of proteins with sorghum tannin: Mechanism, specificity and significance. *J. Am. Oil Chem. Soc.* **1984**, *61*, 916–920.

- (20) Taylor, J.; Bean, S. R.; Ioerger, B. P.; Taylor, J.R.N. Preferential binding of sorghum tannins with gamma-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. *J. Cereal Sci.* **2007**, 46, 22-31.
- (21) Taylor, J.R.N.; Novellie, L.; Liebenberg, N.v.d.W. Protein body degradation in the starchy endosperm of germinating sorghum. *J. Exp. Bot.* **1985**, 36:1287-1295.
- (22) Taylor, J.R.N.; Evans, D.J. Action of sorghum proteinase on the protein bodies of sorghum starchy endosperm. *J. Exp. Bot.* **1989**, 40:763-768.
- (23) Ng'andwe, C.C.; Hall, A.N.; Taylor, J.R.N. Proteolysis of sorghum endosperm proteins when mashing with raw grain plus exogenous protease and potassium metabisulphite. *J. Inst. Brew.* **2008**, 114: 343-348.
- (24) Shull, J.M.; Watterson, J.J.; Kirleis, A.W. Purification and immunocytochemical location of kafirins in *Sorghum bicolor* (L.) Moench endosperm. *Protoplasma* **1992**, 171, 64-74.
- (25) Oria, M.P.; Hamaker, B.R.; Shull, J.M. Resistance of sorghum α -, β -, and γ -kafirins to pepsin digestion. *J. Agric. Food Chem.* **1995**, 43, 2148-2153.
- (26) Hamaker, B.R.; Kirleis, A.W.; Butler, L.G.; Axtell, J.D.; Mertz, E.T. Improving the *in vitro* protein digestibility of sorghum with reducing agents. *PNAS*, **1987**, 84, 626-628.

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

Dr Dirk Hays of Texas A&M University and Dr Zuo-Zhao of Pioneer Hi-Bred International are thanked for supplying the high protein digestibility mutant sorghum and transgenic biofortified sorghums, respectively. This project was funded through the Bill and Melinda Gates Grand Challenges 9, Africa Biofortified Sorghum (ABS) Project. LS da Silva received financial support from the South African National Research Foundation and the Tshwane University of Technology Faculty of Science Research and Innovation Committee.

FIGURE LEGENDS

Figure 1. Representative images of TEM of sorghum protein body preparations by wet milling . a) T1; b) C1; c) HD; d) Macia.

cw = cell wall; p = protein body, arrows show invaginations

Figure 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).

Figure 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) C1; 3) T1; 4) C2; 5) T2; 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).

Figure 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.

Figure 5. Representative images of TEM of pepsin-indigestible residue from cooked protein preparations by starch digestion. a) C1; b) T1; c) Macia; p = protein body; Solid black arrows show proteolysis of protein body periphery; Dashed black arrows show dark staining regions, presumed primarily gamma- and beta-kafirin (X); White arrows show where matrix protein was.

Table 1. Kafirin extracted with 60% (v/v) Tert-butanol 60% followed by (v/v) Tert-butanol plus 5% (v/v) Mercaptoethanol from Transgenic, Null Controls, High Protein Digestibility Mutant and Macia Sorghum

	Kafirin Extraction Yield (g/100 g protein)		
	Kafirin-1	Kafirin-2	Total
NC-P898012-2 (C1)	19.9 ^b ±1.5 (42.1)	27.3 ^c ±2.5 (57.9)	47.2 ^c ±1.0
TG-P898012 (ABS032)-2 (T1)	34.8 ^{d3} ±1.2 ¹ (69.9 ²)	15.0 ^a ±2.3 (30.1)	49.8 ^c ±1.1
NC-P898012-3 (C2)	19.3 ^b ±0.8 (40.4)	28.5 ^c ±1.3 (59.6)	47.8 ^c ±0.4
TG-P898012 (ABS032)-3 (T2)	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

¹Means and standard deviations. ²Values 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) mercaptoethanol. ³Different letters in columns indicate significant difference (p<0.05).

Table 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 (C1)	37.7 ^a ±1.4	52.7 ^a ±2.7	15.0
TG-P898012 (ABS032)-2 (T1)	57.8 ^{bc1,2} ±2.3	65.7 ^b ±3.3	7.9
NC-P898012-3 (C2)	38.8 ^a ±2.0	51.7 ^a ±1.9	12.9
TG-P898012 (ABS032)-3 (T2)	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

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

Figure 1

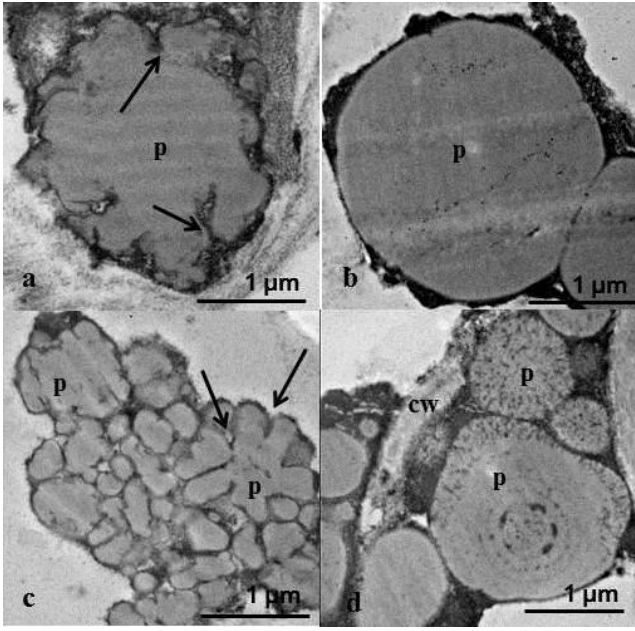


Figure 2

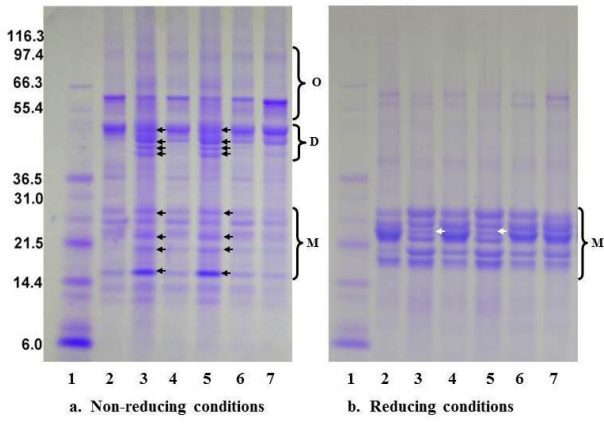


Figure 3

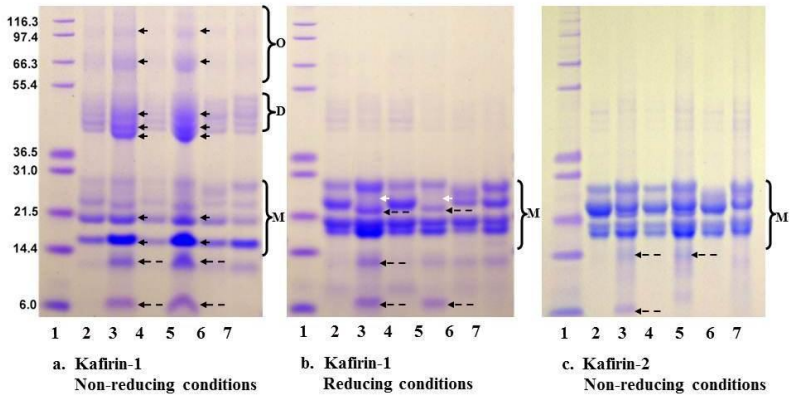


Figure 4

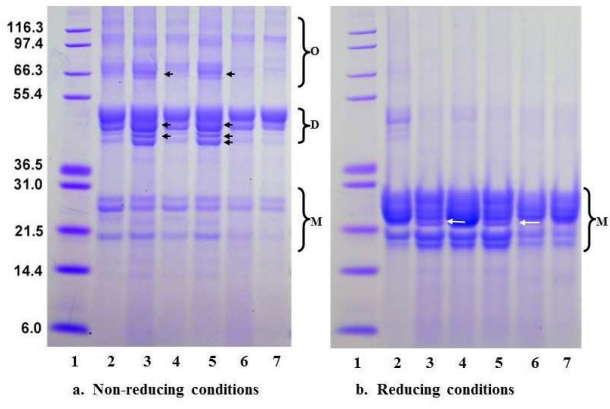


Figure 5

