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Modulation of kernel storage proteins in grain sorghum (*Sorghum bicolor* (L.) Moench)

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Summary

Sorghum prolamins, termed kafirins, are categorized into subgroups α, β, and γ. The kafirins are co-translationally translocated to the endoplasmic reticulum (ER) where they are assembled into discrete protein bodies that tend to be poorly digestible with low functionality in food and feed applications. As a means to address the issues surrounding functionality and digestibility in sorghum, we employed a biotechnology approach that is designed to alter protein body structure, with the concomitant synthesis of a co-protein in the endosperm fraction of the grain. Wherein perturbation of protein body architecture may provide a route to impact digestibility by reducing disulphide bonds about the periphery of the body, while synthesis of a co-protein, with known functionality attributes, theoretically could impact structure of the protein body through direct association and/or augment end-use applications of sorghum flour by stabilizing ß-sheet formation of the kafirins in sorghum dough preparations. This in turn may improve viscoelasticity of sorghum dough. To this end, we report here on the molecular and phenotypic characterizations of transgenic sorghum events that are down-regulated in γ- and the 29-kDa α-kafirins and the expression of a wheat Dy10/Dx 5 hybrid high-molecular weight glutenin protein. The results demonstrate that down-regulation of γ-kafirin alone does not alter protein body formation or impacts protein digestibility of cooked flour samples. However, reduction in accumulation of a predicted 29-kDa α-kafirin alters the morphology of protein body and enhances protein digestibility in both raw and cooked samples.

Introduction

Grain sorghum (*Sorghum bicolor* (L.) Moench) is a major staple for a large portion of the world. The crop ranks fifth among the cereals world-wide with respect to its importance for food and feed applications. To this end, the grain harvested from sorghum, and the millets provides an important source for dietary calories and protein for approximately one billion people in the semi-arid regions of the world (Belton and Taylor, 2004). However, grain sorghum products are known to have relatively poor digestibility, only approximately 50%–70%, in comparison with other grains, such as wheat and maize, which tend to have digestibility percentages over 80% and 70%, respectively (Aboubacar *et al.*, 2001; MacLean *et al.*, 1981). Protein with high digestibility is by definition nutritionally superior owing to the increased availability of amino acids. Digestibility can be impacted by both protein–protein and/or protein–nonprotein interactions (Duodu *et al.*, 2003; Taylor *et al.*, 2007). However, with respect to grain sorghum, it is thought that the major factor influencing digestibility is the former because of high protein cross-linking around the protein body (Duodu *et al.*, 2003).

Protein content of grain sorghum is approximately 13% (Beta *et al.*, 1995), of which the kafirins comprise over 80% of the protein in the endosperm component of the grain (Hamaker *et al.*, 1995). The kafirins are categorized into α, β, γ groupings, with α kafirins approximately 26–27 kDa, and β and γ having molecular masses of 18.7 and 20 kDa, respectively (Belton *et al.*, 2006). The protein component of the endosperm is estimated to contain between 9%–21% γ-kafirin and 66%–84% α-kafirins (de Mesa-Stonestreet *et al.*, 2010). The kafirins are assembled into discrete protein bodies (PB) in the ER, whereby the α-kafirins compose the core and the β- and γ-kafirins decorate the periphery of the PB. It is thought that the organizational structure of the PB has a major impact on protein digestibility of sorghum food and feed products (Hicks *et al.*, 2001). As mentioned above increased cross-linking through disulphide bonds of the β- and γ-kafirins blocks access to the more digestible α-kafirin core (Hamaker *et al.*, 1994; Oria *et al.*, 1995, 2000), which tends to be further exaggerated upon cooking. This model is supported by the observation that the addition of reducing agents during cooking improves the *in vitro* digestibility of sorghum (Arbab and El Tinay, 1997; Hamaker *et al.*, 1987). Moreover, studies evaluating highly digestible sorghum mutants further support the structural role of the PB on this parameter (Weaver *et al.*, 1998). Furthermore, in the sorghum mutant with a high digestible, high lysine (HDHL) phenotype, the PB are highly folded, with reduction in the γ-kafirin around the periphery (Oria *et al.*, 2000).

In addition to digestibility, overall functionality of sorghum flour is relatively poor. Similar to digestibility, functionality is also influenced by both protein–protein and protein–nonprotein interactions. Therefore, to mirror the viscoelastic properties of wheat dough, it is necessary to functionalize the kafirins in such a fashion to allow them to mimic polymeric structures formed during processing between high- and low-molecular weight glutenins and gliadins. Recently, it has been communicated that blending of co-proteins, such as casein, with prolamins, such as zeins and kafirins, during the baking process can stabilize the β-sheet structures assembled during dough formation, thereby mirroring the functionality of wheat glutens (Hamaker *et al.*, 2009). Hence, it maybe feasible to simultaneously improve upon the functionality and digestibility of grain sorghum by attempting to mirror the *in vitro* blending approach described by Hamaker *et al.* (2009) via an *in vivo* strategy whereby perturbation of the PB would expose the α-kafirins, which in turn would theoretically improve digestibility, while the *in vivo* production of a co-protein would aid in the functionalization α-kafirins during the baking process.

We describe here the generation and characterization of novel sorghum genotypes in which deliberate reduction in both γ-kafirin and a predicted 29-kDa α-kafirin storage proteins have been achieved. In addition, we report here on the molecular and phenotypic analyses of transgenic sorghum in which high levels of a prototype co-protein is produced in the endosperm, a wheat high-molecular weight glutenin subunit (HMW-GS).

Results

Characterization of transgenic sorghum events

As a means to address end-use quality of grain sorghum, with respect to nutrition, digestibility and functionality, we designed a strategy for the deliberate down-regulation of both γ- and α-kafirins, alone and in combination, and stacking of these phenotypes with the endosperm-specific expression of a HMW-GS. This in turn will create a gene stack that attempts to alter the rigid structure of the PB, with the simultaneous production of a co-protein, HMW-GS, with the latter addressing functionality and the former, enhanced digestibility, thereby creating transgenic gene stacks in sorghum that will mirror the blending strategy communicated by Hamaker *et al.* (2009). To this end, we assembled a set of binary vectors that harbour genetic elements to specifically down-regulate the γ-kafirin and a 29-kDa α-kafirin. The binary vector designated pPTN915 (Figure S1b) carries the γ-kafirin open reading frame (ORF), under control of its own promoter (Mishra *et al.*, 2008) and terminated by a self-cleaving ribozyme (RZ) derived from a satellite RNA of tobacco ringspot virus. The expression of an ORF terminated with this self-cleaving RZ generates aberrant transcripts, which are retained in the nuclear compartment where they are acted on by an RNA-dependent RNA polymerase, which in turn are processed to siRNAs (Buhr *et al.*, 2002). A total of 14 independent sorghum transformants were derived from transformations conducted with pPTN915. Among the 14 events, two events were significantly reduced in the accumulation of γ-kafirin transcripts. Shown in Figure 1 are the molecular analyses of one pPTN915 event designated 133-3-1-1. A Southern blot analysis on progeny derived from 133-3-1-1 indicates two transgenic loci, at approximately 6.2 kb, are integrated within the genome (Figure 1a). A northern analysis on T_2 seed derived from a single

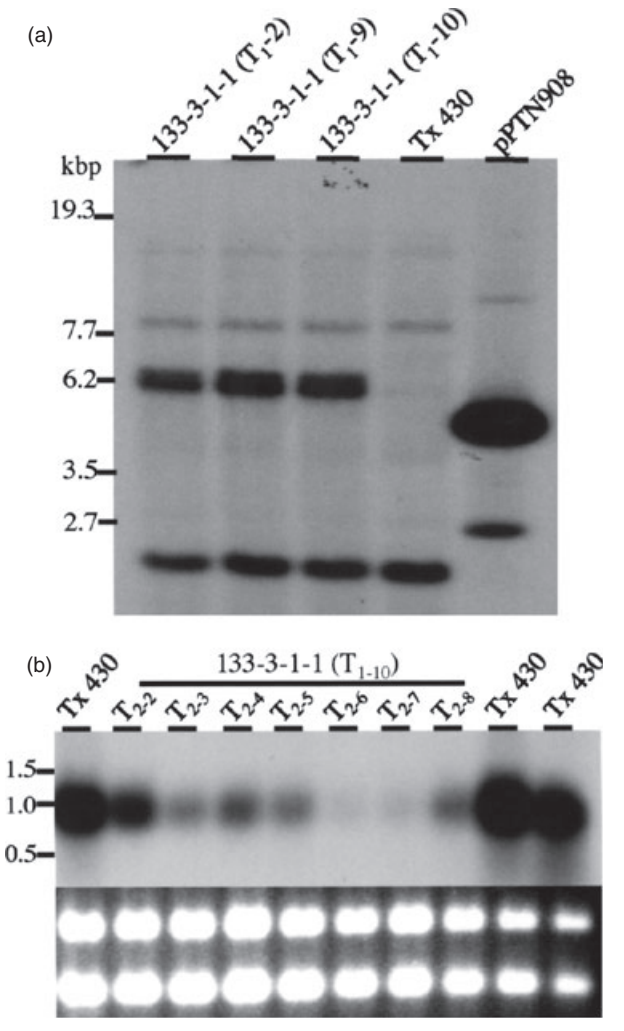

Figure 1 Molecular characterization of down-regulated γ-kafirin event 133-3-1-1. (a) Southern blot analysis on three T_1-derived individuals of event 133-3-1-1. Total genomic DNA (10 μg) was digested with *Sst* I. Blot was hybridized with the γ-kafirin ORF. Lanes 1–3 T_1 plants derived from event 133-3-1-1. Lane 4 contains control wild-type Tx 430 genomic DNA, and Lane 5 contains 50 pg of plasmid pPTN908, an intermediate vector of pPTN915 that harbours γ-kafirin ORF. (b) Northern blot analysis on T_2-derived seed from individual T_1-10 (a) from event 133-3-1-1. Blot hybridized with 800-bp region of γ-kafirin ORF. Lane 1 control RNA from immature Tx 430 seed. Lanes 2–8 RNA isolated from immature T_2 seed derived from T_1-10 event 133-3-1-1. Lanes 9 and 10 RNA isolated from control Tx 430 immature seed (lower panel ethidium bromide gel image of blot).

T_1 individual shows the significant reduction in γ-kafirin transcript accumulation in the developing seed (Figure 1b).

Seed derived from the 133-3-1-1 lineage displayed an opaque phenotype (Figure 2a). To confirm reduction in transcript accumulation translated to lowering of the γ-kafirin protein, 2-D SDS–PAGE gel analysis was conducted on the transgenic and control seed. Differential spots observed in the overview of the proteome of transgenic versus the control seed revealed the absence of a protein spot confirmed to be γ-kafirin by tandem mass spectrometry (Figure 3). Quantification of the γ-kafirin spot indicated that it was approximately 1.1% of the proteins in the 2-D gel (Figure 3a). This is relatively low given the

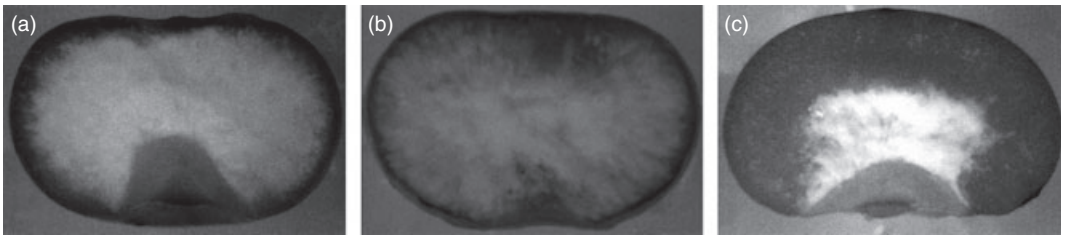

Figure 2 Opaque phenotype observed in down-regulated γ- and 29-kDa α-kafirin seed. (a) Opaque phenotype observed in down-regulated γ-kafirin event 133-3-1-1. (b) Opaque phenotype observed in down-regulated 29-kDa α-kafirin event 285-1-2-1. (c) Opaqueness observed in control Tx 430 seed.

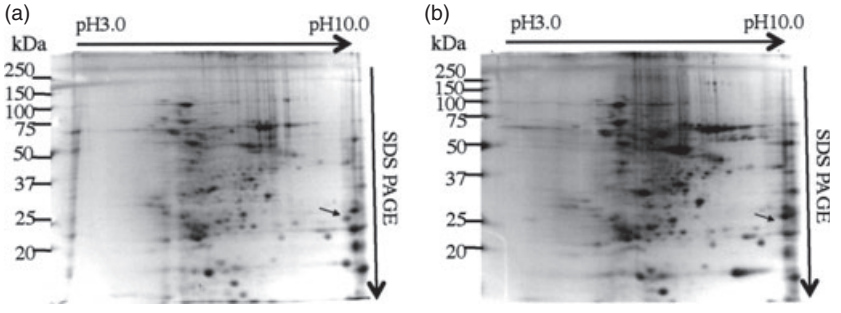

Figure 3 Kernel proteome image of down-regulated γ-kafirin event 133-3-1-1. (a) 2-D gel image of proteome of control Tx 430 seed (arrow indicates confirmed γ-kafirin spot). (b) 2-D gel of proteome of event 133-3-1-1 T_2 seed (arrow indicates location where γ-kafirin spot is expected to reside).

reported levels of γ-kafirin in the literature range from 9% to 21% (de Mesa-Stonestreet *et al.*, 2010). The apparent under-representation of γ-kafirin in the gel likely results from it not entering the IPG strip owing to its aggregation in the IEF sample buffer because of the high cysteine content and its known property of forming intermolecular and intramolecular disulphide bonds.

Down-regulation of a 29-kDa α-kafirin in grain sorghum

As a means to disrupt the central core of the PB, deliberate silencing of a 29-kDa α-kafirin (GenBank accession EU424175) was targeted using an RNAi approach. Here, a binary vector, designated pPTN1017 (Figure S1b), was built that carries a hairpin cassette, with an inverted 500-bp region of the 29-kDa α-kafirin ORF under control of an α-kafirin promoter (DeRose *et al.*, 1989). A total of 12 independent transgenic sorghum events were derived from transformations with pPTN1017. Among the 12 events generated, six displayed down-regulation of the target transcript, and as seen with the down-regulated γ-kafirin events, an opaque phenotype was observed in the seed (Figure 2b). Northern blot analysis on segregating seed derived from two of the events designated 288-1-1-2 and 285-1-2-1 is shown in Figure 4. In these events, the 29-kDa α-kafirin transcript accumulation is clearly significantly reduced. To gain insight on the changes in the proteome at maturity, total protein preparations from null, vitreous seed and down-regulated, opaque seed were separated using 2-D SDS–PAGE (Figure S2). A number of differential spots that were observed in the 2-D SDS–PAGE gel of the vitreous sample and absent in the opaque sample were analysed via tandem mass spectrometry. One of those spots was identified as the 29-kDa α-kafirin with a predicted isoelectric point of 8.97 (Figure S2). The series of spots above and below the location of the 29-kDa α-kafirin ID spot, residing about the upper pI range all had hits with the maize globulin-1 (pI 9.02). This might reflect a tendency of the sorghum globulin proteins to nonspecifically bind to other seed proteins even under the denaturing conditions of the assay. In addition, a number of differential spots, not observed in the vitreous sample were picked from the separated proteins derived from the opaque sample, down-regulated in the 29-kDa α-kafirin, seeds. The IDs of a subset of these are listed in Table 1 and positions on 2-D gel shown in Figure S2b.

The differential proteins identified from the down-regulated 29-kDa α-kafirin sample include an S-like RNAse, 2-cysteine peroxiredoxin, a desiccation-related protein, an isoflavone reductase-like (IRL) along with a glyoxalase I and xylanase inhibitor peptide (Table 1). The common thread underlying these proteins is that their expression pattern can be tied back to stress biology (Dodds *et al.*, 1996, 2010; Haslekås *et al.*, 2003; Kim *et al.*, 2003). The opaque endosperm phenotype in maize associated with either *floury2* (*fl2*) mutation (Gillikin *et al.*, 1997), *defective endosperm** (*De*-B30*) mutation (Kim *et al.*, 2004) and the *Macronate* (*Mc*) mutation (Kim *et al.*, 2006) trigger up-regulation of proteins linked with endoplasmic reticulum (ER) stress (Schröder, 2006) as a consequence of harbouring mutations in the signal peptides of the 22-kDa α-zein, 19-kDa γ-zein, and the 16-kDa γ-zein in *fl2*, *De*-B30* and *Mc* mutants, respectively. In such situations, the ER stress-induced cellular response is designed to aid in enhancing proper protein folding and hence the observed increase in transcript levels of various molecular chaperones such as BiP, hsp70 and PDI in *fl2* and *Mc* (Hunter *et al.*, 2002) and in BiP peptide accumulation in *De*B30* (Kim *et al.*, 2004).

In contrast to the kernel opaque phenotype in maize manifested by the various signal peptide mutations that reside in *fl2*,

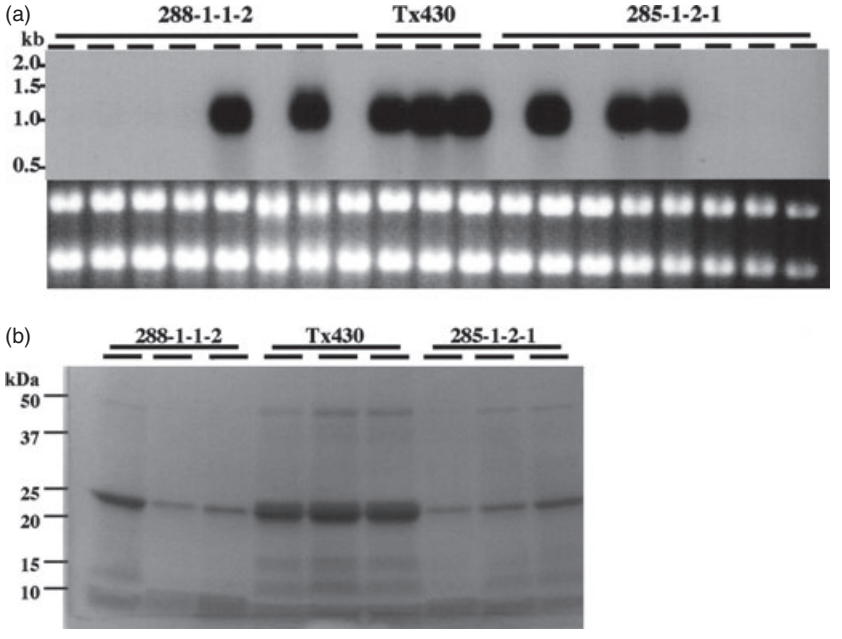

Figure 4 Northern blot and kernel storage protein accumulation visualization in down-regulated 29-kDa α-kafirin events. (a) Northern blot analysis on RNA derived from immature kernels. Lanes 1–8 event 288-1-1-2. Lanes 9–11 Tx 430 control samples. Lanes 12–19 event 285-1-2-1. Lower panel is an image of the corresponding ethidium bromide gel. Probe used in the hybridization is 500-bp region of the 29-kDa α-kafirin ORF. (b) 1-D SDS–PAGE gel image of kernel proteome. Lanes 1–3, a 1-D image of kernel proteome of pPTN1017 event 285-1-2-1. Lanes 4–6, a 1-D image of kernel proteome image of Tx 430 controls and lanes 7–9 a 1-D image of kernel proteome of pPTN1017 event 288-1-1-2.

Table 1 Novel proteins identified in kernel proteome of down-regulated 29-kDa α-kafirin events

Protein	ID	IP	% Coverage	Score
S-like RNAse	gi242081561	5.32	72	722
2-cysteine peroxiredoxins	gi195626524	5.81	27	394
Desiccation PCC 13-62	gi242075650	5.06	37	619
Isoflavone reductase	gi242052385	5.38	66	1197
Glyoxalase I	gi1808684	5.73	24	404
Xylanase inhibitor	gi242047612	6.59	56	785

Protein column refers to name of the identified protein. The ID and IP columns indicate the GenInfo Identifier (gi) number and predicted isoelectric point of the corresponding protein, while the percent coverage and score columns refer to the percent amino acid sequence coverage and probability score from mass spec analysis data.

De-B30* and *Mc*, the *opaque2* (*o2*) mutant is because of a defect in a basic leucine zipper type transcription factor (Lohmer *et al.*, 1991; Schmidt *et al.*, 1992), which governs expression of a number of maize endosperm transcripts, including that which encodes the 22-kDa α-zein. Hence, changes in the transcriptome and proteome of the kernel triggered by silencing a major storage protein, such as the 29-kDa α-kafirin, described herein or the maize zeins (Huang *et al.*, 2006) would more likely mirror that associated with *o2* than the ER stress triggering opaque mutants. Indeed, transcript profiling of *Mc*, *fl2* and *o2* revealed the two ER stress-inducing mutants display similar transcript accumulation of the molecular chaperonins, BiP, hsp70 and cyclophilin, while *o2* is more like wild type with respect to these chaperonins (Hunter *et al.*, 2002). Moreover, an IRL transcript was found to be significantly up-regulated in the embryo of α-zeins silenced maize event (Frizzi *et al.*, 2010), one of the differential protein spots identified in the silenced 29-kDa α-kafirin sorghum (Table 1).

Production of wheat HMW-GS

As a prototype genetic approach to introduce a co-protein with potential to impact functionality and/or digestibility of sorghum grain a binary vector, designated pPTN883 (Figure S1c), was assembled that carried a HMW-GS cassette that has the promoter, 5′ UTR and the genetic region to encode for the first 124 amino acids residues of the Dy10 HMW-GS, fused to the C-terminal portion of the Dx5 HMW-GS encompassing amino acid residues 130–848, coupled with the 3′ polyadenylation element of the Dx5 HMW-GS (Blechl and Anderson, 1996). A total of 23 independent transgenic sorghum events were derived from transformations with pPTN883. Relative transcript levels of the HMW-GS gene were monitored via Northern blot analysis. Expression of the gene in a subset of the events is shown in Figure 5. It is noteworthy that the expression of this HMW-GS under control of the Dy10 promoter in sorghum grain is extremely strong, the hybridization signals on the autoradiograph seen in Figure 5 were observed within 4 h of exposure. Moreover, a comparative proteome image between wild-type and transgenic seed reveals major new protein spots in the transgenic sample, verified to be HMW-GS via tandem mass spectrometry (Figure 6).

A sequential extraction scheme was utilized to investigate the level of the HMW-GS cross-linking into high-molecular weight protein structures. Initial extraction of ground kernels under nonreducing conditions removes primarily monomeric kafirins, and any monomeric forms of the HMW-GS, along with lower-molecular weight disulphide bonded polymeric protein structures, which together comprise the 'soluble protein' component

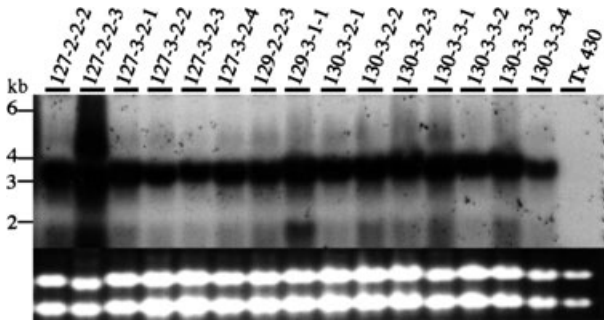

Figure 5 Northern blot on sorghum events carrying the wheat HMW-GS cassette. Lanes 1–15 RNA isolated from immature seed derived from pPTN883 sorghum events. Lane 16 RNA isolated from immature seed derived from control Tx 430 (lower panel ethidium bromide image of blot).

of the kernel (Shimoni *et al.*, 1997). The remaining pellet was subsequently extracted under reducing conditions incorporating ß-mercaptoethanol as the reducing agent. Under these conditions, disulphide bonded larger molecular weight polymeric protein structures are recovered, which are categorized as 'insoluble proteins' (Shimoni *et al.*, 1997). The soluble and insoluble protein components were analysed via SDS–PAGE. As can be seen in Figure 7, the vast majority of the HMW-GS resides in the insoluble protein component, implying that this transgenically expressed protein is either directly disulphide bonded to the kafirin/PB complex or is self-disulphide bonded in extremely large molecular weight molecules.

Imaging of PB and amino acid profiles of transgenic events

Imaging of PB in immature kernels was monitored via transmission electron microscopy (TEM). The morphology of the PB present in immature kernels derived from transgenic events either expressing the HMW-GS (pPTN883) or displaying a down-regulation of γ-kafirin (pPTN915) was not altered in morphology as compared to control, wild-type (nontransgenic) PB imaged at the same time point in development (data not shown). However, visualization of the PB in immature grain in which 29-kDa α-kafirin was down-regulated (pPTN1017) revealed distortion of the PB (Figure 8). The major morphological change observed was deep invaginations to the central core of the PB, reminiscent of PB structure found in the highly digestible sorghum mutant (Oria *et al.*, 2000).

Total amino acid profile of mature grain was determined for the selected pPTN883, pPTN915 and pPTN1017 events. As can be seen in Figure 9, down-regulation of γ-kafirin led to decreases in proline, glutamate and leucine, while down-regulation of 29-kDa α-kafirin enhanced lysine, arginine and aspartate, along with slight reductions in glutamate and leucine. With respect to the pPTN883 events, production of the HMW-GS had minimal impact on the amino acid profile of the grain, with only a slight lowering of leucine observed (Figure 9).

Influence on digestibility by the targeted alterations in protein composition of sorghum grain

In vitro digestibility was carried out on flour derived from a subset of the transgenic events. Grain protein extracts were first characterized via reversed phase HPLC (RP-HPLC; Bean *et al.*, 2011). Traces obtained from events 128-2-1-1 (pPTN883), 133-3-1-1 (pPTN915) and wild type are shown in Figure 10a. A novel peak falls out just under 4 min in the 128-2-1-1 event, and the known γ-kafirin peak is drastically diminished in the 133-3-1-1 event (Figure 10a). RP-HPLC traces on ground seed samples derived from progeny of the down-regulated 29-kDa α-kafirin event 288-1-1-2 are shown in Figure 10b, and corresponding Coomassie gel in Figure 4b. The traces reveal drastic changes between the 8.5 and 12.0 min ranges, which are reflected in the loss of major peaks, which correspond to the reduced α-kafirin band imaged in Figure 4b.

Seed samples, ground to uniformity, were subjected to a pepsin digestion assay (Mertz *et al.*, 1984). *In vitro* digestibility was measured in both raw flour and cooked samples. Although variation in per cent protein digestibility of control (genotype Tx 430), raw flour samples was observed, when compared to the corresponding transgenics samples, assayed at the same time period, raw flour derived from some of the HMW-GS and down-regulated γ-kafirin events displayed improvement in this parameter over 10% (Table 2). However, upon cooking *in vitro* digestibility of the HMW-GS and silenced γ-kafirin transgenic events and control samples did not vary significantly, with percentage of digested protein observed ranging from 24% to just under 30% across these samples assayed (Table 2). In regards to the pPTN883 events assayed, correlating HMW-GS production in the seed, based on RP-HPLC peak area (Figure 10a), with raw digestibility of raw flour, a slight but positive relationship was observed ($r^2 = 0.39$). This data, along with the results observed from the sequential extraction procedure is suggestive that indeed the HMW-GS is directly interacting with the PB.

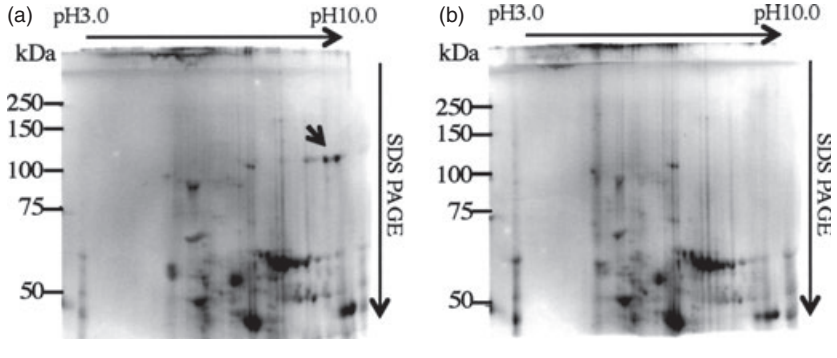

Figure 6 Kernel proteome image of wheat HMW-GS sorghum event. (a) kernel proteome image from pPTN883 sorghum event. Arrow highlights distinct spots identified as the wheat HMW-GS. (b) kernel proteome image of Tx 430 control kernel.

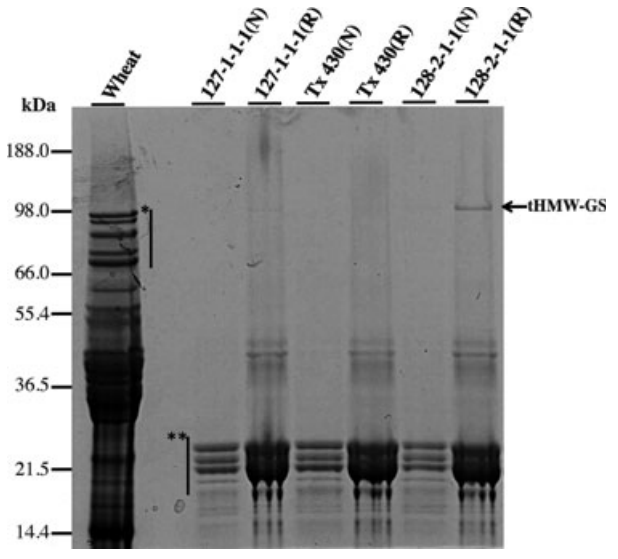

Figure 7 Sequential extraction of HMW-GS from sorghum. Wheat lane refers to extracts from wheat seed under reducing conditions. Lanes 127-1-1-1 (pPTN883 event), Tx 430 (control seed) and 128-2-1-1 (pPTN883 event). N and R indicate nonreducing and reducing conditions, respectively. Highlight the endogenous wheat *HMW-GS and **sorghum kafirins.

In vitro digestibility of raw flour derived from two independent events down-regulated in 29-kDa α-kafirin (pPTN1017) designated 285-1-2-1 and 288-1-1-2 also displayed improvement in this parameter, including over 30% improved digestibility of the cooked samples (Table 2). Moreover, size exclusion chromatography revealed that silencing of 29-kDa α-kafirin led to a significant reduction in percentage of the insoluble protein component of the flour (Table 3). Importantly, owing to the compensation mechanism of seeds, overall total protein levels were not changed (Table 2).

Discussion

A set of transgenic sorghum events has been generated with targeted modulation of kernel storage protein accumulation. The long-term goal is to assemble a series of creative gene stacks from these transgenic events as a means to simultaneously alter PB morphology and express a co-protein, HMW-GS, as a means to address improvements in both digestibility and viscoelasticity of the flour for end-use food and feed applications. Thereby creating a genetic approach to mirror the *in vitro* blending strategy described by Hamaker *et al.,* (2009). To this end, we have used selected events, 285-1-2-1 (pPTN1017), 133-3-1-1 (pPTN915) and 128-2-1-1 (pPTN883) in a series of crossing blocks and have successfully generated double stack combinations along with the triple gene stack. The novel sorghum events described here will aid in dissecting out some of the parameters influencing protein and perhaps indirectly starch (Wong *et al.*, 2009) digestibility, leading to improved functionality of grain sorghum end-use applications for feed and food. For example, a long-standing hypothesis has been that disulphide cross-linking of the storage proteins plays a negative role in digestibility of both uncooked and cooked sorghum (Duodu *et al.*, 2003; El Nour *et al.*, 1998; Winn *et al.*, 2009). In regards to cooked sorghum samples, it has been shown that disulphide-linked polymeric kafirins are formed which are more resistant to digestion (Emmambux and Taylor, 2009). In a study that partitioned the level of polymeric proteins in vitreous and opaque components of sorghum kernels, it was shown that the level of γ-kafirin in the vitreous portion of the endosperm was negatively correlated with amount of soluble proteins and γ-kafirin levels appear to be a strong indicator of polymeric proteins in the vitreous portion of the kernel (Ioerger *et al.*, 2007). In a related study employing both statistical and biochemical approaches as a means to identify genetic targets for the improvement of end-use quality of grain sorghum, it was concluded that γ-kafirin is the most resistant to digestion and the β-kafirin, the other storage protein about the periphery of the PB, is readily digested, and therefore, based on the analyses, the former was a logical target for down-regulation as a means to improve digestibility (Wong *et al.*, 2010). However, the event 133-3-1-1 serves as the direct test of this hypothesis, and while a modest increase (approximately 11%) in protein digestibility was observed in raw flour preparations, no change was seen upon cooking (Table 2). Moreover, no change in PB morphology was observed in the 133-3-1-1 event, indicating reduction in γ-kafirin alone is not sufficient to impact PB shape or digestibility. In maize, reduction in both peripheral prolamins around the PB, β- and γ-zeins, is required to alter PB formation

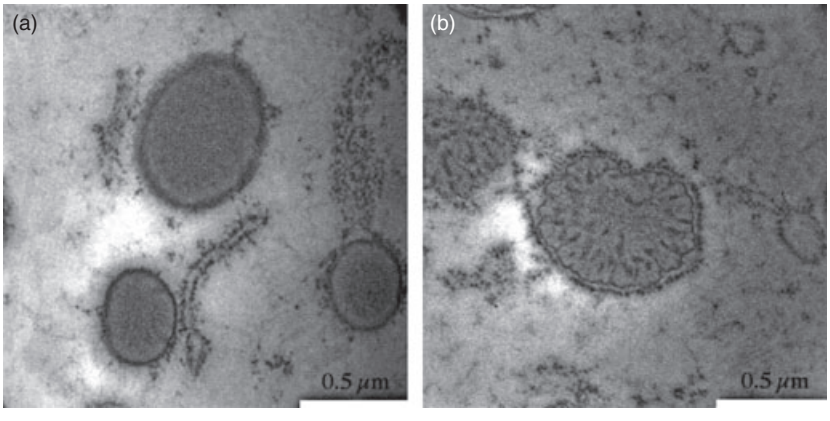

Figure 8 Transmission electron microscopy image of sorghum protein bodies. (a) TEM image (30×) of PB in endosperm of Tx 430 kernel 12 days postanthesis. (b) TEM image (30×) of PB in endosperm of down-regulated 29-kDa α-kafirin event (pPTN1017) kernel 12 days postanthesis.

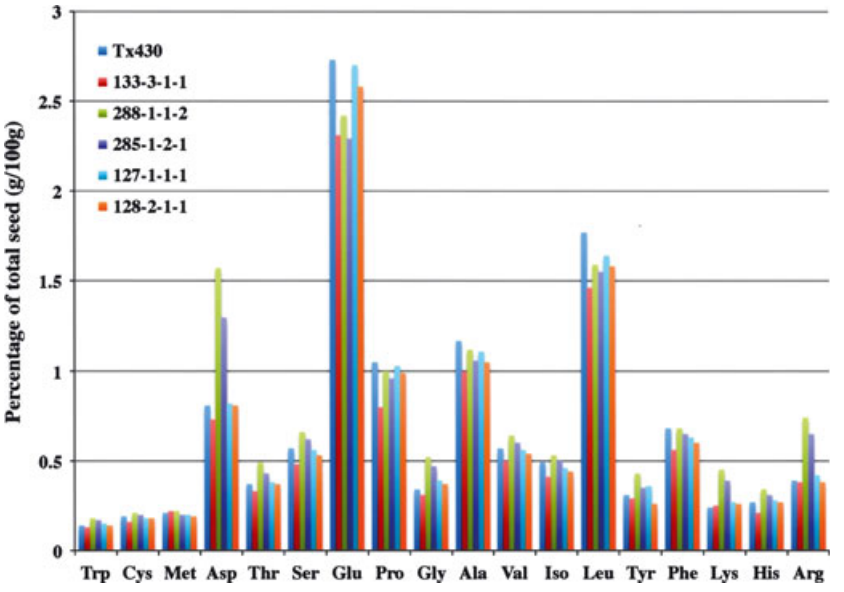

Figure 9 Amino acid profiles obtained from whole kernels. Numbers corresponding to the respective amino acids are percentages (g/100 g) of total kernel. Amino acid profiles were ascertained from ground kernel samples derived from 133-3-1-1 (pPTN915), 288-1-1-2 (pPTN1017), 285-1-2-1 (pPTN1017), 127-1-1-1 (pPTN883) and 128-2-1-1 (pPTN883).

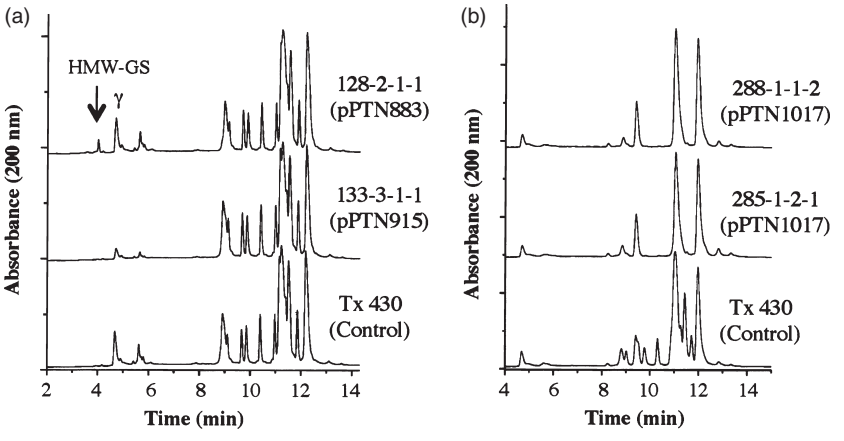

Figure 10 RP-HPLC chromatograms derived from sorghum kernel samples. (a) Displays trace obtained from sorghum event 128-2-1-1 accumulating wheat HMW-GS (arrow), middle trace obtained from sorghum event 133-3-1-1 (pPTN915) showing distinct reduction in γ-kafirin peak and bottom trace corresponding to a Tx 430 control sample. (b) Displays two RP-HPLC traces obtained from the two pPTN1017 events and lower trace corresponds to control Tx 430.

(Wu and Messing, 2010). Hence, the next logical approach in sorghum would be to explore the impact on PB morphology and digestibility in events silenced in β-kafirin level alone and stacked with down-regulated γ-kafirin or exploit the genetic variation present in the sorghum germplasm for β-kafirin (Laidlaw *et al.*, 2010) to pyramid with the respective silenced kafirin events described here.

The opaque phenotype observed here in the down-regulated γ- and 29-kDa α-kafirin events (Figure 2) is also seen when the corresponding maize homologues are silenced (Wu and Messing, 2010); however, the penetrance of the phenotype is greater in the γ-kafirin silenced events, as compared to 29-kDa α-kafirin silenced events (Figure 2), while the opposite effect is noticed in maize. However, the observation that reduction in the major kernel storage protein, 29-kDa α-kafirin, leads to accumulation of nonkafirin proteins, which in turn translates to amino acid changes (Figure 9), without compromising total protein levels (Table 2), is in agreement with the findings in other grains such as maize (Frizzi *et al.*, 2010) and rice (Kawakatsu *et al.*, 2010) and is the underlying biology that accounts for the seminal report communicated by Mertz *et al.* (1964).

The ability of plants to compensate for the loss of a major seed storage protein by up-regulating nonstorage protein genes is a phenomenon that has been observed in both dicotyledonous (Schmidt and Herman, 2008) and monocotyledonous (Hunter *et al.*, 2002; Kawakatsu *et al.*, 2010) species. We under took a proteomics approach to get a glimpse of the nonkafirin proteins that accumulate in mature sorghum grain upon down-regulation of the 29-kDa α-kafirin. All the proteins identified are linked to a generalized stress response (Table 1). Changes in

Table 2 *In vitro* digestibility of flour samples derived from sorghum kernels

Event	Construct	% Protein (Pre-R)	% Digestibility (Raw)	% Digestibility (Cooked)
125-4-3-3	pPTN883	12.08 ± 0.09	51.33 ± 1.51	25.77 ± 4.39
126-4-1-3	pPTN883	13.80 ± 0.14	35.67 ± 0.56	26.99 ± 0.88
127-1-1-1	pPTN883	14.48 ± 0.09	43.57 ± 1.41	24.59 ± 7.07
128-2-1-1	pPTN883	12.64 ± 0.13	50.14 ± 2.10	29.36 ± 1.83
128-2-1-1	pPTN883	14.97 ± 0.06	66.57 ± 0.23	28.98 ± 0.90
133-3-1-1	pPTN915	14.55 ± 0.03	59.76 ± 4.19	28.46 ± 4.74
288-1-1-2	pPTN1017	15.76 ± 0.11	67.33 ± 2.98	35.27 ± 2.09
285-1-2-1	pPTN1017	18.52 ± 0.19	63.04 ± 0.61	39.20 ± 1.91
Tx 430	—	13.62 ± 0.75	44.87 ± 5.60	24.48 ± 1.99

Event column indicates from which sorghum event flour sample was derived. Construct column refers to the respective plasmid used for transformation. Percent protein (Pre-R) refers to mean protein percentage ± standard deviation in pre *in vitro* digested raw flour. Percent digestibility (Raw) and (Cooked) columns indicate the mean percentage of digested protein ± standard deviation, following *in vitro* digestion assay.

Table 3 Percentage of soluble proteins present in derived sorghum flour

Event	Construct	%SP	%IP	%RP
127-1-1-1	pPTN883	40.8 ± 0.25	45.5 ± 3.93	13.7 ± 3.67
128-2-1-1	pPTN883	40.8 ± 0.10	43.9 ± 3.45	15.3 ± 3.35
133-3-1-1	pPTN915	49.0 ± 0.60	42.7 ± 3.08	8.2 ± 2.47
288-1-1-2	pPTN1017	44.6 ± 5.30	17.4 ± 4.32	38.0 ± 4.56
285-1-2-1	pPTN1017	40.1 ± 2.27	20.6 ± 3.66	39.3 ± 2.25
Tx 430	WT	47.0 ± 0.53	41.4 ± 4.63	11.59 ± 4.34

Event column indicates the origin of flour samples analysed, while construct column reflects corresponding plasmid. Per cent SP, IP and RP refer to mean (±standard deviation) percentage of soluble, insoluble and residual proteins, respectively.

carbon flux, at the initiation of synthesis of storage reserves, typically 14–18 days after pollination in maize, include up-regulation of a number of stress related transcripts in the various maize mutants with an opaque phenotype (Hunter *et al.*, 2002) and silenced α-zeins through an RNAi approach (Frizzi *et al.*, 2010). However, the global changes, in immature kernels of maize, in both transcript and protein levels that occur upon reduction in one or more major kernel storage proteins covers a much broader array of function (Frizzi *et al.*, 2010; Hartings *et al.*, 2011; Hunter *et al.*, 2002).

Elucidation of the mechanism controlling these global changes in carbon flux will aid in our understanding of the compensation mechanism of seeds (Kawakatsu *et al.*, 2010; Schmidt and Herman, 2008), which in turn will translate to designing of optimal genetic strategies for targeted improvements in seed quality, nutritional content and end-use functionality. Having a snapshot of the proteome changes at various stages of seed development will provide insight into the regulation underpinning this phenomenon. With respect to the differential up-expressed spots identified in mature sorghum kernels in which the 29-kDa α-kafirin is down–regulated the identified proteins fall under a single functionality class, stress response. For example, a S-like RNAse protein was one of the identified up-regulated spots observed. Homologs of S-like RNAse have been communicated to be elevated in expression upon leaf senescence and phosphate starvation (Liang *et al.*, 2002) and pathogen ingress (Dodds *et al.*, 1996). The other novel spots identified in the 29-kDa α-kafirin down-regulated event include 2-cysteine peroxiredoxin whose activity is associated with maintenance of proper redox potential of a cell (Dietz *et al.*, 2002), protein PCC 13-62 putatively linked to desiccation (Bartels *et al.*, 1990), isoflavone reductase-like genes implicated in both biotic and abiotic stress responses (Kim *et al.*, 2003, 2010), glyoxalase 1 which has been shown to combat salt stress (Singla-Pareek *et al.*, 2003) and xylanase inhibitors which play a role in abating fungal pathogenesis (Dornez *et al.*, 2010).

The wheat HMW-GS (Blechl and Anderson, 1996) was selected as co-protein for downstream investigations into potential impacts on improving leavening properties of sorghum flour. This co-protein was chosen because of the known endogenous influence that HMW-GS have on functionality of wheat flour (Shewry, 2009). Grain sorghum is often a sought after ingredient in formulating diets for individuals who suffer from coeliac disease (de Mesa-Stonestreet *et al.*, 2010). The prevalence of coeliac disease throughout the world is generally under 2%, but has been reported to be up to 5.6% (Tack *et al.*, 2010). One of the proteins thought to play a role in the triggering of coeliac disease are the wheat HMW-GS (Sollid, 2002). Hence, if this co-protein proves to be successful in aiding in the leaven properties of baked goods with sorghum products, then proper identity preservation from conventional sorghum grain may need to be implemented. Moreover, products with wheat ingredients fall under the Food Allergen Labeling Consumer Act (FALCPA), therefore, within the US food labels would be required to list a sorghum flour with HMW-GS as wheat-based ingredient. On the flip side, utilizing such a sorghum biological as the pPTN883 events described herein, can serve as a useful tool for elucidating the dietary components that trigger coeliac disease by isolating a putative coeliac disease trigger away from its endogenous seed matrix, which is thought to influence other, more serious, food allergens (van Wiljk *et al.*, 2005).

When over expressed in wheat, the hybrid HMW-GS (Blechl and Anderson, 1996) was shown to have the capacity to form intramolecular disulphide bonds, because of the unique position of cysteine residues at the N- and C-terminal regions of the peptide; however, a portion of the transgenically expressed peptide was still present within glutenin polymers of the grain (Shimoni *et al.*, 1997). Employing a sequential extraction scheme, we have shown that the vast majority of the hybrid HMW-GS is present in reduced extracts (Figure 7) of sorghum, suggestive of being incorporated into large polymeric structures, most likely the PB. However, we cannot rule out the possibility that the HMW-GS is competent to form large self-bonded polymeric structures in sorghum kernels. Either way, pyramiding of this trait with the altered PB phenotype observed in the pPTN1017 events (Figure 8) will in essence assemble the *in vivo* blending of a co-protein in a sorghum kernel matrix with improved digestibility (Table 2), thereby creating a novel sorghum flour composite with potential improvements in end-use baking applications.

In both maize and sorghum, the ratio of vitreous and opaqueness of the kernel will influence texture of the endosperm. A hard endosperm typically will possess a larger vitreous

area about an opaque core of the kernel, while a softer endosperm will have a significantly larger area of opaqueness. Endosperm hardness is a desired trait for selection in cereal breeding programs because of its association with improved functionality and positive correlation with yield and grain resistance to fungal ingress (Tesso *et al.*, 2006; Wu *et al.*, 2010). On the other hand, soft endosperm has been linked with improved digestibility and nutritional quality in sorghum (Oria *et al.*, 2000) and maize (Sofi *et al.*, 2009; Wu *et al.*, 2010). The maize *opaque2* (*o2*) mutant is a lesion in a transcription factor that among its targets is the 22-kDa α-zein (Habben *et al.*, 1993). Maize germplasm carrying *o2* possess enhanced nutritional quality, but lack desired grain hardness. The stacking of *o2* with various *o2* modifiers has resulted in selections of vitreous kernels that maintain the enhanced nutritional quality of such *o2* combinations, without compromising agronomic performance of the crop, this germplasm is referred to as quality protein maize (QPM; Crow and Kermicle, 2002). QTLs linked to the *o2* modifiers have been mapped to chromosomes, 1, 7 and 9 (Holding *et al.*, 2008), and interestingly, the *o2* modifying phenotype is associated with increased levels of γ-zeins (Wu *et al.*, 2010). Importantly, the genetic dissection of the *o2* modifying phenotype has permitted the development of molecular markers that have greatly aided maize breeding programs in introgression of the QTLs associated with *o2* modifiers into elite genotypes (Gupta *et al.*, 2009; Sofi *et al.*, 2009).

The high digestible/high lysine sorghum germplasm is currently following an analogous path as that of the *o2*/QPM story but some hurdles still remain (Tesso *et al.*, 2006, 2008). Nonetheless, the genetic loci associated with the sorghum endosperm modifiers (Tesso *et al.*, 2008) may complement the biotechnology strategy outlined herein thereby laying the foundation towards the development of quality protein sorghum coupled with enhanced digestibility and improved functionality.

Experimental procedures

Assembly of binary vectors

The 29-kDa α-kafirin gene was isolated via RT-PCR (genotype Tx 430) from mRNA derived from immature seed. Primers used in the RT-PCR reaction were Kaf-5: ATGGCTACCAAGATATTTG-TCCTCCTTGCG and Kaf-3: AATCTAGAAGATGGCACTTCCAAC-GATGGG, based on GenBank accession number EU424175. A 500-bp element derived from the PCR product was subsequently re-amplified to incorporate convenient cloning sites at the 5′ and 3′ ends, *Hpa* I and *Xba* I, along with *Sst* I and *Xho* I, respectively. The 29-kDa α-kaf 500-bp elements, with incorporated restriction sites were cloned as inverted repeats into pUC18-RNAi (gift H. Cerutti U. of Nebraska), a plasmid which harbours the second intron of the *Arabidopsis* small nuclear ribonuclear protein D1 (locus At 4g02840). The intron is delineated by a set of restriction sites that facilitates assembly of inverted repeat elements. The derived 29-kDa α-kafirin RNAi element was then subcloned downstream of the 832-bp α-kafirin promoter (GenBank accession X16104), which was cloned out by PCR using primer set p-alphakaf-1: AGA-CCTCCCAACCCATGCTCGCCACGTTTG and p-alphakaf-2: TTG-GAAGGACGTTGCTAGTTCGTTTCAC. The RNAi cassette was terminated by the CaMV 35S polyadenylation signal. The resultant RNAi cassette was sublconed into the binary vector pPZP212 (Hajdukiewicz *et al.*, 1994) to make the final vector designated pPTN1017 (Figure S1a).

The γ-kafirin promoter was isolated by PCR using primer set gKaf-2-5:CCGTGTACAACGAAGTGGTGAGTCATGAG and gKaf-2-3:GGTGTCGAGTTCTTGTCTGCTCTG based on GenBank accession number X62480, which amplified a 493-bp region upstream of the translational start site of the gene. The γ-kafirin ORF was cloned using RT-PCR derived from mRNA from immature seed (Tx 430) using primer set, based on GenBank accession number X62480, gKaf5: ATGAAGGTGTTGCTC-GTTGCCCTCGCTC and gKaf3: TTAATAGTGGACACCACCGG-CAAAAGG. The γ-kafirin promoter and ORF were assembled into a cassette terminated by a self-cleaving ribozyme derived from the satellite RNA of tobacco ringspot virus (Buhr *et al.*, 2002). The resultant element was subcloned into the binary vector pPZP212 (Hajdukiewicz *et al.*, 1994) and the final vector designated pPTN915 (Figure S1b).

A hybrid HMW-GS cassette consisting of the 5′ UTR and first 124 amino acid residues of the Dy 10 HMW-GS, fused to the C-terminal portion of the Dx5 HMW-GS encompassing amino acids residues 130–848, coupled with the 3′ polyadenylation element of the Dx5 HMW-GS (Blechl and Anderson, 1996) was subcloned as an *Eco* RI fragment into the binary vector pPZP211 (Hajdukiewicz *et al.*, 1994) and the resultant plasmid referred to as pPTN883 (Figure S1c).

The three final binary vectors, pPTN883, pPTN915 and pPTN1017 were mobilized in to *A. tumefaciens* strain NTL_4/pKPSF2 (Palanichelvam *et al.*, 2000) and the transconjugants used for sorghum transformation, genotype Tx 430, as previously described (Howe *et al.*, 2006).

Molecular characterization of transgenic sorghum events

Total genomic DNA was isolated from sorghum leaves 133-3-1-1 event (pPTN915) following a modification of the protocol described by Dellaporta *et al.* (1983). Genomic DNA was restriction digested with *Sst* I, which has a single recognition site within the T-DNA of pPTN915. The membrane was hybridized with $dCT^{32}P$ labelled γ-kafirin ORF (Prime-It II kit Agilent Technologies Cat # 300385, La Jolla, CA).

Northern blot analyses were conducted on total RNA isolated from individual seeds harvested 20 days postanthesis. RNA sample isolation, separation and hybridizations were carried out as previously described (Buhr *et al.*, 2002). Membranes were hybridized with $dCT^{32}P$ labelled HMW-GS, 2.2 kb region of the ORF (pPTN883) and 500-bp region of 29-kDa α-kafirin ORF (pPTN1017) or 820-bp region of γ-kafirin ORF (pPTN915).

Seed protein extraction

Individual seeds were ground in liquid nitrogen. Protein extraction buffer was added to the ground seed and thoroughly mixed. The extraction buffer was composed of 0.1 M Trizma base (pH 8.0), 10 mM EDTA, 0.9 M sucrose and 0.4% (v/v) ß-mercaptoethanol. The mixture was subsequently extracted for 30 min at 4 °C, with slight agitation, with an equal volume of Tris-saturated phenol. Following the extraction step, the suspension was centrifuged at 4350 ***g*** in a Beckman JA-20 rotor for 10 min. The upper phenol phase was collected and precipitated overnight at −20 °C by the addition of 5× volume of 0.1 M ammonium acetate in 100% methanol. Precipitated proteins were pelleted at 17,400 ***g*** in a Beckman JA-20 rotor for 10 min. The protein pellet was subsequently washed with 0.1 M

ammonium acetate in 100% methanol, followed by washings in 80% (v/v) acetone and 70% (v/v) ethanol. The washed protein pellets were suspended in 8 M urea, 2 M thiourea, 2% (w/v) CHAPS and 2% (v/v) Triton X-100. Protein concentrations were quantified using the Bradford assay (Bio-Rad Cat# 500-0116, Hercules, CA).

Two-dimensional gel electrophoresis

A total of 300 μg of protein was loaded onto each 7 cm pH 3.0–10.0 Ready Strip IPG strip (Bio-Rad Cat# 163-2000). Following focusing in the first dimension, the strips were run in the second dimension using 14% (w/v) SDS–PAGE and the gel was subsequently stained with Coomassie G-250. Differential spots selected were analysed using tandem mass spectrometry.

Imaging of protein bodies

Immature seeds harvested 12 days after anthesis were transversely sliced into 1–2 mm pieces and fixed in 4% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 50 mM potassium phosphate buffer (pH 6.8) at 4 °C for 16 h. The samples were stained with 2% (w/v) osmium tetroxide in 50 mM potassium phosphate buffer (pH 6.8), followed by three washes in buffer. Following the last wash, the samples were dehydrated in a graded ethanol series (v/v) for 15 min at each step from 10%, 30%, 50%, 70%, 90%, 95% and 100% ethanol. Following the dehydration step, samples were infiltrated for 2 h at each gradient in 20%, 40%, 60% and 80% LR White resin in (w/v) ethanol, followed by an over night infiltration in 100% LR white resin. The 100% LR white resin infiltration was continued for 48 h with 12 h changes in resin. Following the infiltration procedure, the samples were placed in plastic moulds and polymerized for 2 days at 55 °C. The prepared specimens were sectioned with a diamond knife using an ultramicrotome (LKB Ultratome III, Stockholm, Sweden). The sections were overlaid to copper grids coated with formvar membrane and carbon. Sections were poststained for 5 min with 2.5% (w/v) uranyl acetate, followed by 3 min step in 0.1% (w/v) lead citrate. The prepared samples were imaged with a Hitachi H7500 transmission electron microscope (Hitachi, Tokyo, Japan) at 80 kV at the University of Nebraska's Microscopy Core Research Facility.

In vitro digestibility assay, RP-HPLC and SDS–PAGE analyses of storage protein extracts

Kafirin fractions were isolated from 100 mg seed samples that were uniformly ground using a Udy mill (Udy Corp., Fort Collins, CO). Protein extractions and kafirin selective isolation procedures were conducted as previously described (Bean *et al.*, 2011), with HPLC separations carried out on a Agilent 1100 HPLC, and reverse-phase separations using a Poroshell C18 column following the procedure outlined by Bean *et al.* (2000, 2011).

In vitro pepsin digestibility assay was conducted with duplicated samples. The samples were reground with a UDY mill coupled with a 0.5-m screen. Pepsin digestion assay was conducted as outlined by Mertz *et al.* (1984). Digestibility was measured in raw flour and in some cases cooked as porridge. Amino acid profile of the ground grain was ascertained through a commercial source (Eurofins, Des Moines, IA).

To determine whether the HMW-GS resides primarily as a monomer or polymeric protein structures within the cell a sequential extraction scheme was used based on that reported on by Shimoni *et al.* (1997). Albumin and globulin proteins were pre-extracted from the samples as previously described (Bean *et al.*, 2011). The residual remaining following the removal of albumin and globulin fractions was subsequently extracted with 1 mL of 60% (w/v) t-butanol containing 0.5% (w/v) sodium acetate for 5 min with continuous vortexing. This extraction step was repeated and supernatants from each extraction were pooled in a 1 : 1 ratio. Samples were then analysed by SDS–PAGE using 4–12% NuPAGE® gel (InVitrogen, Grand Island, NY) with MOPS runner buffer. Prior to SDS–PAGE analysis, samples were mixed with Novex sample buffer (InVitrogen) in a 1 : 4 ratio containing 2% (v/v) ß-mercaptoethanol and boiled for 5 min. Wheat proteins from the cultivar Karl 92 were extracted with 50% (v/v) 1-propanol plus 2% (v/v) ß-mercaptoethanol and mixed with Novex sample buffer. Ten microlitres from each sample was loaded per lane and gels run at 200 V until the dye front entered the buffer. Gels were stained using Imperial™ protein stain (Thermo Scientific, Rockford, IL) following the manufacturer's directions.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Diagrammatic representation of transgenic cassettes with the T-DNA elements of the respective binary vectors.
Figure S2 Kernel proteome image of down-regulated 29-kDa α-kafirin event.

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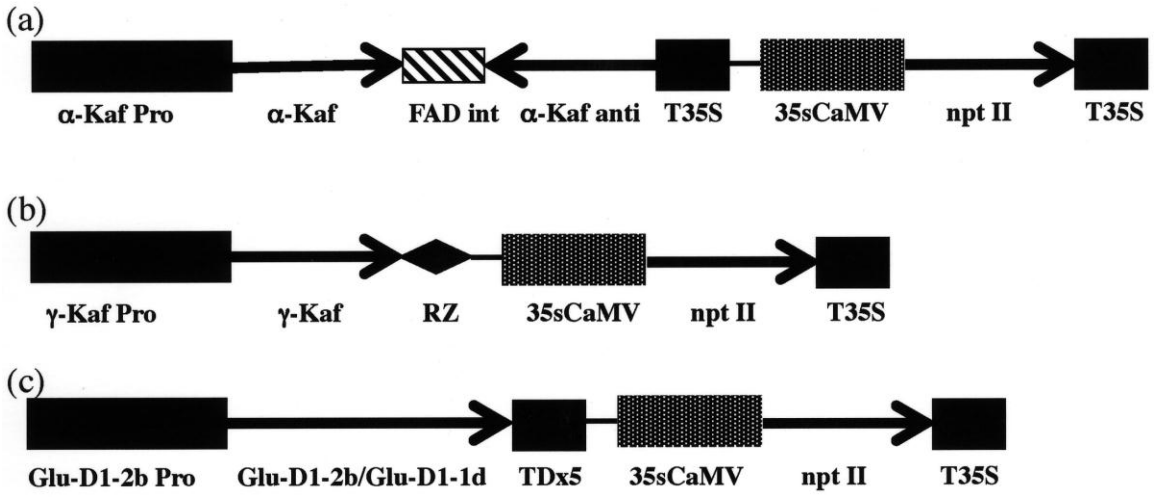
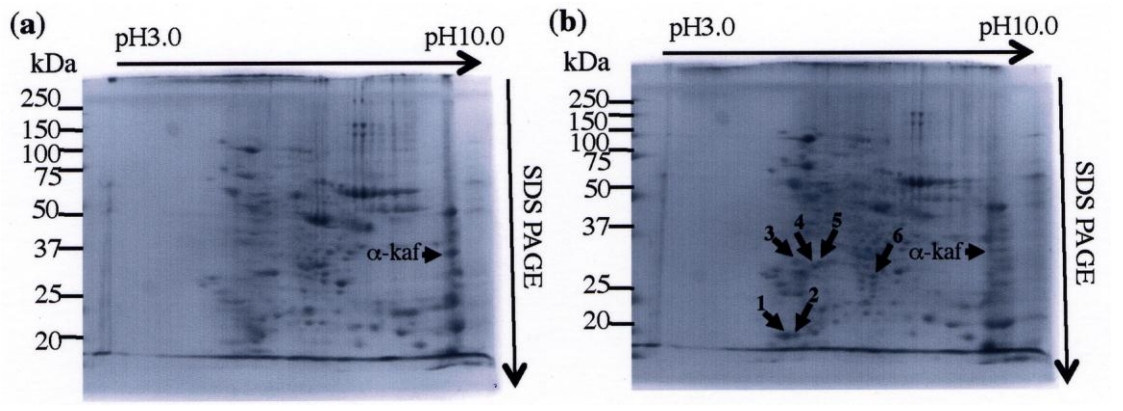


Supplemental Figure 1: Diagrammatic representation of transgenic cassettes with the T-DNA elements of the respective binary vectors

(S1a**)**: Genetic elements that reside within the T-DNA region of the binary vector pPTN1017, including the RNAi cassette and npt II plant selection cassette. (S1b): Genetic elements that reside within the T-DNA of the binary vector pPTN915, including the plus sense gamma kafirin terminated by self-cleaving ribozyme, and the npt II selectable marker gene cassette. **(**S1c**)**: Genetic elements that reside within the T-DNA region of the binary vector pPTN883, including the HMW-GS cassette and the npt II plant selectable marker cassette. **Note**: Genetic elements are not drawn to scale.



Supplemental Figure 2 : Kernel proteome image of down-regulated 29 kDa α-kafirin event.

(a): 2-D gel image of proteome of control Tx 430 mature seed (arrow indicates confirmed α-kafirin spot). (b): 2-D gel of proteome of mature 29 kDa α-kafirin down regulated T_2 seed. The α-kaf labeled arrow indicates location where α-kafirin spot is expected to reside, arrows number 1 through 6 highlight differential spots identified (Table 1), S-Like RNAse, 2-csyteine peroxiredoxins, desiccation PCC 13-62, isoflavone reductase, glyoxalase I and xylanase inhibitor, respectively.