Impacts of Kafirin Allelic Diversity, Starch Content, and Protein Digestibility on Ethanol Conversion Efficiency in Grain Sorghum

Julia E. Cremer,[1,2] Liman Liu,[3] Scott R. Bean,[4] Jae-Bom Ohm,[5] Michael Tilley,[4] Jeff D. Wilson,[4] Rhett C. Kaufman,[4] Thanh H. Vu,[6] Edward K. Gilding,[7] Ian D. Godwin,[1] and Donghai Wang[3]

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ABSTRACT

Seed protein and starch composition determine the efficiency of the fermentation process in the production of grain-based ethanol. Sorghum, a highly water- and nutrient-efficient plant, provides an alternative to fuel crops with greater irrigation and fertilizer requirements, such as maize. However, sorghum grain is generally less digestible because of extensive disulfide cross-linking among sulfur-rich storage proteins in the protein–starch matrix. Thus, the fine structure and composition of the seed endosperm directly impact grain end use, including fermentation performance. To test the hypothesis that kafirin (prolamin) seed storage proteins specifically influence the efficiency of ethanol production from sorghum, 10 diverse genetic lines with allelic variation in the β-, γ-, and δ-kafirins, including three β-kafirin null mutants, were tested for ethanol yield and fermentation efficiency. Our selected lines showed wide variation in grain biochemical features, including total protein (9.96–16.47%), starch (65.52–74.29%), and free amino nitrogen (FAN) (32.84–73.51 mg/L). Total ethanol yield (ranging from 384 to 426 L/metric ton), was positively correlated to starch content (R^2 = 0.74), and there was a slight positive correlation between protein digestibility and ethanol yield (R^2 = 0.52). Increases in FAN content enhanced fermentation efficiency (R^2 = 0.65). The highest ethanol producer was elite staygreen breeding line B923296, and the line with the highest fermentation efficiency at the 72 h time point was inbred BT×623. A large-seeded genotype, KS115, carrying a novel γ-kafirin allele, was rich in FAN and exhibited excellent short-term fermentation efficiency at 85.68% at the 20 h time point. However, the overall ethanol yield from this line was comparatively low at 384 L/metric ton, because of insufficient starch, low digestibility, and high crude protein. Multivariate analysis indicated an association between the β-kafirin allele and variation in grain digestibility (P = 0.042) and FAN (P = 0.036), with subsequent effects on ethanol yield. Reversed-phase HPLC profiling of the alcohol-soluble kafirin protein fraction revealed diversity in protein content and composition across the lines, with similarities in peak distribution profiles among β-kafirin null mutants compared with normal lines.

Commercial production of plant-derived fuels presents an effective strategy for reducing our reliance on fossil fuels and increasing energy security (U.S. Department of Energy 2012). Grain ethanol production involves the conversion of starch to ethanol with the enzyme α-amylase to aid gelatinization and liquefaction and glucoamylase for production of fermentable sugars or saccharification. Bioethanol can be employed as a gasoline extender and fuel oxygenate, and distribution is aided by existing infrastructure (U.S. Department of Energy 2006). The fermentation process generates valuable by-products, including distillers dried grains with solubles (DDGS), which are marketed as high-quality feed products. Under the Renewable Fuels Standard Energy Independence and Security Act (Renewable Fuels Association [RFA] 2007), the recommendation has been for an increase in the production of bioethanol to 136 billion liters by 2022, with government legislation in the European Union, the United States, Brazil, Australia, and others mandating increased fuel ethanol components of up to 30% by 2025 (Plaza 2012). The largest producers of bioethanol are the United States and Brazil, accounting for almost 90% of global production (Lichts 2011). Maize is the major feedstock for the bioethanol industry in the United States, representing 95% of the total 56 billion liters produced there in 2011 (RFA 2012). In Brazil, nearly half the nation's vehicles run on fuel ethanol produced from sugarcane. However, in recent years negative impacts have been associated with the production of some forms of biofuels. For example, maize cropping requires relatively high irrigation and fertilizer inputs, causing drought susceptibility and nitrification of waterways (Farré and Faci 2006; Donner and Kucharik 2008). Additionally, it has been reported that burning cane trash for fuel production generates carbon emissions similar to those associated with fossil fuels (Tsao et al 2012). Thus, sustainable alternatives to these bioethanol feedstocks are being sought, particularly in regions of low water availability. One viable solution is biofuel from sorghum grain. An efficient C4 assimilator with less fertilizer inputs required to achieve optimal yields compared with other crops (Kim and Day 2011), sorghum tolerates a range of soil conditions and exhibits a high level of drought tolerance, requiring half the water needed to grow corn and a quarter the water required for sugar cane (Pedersen and Rooney 2004). Ethanol produced from grain sorghum at facilities that use biogas digesters and specifically combined heat and power technology qualifies as an advanced biofuel, meeting greenhouse gas emissions reduction thresholds (U.S. Environmental Protection Agency 2012).

The efficiency with which grain is converted into fuel is largely dependent on the structural features and chemical composition of the seed endosperm. Factors directly affecting fermentation efficiency, yield, and DDGS quality include starch content and composition, proportion of amylose to amylopectin, flour viscosity, crude protein content, digestibility, and condensed tannins in tannin-containing sorghum (Wu et al 2006, 2007; Yan et al 2009, 2010). These traits are genetically controlled but are also strongly influenced by agronomic factors such as water and nutrient availability. Sorghum digestibility is reduced by extensive cross-linking among proteins in the grain endosperm (Duodu et al 2003). This reduced digestibility may limit enzymatic accessibility to starch in grain sorghum relative to maize, impacting on ethanol conversion efficiency. Storage proteins are synthesized on the endoplasmic reticulum and deposited as vacuolar protein aggregates, which develop into protein bodies, encasing starch in the

[1] School of Agriculture and Food Sciences, The University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia.
[2] Corresponding author. Phone: +61-3365-2141. Fax: +61-3365-1177. E-mail: j.cremer@uq.edu.au
[3] Department of Biological and Agricultural Engineering, Kansas State University, Manhattan, KS 66506, U.S.A.
[4] U.S. Department of Agriculture, Agriculture Research Service (USDA-ARS), Center for Grain and Animal Health Research, Manhattan, KS 66502, U.S.A. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.
[5] USDA-ARS Cereal Crops Research Unit, Fargo, ND 58102, U.S.A.
[6] Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, U.S.A.
[7] Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia.

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seed (De Mesa-Stonestreet et al 2010). Sorghum and maize endosperm contain relatively high proportions of proline-rich prolamins, which are hydrophobic in nature and develop highly folded, complex tertiary structures, reducing in vitro endosperm solubility (Shull et al 1992; Momany et al 2005). Sorghum and maize prolamins, referred to as kafirins and zeins, respectively, exhibit extensive sequence homology and a similar relative distribution in the protein bodies (DeRose et al 1989). However, variation in the functional characteristics and degree of polymerization of the proteins accounts for differences in grain quality traits such as digestibility and ethanol conversion across maize and sorghum (Hamaker and Bugusu 2003; Zhao et al 2008). Immunolocalization studies indicate that cysteine-rich β- and γ-kafirins are located on the periphery of sorghum protein bodies, whereas the α-kafirins fill the interior of the structure, along with small amounts of δ-kafirin (Oria et al 2000). A line of high-digestibility sorghum mutants were found to exhibit an altered protein body structure in the grain, with the more hydrophobic β- and γ-kafirins relocated from the periphery of the bodies into folds in the structure, increasing centrally located α-kafirin exposure to protease activity (Oria et al 2000; Tesso et al 2008). Improvements in endosperm digestibility in these lines was directly correlated to the high-digestibility mutant allele dosage and translated into higher ethanol production efficiency in fermentation studies (Wu et al 2010). Sequencing and molecular analysis of the β-, γ-, and δ-kafirin genes across a variety of sorghum commercial hybrids and wild relatives revealed a wide range of allelic diversity (Izquierdo and Godwin 2005; Laidlaw et al 2010). A mutation in the β-kafirin gene was identified in several sorghum varieties, in which a single cytosine insertion results in a frame shift and early termination codon. Functional analysis showed that β-kafirin null line QL12 exhibits altered flour viscosity, presumably because of changes in β-kafirin levels in endosperm protein bodies. Genetic characterization of the kafirins across diverse sorghum lines has facilitated investigation of the effects of variation in seed storage proteins on ethanol production in sorghum grain. Thus, the aim of this study is to evaluate the impact of kafirin allelic diversity on ethanol conversion efficiency across a selection of sorghum lines, characterized for kafirin genetic background, seed biochemistry, and composition. The study will carry forward research into sorghum-based ethanol production and subsequently allow for identification of key factors affecting ethanol bioconversion, illustrating how they are influenced by the composition of the protein–starch matrix.

MATERIALS AND METHODS

Plant Genotypes, Seed Weight, and Sample Preparation. Mature grain from 10 commercial grain sorghum hybrid parent inbreds (Table I) with varying genetic backgrounds for the kafirin seed storage proteins was harvested at the University of Queensland Gatton campus during the 2011–2012 summer cropping season. Sorghum varieties were selected from a sample population previously characterized for allelic variation in β-, γ-, and δ-kafirins. Lines with sequenced genomes and additional reported resistance to environmental stress were included in the panel. Seed weight was measured in grams per hundred grains and then averaged to milligrams per grain. Whole grain was milled through a UDY sample mill (UDY Corp., Fort Collins, CO, U.S.A.) fitted with a 0.5 mm mesh screen for all analytical procedures.

Starch Analysis. Total starch in 200 mg of milled sorghum samples was determined in duplicate with a colorimetric technique following AACC International Approved Method 76-13.01 utilizing a dimethyl sulfoxide pretreatment for resistant starch (K-TSTA, Megazyme International Ireland, Bray, Ireland). Starch was isolated from the milled samples by the sonication method of Park et al (2006). Amylose content was determined on the isolated starch samples by the concanavalin A precipitation assay (K-AMYL, Megazyme).

Crude Protein Digestibility. Protein digestibility was determined in duplicate following a previously described method for measuring in vitro pepsin digestibility (Mertz et al 1984). Briefly, 200 mg of milled sorghum flour per sample was mixed in 35 mL of pepsin solution (1.5 mg/mL in 0.1M phosphate buffer containing KH_2PO_4 and H_3PO_4, pH 2) and incubated at 37°C for 2 h. After incubation, 2 mL of 2M NaOH was added, and the sample was vortexed and centrifuged (3,220 × *g*, 15 min). The supernatant was then discarded. Residue was washed in 10 mL of 0.1M phosphate buffer, pH 2, centrifuged (3,220 × *g*, 15 min), and the supernatant discarded. Washing steps were repeated, and after the second wash and centrifugation the samples were placed in a –80°C freezer (Romulus Holding Company, New York, NY, U.S.A.). Prior to nitrogen analysis, samples were lyophilized (Labconco, Kansas City, MO, U.S.A.), and total protein content was measured through nitrogen combustion (LECO Corporation, St. Joseph, MI, U.S.A.).

Flour Moisture Content. Moisture readings were taken from 1 g of milled sorghum flour per sample in duplicate with an MX-50 moisture analyzer (A&D, Tokyo, Japan). Average moisture content was calculated as the mean across the duplicates.

Ethanol Production and Fermentation Efficiency. Analysis of total ethanol production and fermentation efficiency was carried out as previously described (Wu et al 2008). Ground sample (30 g dry mass) was combined with 100 mL of heated (≈60–70°C) enzyme solution (0.1 g of KH_2PO_4 and 20 μL of Liquozyme heat-stable α-amylase containing enzyme preparation per liter) (Novozymes North America, Franklinton, NC, U.S.A.) in an Erlenmeyer flask to form a uniform slurry with shaking (180 rpm) at 70°C. Liquefaction of the slurry was initiated by increasing temperature from 70 to 90°C for 30 min, holding at 90°C for 5 min, and then reducing the temperature to a constant 86°C for a further 60 min. Material on the sides of flasks was pushed back into slurry and rinsed with 3–5 mL of dH_2O. The mashes were cooled to room temperature and adjusted to pH 4.2 with 2N HCl. Dry ethanol yeast (Ethanol Red, Lesaffre Yeast Co., Milwaukee, WI, U.S.A.) was activated with 1 mL of preculture broth (20 g of

TABLE I
Sorghum Grain Lines Characterized for Allelic Variance in the Kafirin Storage Proteins and Tested for Ethanol Production Efficiency

Lines/Genotypes	β-Kafirin Allele	γ-Kafirin Allele	δ-Kafirin Allele	Origin	Comments
QL12	GU732403 null	M73688	AY834250	Australia	Staygreen male parental line, yellow endosperm
B923296	GU732401	M73688	AY834250	Australia	Elite staygreen female parental line
ICSV400	GU732404	M73688	AY834250	Mali	Breeding line for malting and grain yield
M35	GU732401	GU732407	AY834250	India	Drought resistant, cooking-quality landrace
KS115	GU732401	GU732408	AY834250	United States	Breeding line, large seed, yellow endosperm
IS17214	GU732403 null	M73688	AY043223	Nigeria	Landrace
BOK11	GU732401	M73688	AY043223	United States	Inbred breeding line: dwarf hydro × rice, kafir
BT×623	AJ717660	M73688	AY834250	United States	Inbred breeding line, zerazera-caudatum
296B	GU732401	GU732407	AY834250	India	Inbred female parental line: dwarf, high yielding
RT×2737	GU732403 null	M73688	AY834250	United States	Commercial hybrid breeding line, staygreen

glucose, 5 g of peptone, 3 g of yeast extracts, 1 g of KH_2PO_4, and 5 g of $MgSO_4 \cdot 7H_2O$ per liter) and incubated at 38°C for 25–30 min with shaking at 200 rpm. Simultaneous saccharification and fermentation was initiated with 1 mL of activated yeast culture, 100 µL of Spirizyme glucoamylase-containing enzyme preparation (Novozymes North America), and 0.3 g of yeast extract. Flasks were sealed with an S-airlock filled with mineral oil. Fermentation was carried out at 30°C for 72 h with shaking at 150 rpm. Fermentation efficiency was calculated by monitoring weight loss of the mash as a result of CO_2 diffusion during the fermentation process. Ethanol concentration following distillation (conducted as described in Yan et al 2010) was quantified by HPLC with a Rezex RCM-monosaccharide column (300 × 7.8 mm) and a reflective index detector (RID-10A, Shimadzu, Columbia, MD, U.S.A.). The mobile phase through the column was 0.6 mL/min of dH_2O at a constant temperature of 80°C. Fermentation efficiency was calculated according to theoretical yield of 56.72 g from 100 g of dry starch.

Free Amino Nitrogen (FAN) Analysis. FAN was determined according to previously described methods (European Brewery Convention 1987) with modification. Sorghum flour (150 mg) was mixed with 1.5 mL of deionized distilled water in a 2.5 mL microcentrifuge tube, vortexed five times in 10 min, and then centrifuged at 12,000 rpm for 20 min. A 1.0 mL aliquot of supernatant was diluted with 9.0 mL of distilled water and then analyzed for FAN.

Reversed-Phase HPLC (RP-HPLC). Alcohol-soluble proteins (prolamins) were isolated from milled flour samples as described in Bean et al (2010). Briefly, flour was dissolved in 1 mL of extraction solvent (60% tertiary butanol, 0.5% sodium acetate, and 2% β-mercaptoethanol). The pellet was then vortexed for 5 min and centrifuged at 10,000 rpm for 4 min, and 500 µL of supernatant was transferred to a new tube. The procedure was repeated once, and a further 500 µL of supernatant was collected and pooled 1:1. Finally, 33 µL of 4-vinylpyridine was added to each prolamin sample and vortexed for 10 min for alkylation of proteins. Protein samples (5 µL injections) were analyzed on an Agilent 1100 HPLC system (Agilent, Foster City, CA, U.S.A.) fitted with a Poroshell C-18 column, 2.1 × 75 mm (Agilent), with a previously described gradient (Bean et al 2010). Detection was by UV at 214 nm.

Lab on a Chip. The lab-on-a-chip procedure was carried out on the Agilent 2100 bioanalyzer with alcohol-soluble protein samples extracted as for the RP-HPLC methods described earlier and processed with a Protein 80 assay kit (Agilent). A 4 µL aliquot of each protein sample was combined with 2 µL of denaturing buffer containing β-mercaptoethanol in a 0.5 mL microcentrifuge tube. Sample tubes and an additional tube containing 6 µL of ladder were heated to 95°C for 5 min, cooled, and centrifuged. dH_2O (84 µL) was added to each tube, and samples were vortexed and spun briefly. Protein samples (6 µL), ladder (6 µL), and gel dye (12 µL) were loaded into the appropriate well on the chip. The chip was inserted into the bioanalyzer and analyzed according to the manufacturer's instructions.

Statistical Analysis. Significance of correlation among factors affecting ethanol conversion was illustrated with Microsoft Excel. Letters indicating significance of differences (Table II) were generated in Minitab by using an ANOVA general linear model with pairwise comparisons according to Tukey methods with 95% confidence. Principal component analysis (PCA) was carried out for seed parameters (outlined in Table II) in Minitab by using multivariate analysis within a correlation matrix. In addition, generalized linear mixed models were applied in the R platform (fitted with a Poisson distribution) for multivariate analysis of the relationship between seed biochemical features, kafirin alleles, and ethanol production efficiency.

RESULTS AND DISCUSSION

Genotypic Associations with Fermentation Efficiency and Ethanol Yield. The performance of a selection of sorghum genotypes (Table I) was evaluated for fermentation efficiency and total ethanol yield to determine how variation in endosperm protein–starch matrix impacts on ethanol conversion. Total ethanol yields across the lines varied by 10.8%, and fermentation efficiency varied by 26% at 20 h and by 5.4% at 72 h (Table II). The grain types produced between 384 and 426 L of ethanol per metric ton (Fig. 1), with fermentation efficiencies of 68–85% at 20 h and 87–92% at 72 h, similar to values obtained in comparable studies (Wu et al 2007, 2010). Total starch ranged between 65.52 and 74.29%, similar to other studies (Corredor et al 2006; Wu et al 2013a). Commercial line B923296 was competitive with maize in terms of ethanol yield (Mueller and Kwik 2013), producing the highest level of ethanol across the lines at 426 L/metric ton (2.86 gallons per bushel). This genotype displayed high fermentation efficiency at the 20 and 72 h time points, high starch, and good digestibility, traits that likely combined to enhance yield (Table II). Genotypes IS17214 and ICSV400 also yielded well (420 L/metric ton each), both with a high starch content and moderately large seed size, although fermentation efficiency was relatively low in these lines. KS115 and BT×623, each with a high crude protein content, low digestibility, and low starch, produced the lowest overall ethanol yields at 384 and 395 L/metric ton, respectively. However, the proportion of ethanol produced per gram of starch (Table II) was comparable to most other genotypes, signifying that low starch content is the major factor re-

TABLE II
Kafirin Allelic Profiles, Biochemical Measurements, and Ethanol Fermentation Characteristics for Selection of Sorghum Grain Cultivars, with Mean Values and Standard Deviations (SD) Calculated Across Replicate Sample Measurements[z]

Cultivar	Seed Weight (mg)	Crude Protein (%)	Protein Digestibility (%)	Starch (%, dry basis)	Amylose (%)	FAN (mg/L)	Fermentation Efficiency (%) at 20 h	Fermentation Efficiency (%) at 72 h	Ethanol Yield (L/metric ton)	Ethanol (mL/kg of Starch)
QL12	28.6	14.57b	58.42	70.11b	21.76bc	33.0e	70.44	88.40cde	410.69c	585.79abc
B923296	28.0	11.9d	61.16	71.0b	23.58a	32.84e	73.63	90.42ab	426.91a	601.41a
ICSV400	44.6	11.95d	56.93	71.50b	21.93bc	39.67e	69.97	88.57bcde	420.94ab	588.71ab
M35	30.4	12.93c	48.65	71.06b	21.53c	36.54e	69.69	89.55bcd	415.80bc	585.13abc
KS115	72.5	16.47a	40.83	65.52c	23.19ab	73.51a	85.68	90.21abc	384.37e	586.61abc
IS17214	43.0	11.43d	59.22	74.29a	23.31ab	37.61e	67.98	88.14de	420.30ab	565.78cd
BOK11	29.1	11.67d	65.03	70.34b	22.60abc	51.33c	72.55	88.19de	414.11bc	588.74ab
BT×623	32.2	12.93c	50.8	66.29c	22.47abc	48.13cd	75.13	92.05a	395.66d	596.83ab
296B	31.1	9.96e	68.21	73.85a	19.74d	64.27b	74.24	87.67de	415.67bc	562.88d
RT×2737	31.1	13.3c	58.12	71.4b	19.74cd	40.45de	73.36	87.36e	413.45bc	579.06bcd
Mean	37.1	12.7	56.7	70.5	22.0	45.7	73.3	89.1	411.8	584.1
SD	…	0.127	8.059	0.350	0.381	1.452	…	0.339	1.551	3.959

[z] Means that do not share a letter are significantly different ($P < 0.5$). FAN = free amino nitrogen.

stricting yield in these lines. Fermentation efficiency in KS115 of 85.68% at 20 h exceeded efficiencies of other genotypes by more than 10%, indicating that this line could perform well in a short-term fermentation system, particularly if starch content were improved.

Endosperm Starch and Protein Effects on Fermentation. The structure and degree of cross-linking among protein bodies in the starch matrix has significant effects on digestibility, starch accessibility, and ethanol conversion (Duodu et al 2003; Wu et al 2007). Crude protein and starch levels across the lines in the current study were similar to those attained in previous analyses (Wu et al 2008; Zhao et al 2008; Yan et al 2011). Starch content was shown to have a major influence on grain ethanol production, in which a strong positive linear correlation was observed between total starch and ethanol yield (R^2 = 0.736) (Fig. 2), as observed in past studies (Wang et al 2008; Yan et al 2011). Of the genotypes tested, the β-kafirin null line IS17214 contained the highest amount of total starch and produced the second highest ethanol yield, with above-average protein digestibility and medium-level fermentation efficiency. β-Kafirin null allelic variants QL12 and RT×2737 contained lower starch compared with IS17214, which is likely to be the main factor accounting for their reduced ethanol yield, comparatively, because protein digestibility was above average in these lines.

With regard to starch composition, all the genotypes tested in the study were normal in terms of the amount of amylose present

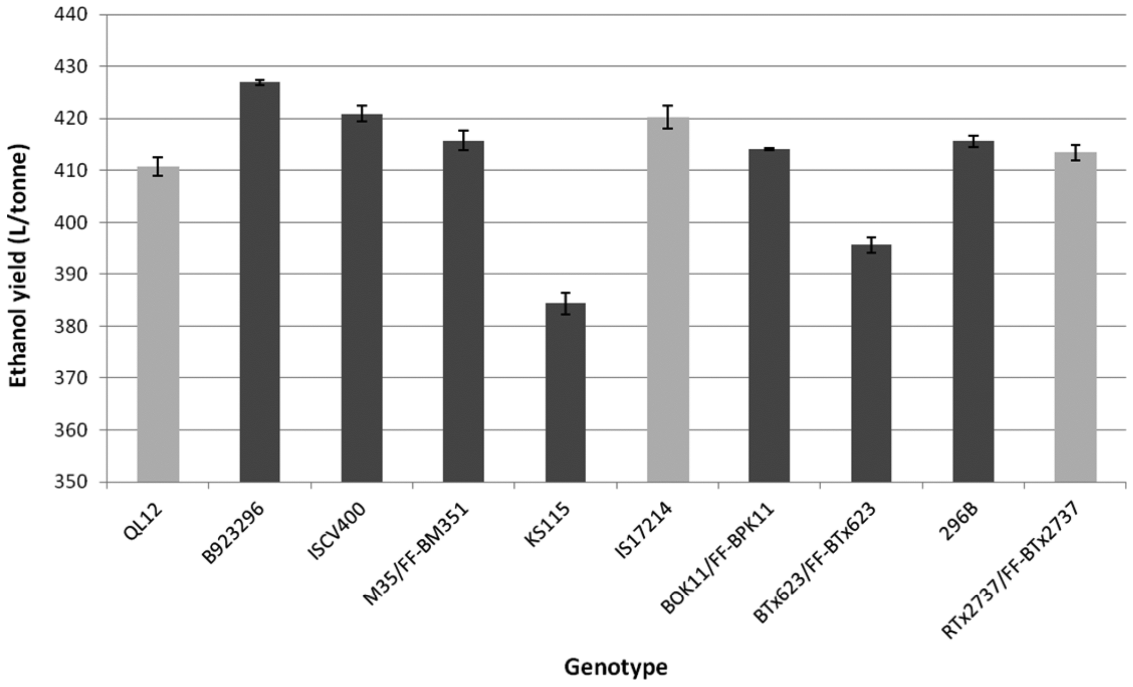

Fig. 1: Ethanol yield (L/metric ton) produced by 10 sorghum lines with allelic variation in the kafirin storage proteins. β-Kafirin null mutants IS17214 and RT×2737 (light gray) produced above-average ethanol yields, with QL12 producing average yields.

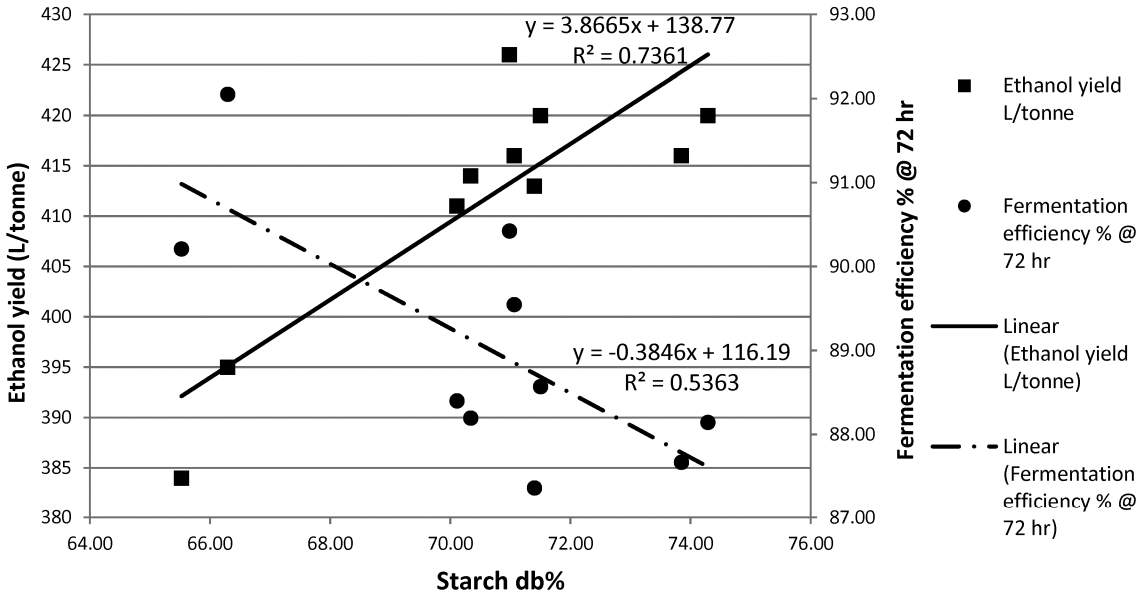

Fig. 2: Relationship between starch content, fermentation efficiency, and ethanol yield across 10 sorghum genotypes. A significant positive correlation (R^2 = 0.74) was observed between total starch (% dry basis) and ethanol yield (L/metric ton). There was also a weak, although significant, negative correlation (R^2 = 0.54) between starch content and fermentation efficiency at 72 h.

(Table II), that is, none of the samples were waxy genotypes. Only minor differences in amylose content (≈19–24%) were found across all the genotypes. There was no correlation between amylose content and ethanol yield (data not shown), suggesting that within this normal range, amylose-to-amylopectin ratio did not play a role in determining ethanol fermentation properties of the sorghum genotypes used in this study. It has been demonstrated that more subtle changes in starch structure contribute to enhanced digestibility (Gilding et al 2013). These changes were not measured in this study; however, it is conceivable that starch structural differences associated with higher digestibility may, at least in part, also contribute to ethanol yield differences.

Protein digestibility and ethanol yield were positively correlated across the genotypes (P = 0.019) (Fig. 3), as previously observed (Zhan et al 2006; Wu et al 2007; Zhao et al 2008). Additionally, β-kafirin had significant, although relatively small, impacts on digestibility as observed with multivariant analysis (P = 0.0416), in which digestibility was slightly higher than average in null β-kafirin mutant lines. Previously, Zhao et al (2008) showed that increases in ethanol yield and conversion efficiency occur as the amount of extractable proteins from sonication of mashed samples increases, indicating that endosperm digestibility impacts directly on ethanol production. Conformational changes in endosperm protein structure following cooking further influence the accessibility of amylolytic enzymes to starch. Zhan et al (2006) demonstrated this change with supercritical fluid extrusion cooking of sorghum grain prior to fermentation, showing that alterations to the protein matrix that enhance starch accessibility also increase ethanol yield.

FAN Impact on Fermentation Efficiency. Fermentation requires an adequate supply of nitrogenous compounds to fuel yeast growth and proliferation. Release of FAN during enzymatic breakdown of endosperm proteins provides a grain-specific source of amino nitrogen. Nitrogen deficiencies have been reported as a major cause for a slow fermentation (Moreira et al 2011; Peralta-Contreras et al 2013). Low FAN levels can be supplemented in the industrial fermentation process with a mix of amino acids and ammonium sulfate at the exponential phase of yeast growth, enhancing fermentation rate and ethanol conversion efficiency but increasing commercial costs. A strong positive association was observed in the current study between FAN and fermentation efficiency at the 20 h time point (R^2 = 0.647), in agreement with past work (Yan et al 2009, 2010; Wu et al 2010). This relationship was exemplified by KS115, which displayed relatively high levels of FAN and performed well in the early stages of the fermentation process (Fig. 4). An antagonistic relationship appears to occur among factors affecting ethanol yield and fermentation efficiency, as illustrated through PCA (Fig. 5), in which protein digestibility and total starch are positively associated with ethanol yield, whereas FAN content and total protein are associated with increased fermentation efficiency. Moreover, a significant correlation was identified between the β-kafirin allele and FAN levels (P = 0.0357), indicating that diversity at this locus will have a potential effect on fermentation efficiency. In a previous analysis of normal versus waxy sorghums, genotypes with high crude protein and FAN content exhibited high fermentation efficiencies but generally with reduced ethanol yields compared with low-protein, high-digestibility varieties (Yan et al 2011). KS115, a large-seeded grain type with a high proportion of FAN-rich embryo, exhibited remarkably high fermentation efficiency in the early stages of the fermentation process (Fig. 4) but performed poorly in terms of overall ethanol yield.

Kafirin Allelic Effects on Ethanol Conversion Efficiency. The interconnectivity of seed storage proteins governs the susceptibility of matrix components to proteolysis, impacting on starch availability and fermentation profile (Duodu et al 2003). Previous work has shown that grain kafirin content is significantly linked to variation in seed biochemistry, including fat, protein, and starch content, as well as seed weight (Hicks et al 2001). In the current investigation, the β-kafirin null lines IS17214 and RT×2737 produced higher than average yields, with QL12 producing average yields (Table II). Digestibility, starch, and FAN profiles were similar for lines carrying a β-kafirin null allele, indicating that an analogous kafirin profile may produce corresponding similarities in grain characteristics. Among the genotypes, KS115 exhibited a significantly higher seed weight. Seed weight is controlled by a

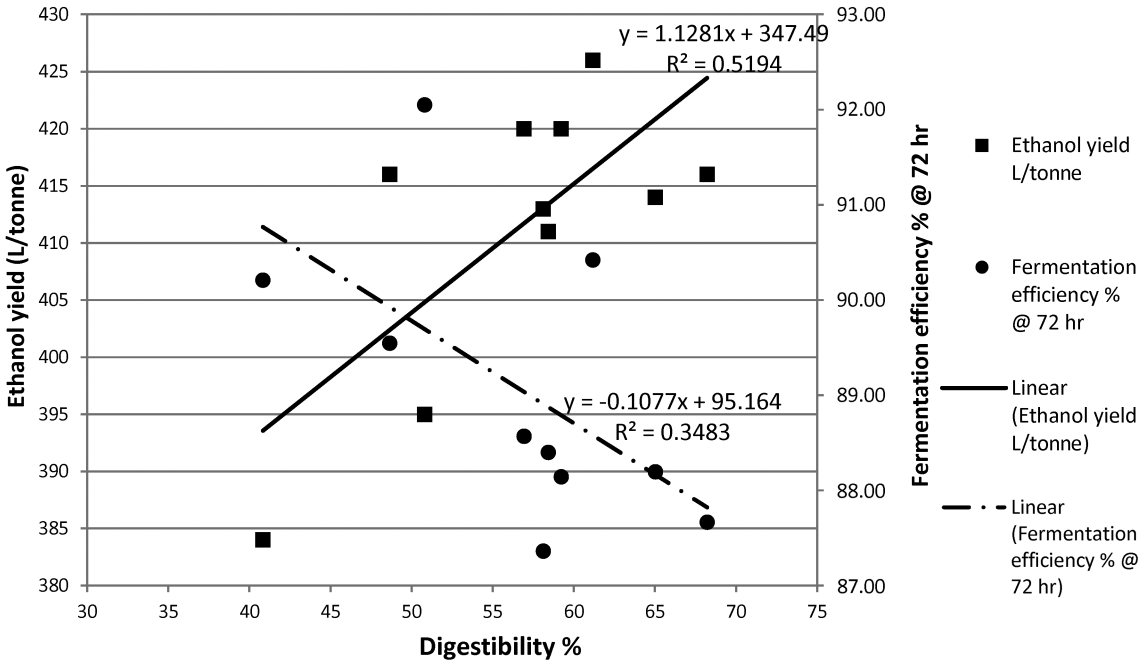

Fig. 3: Associations between protein digestibility (%), fermentation efficiency at 72 h (%), and ethanol yield (L/metric ton) across 10 sorghum genotypes. A positive linear relationship was observed between digestibility and ethanol yield (R^2 = 0.5194), indicating a low, yet significant, correlation, also shown with multivariate analysis (P = 0.019). A trend toward an association between digestibility and fermentation efficiency was also observed (R^2 = 0.3483), although this was not a significant correlation (P = 0.072) within this data set.

number of factors, including various embryo- and endosperm-specific regulators (Sundaresan 2005). However, the composition of endosperm storage proteins, such as the kafirins, is also linked to the large-seeded trait (Hicks et al 2001). KS115 carries a novel γ-kafirin allele and exhibited high fermentation efficiency in the early stages of the conversion process but with low ethanol yields produced overall.

Alterations in the positioning of the kafirins located on the periphery of the protein bodies was shown to increase grain digestibility and ethanol production efficiency, indicating that regulatory changes to β- or γ-kafirin expression may instigate these changes, similar to the MW 22,000 α-kafirin mutation in high-digestibility lines (Wu et al 2013b). Lines M35 and B923296 each displayed higher digestibility and starch content and lower FAN compared with KS115. Variation in γ-kafirin allelic background, in which β- and δ-kafirin alleles are identical between the poor ethanol producer KS115 and mid- to high-yielding M35 and B923296, indicates a possible link between kafirin genetic background and ethanol conversion. The various γ-kafirin alleles encode for identical protein sequences. However, kafirin expression may be differentially regulated at the transcriptional level, resulting in variable grain kafirin content. In any case, significant diversity was ob-

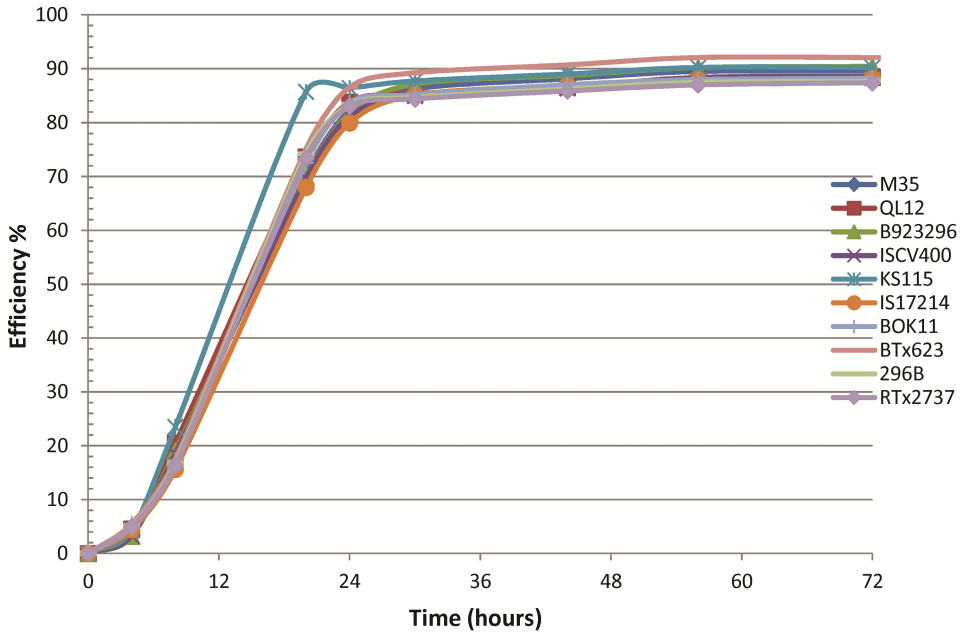

Fig. 4: Fermentation efficiency (%) versus fermentation time. KS115 is an outlier that exhibits high efficiency in the early stages of the fermentation process relative to other genotypes.

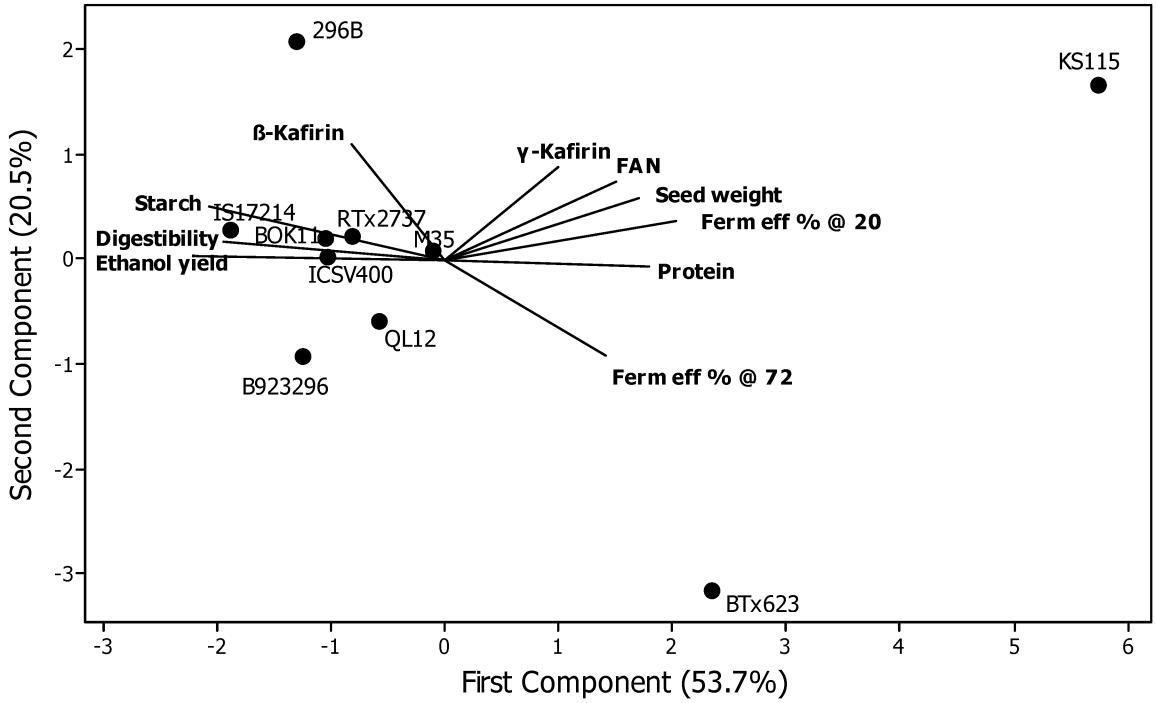

Fig. 5: PCA of parameters affecting fermentation efficiency and ethanol yield. Starch and protein digestibility are positively correlated to yield, and crude protein content and FAN levels are positively correlated to fermentation efficiency. The β-kafirin allele is more strongly related to digestibility and ethanol yield, whereas γ-kafirin shows a positive association with fermentation efficiency and free amino nitrogen (FAN).

served in the kafirin RP-HPLC peak distribution profile for KS115 compared with other genotypes, with a greater number of peaks eluting at 10–12 min in KS115 and an additional peak appearing at 12.5 min that was not observed in other lines (Fig. 6). The observed differences on profiles raise the question as to whether there is a specific kafirin allelic combination linked to increased protein digestibility and ethanol conversion efficiency. Through this study, it appears that the content of β-kafirin in the seed significantly impacts on ethanol production and that γ-kafirin could also play a distinct role, as observed in KS115.

Regulation of Kafirin Seed Storage Proteins. Cosuppressing the synthesis of various prolamin subclasses in sorghum, including α-, β-, and γ-kafirins, has been shown to increase grain protein digestibility (da Silva et al 2011). Kumar et al (2012) reported that down-regulation of α-kafirin in the sorghum endosperm resulted in increased endosperm digestibility; however, altering γ-kafirin expression in isolation had no apparent phenotypic effects on protein body morphology or cooked flour digestibility (Wu and Messing 2010; Kumar et al 2012). In maize, quantitative trait locus analysis correlated starch digestibility to chromosome regions already linked to the zeins (Lebaka et al 2007). Mutations in the maize *opaque-2* gene resulted in a 50% reduction in zein, exhibiting a floury or opaque endosperm, with improved in situ starch digestibility and ethanol conversion (Aukerman et al 1991). Variation in the β-kafirin allele across the collection of grain types analyzed in the present study resulted from altered expression of the gene, such as in QL12, in which production of a truncated protein caused distinct changes to seed biochemistry, including digestibility. The relatively small but significant increase in digestibility and ethanol yield in the β-kafirin null lines compared with normal lines indicated that some degree of functional redundancy may exist between β- and γ-kafirin. The relationship between seed kafirin content and grain quality parameters, such as digestibility and ethanol conversion efficiency, observed in this study justifies further investigation into interactions among the kafirins and the mechanisms regulating their targeting to the endosperm.

Effects of Seed Size on Ethanol Conversion. Large-seeded hybrids often contain higher levels of crude protein and fat and less starch than small-seeded lines and hybrids (Kriegshauser et al 2006). KS115, which has a high seed weight, is rich in protein and fat, and is low in starch (Hicks et al 2002), produced the lowest ethanol yield across the lines. Grain containing low levels of amylose (for example, waxy or heterozygous waxy types) are known to perform better in the fermentation process (Wu et al 2006, 2010; Wang et al 2008). Ethanol conversion may have been impeded in this line by low total starch content or by relatively high levels of amylose-lipid complex in the grain endosperm. The KS115 seed contains a higher proportion of protein-rich embryo relative to starchy endosperm (Yang et al 2009), providing less starch for conversion to ethanol, despite the large grain size. It is unknown whether the large-seeded structure is related to changes in storage protein composition, such as increased kafirin content, or to changes in the regulation of protein aggregation. In rice, the polycomb complex *OSFIE2* has been linked with certain aspects of grain filling and seed size, including regulation of the starch synthesis rate-limiting step and multiple storage compounds (Nallamilli et al 2013). Homologs to the polycomb complex gene family were identified in other major cereals, such as maize, indicating that large-seeded sorghum may carry an alternate allele for an *OSFIE2* ortholog, causing specific changes to seed morphology. RP-HPLC analysis of protein content and composition across

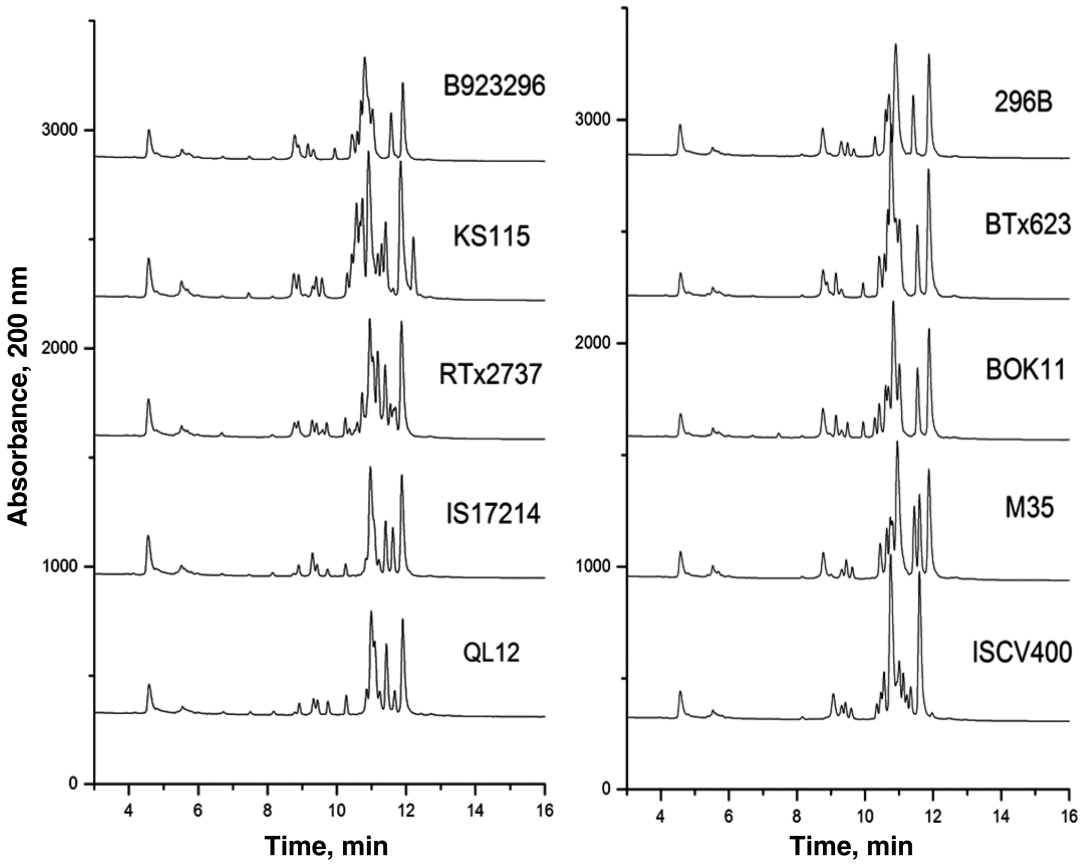

Fig. 6: Reversed-phase HPLC peak distributions for the alcohol-soluble kafirin protein fraction across 10 sorghum genotypes evaluated for ethanol production efficiency. Peak profiles for β-kafirin null allelic variants QL12, IS17214, and RT×2737 are similar in size and distribution, indicating similarities in protein composition. Chromatograms for low-digestibility lines, such as KS115 and BT×623, show large peak heights and a relatively diverse peak distribution profile.

the genotypes showed that levels of alcohol-soluble protein were higher in KS115 grain than in the more digestible, higher yielding lines, with the KS115 chromatogram exhibiting larger peak areas for the kafirin-containing fraction and a more diverse peak distribution profile (Fig. 6). A greater proportion of kafirin in the grain endosperm is likely to contribute to the low digestibility and poor ethanol yield through reduced enzyme accessibility to starch. Lab-on-a-chip size-based separation of alcohol-soluble proteins across the genotypes provided additional evidence of the high content of insoluble protein in KS115, relative to other lines (Fig. 7). KS115 grain has been recommended as a valuable component in animal feed, because of its high fat content, which provides a major energy source for livestock (Hicks et al 2002). However, with a low starch content and high susceptibility to grain molds, breeding strategies for large-seeded cultivars for fuel ethanol would likely involve introgression of genes for large seededness and embryo size into backgrounds with high starch content and protein digestibility coupled with improved stress resistance.

Variation in grain prolamin profile has been reported to have major impacts on grain quality, accounting for differences in digestibility and ethanol conversion rates across different genotypes (Wu et al 2006; Wong et al 2009). RP-HPLC peak distribution profiles for alcohol-soluble protein from β-kafirin null allelic variants (QL12, IS17214, and RT×2737), compared with genotypes expressing a functional form of β-kafirin, revealed that the mutation appears to be associated with the disappearance of a major set of protein peaks eluted around 10.5–11 min (Fig. 6). Further investigation beyond the scope of this paper into the precise identity of the missing alcohol-soluble proteins in β-kafirin null lines and their impact on grain quality is warranted. QL12 crude protein levels were relatively high (Table II) compared with other genotypes, which was not reflected in RP-HPLC and lab-on-a-chip analysis of the alcohol solubles. This difference may be attributed to a high content of albumins, globulins, or glutelins, as observed with subsequent analysis of water/salt-soluble protein for this line (data not shown).

Candidate Traits for Sorghum Grain Biofuels Breeding Program. A number of quality traits contribute to the efficiency with which starch is converted to ethanol in the production of grain-based fuels. This investigation revealed that differential expression of the β-kafirin gene had significant, although conservative, impacts on ethanol production through changes to protein digestibility and FAN content. The important role of starch in determining the suitability of a grain crop for conversion to bioethanol was further verified, in which a tight positive correlation was observed between total starch content and ethanol yield within this dataset. Lines BT×623 and KS115 had the lowest starch contents and, subsequently, the lowest ethanol yields. Elite Australian line B923296 produced high ethanol yields with an efficient fermentation profile and was the third most digestible grain among the genotypes tested, indicating its value as a potential bioethanol feedstock, among other high-yielding lines in the study. B923296 was previously incorporated into breeding programs for staygreen traits and midge resistance. The β-kafirin null line QL12 and the Indian line M35 were also incorporated into breeding programs as staygreen parental varieties and were efficient ethanol producers, although they exhibited slightly lower starch and digestibility relative to B923296. Large-seeded types, exemplified by KS115, fermented efficiently in the initial stages of the conversion process but produced very low yields because of insufficient starch. However, the line did appear to produce ethanol efficiently per kilogram of starch available, compared with other lines (Table II). These lines represent a valuable source of germplasm for use in research and development and could be exploited to contribute useful ethanol-related traits to breeding

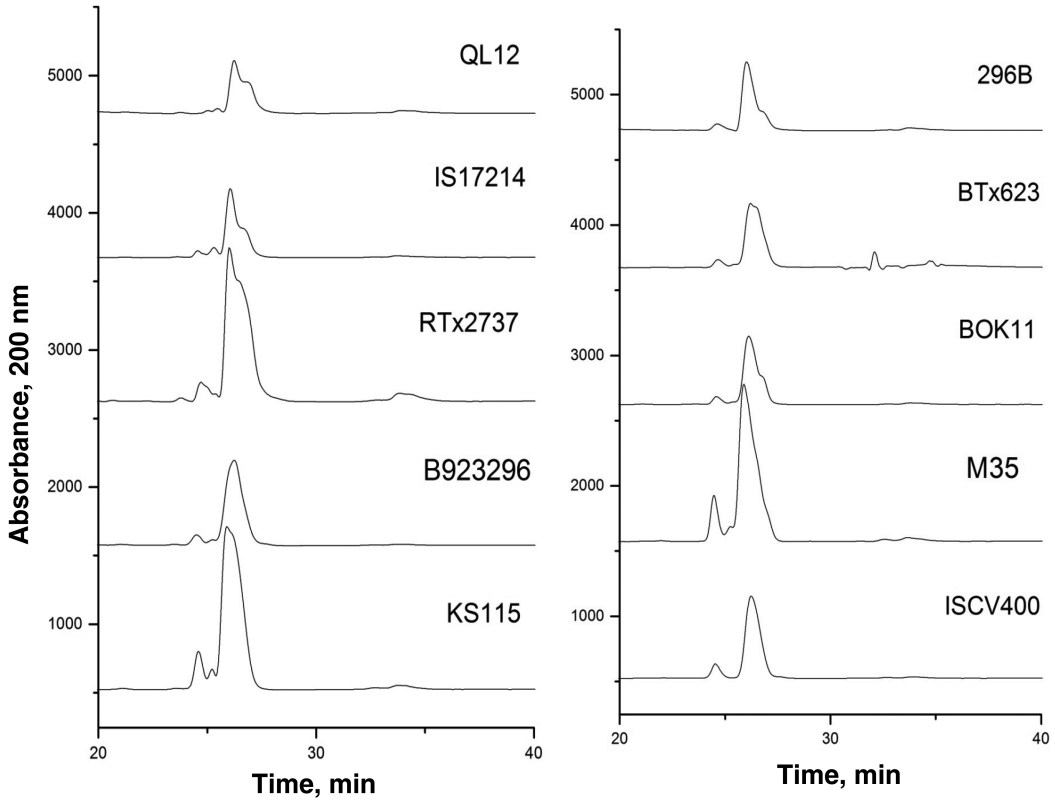

Fig. 7: Lab-on-a-chip size-based separation of the alcohol-soluble kafirin fraction in sorghum lines evaluated for ethanol fermentation. β-Kafirin null mutants QL12 and IS17214 have low kafirin content, similar to highly digestible line 296B. β-Kafirin null RT×2737 shows higher levels of kafirin protein comparatively, as was observed with reversed-phase HPLC, in which peaks areas were larger and distribution profile was more diverse. KS115 and M35 exhibited relatively high kafirin content, contributing to the reduced digestibility of these lines.

programs aimed particularly at increasing seed size and improving early stage ethanol production efficiency in sorghum.

CONCLUSIONS

Endosperm protein structure and composition play an important role, in addition to starch, in determining the suitability of a grain crop for bioethanol production. Here it was illustrated that fermentation yield and efficiency are determined by key quality parameters, such as starch and protein content. These traits are strongly influenced by the specific expression and interaction of endosperm storage proteins. Genetic variation in β-kafirin alters digestibility and FAN content, with subsequent effects on ethanol conversion. This work recommends sorghum with high starch content, high digestibility, and low levels of β-kafirin for further development in the grain-based ethanol industry. Large-seeded varieties produce ethanol more efficiently in the short term owing to a high FAN content and possible variation in the regulation of γ-kafirin, providing a valuable source of germplasm for breeding initiatives aimed at improving fermentation rate. Specific investigation of correlations between the γ-kafirin allele and the large-seeded phenotype will be useful in deciphering the effects of variation in kafirin genetic background on end-use traits. Furthermore, transcriptional profiling of the kafirin genes across these genotypes will contribute to our understanding of the impact of this genetic diversity on seed biochemical traits and ethanol production. Sorghum currently represents ≈5% of the grain ethanol market in the United States (RFA 2007), but it is ideally positioned for expansion in the industry. Genotypes exhibiting optimal endosperm composition and storage protein profile for converting ethanol offer commercially competitive alternatives to fuel crops with greater environmental impacts, such as maize and sugarcane.

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