Howard Hughes Medical Institute

Published as: *Crop Sci*. 2015 ; 55(6): 2813–2825.

Mutations in Durum Wheat *SBEII* Genes affect Grain Yield Components, Quality, and Fermentation Responses in Rats

Brittany Hazard,
Dep. of Plant Sciences, Univ. of California, Davis, CA 95616

Xiaoqin Zhang,
Dep. of Plant Sciences, Univ. of California, Davis, CA 95616

Mahmoudreza Naemeh,
Dep. of Plant Sciences, Univ. of California, Davis, CA 95616

M. Kristina Hamilton,
Dep. of Anatomy, Physiology and Cell Biology, UC Davis School of Veterinary Medicine, Davis, CA 95616

Bret Rust,
Dep. of Nutrition, Univ. of California, Davis, CA 95616

Helen E. Raybould,
Dep. of Anatomy, Physiology and Cell Biology, UC Davis School of Veterinary Medicine, Davis, CA 95616

John W. Newman,
Dep. of Nutrition, Univ. of California, Davis, CA 95616. USDA-ARS, Obesity and Metabolism Research Unit, Western Human Nutrition Research Center, Davis, CA 95616

Roy Martin, and
Dep. of Nutrition, Univ. of California, Davis, CA 95616. USDA-ARS, Obesity and Metabolism Research Unit, Western Human Nutrition Research Center, Davis, CA 95616

Jorge Dubcovsky★
Dep. of Plant Sciences, Univ. of California, Davis, CA 95616. Howard Hughes Medical Institute, Chevy Chase, MD 20815

Abstract

Increased amylose in wheat (*Triticum* ssp.) starch is associated with increased resistant starch, a fermentable dietary fiber. Fermentation of resistant starch in the large intestine produces short-chain fatty acids that are associated with human health benefits. Since wheat foods are an important component of the human diet, increases in amylose and resistant starch in wheat grains have the potential to deliver health benefits to a large number of people. In three replicated field trials we found that mutations in starch branching enzyme II genes *(SBEIIa* and *SBEIIb*) in both A

★Corresponding author: jdubcovsky@ucdavis.edu.

and B genomes (*SBEIIa/b*-AB) of durum wheat [*T. turgidum* L. subsp. *durum* (Desf.) Husn.] resulted in large increases of amylose and resistant starch content. The presence of these four mutations was also associated with an average 5% reduction in kernel weight ($P = 0.0007$) and 15% reduction in grain yield ($P = 0.06$) compared to the wild type. Complete milling and pasta quality analysis showed that the mutant lines have an acceptable quality with positive effects on pasta firmness and negative effects on semolina extraction and pasta color. Positive fermentation responses were detected in rats (*Rattus* spp.) fed with diets incorporating mutant wheat flour. This study quantifies benefits and limitations associated with the deployment of the *SBEIIa/b*-AB mutations in durum wheat and provides the information required to develop realistic strategies to deploy durum wheat varieties with increased levels of amylose and resistant starch.

Wheat food products are a significant source of carbohydrates in the human diet and provide more than 500 calories per capita per day worldwide (FAOSTAT, 2014). Wheat is very versatile and can be processed into a wide variety of foods including bread, cookies, cakes, noodles, and pasta (Sestili et al., 2014). Durum wheat is the preferred raw material for making pasta and is also used to make couscous and flatbreads (Sissons, 2008; Ma et al., 2013).

Starch is the major carbohydrate present in the mature wheat grain and is comprised of the two glucose polymers amylopectin (70–80%) and amylose (20–30%). Amylopectin has a highly branched structure and is readily digested by human α-amylases whereas amylose exists in linear helical chains and forms complexes resistant to α-amylase digestion. Starch with increased amylose content is associated with increased resistant starch (RS), a fermentable dietary fiber. Resistant starch is currently defined as the combination of starch and starch degradation products that are not absorbed in the small intestine of healthy individuals (Champ, 2013). Most RS is ultimately metabolized in the large intestine by gut microflora, which produce short-chain fatty acids (SCFAs). The SCFAs are associated with health benefits in the colon as well as systemic health benefits (Sestili et al., 2014). Substituting readily digested starches with RS also decreases the glycemic load of foods which in turn reduces insulin secretion and is important for obesity and diabetes prevention (Champ, 2013).

The demand for fiber-rich foods is growing due to the increased awareness of their positive health benefits, however many consumers do not fulfill the recommended daily intake requirements (Yong-Cheng and Maningat, 2013). Since food products made with high-amylose flours contain more RS, increasing amylose content in wheat grains is a valuable objective for both durum and common wheat (*T. aestivum* L.) breeding programs. Amylose levels in wheat starch can be increased substantially by the simultaneous downregulation of duplicated starch branching enzyme II genes (*SBEIIa* and *SBEIIb*) (Regina et al., 2006, 2015; Sestili et al., 2010, 2015; Botticella et al., 2011; Hazard et al., 2012, 2014; Slade et al., 2012). We have previously shown that durum wheat germplasm carrying loss-of-function mutations in *SBEIIa* and *SBEIIb* in both the A and B genomes (*SBEIIa/b*-AB; PI 670160) had a 66% increase in amylose content and a 7- to 10-fold increase in RS content relative to a wild-type sib control (Hazard et al., 2014). The same study showed that the increases in amylose and RS were associated with decreases in total starch content (−7%) and kernel

weight (−8%). Similar reductions have been reported in other studies using both mutants and RNA interference (RNAi) transgenic plants with reduced levels of *SBEII* transcripts (Regina et al., 2006, 2015; Sestili et al., 2010, 2015; Slade et al., 2012). These results suggested the possibility that the benefits of increased RS might be partially offset by negative pleiotropic effects on grain yield.

In this study we provide a precise quantification of the effects of these *SBEII* mutant alleles on different grain yield components in three replicated field studies. We also provide a detailed comparison of semolina and pasta characteristics in the *SBEII* mutant and wild-type sib line. Finally, we incorporated semolina from *SBEIIa/b*-AB mutant grains into diets for a rat-feeding study, and demonstrated a positive gastrointestinal fermentation response relative to the wild-type sib line. The data presented in this study will inform the development of balanced strategies to deploy durum wheat varieties carrying this beneficial trait.

MATERIALS AND METHODS

Experimental Materials and Growth Conditions

The identification, selection, and combination of the mutations in the *SBEIIa* and *SBEIIb* genes in both the A and B genomes to develop the *SBEIIa/b*-AB quadruple mutant used in this study has been described previously (Hazard et al., 2012, 2014). The four individual mutants used to develop *SBEIIa/b*-AB were backcrossed twice to the wild-type parent Kronos to reduce the background mutations. This germplasm has been previously deposited in the National Small Grain Collection under accession number PI 670160 (Hazard et al., 2014; USDA-ARS NGRP, 2014).

The *SBEIIa/b*-AB mutant and the wild-type sib control were grown in three locations during the 2013–2014 growing season: Sacramento Valley at the UC Experimental Field Station in Davis, CA (38° 32′ N, 121° 46′ W), San Joaquin Valley at the UC Research and Extension Center (REC) in Five Points, CA (36° 20′ N, 120° 6′ W), and in Imperial Valley at the Desert REC in Holtville, CA (32° 48′ N, 115° 26′ W). Each experiment was set up in a randomized complete block design with six blocks (replicates).

In Davis, plots were sown at a 3 million seeds ha^{-1} seeding rate during November (fall planting) in a Yolo soil (fine-silty, mixed, superactive, nonacid, thermic Mollic Xerofluvent). Fertilization in Davis consisted of a pre-planting application of 112 kg ha^{-1} N and a top-dress application of 67 kg ha^{-1} N at tillering. Plots in Davis were flood irrigated three times. In Five Points, plots were sown at a 2.5 million seeds ha^{-1} seeding rate during December (winter planting) in a Panoche soil (fine-loamy, mixed, superactive, thermic Typic Haplocambid). Fertilization in Five Points included a pre-planting application of 134.5 kg ha^{-1} N, a top-dress application of 56 kg ha^{-1} N at tillering and a top-dress application of 44.8 kg ha^{-1} N at flowering. Plots in Five Points were sprinkler-irrigated two times followed by four flood irrigations. In Holtville, plots were sown at a 2.5 million seeds ha^{-1} seeding rate in December (winter planting) in Glenbar soil (fine-silty, mixed, superactive, calcareous, hyperthermic Typic Torrifluvent) and Imperial soil (fine, smectitic calcareous, hyperthermic Vertic Torrifluvent). Fertilization in Holtville consisted of a pre-planting application of 38.4 kg ha^{-1} P and a top-dress application of 107.6 kg ha^{-1} N at planting. During growth 4 more

top-dress applications of N were used (135.5, 100.8, 89.6, and 56 kg ha^{-1}). Plots in Holtville were irrigated six times.

Yield and Yield Components

Before harvest spike density was estimated by counting the number of spikes in a randomly selected 30.5 cm^2 area within each plot. Spikelet number and kernel number were calculated for each plot by counting spikelets per spike and kernels per spike using 10 randomly collected spikes. Mature grains were harvested in May 2014 in Holtville and in June 2014 in Davis and Five Points. Grain weights were recorded and kernel weight was measured using 1000 kernels per plot.

Quality Evaluations

Evaluation of grain, milling and pasta quality followed the standard American Association for Cereal Chemist International (AACCI, Approved methods of analysis, 11th edition, St. Paul, MN) approved methods used at the California Wheat Commission (CWC) Milling and Baking Lab. Grain measurements included test weight (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-55-10.01), moisture (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-44-15.0), protein (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-46-30.01) and ash (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-08-01.01). Black tip was evaluated by visual inspection using 10 g samples.

Semolina was produced by tempering to final moisture of 16% and milling durum wheat grains following approved AACCI method 26-42.01 for experimental milling (AACCI, http://methods.aaccnet.org/summaries/26-42-01.aspx). Total extraction and semolina extraction were recorded and semolina evaluations included moisture (AACCI http://dx.doi.org/10.1094/AACCIntMethod-44-15.02), protein (http://dx.doi.org/10.1094/AACCIntMethod-46-30.01), ash (http://dx.doi.org/10.1094/AACCIntMethod-08-01.01), wet gluten and gluten index (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-38-12.02) and falling number (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-56-81.03). Dough quality parameters were evaluated using an alveograph (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-54-30.02) (CHOPIN Technologies, Villeneuve-la-Garenne Cedex, France).

The higher levels of fiber present in the *SBEIIa/b*-AB mutant flour relative to the wild type require greater hydration levels to achieve similar dough consistencies. Therefore, for alveograph measurements flour from the *SBEIIa/b*-AB mutant was hydrated to 65% (as done in whole grain pasta flours) whereas flour of the wild-type sib was hydrated at 52% (as standard durum wheat) (Figure 1A).

AACCI method 66-42.01 for micro-scale pasta processing (AACCI, http://methods.aaccnet.org/summaries/66-42-01.aspx) was used to make spaghetti with 1-kg samples of semolina from Imperial Valley and San Joaquin Valley. Water added to semolina was adjusted based on the *P* value (tenacity) obtained from the alveogram; which provides an adjusted absorption value. Semolina and water were mixed for 5 min (1 min low speed, 4 min high speed) using a Hobart mixer (Hobart Corp., Troy, OH). The following extrusion conditions were used: 45°C, 46 cm of Hg in the mixing chamber, and a 25 rpm auger speed.

Extruded spaghetti was dried in a laboratory pasta dryer for 20.5 h using a low temperature (50°C) drying cycle and a maximum relative humidity of 90%. Pasta quality evaluations included cooked weight, cooking loss, firmness, and color. Cooked weight was determined by cooking 10 g of spaghetti (broken into lengths of 5 cm) for 12 min in 350 mL of distilled water. The cooked spaghetti was drained and weights were recorded. For cooking loss, the cooking water was collected from each sample and evaporated to dryness overnight in a forced-air oven at 130°C. The remaining residue was weighed and reported as a percentage of the original sample. Firmness of 5 pasta strands was measured using a TA.XTplus Texture Analyzer (Stable Micro Systems, Godalming, UK) following accepted methods (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-66-50.01). Results were reported in the amount of work (g·cm) to shear one strand of pasta. Pasta color (AACCI, http://dx.doi.org/10.1094/AACCIntMethod-14-22.01) was measured using a Minolta Chroma Meter CR-310 following manufacturer's instructions.

Starch Properties

Rapid visco analysis (RVA) was performed following the manufacturer's RVA Durum Method (Newport Scientific Method 11, Version 5, December 1997). Relative amylose content (amount of amylose as a percentage of total starch) was measured for 25-mg samples of semolina using the AMYLOSE/AMYLOPECTIN kit developed by Megazyme International (Wicklow, Ireland; catalogue number K-AMYL, http://secure.megazyme.com/files/BOOKLET/K-AMYL_1107_DATA.pdf, accessed August 2014) following the manufacturer instructions. The RS content (amount of RS measured on a g/kg "as is" basis) was measured for 100-mg samples of semolina using the RESISTANT STARCH kit from Megazyme International (Wicklow, Ireland; catalogue number K-RSTAR, http://secure.megazyme.com/files/BOOKLET/K-RSTAR_1108_DATA.pdf, accessed July 2014). Total starch content was measured for 100-mg samples of semolina using the TOTAL STARCH kit developed by Megazyme International (Wicklow, Ireland; catalogue number K-TSTA, http://secure.megazyme.com/files/BOOKLET/K-TSTA_1107_DATA.pdf, accessed August 2014) following the manufacturer's instructions for the recommended KOH assay format.

Fermentation Response in Rats

Twenty-four 8-wk-old Wistar rats were purchased from Harlan Company (Indianapolis, IN) and housed in individual cages in a room with controlled temperature (22°C) and 12:12 h light–dark cycle. Upon arrival they were acclimated for 6 d and fed a chow diet. Following acclimation, the rats were weighed and assigned randomly to diet treatment groups: *SBEIIa/b*-AB wheat, control wheat, and the references cellulose and inulin ($n = 6$, Supplementary Table S1) (Research Diets Inc., New Brunswick, NJ). Cellulose is an important component of plant cell walls and was included as a reference for an indigestible carbohydrate. Inulin is a naturally occurring storage carbohydrate in plants and was included as a reference for a functional dietary fiber readily fermented by bacteria in the colon (Roberfroid, 2005).

The mean weight of all rats assigned to a particular diet was not significantly different from the mean weights of rats assigned to other diet groups. Semolina flour was the main source

of carbohydrates in the wheat diets and corn starch was used to normalize the total starch content in the *SBEIIa/b*-AB diet to match the control diet. Food intake and body weight were measured three times a week during the 4-wk study. The protocol was approved by the Institutional Animal Care and Use Committee (University of California, Davis).

At the end of the study, final body weight was recorded and necropsies were performed using isoflurane anesthesia on non-fasting rats. Blood was retrieved by cardiac puncture for serum collection using the BD P700 Blood Collection System for Plasma GLP-1 Preservation containing a Dipeptidyl Peptidase IV protease inhibitor (Franklin Lakes, NJ). Blood samples were centrifuged at 10,000 rpm to isolate serum which was frozen for later use. The gastrointestinal tract was removed from the end of the stomach to the anus, fat removed, and cecum weighed (with and without contents). Before freezing, cecal contents were homogenized in 3 mL of distilled water and pH was measured using an accumet AB15 pH meter (Fisher Scientific).

Gut peptide hormones glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits on thawed serum samples following manufacturer's instructions (ALPCO Diagnostics, Salem, NH). Sample absorbance was analyzed on Spectramax M2 (Molecular Devices).

For SCFA analysis, cecal contents were thawed and homogenized in analytical grade water (10:1 dilution) using a Geno/Grinder 2000 (SPEX SamplePrep, Metuchen, NJ). Solids were separated by centrifugation at 4500 rpm for 10 min and 650 μL of supernatant was transferred to a 45 μm PVDF filter tube (Millipore, Billerica, MA) and spiked with 13 μL deuterated surrogates, including d3-acetate (524 mM), d3-propionate (27 mM) and d3-butyrate (22 mM). The filtrate was acidified with 13 μL 6N HCL and 500-μL samples were extracted in diethyl ether. Samples were derivitized for 2 h using 10 μL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane. Following derivitization, solvent was removed by centrifugal vacuum evaporation (GenVac EZ2). Residues were reconstituted in 5μL of silylating reagent and 100 μL of hexane and enriched with a 15:1 n5 methyl ester in 10 μL hexane for a total volume of 115 μL. Reconstituted samples were allowed to derivatize again for 24 hours. Five point calibration solutions containing analytes and surrogates were prepared in real time with sample extracts.

Samples were analyzed by gas chromatography mass spectrometry (GC–MS) on an Agilent 5890 GC equipped with 30 m by 0.25 mm by 0.25 μm DB-225ms column interfaced with an Agilent 5973 mass spectral detector run with electron impact ionization and selective ion monitoring/full scan mode. A 2-μL aliquot was introduced into the injection port (230°C) run with a 50:1 split. The oven initial temperature of 80°C was held for 0.5 min, ramped at 35°C/min to 200°C, at 25°C/min to 220°C, and at 20°C/min to 240°C and held at this temperature for 10.27 min. Both front and rear injection ports were under electronic pressure control, and the rear injection port was configured to perform as a backflush module, connected to the chromatographic column with a 0.5 m length of deactivated fused silica attached to a column splitter installed 0.5 m before the mass spectrometer transfer line. The initial chromatographic column flow of 1.5 mL/min was reduced to 1 mL/min at 2.85 min at 1 mL/min^2, and held at 1 mL/min for 5.65 min at which time (9.0 min) the flow was reduced

to 0.1 mL/min at 10mL/min^2. The rear port was maintained at 2 psi from initial to 9.0 min, and increased at 50 psi/min to 35 psi, and held until 7 min, providing an isothermal back flushing of the chromatographic column. The diluted supernatants were diluted again at 2:1 and re-injected to assess accuracy of linear calibration curve extrapolation and intra-assay precision. Average values from both assays are reported. Quantification was based on calibrations using unique ions collected in selected ion monitoring (Supplementary Table S2) and corrected for deuterated surrogate recoveries. Peak identification was confirmed by secondary ion relative abundance equivalent retention time to the authentic standards.

Data Analysis

Differences in S*BEIIa/b*-AB and wild-type sib control lines for yield and yield components, grain, milling, and pasta quality and starch properties were analyzed using the general linear model (SAS Institute, 2013). In the combined analysis the three locations were considered as fixed effects and blocks were considered nested within location. Individual ANOVAs were also reported separately for each location to show the consistency of the results across environments. Effects of diets on fermentation response were compared using ANOVA with food intake as a co-variable. ANOVA assumptions were tested using Shapiro–Wilk's test for normality of residuals.

RESULTS

Grain Yield and Yield Components

The ANOVA model comparing genotypes across the three locations explained 88 and 83% of the variation in kernel weight and grain yield, respectively. Significant differences in kernel weight were detected between genotypes ($P = 0.0007$) and among locations ($P < 0.0001$). The significant interaction between genotype and locations ($P = 0.03$, Table 1) suggests that the effect of the *SBEIIa/b*-AB mutations on kernel weight was modulated by the environment. On average, the kernel weight of the *SBEIIa/b*-AB mutants was 5.2% smaller than the kernel weight of the wild-type sib lines (Table 1). When analyzed separately by location significant decreases were only present in Imperial Valley (−9.8%, $P = 0.0042$), though a decreasing trend was observed in all locations (−1.5% in Sacramento Valley and −4.1% in San Joaquin Valley) (Table 1).

The ANOVA for total grain yield showed similar trends as the results described above for grain weight, but the higher variability of this trait resulted in less statistical power. Significant differences were detected among locations but not between genotypes or for the interaction between genotype and locations (Table 1). However, it is important to indicate that plants carrying the *SBEIIa/b*-AB mutations showed an average reduction in grain yield of 15.4% relative to the wild-type sib lines, and that these differences were marginally nonsignificant ($P = 0.06$, Table 1). When analyzed separately by location yield penalties were observed in all three locations, though none of these reductions were significant (Table 1). Yield penalties were similar in the Imperial Valley (−8.8%) and Sacramento Valley (−7.6%) trials and greater in the San Joaquin Valley trial (−26.3%, Table 1).

No significant differences among genotypes were detected for spike density, spikelet number per spike, and kernel number per spike (Supplementary Table S3). However, the interaction between kernel number per spike and location was significant ($P = 0.03$). This interaction was the result of a positive effect of the *SBEIIa/b*-AB mutations on kernel number per spike in Imperial Valley (+ 22.1%) and Sacramento Valley (+ 10.7%) that partially compensated the reduced kernel weight, and a negative effect in the San Joaquin Valley (−9.6%, Supplementary Table S3) that, together with the reduction in kernel weight explains the larger reduction in total grain yield in this location (Table 1).

Starch Properties

The ANOVAs for RS and amylose content in semolina showed significant differences both among genotypes and location but not genotype × location interactions, indicating consistent effects across locations (Table 2). The average amylose content in the *SBEIIa/b*-AB mutants was 61% higher than in the wild-type, with similar increases in Imperial Valley (57%), Sacramento Valley (68%) and San Joaquin Valley (58%, Table 2). The increases in amylose content resulted in even larger increases in RS (average 851%), which vary from 1010% in Imperial Valley to 760 and 749% in San Joaquin and Sacramento Valleys, respectively (Table 2).

The changes in the proportions of amylose and RS were associated with an average decrease of 6% in total starch in the *SBEIIa/b*-AB mutants relative to the wild-type ($P = 0.028$, Table 2). Significant differences in total starch were also detected among locations ($P = 0.0012$) but not for the genotype × location interaction (Table 2). When locations were analyzed separately, the samples from the Sacramento Valley had the greatest decreases in total starch (−8%, $P = 0.0004$) (Table 2).

The significant changes in the proportion of amylose and RS were also associated with overall changes in starch viscosity, as shown in the RVA viscograph (Fig. 1B). Significant decreases were observed for all viscosity parameters including peak viscosity (−50%), trough viscosity (−39%), final viscosity (−35%), breakdown (−85%) and setback (−30%) (Supplementary Table S4). In the *SBEIIa/b*-AB mutants, peak time increased by 20% ($P < 0.0001$) and pasting temperature increased by 11% ($P = 0.056$, Supplementary Table S4).

Grain Quality

Differences in test weight were comparable to the changes reported above for kernel weight, with significant differences for genotype, location, and genotype × location interactions (Table 3). On average, the *SBEIIa/b*-AB mutants showed a 3.8% decrease in test weight relative to the wild-type sib lines ($P < 0.0001$).

An unexpected result in the grain quality analysis was the presence of higher levels of black tip in the *SBEIIa/b*-AB mutants relative to the wild-type sib lines ($P = 0.0018$, Table 3). Increases in black tip were observed in all locations, but when analyzed separately only Sacramento Valley and San Joaquin Valley showed significant differences (data not shown). In Sacramento Valley the proportion of grains with black tip was more than threefold higher than in the other two locations (Table 3). The Kronos parent line used in this study is known

to be more susceptible to black tip in the Sacramento Valley, and that is one of the reasons this variety is commercially grown in the two other locations.

Milling and Semolina Quality

The percent of semolina extracted from the grains after milling was 4.6% lower on average in the *SBEIIa/b*-AB mutants ($P < 0.0001$) relative to the wild-type, an expected result given the lower test weight of the mutants. The reductions in kernel weight and total starch in the *SBEIIa/b*-AB mutant also predicted increases in the concentration of other grain components. This prediction was confirmed by significant increases in semolina protein (11.4%, $P < 0.0001$) and ash (44.4%, $P = 0.0004$, Table 3). Since increases in protein and ash content in whole grain samples were very similar to those observed in semolina, only the latter are reported in Table 3. Wet gluten increased by 8.7% on average ($P < 0.0001$) which was consistent with increases in semolina protein (Table 3). Despite increases in protein content and wet gluten in *SBEIIa/b*-AB mutants, there were no significant increases in gluten index (Table 3).

Falling number in *SBEIIa/b-AB* mutants was on average 38% higher than in the wild-type sib lines ($P < 0.0001$, Table 3). The ANOVA model, which explained 88% of the variation in falling number, showed significant differences among genotypes and locations and borderline significant values for genotype × location interactions (Table 3).

On average, alveograph parameters measuring dough strength (W), resistance/tenacity (P) and extensibility (L) ratios (P/L) increased among *SBEIIa/b*-AB mutants compared to the wild-type controls, but the differences were significant only for the P/L values. The P/L values showed significant differences among genotype and locations, but nonsignificant interactions (Table 3). The P/L increases varied from 80% in the Imperial and Sacramento Valley samples to 140% in the San Joaquin Valley samples (Table 3).

The comparison of W values is complicated by the different levels of hydration required in samples from the two genotypes to obtain similar dough properties. Even when the samples from the *SBEIIa/b*-AB mutants were hydrated at 65% and the wild-type samples were hydrated at 52%, the *SBEIIa/b*-AB mutants showed higher but not significant W values than the control (Table 3). However, when *SBEIIa/b*-AB and control semolina samples were both hydrated at 52% changes in alveograms were much greater and visually apparent (Fig. 1A). It was not possible to compare alveograph parameters at 52% hydration, because dough samples from the *SBEIIa/b-AB* mutants were too dry for recording accurate data on the alveograph instrument at this level of hydration.

Pasta Quality

The large amount of grain required for full pasta extrusion and quality tests, limited these analyses to the Imperial Valley and San Joaquin Valley locations. In the combined ANOVA including both locations, significant changes were observed between *SBEIIa/b*-AB mutant and control lines for all pasta quality parameters measured (Table 4). Positive changes included increased firmness levels in the *SBEIIa/b*-AB mutants, which showed on average 12.4% increases in firmness values relative to the wild-type sib lines (Table 4). *SBEIIa/b*-AB mutants also displayed significant pasta quality penalties compared to control lines including

an average 20.4% increase in cooking loss ($P < 0.0001$), a 6.1% reduction in cooked weight ($P = 0.0005$), and a 10.4% decrease in overall color score ($P = 0.0005$) (Table 4). The negative impact of the *SBEIIa/b*-AB mutants on color was the combined effect of reductions in Color b (yellow–blue, $P < 0.0001$) and Color L (black–white, $P = 0.016$) values, and increased values of Color a (red–green, $P = 0.0019$, Table 4).

Fermentation Response in Rats

The inclusion of either *SBEIIa/b*-AB or wild-type wheat flour in the diets of rats for 4 wk (Supplementary Table S1) was sufficient to generate significant differences in fermentation responses. Cecal weights in the rats fed the *SBEIIa/b*-AB wheat diet were 37% higher than in those fed the control wheat diet ($P = 0.012$) (Table 5). In addition, the cecal pH of the samples of rats fed *SBEIIa/b*-AB wheat (pH 6.88) was significantly lower than the control (pH 7.25, $P = 0.029$, Table 5). Bodyweight ($P = 0.6$) and food intake ($P = 0.5$) showed no significant differences between diets.

The more acidic pH of the cecal contents from rats fed with *SBEIIa/b*-AB mutant wheat was associated with a significant increase in total SCFAs. On average, the total SCFA content in the rats fed with *SBEIIa/b*-AB mutant wheat increased by 60% relative to the rats fed with the control wheat grains ($P < 0.0001$, Table 5). The most prevalent SCFA was acetate which increased by 56% compared to the control (Table 5). Propionate increased over twofold in the *SBEIIa/b*-AB group but the differences were non-significant ($P = 0.1$, Table 5). Changes in butyrate were also not significant (but consistent with the other increasing trends in acetate and propionate, Table 5). The intra-assay precision for the GC–MS analysis was approximately 20%.

The levels of gut peptide satiety hormones GLP-1 and PYY were higher in the *SBEIIa/b*-AB serum samples relative to the controls (161 and 21%, respectively) but the increases were not significant (Table 5).

In addition to *SBEIIa/b*-AB and control wheat treatment groups, cellulose and inulin diets were included in the rat feeding study as references to compare the fermentation responses to other classes of dietary fiber (Supplementary Table S1). Cecal pH of rats fed cellulose diets were the highest and cecal weights and SCFA contents the lowest compared to all other diet treatments, consistent with the poor fermentability of cellulose (compare Table 5 and Supplementary Table S5). Rats fed diets with inulin as the primary source of dietary fiber showed the lowest cecal pH values and largest cecal weights and SCFA contents, consistent with the high fermentability of inulin (compare Table 5 and Supplementary Table S5).

DISCUSSION

Resistant starch serves as a prebiotic, defined as a non-digestible food ingredient that generates beneficial effects by feeding indigenous bacteria living in human large intestines. The prebiotic nature of RS is associated with significant production of SCFAs and lower colonic pH which has been suggested to provide protection from cancer formation (Fuentes-Zaragoza et al., 2011). In addition to prebiotic functions, foods with high levels of RS have lower glycemic indices and caloric values and provide increased satiety which is important

for diabetes and obesity prevention (Lunn and Buttriss, 2007). Wheat with increased levels of amylose and RS can provide these nutritional benefits in the form of food products that represent a large proportion of current human diets in many societies (Sestili et al., 2014).

Although no daily intake recommendations on RS are in place in the United States, the National Medical Research Council in Australia recommends consuming 25 to 30 g per day, more than five times the current U.S. intake (4.9 g per day) (Murphy et al., 2008; Colyer et al., 2013). Americans consume 8.8 kg of pasta per capita on average ("IPO World Pasta Industry Status Report," 2013), so the 7- to 10-fold increases in RS in wheat semolina extracted from the *SBEIIa/b*-AB mutants could have a significant impact in the overall consumption of RS in the United States. Global consumers will also reap the benefits since the United States produces nearly 2 Tg of durum wheat annually, is the second largest producer of pasta worldwide (IPO World Pasta Industry Status Report, 2013) and is one of the major exporters of durum wheat (Hausmann et al., 2011).

However, the deployment of *SBEIIa/b*-AB mutations in commercial pasta wheat varieties will require a better understanding of potential pleiotropic effects of the *SBEII* mutations on agronomic performance, pasta quality, and health benefits. The implications of the results obtained in this study in each of these three areas are discussed below.

Grain Yield and Grain Quality Penalties Associated with *SBEIIa/b*-AB Wheat

The three field experiments described in this study consistently show that the presence of loss-of-function mutations in all *SBEIIa* and *SBEIIb* homoeologs in the *SBEIIa/b*-AB mutant is associated with penalties in total starch, kernel weight, and test weight. A positive correlation between total starch and test weight has been also observed in previous durum wheat studies (El-Khayat et al., 2006). Variation in test weight is of particular interest to durum millers because it is usually positively correlated with semolina yield (Matsuo and Dexter, 1980) although these relationships can be affected by genotype and environment (Troccoli and Di Fonzo, 1999). A similar association was observed in this study, where the 3.8% average reduction in test weight was associated with a 4.6% decrease in semolina extract (Table 3). As in previous studies, the effects of genotype on test weight were modulated by the environment (e.g., see significant interaction in Table 3). In a previous field trial we found a 7% reduction in total starch associated with an 8% reduction in kernel weight (Hazard et al., 2014).

The reductions in grain weight observed in the Sacramento Valley and Imperial Valley were partially compensated by slight increases in the number of grains per spike (Supplementary Table S3), resulting in nonsignificant reductions in total grain yield (Table 1). The largest reduction in grain yield was observed in San Joaquin Valley, where lines carrying the *SBEIIa/b*-AB mutations showed reductions in both kernel weight and kernel number. The differences observed in this study among locations suggest that a careful evaluation of growing locations and field management practices can reduce the penalties in test weight and total grain yield associated with the presence of the *SBEIIa/b*-AB mutations. It is possible that the different fertilization and irrigation regimes contributed to the differences in yield and grain weight between *SBEIIa/b*-AB mutants and controls observed among the different locations. The need to select dedicated areas for the production of these high-RS

durum varieties is also highlighted by the increased susceptibility of the Kronos *SBEIIa/b*-AB mutants to black tip (Table 3) and the high incidence of this problem in the Sacramento Valley. We are currently introgressing the *SBEIIa/b*-AB mutants into the durum wheat variety Desert King, which has lower levels of black tip than Kronos (Jackson, 2011). The combination of more resistant varieties and of locations with low incidence of black tip (e.g., the San Joaquin and Imperial valleys) can be used to control this particular problem.

Mutations in the *SBEII* genes or reductions in their expression in RNAi transgenic plants have been associated with reduction in total starch and grain weight in different genetic backgrounds. These results suggest that these are pleiotropic effects of the modifications of the *SBEII* genes rather than linkage drag effects or residual mutations in other parts of the genome. Slade et al. (2012), reported 11.4% decreases in total starch in durum wheat with mutations only in the *SBEIIa* genes (47% amylose). Similarly, Botticella et al. (2011) reported approximately 5% decreases in total starch in common wheat with loss-of function mutations in two of the three *SBEIIa* homoeologs (40% amylose). More recently Sestili et al. (2015) reported larger decreases in total starch (−17.5%) and kernel weight (−10.5%) in durum *SBEII* mutants with approximately 52% amylose and up to eightfold increases in RS. Regina et al. (2015) also reported 17% decreases in kernel weight and 33% decreases in total starch in lines with more than 80% amylose and more than 10-fold increases in resistant starch. Interestingly, the Imperial Valley desert location showed the highest levels of resistant starch but the lowest amylose content (Table 2). It is possible that the higher average temperatures observed in Imperial Valley have differential effects on amylose and resistant starch content. For example, shorter glucan chain lengths (that can contribute to increased resistant starch) have been observed in wheat under heat stress (Beckles and Thitisaksakul, 2014).

The negative impacts of increased amylose and RS levels on total starch, test weight, and grain yield observed here and in previous studies suggest that economic incentives will be required to encourage growers to produce high-RS durum wheat varieties. Variation observed in the magnitude of the negative effects of the *SBEII* mutations on grain weight and yield in different studies suggest that efforts to select the most compatible genetic backgrounds and the optimum growing regions may be able to mitigate these negative effects.

Compositional Changes in *SBEIIa/b*-AB Wheat Affect Semolina and Dough Characteristics

Ash and Protein Content—The reduced levels of total starch in the grain and the associated reduction in kernel weight in the *SBEIIa/b*-AB mutants likely contributed to the increased concentration in protein and ash content in the grain. Regina et al. (2015) also reported dramatic increases in protein (>70%) in mutant *SBEII* lines with concomitant decreases in kernel weight and total starch. Since ash content is an important quality parameter for premium quality durum wheat (Troccoli et al., 2000), the 40% increase in ash content in the *SBEIIa/b*-AB mutants (Table 3) is of concern. Therefore, durum wheat varieties that will be used as recurrent parents for the introgression of the *SBEIIa/b*-AB mutations should be selected for low levels of ash content, to maintain this trait within the

regulatory thresholds established for premium pasta quality production (Troccoli et al., 2000).

In contrast to the negative impacts of increased ash content on pasta quality, the increases in protein content observed in the *SBEIIa/b*-AB mutants are associated with beneficial effects on pasta quality (Sissons, 2004). Increased levels of protein are desirable because the protein matrix formed during cooking holds the starch granules allowing the pasta to swell, maintain firmness, and reduce cooking loss (Sissons, 2008). We also observed increased levels of wet gluten. However, those differences became not significant when protein content was used as a co-variable in an ANCOVA, suggesting that the differences in wet gluten were determined, at least in part, by the increased protein content of the *SBEIIa/b*-AB mutants.

Dough Strength—Typically strong gluten makes dough with better extrusion properties and cooked textural characteristics while weak and inelastic gluten promotes poor pasta cooking quality (Sissons, 2008). Gluten strength is determined by both protein content and protein quality. Although increases in protein content were observed in *SBEIIa/b*-AB mutants both the ANOVA and ANCOVA (with protein as a co-variable) showed no significant differences in gluten index between mutant and wild-type genotypes, which suggests that the *SBEIIa/b*-AB mutations do not affect protein quality.

To test if the observed increases in overall dough strength (alveograph W) were the result of the differences in semolina protein content described above, we compared the results from the ANOVA for W values with an ANCOVA analysis for the same parameter using semolina protein as a co-variable. We did not detect any significant differences in either analysis, likely due to the high variability of the W values among replications.

The presence of the *SBEIIa/b*-AB mutations was associated with an increase in resistance and extensibility, as indicated by highly significant increases in P/L values ($P < 0.0001$) that were consistent across all three locations (Table 3 and Fig. 1A). Both the ANOVA and ANCOVA using protein content as covariable showed significant differences among genotypes ($P < 0.0001$) suggesting that the differences were not only mediated by changes in protein content. This was further reflected in the fact that the regression of P/L and protein content was not significant ($P = 0.2$) which explains the similar results from the ANOVA and ANCOVA analyses.

Traditionally, alveograph tests on durum wheat show semolina dough to be relatively inelastic with higher tenacity/elasticity (P) than extensibility (L), resulting in P/L values above 1.5 (Sissons, 2008). The alveograph P/L value for the Kronos wild-type sib controls (P/L = 1.8) was close to this reference value. By contrast, the P/L value for the *SBEIIa/b*-AB mutant was more than twice the reference value (P/L = 3.7, Table 3). The dough of *SBEIIa/b*-AB mutants was too dry to measure properly at a 52% hydration level. The increased water absorption and dryness of the dough was likely due to the increased fiber content in the high-amylose flour (Sissons, 2008). Therefore, we increased hydration to 65%, which is a level used frequently for testing whole grain flours with increased fiber content. It is possible that the increased water absorption of the *SBEIIa/b*-AB mutants contributed more than the protein changes to the observed increases in overall strength and

extensibility. It is also important to note that changes in amylopectin structure, such as modified chain lengths and degree of branching, can alter starch architecture and physicochemical properties of the dough (Regina et al., 2015).

Taken together these data suggest that the *SBEIIa/b*-AB mutations have limited effect on gluten strength beyond an indirect effect on wet gluten mediated by the effect in protein content. The effect of the *SBEII* mutations on dough resistance and extensibility appears to be independent of the effect of the mutations on protein content. In summary, end users must be aware that semolina from the *SBEIIa/b*-AB mutants absorbs more water and should adjust their recipes and processing protocols accordingly to accommodate these increased hydration requirements.

Rapid Visco Analyses—Changes in the chemical-physical properties of starch in the *SBEIIa/b*-AB mutants have strong effects on starch properties as demonstrated in the contrasting RVA viscosity profiles from the mutant and wild-type control sib lines (Fig. 1B). Viscosity levels of the *SBEIIa/b*-AB mutants were consistently lower than those of the wild-type sib controls across the entire pasting curves (Supplementary Table S4). These results were comparable to previous studies of *SBEIIa*-silenced mutants as well as wheat flour mixtures enriched by the addition of exogenous RS (Regina et al., 2006, 2015; Fu et al., 2008; Sestili et al., 2010).

The average pasting temperature in the *SBEIIa/b*-AB semolina was nearly eight degrees higher than the wild-type control (Supplementary Table S4). Pasting temperatures can provide an indication of the minimum temperature required for cooking, thus incorporating *SBEIIa/b*-AB wheat semolina into a cooking formula could potentially alter the thermal stability of other ingredients. During heating, starch typically absorbs water causing the granules to swell and rupture eventually. This is followed by dissolution of the starch which produces a rapid increase in viscosity. However, increased amylose content is known to suppress swelling and this is a probable explanation for the observed 50% decrease in peak viscosity in the *SBEIIa/b*-AB mutant relative to the wild-type control (Sissons, 2008).

During the hold period of the RVA test there is usually a breakdown in viscosity to a holding strength/trough viscosity as seen in the wild-type sib controls (40.6 rapid visco units [RVU]). Conversely, *SBEIIa/b*-AB semolina underwent little breakdown (6 RVU) (Fig. 1B and Supplementary Table S4). Lower breakdown values are associated with the ability of the semolina to withstand heating and shear stress during mixing which is important for processing. Thus, the decreased breakdown in *SBEIIa/b*-AB semolina may provide favorable increases in heating and mixing tolerance. During the cooling and retrogradation phase setback values decreased in *SBEIIa/b*-AB wheat (78.0 RVU, control: 111.2 RVU). Setback can be correlated with texture of final products and high setback is also associated with syneresis during freeze/thaw cycles. Thus, frozen food products incorporating *SBEIIa/b*-AB wheat may undergo undesirable weeping on thawing (Freschi et al., 2014).

Final viscosity (*SBEIIa/b*-AB: 155.7 RVU, control: 238.0 RVU) is the most common RVA parameter used to define starch quality and indicates the ability of a material to form a viscous paste after cooking and cooling. Overall, the observed decreases in viscosity

parameters will change the functional characteristics in food products made with *SBEIIa/b*-AB wheat flours and may be favorable or unfavorable depending on the cooking process and characteristics desired in the end product.

Falling Number—A falling number of 300 s or higher indicates minimal amylase activity due to sprout damage (Wheat and flour testing methods: a guide to understanding wheat and flour quality, version 2, 2008). Too much enzyme activity and sprouting can result in sticky dough and have undesirable effects on pasta quality such as reduced shelf life, increased cooking loss and softer pasta (German, 2006). Both *SBEIIa/b*-AB mutants and wild-type controls had falling numbers over this threshold, which is characteristic of durum wheat, but the values were significantly higher in the *SBEIIa/b*-AB mutants than in the wild-type control. Although differences in falling number between the two genotypes were observed in all locations, the magnitude of the differences varied across locations, a fact that was reflected in a highly significant genotype × location interaction (Table 3). These large differences in falling number are likely associated with decreased digestibility of amylose and RS by α-amylases in the grain. Although the high falling numbers of *SBEIIa/b*-AB flours may not have direct implications in pasta quality, use of these mutations in common wheat may have favorable effects in bread making varieties with low falling numbers but undesirable effects in soft wheat flours for baking. Lower enzyme activities may not produce enough sugar for proper dough development and additions of enzymes may be necessary.

Pasta Quality in Spaghetti Made with *SBEIIa/b*-AB Wheat

Color, cooking quality, and cooked texture are key quality traits of pasta. The compositional changes in *SBEIIa/b*-AB semolina produced significant differences in all the pasta quality parameters measured in this study (Table 3). Pasta made with *SBEIIa/b*-AB wheat showed favorable increases in firmness, an important indicator of pasta texture. A possible explanation for the higher firmness values is the ability of high-amylose starch granules to resist rupture and deformation on swelling. Additionally, the increased protein content in *SBEIIa/b*-AB mutants could contribute to an enhanced matrix that holds starch granules during cooking. This result is consistent with other pasta quality evaluations using high-amylose and RS flours (Soh et al., 2006; Aravind et al., 2013). Specifically, Soh et al. (2006) conducted a reconstitution study in durum wheat semolina that incorporated high-amylose maize flour and showed a positive correlation between amylose content and pasta firmness. The study reported that optimum pasta firmness was observed for flour including 32 to 44% amylose (Soh et al., 2006). On average, *SBEIIa/b*-AB mutants produced semolina with 44.9% amylose, which is closer to the upper limit of the reported optimal range. By contrast, the wild-type sib controls showed amylose levels below the optimal range (27.9% amylose).

Despite the favorable increases in firmness, pasta made from semolina extracted from *SBEIIa/b*-AB mutants also exhibited undesirable increases in cooking loss as well as decreases in cooked weight and overall color score (Table 4). The increase in cooking loss may be attributable to the soluble nature of amylose and ability to leach off during cooking. Cooked weight, an indicator of water-binding capacity is normally three times the dry weight of spaghetti (Dick and Youngs, 1988). Although decreases were observed in spaghetti made with semolina from the *SBEIIa/b*-AB mutants, values were still close to 30 g

of cooked weight for 10-g samples (Table 4). The reductions in cooked weight observed in the *SBEIIa/b*-AB mutants may also be due to decreased water uptake as a result of amylose restricting the swelling of starch granules (Sissons, 2008).

The yellowness of pasta is of aesthetic importance for consumer acceptance and marketing whereas redness and brownness are considered undesirable (Dick and Youngs, 1988). Increases in redness and brownness in *SBEIIa/b*-AB pasta are likely due to increased ash content in semolina which is positively correlated with color a (red–green) value (Table 4).

Overall, the pasta produced from high-RS semolina extracted from the *SBEIIa/b*-AB mutants was of acceptable quality. The negative effect of these mutations on color and ash content remain a concern that will need to be addressed to increase consumer acceptance of the high-RS pasta products. Deployment of the *SBEIIa/b*-AB mutations in genetic backgrounds with reduced ash content and optimum color scores may help mitigate the negative impact of these pleiotropic effects.

Enhanced Fermentation Response in Rats Fed *SBEIIa/b*-AB Wheat

The fundamental incentive to develop wheat with increased amylose and RS is to create a widespread food source of RS that can provide nutritional and health benefits to consumers. Thus an important goal of this study was to confirm that the increases in amylose and RS in *SBEIIa/b*-AB grain translated to favorable changes in nutritional value and promoted beneficial changes in gastrointestinal fermentation. Due to limited materials, the initial trial was limited to six animals per treatment and lasted 4 wk. This experiment was sufficient to show enhanced fermentation in rats fed *SBEIIa/b*-AB diets compared to controls. The differences included significant increases in cecal contents, decreases in cecal pH, and increases in cecal SCFAs (Table 5).

The addition of cellulose and inulin treatment groups demonstrated the broad range of fermentability associated with different dietary fibers (Table 5 and Supplementary Table S5). Fermentation responses were the lowest in the cellulose diet and the highest in the inulin diet, confirming that they were adequate references for the wheat diet groups. The fermentation responses to the *SBEIIa/b*-AB wheat diet were significantly higher than those to the wild-type wheat diet but not as high as those observed in the inulin diet. Still, the effects were large enough to suggest that flours from *SBEIIa/b*-AB wheat may be a suitable alternative to inulin additives in pasta, which have been suggested to negatively affect the formation of starch-protein matrices (Bustos et al., 2015).

Resistant starch has been shown to have an effect on appetite and satiety, yet the exact mechanism is currently unknown. Various mechanisms have been proposed, suggesting GLP-1 and PYY (satiety hormones) may be produced in response to SCFA production in the colon (Bodinham and Robertson, 2013). Although increases in GLP-1 and PYY were not significant in this study (Table 5), increases in these hormones have been reported in other RS animal feeding studies (Bodinham and Robertson, 2013).

Results of this study are similar to the fermentation response demonstrated in high-amylose wheat flours from grains of *SBEII*-RNAi transgenic plants with reduced transcript levels of

SBEII genes (Regina et al., 2006). The amylose content in flours from the transgenic common wheat lines was 74% (Regina et al., 2006), which is significantly more than the 44.9% amylose detected in the in *SBEIIa/b*-AB mutants in this study. In spite of the higher levels of amylose in the common wheat transgenic plants relative to the mutants characterized in this study, we observed comparable decreases in pH (0.37 here vs. 0.33 in the 74% amylose wheat) and increases in SCFAs (120 μmol here vs. 114 μmol in the 74% amylose wheat) (Regina et al., 2006). However, it is important to note that the amylose quantification methods used in these two studies were different, and may have contributed to some of the observed changes. Additional studies to determine the optimal level of amylose and RS to satisfy both nutritional and quality values would be useful to select optimum targets for RS in wheat products. It would be also interesting to investigate the effect of *SBEIIa/b*-AB wheat diets on obese or diabetic animal models.

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, the *SBEIIa/b*-AB mutations present in the publicly available line (PI 670160) seem to be appropriate for commercial production of high RS semolina and pasta products. However, economic incentives may be required to compensate for the negative effects of these mutations on grain yield (average 15%). In addition, durum varieties with low ash content and optimum color scores should be selected as recurrent parents to mitigate the negative impact of the *SBEII* mutations on these two quality traits. The careful selection of the production areas may also ameliorate some of the negative yield effects. The negative pleiotropic effects associated with the *SBEIIa/b*-AB mutants are compensated by the positive effects on grain protein content and pasta firmness and, most importantly, by the 7- to 10-fold increases in RS. The rat feeding study presented here suggests that these increases in RS can likely be translated into health benefits to wheat consumers, a suggestion also supported by studies with reconstituted high-RS flours (Flint, 2013).

We are currently introgressing these *SBEIIa/b*-AB alleles into different durum varieties to study the effect of genetic backgrounds in the modulation of the different effects described in this study. We have also transferred the *SBEIIa/b*-AB mutations from tetraploid wheat into hexaploid wheat, and we are now combining them with a mutation in the D genome copy of *SBEIIa* to develop quintuple *SBEII* mutants with increased RS in common wheat.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by the National Research Initiative Competitive Grant 2011-68002-30029 (Triticeae-CAP) from the USDA National Institute of Food and Agriculture, the Howard Hughes Medical Institute, and the Gordon & Betty Moore Foundation grant number GBMF3031. Additional support was provided by USDA Intramural Project 2032-51530-022-00D. The USDA is an equal opportunity provider and employer. Brittany Hazard acknowledges the generous support from the Colorado Wheat Research Foundation (CWRF) and ConAgra Mills through a fellowship for her Ph.D. studies. We also acknowledge Claudia Carter and Teng Vang at the California Wheat Commission for training and use of their facilities. Research Diets supplied the diets used in the rodent studies.

Abbreviations

AACCI	American Association of Cereal Chemists International
CWC	California Wheat Commission
ELISA	enzyme-linked immunosorbent assay
GC–MS	gas chromatography- mass spectrometry
GLP-1	glucagon-like peptide 1
L	alveograph length
P	alveograph tenacity
PYY	peptide tyrosine tyrosine
REC	research and extension center
RS	resistant starch
RVA	rapid visco analysis
RVU	rapid visco units
SCFA	short-chain fatty acid
UC	University of California
W	alveograph overall strength

References

Aravind N, Sissons M, Fellows CM, Blazek J, Gilbert EP. Optimisation of resistant starch II and III levels in durum wheat pasta to reduce in vitro digestibility while maintaining processing and sensory characteristics. Food Chem. 2013; 136:1100–1109.10.1016/j.foodchem.2012.08.035 [PubMed: 23122168]

Beckles DM, Thitisaksakul M. How environmental stress affects starch composition and functionality in cereal endosperm. Starch/Staerke. 2014; 66:58–71.

Bodinham, CL.; Robertson, MD. The acute effects of resistant starch on appetite and satiety in overweight individuals. In: Shi, Y.; Maningat, CC., editors. Resistant starch: Sources, applications and health benefits. 1. John Wiley & Sons; Chichester, West Sussex, UK: 2013. p. 215-227.

Botticella E, Sestili F, Hernandez-Lopez A, Phillips A, Lafiandra D. High resolution melting analysis for the detection of EMS induced mutations in wheat *sbeIIa* genes. BMC Plant Biol. 2011; 11:156.10.1186/1471-2229-11-156 [PubMed: 22074448]

Bustos MC, Perez GT, Leon E. Structure and quality of pasta enriched with functional ingredients. RSC Adv. 2015; 5:30780–30792.10.1039/C4RA11857J

Champ, M. Metabolic effects of resistant starch. In: Shi, Y.; Maningat, CC., editors. Resistant starch: Sources, applications and health benefits. 1. John Wiley & Sons; 2013. p. 229-249.

Colyer, CGB.; Landon, S.; Salman, H. The resistant starch report. Food Australia; 2013. http://foodaust.com.au/wp-content/uploads/2012/04/Hi_Maize-supplement_web.pdf [accessed 18 Feb. 2015]

Dick, JW.; Youngs, VL. Evaluation of durum wheat, semolina, and pasta in the United States. In: Fabriani, G.; Lintas, C., editors. Durum wheat: Chemistry and technology. Am. Assoc. of Cereal Chemists; St. Paul, MN: 1988. p. 237-248.

El-Khayat GH, Samaan J, Manthey FA, Fuller MP, Brennan CS. Durum wheat quality I: Some physical and chemical characteristics of Syrian durum wheat genotypes. Int J Food Sci Technol. 2006; 41(Supplement 2):22–29.

FAOSTAT. [accessed 18 Feb. 2015] Food supply-Crops primary equivalent. FAO of the United Nations. 2014. http://faostat3.fao.org/download/FB/CC/E

Flint, HJ. The microbiology of resistant starch fermentation in the human large intestine: A host of unanswered questions. In: Shi, Y.; Maningat, CC., editors. Resistant starch: Sources, applications and health benefits. 1. John Wiley & Sons; Chichester, West Sussex, UK: 2013. p. 251-265.

Freschi J, Doran L, Malumba P, Blecker C. Impact of freezing and thawing processes on wheat and potato starch gel syneresis. Starke. 2014; 66:208–215.10.1002/star.201200294

Fu L, Tian J, Sun C, Li C. RVA and farinograph properties study on blends of resistant starch and wheat flour. Agric Sci China. 2008; 7:812–822.10.1016/S1671-2927(08)60118-2

Fuentes-Zaragoza E, Sánchez-Zapata E, Sendra E, Sayas E, Navarro C, Fernández-Lõpez J, Pérez-Alvarez JA. Resistant starch as prebiotic: A review. Starch/Staerke. 2011; 63:406–415.

German, CL. Extension crops marketing specialist. Univ. of Delaware; Newark: 2006. Understanding the falling number wheat quality test. www.udel.edu/FREC/PUBS/ER06-02.pdf [accessed 18 Feb. 2015]

Hausmann, R.; Hidalgo, C.; Bustos, S.; Coscia, M.; Chung, S.; Jimenez, J.; Simoes, A.; Yildirim, M. The Atlas of economic complexity. Puritan Press; Cambridge, MA: 2011. http://atlas.media.mit.edu/explore/geo_map/sitc/export/show/all/0411/2011/ [accessed 18 Feb. 2015]

Hazard B, Zhang X, Colasuonno P, Uauy C, Beckles DM, Dubcovsky J. Induced mutations in the starch branching enzyme II (*SBEII*) genes increase amylose and resistant starch content in durum wheat. Crop Sci. 2012; 52:1754–1766. [PubMed: 26924849]

Hazard B, Zhang X, Naemeh M, Dubcovsky J. Registration of durum wheat germplasm lines with combined mutations in *SBEIIa* and *SBEIIb* genes conferring increased amylose and resistant starch. J Plant Reg. 2014; 8:334.

IPO world pasta industry status report. Int. Pasta Organ; Madison, WI: 2013. http://www.internationalpasta.org/resources/WorldPasta Industry Survey/IPOstatreport2013.pdf [accessed 18 Feb. 2015]

Jackson, L. Wheat cultivars for California. Div. of Agric. and Natural Resources, Univ. of California; 2011. http://smallgrains.ucdavis.edu/ [accessed 18 Feb. 2015]

Lunn J, Buttriss JL. Carbohydrates and dietary fibre. Nutr Bull. 2007; 32:21–64.10.1111/j.1467-3010.2007.00616.x

Ma J, Zhang CY, Yan GJ, Liu CJ. Improving yield and quality traits of durum wheat by introgressing chromosome segments from hexaploid wheat. Genet Mol Res. 2013; 12:6120–6129.10.4238/2013.December.2.9 [PubMed: 24338405]

Matsuo RR, Dexter JE. Relationship between some durum wheat physical characteristics and semolina milling properties. Can J Plant Sci. 1980; 60:49–53.10.4141/cjps80-007

Murphy MM, Douglass JS, Birkett A. Resistant starch intakes in the United States. J Am Diet Assoc. 2008; 108:67–78.10.1016/j.jada.2007.10.012 [PubMed: 18155991]

Regina, A.; Berbezy, P.; Kosar-hashemi, B.; Li, S.; Cmiel, M.; Larroque, O., et al. [accessed 23 July 2015] A genetic strategy generating wheat with very high amylose content. Plant Biotechnol J. 2015. http://onlinelibrary.wiley.com/doi/10.1111/pbi.12345/abstract

Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, et al. High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proc Natl Acad Sci USA. 2006; 103:3546–3551.10.1073/pnas.0510737103 [PubMed: 16537443]

Roberfroid MB. Introducing inulin-type fructans. Br J Nutr. 2005; 93(S1):S13–S25.10.1079/BJN20041350 [PubMed: 15877886]

SAS Institute. PROC GLM, SAS 9.3. SAS Inst; Cary, NC: 2013.

Sestili, F.; Botticella, E.; Lafiandra, D. TILLING for Improved starch composition in wheat. In: Tuberosa, R.; Graner, A.; Frison, E., editors. Genomics of plant genetic resources. Springer; Netherlands, Dordrecht: 2014. p. 467-487.

Sestili F, Janni M, Doherty A, Botticella E, D'Ovidio R, Masci S, et al. Increasing the amylose content of durum wheat through silencing of the *SBEIIa* genes. BMC Plant Biol. 2010; 10:144.10.1186/1471-2229-10-144 [PubMed: 20626919]

Sestili F, Palombieri S, Botticella E, Mantovani P, Bovina R, Lafiandra D. Plant science TILLING mutants of durum wheat result in a high amylose phenotype and provide information on alternative splicing mechanisms. Plant Sci. 2015; 233:127–133.10.1016/j.plantsci.2015.01.009 [PubMed: 25711820]

Sissons, MJ. Pasta. In: Corke, H.; Wrigley, C., editors. Encyclopedia of grain science. Elsevier; Boston, MA: 2004. p. 409-418.

Sissons MJ. Role of durum wheat composition on the quality of pasta and bread. Food. 2008; 2:75–90.

Slade AJ, McGuire C, Loeffler D, Mullenberg J, Skinner W, Fazio G, et al. Development of high amylose wheat through TILLING. BMC Plant Biol. 2012; 12:69.10.1186/1471-2229-12-69 [PubMed: 22584013]

Soh HN, Sissons MJ, Turner MA. Effect of starch granule size distribution and elevated amylose content on durum dough rheology and spaghetti cooking quality. Cereal Chem. 2006; 83:513–519.10.1094/CC-83-0513

Troccoli A, Borrelli GM, De Via P, Fares C, Di Fonzo N. Mini review: Durum wheat quality: A multidisciplinary concept. J Cereal Sci. 2000; 32:99–113.

Troccoli A, Di Fonzo N. Relationship between kernel size features and test weight in triticum durum. Cereal Chem. 1999; 76:45–49.10.1094/CCHEM.1999.76.1.45

USDA-ARS NGRP. [accessed 18 Feb. 2015] USDA-ARS National germplasm system small grains collection. USDA ARS, Natl Ctr for Genetic Resources Preservation. 2014. www.ars-grin.gov/npgs

Yong-Cheng, S.; Maningat, CC. Resistant starch: Sources, applications and health benefits. John Wiley & Sons; Chichester, West Sussex, UK: 2013.

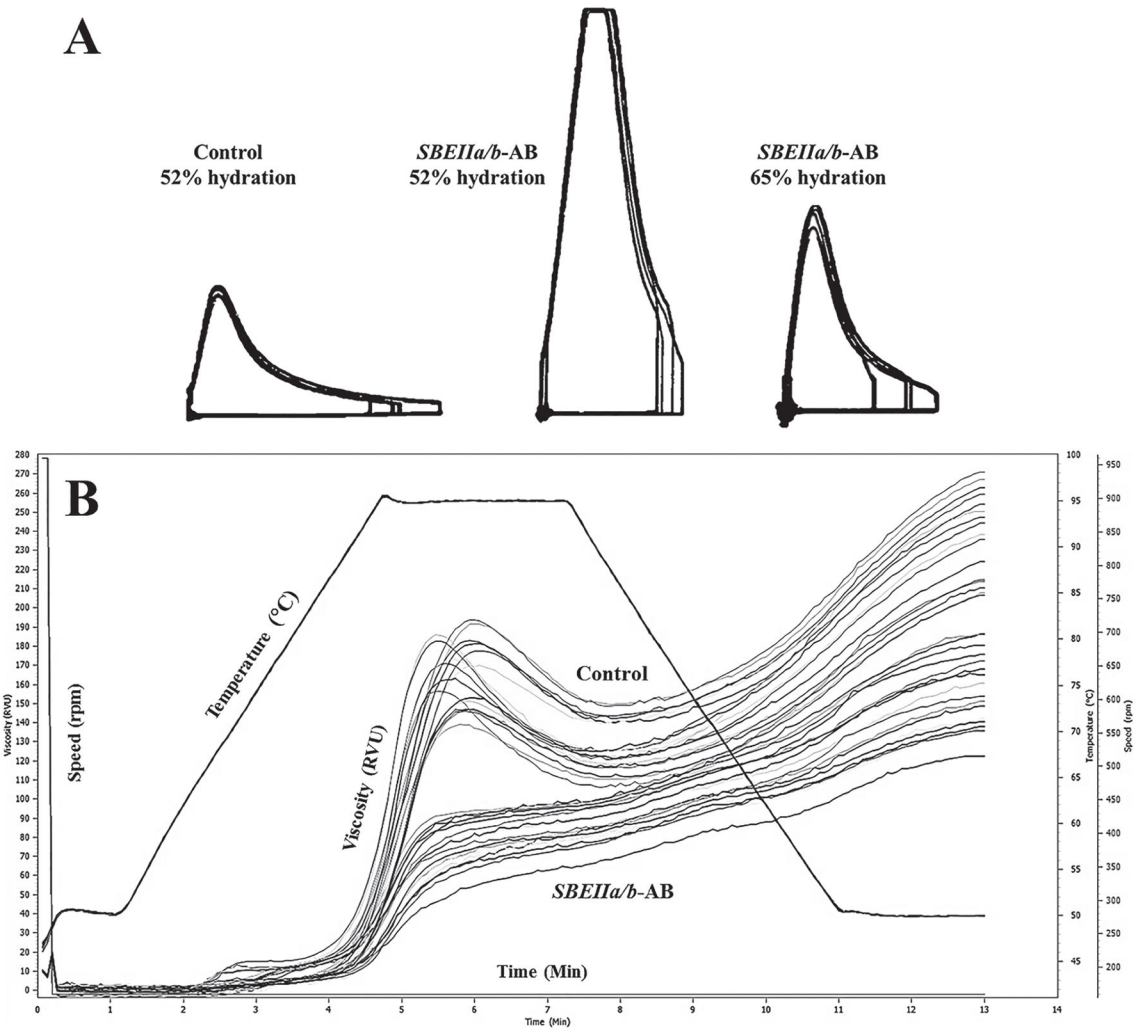

Figure 1.

(Panel A) Alveograms of wild-type control semolina at 52% hydration and *SBEIIa/b*-AB semolina at 52% and 65% hydration. (Panel B) RVA viscogram of wild-type control and *SBEIIa/b*-AB semolina depicting viscosity curves (RVU), temperature profiles (°C) and speed (rpm) for RVA analyses.

Table 1

Yield and kernel weight of *SBEIIa/b*-AB mutants and wild-type sib control lines with fixed model ANOVAs for combined locations. Untransformed arithmetic means are reported for the three locations combined and separately.

Location	Genotype	Yield	Kernel weight
		kg ha^{-1}	mg kernel^{-1}
All locations	*SBEIIa/b*-AB	3380.7 ± 325.1	53.3 ± 1.3
	Wild type	3995.3 ± 356.7	56.2 ± 0.8
Imperial Valley	*SBEIIa/b*-AB	4735.4 ± 255.7	52.2 ± 1.3
	Wild type	5189.8 ± 231.9	57.8 ± 0.7
Sacramento Valley	*SBEIIa/b*-AB	1957.9 ± 282.4	57.6 ± 0.8
	Wild type	2118.5 ± 239.2	58.5 ± 1.1
San Joaquin Valley	*SBEIIa/b*-AB	3448.9 ± 400.3	50.1 ± 0.7
	Wild type	4677.6 ± 321.9	52.3 ± 0.9
Source of Variation			
Genotype† (*P*)		0.06	0.0007
Location‡ (*P*)		<0.0001	<0.0001
Genotype × Location† (*P*)		0.4	0.032
Block(Location)† (*P*)		0.1	0.2
Variation Explained (R^2)		0.83	0.88

† Error used = MS(Error).

‡ Error used = MS(block(location)).

Table 2

Resistant starch (“as is” basis), amylose, and total starch (dry-weight basis) of *SBEIIa/b*-AB mutants and wild-type sib control lines with fixed model ANOVAs for combined locations. Untransformed arithmetic means are reported for three locations combined and separately.

Location	Genotype	Resistant starch	Amylose	Total starch
		g/kg	%	g/kg
All Locations	*SBEIIa/b*-AB	61 ± 5	44.9 ± 2.5	668 ± 11
	Wild type	6 ± 0.4	27.9 ± 0.4	708 ± 15
Imperial Valley	*SBEIIa/b*-AB	80 ± 8	40.9 ± 6.8	635 ± 17
	Wild type	7 ± 1.0	26.1 ± 0.8	666 ± 19
Sacramento Valley	*SBEIIa/b*-AB	44 ± 2	47.6 ± 7.9	652 ± 9
	Wild type	5 ± 0.2	28.4 ± 0.4	710 ± 9
San Joaquin Valley	*SBEIIa/b*-AB	59 ± 5	46.1 ± 7.8	717 ± 11
	Wild type	7 ± 0.5	29.2 ± 0.3	749 ± 35
Source of Variation				
Genotype† (*P*)		<0.0001	<0.0001	0.028
Location‡ (*P*)		0.0003	<0.0001	0.0012
Genotype × Location† (*P*)		0.2	0.4	0.8
Block(Location)† (*P*)		0.6	0.8	0.7
Variation explained (R^2)		0.98	0.97	0.70

†Error used = MS(Error).

‡Error used = MS(block(location)).

Table 3

Grain, milling and dough quality parameters of *SBEIIa/b*-AB mutants and wild-type sib control lines with fixed model ANOVAs for combined locations. Untransformed arithmetic means are reported for three locations combined and separately. Protein is reported on a 12% moisture basis and ash is reported on an "as is" basis.

Location	Genotype	Test weight	Black tip	Semolina extract	Semolina ash	Falling number	Semolina protein	Gluten index	Wet gluten	Alveo-graph W	Alveo-graph P/L
		kg/hL		———%———		s		———%———		$\times 10^{-4}$ J	P/L
All locations	*SBEIIa/b*-AB	77.2 ± 0.4	4.7 ± 1.0	58.6 ± 0.7	1.4 ± 0.1	822.2 ± 47.6	16.0 ± 0.5	24.6 ± 3.5	45.0 ± 1.5	94.5 ± 8.5	3.7 ± 0.4
	Wild type	80.3 ± 0.3	2.6 ± 0.7	61.4 ± 0.7	1.0 ± 0.04	595.1 ± 24.2	14.3 ± 0.5	21.9 ± 4.4	41.4 ± 1.5	85.1 ± 6.5	1.8 ± 0.2
Imperial	*SBEIIa/b*-AB	77.8 ± 0.1	2.7 ± 0.7	62.6 ± 0.5	1.7 ± 0.4	997.7 ± 84.9	16.4 ± 0.4	13.4 ± 4.0	49.4 ± 0.8	63.0 ± 7.1	1.7 ± 0.1
	Wild type	80.1 ± 0.2	1.7 ± 0.3	64.1 ± 0.6	1.0 ± 0.08	702.8 ± 26.9	15.4 ± 0.1	8.7 ± 0.4	46.1 ± 0.7	67.4 ± 2.9	0.9 ± 0.1
Sacramento	*SBEIIa/b*-AB	75.2 ± 0.2	9.8 ± 0.9	56.4 ± 0.5	1.3 ± 0.02	776.2 ± 71.0	18.0 ± 0.2	22.2 ± 5.8	48.4 ± 1.7	97.2 ± 13.1	3.5 ± 0.4
	Wild type	78.9 ± 0.4	6.0 ± 1.3	60.5 ± 0.8	1.0 ± 0.03	487.0 ± 12.5	16.2 ± 0.2	10.3 ± 0.6	45.0 ± 1.0	83.1 ± 14.8	1.9 ± 0.2
San Joaquin	*SBEIIa/b*-AB	78.6 ± 0.4	1.5 ± 0.3	56.9 ± 0.7	1.2 ± 0.03	692.7 ± 30.2	13.5 ± 0.4	38.2 ± 3.6	37.2 ± 1.4	123.3 ± 12.1	5.8 ± 0.3
	Wild type	81.8 ± 0.2	0.2 ± 0.2	59.7 ± 1.4	0.9 ± 0.07	595.3 ± 20.3	11.4 ± 0.2	46.6 ± 3.2	33.1 ± 0.9	104.9 ± 7.8	2.4 ± 0.2
Source of Variation											
Genotype[†] (*P*)		<0.0001	0.0018	<0.0001	0.0004	<0.0001	<0.0001	0.4	<0.0001	0.2	<0.0001
Location[‡] (*P*)		<0.0001	<0.0001	0.0001	0.5	0.0002	<0.0001	<0.0001	<0.0001	0.0053	<0.0001
G × L[§] (*P*)		0.034	0.1	0.2	0.9	0.049	0.040	0.031	0.9	0.3	0.1
B(L)[¶] (*P*)		0.1	0.2	0.1	0.6	0.4	0.021	0.5	0.012	0.038	0.3
R^2		0.97	0.92	0.90	0.70	0.88	0.98	0.89	0.96	0.85	0.95

[†]Error used = MS(Error).

[‡]Error used = MS[block(location)].

[§]G × L = Genotype × Location.

[¶]B(L) = Block (Location).

Table 4

Pasta quality parameters of *SBEIIa/b*-AB mutants and wild-type sib control lines with fixed model ANOVAs for combined locations. Untransformed arithmetic means are reported for two locations combined and separately.

Location	Genotype	Cooked weight	Cooking loss	Firmness	Color score	Color b	Color L	Color a
					score	(yellow–blue)	(black–white)	(red–green)
		g	%	g × cm				
All locations	*SBEIIa/b*-AB	27.0 ± 0.2	6.6 ± 0.1	8.5 ± 0.4	7.8 ± 0.1	38.2 ± 0.3	54.4 ± 0.5	3.2 ± 0.2
	Wild type	28.8 ± 0.2	5.4 ± 0.1	7.6 ± 0.3	8.7 ± 0.2	40.4 ± 0.4	55.6 ± 0.4	1.9 ± 0.3
Imperial Valley	*SBEIIa/b*-AB	27.2 ± 0.4	6.4 ± 0.1	9.2 ± 0.6	7.8 ± 0.1	38.4 ± 0.2	53.0 ± 0.2	3.4 ± 0.4
	Wild type	28.4 ± 0.3	5.4 ± 0.1	8.5 ± 0.1	8.3 ± 0.2	39.3 ± 0.2	54.6 ± 0.5	2.6 ± 0.3
San Joaquin Valley	*SBEIIa/b*-AB	26.8 ± 0.2	6.7 ± 0.2	7.7 ± 0.5	7.8 ± 0.2	37.9 ± 0.5	56.1 ± 0.5	2.9 ± 0.2
	Wild type	29.2 ± 0.4	5.5 ± 0.1	6.6 ± 0.2	9.2 ± 0.2	41.7 ± 0.3	56.8 ± 0.3	1.1 ± 0.3
Source of Variation								
Genotype† (*P*)		0.0005	<0.0001	0.053	0.0005	<0.0001	0.016	0.0019
Location‡ (*P*)		0.6	0.2	0.0013	0.041	0.032	<0.0001	0.0096
Genotype × Location† (*P*)		0.1	0.5	0.6	0.043	0.0006	0.4	0.1
Block(Location)† (*P*)		0.6	0.6	0.7	0.7	0.3	0.7	0.5
Variation explained (R^2)		0.81	0.86	0.75	0.83	0.93	0.87	0.82

†Error used = MS(Error).

‡Error used = MS(block(location).

Table 5

Fermentation response indices in rats fed *SBEIIa/b*-AB and control wheat diets. Untransformed arithmetic means and *P* values are reported.

Cecal contents	*SBEIIa/b*-AB	Control	*P* value
Weight, g	2.72 ± 0.16	1.99 ± 0.18	0.012
pH	6.88 ± 0.14	7.25 ± 0.06	0.029
SCFAs[†], μmol			
Total[‡]	319.8 ± 37.7	200.1 ± 28.6	<0.0001
Acetate	222.3 ± 28.0	142.5 ± 19.6	0.042
Propionate	51.8 ± 15.7	23.6 ± 4.3	0.1
Butyrate	45.7 ± 10.3	34.0 ± 5.9	0.3
Serum gut peptides			
GLP-1, pmol/L	0.31 ± 0.13	0.12 ± 0.02	0.2
PYY, ng/mL	0.66 ± 0.17	0.55 ± 0.05	0.2

[†]SCFAs, short-chain fatty acids.

[‡]Total SCFAs include acetate, propionate, and butyrate.