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Registration of Durum Wheat Germplasm Lines with Combined Mutations in *SBEII*a and *SBEIIb* Genes Conferring Increased Amylose and Resistant Starch

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Abstract

Durum wheat [*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.], used in pasta, couscous, and flatbread production, is an important source of starch food products worldwide. The amylose portion of the starch forms resistant starch complexes that resist digestion and contribute to dietary fiber. Increasing the amount of amylose and resistant starch in wheat by mutating the *STARCH BRANCHING ENZYME II* (*SBEII*) genes has potential to provide human health benefits. Ethyl methane sulfonate mutations in the linked *SBEIIa* and *SBEIIb* paralogs were combined on chromosomes 2A (*SBEIIa/b*-A; Reg. No. GP-968, PI 670159), 2B (*SBEIIa/b*-B; Reg. No. GP-970, PI 670161), and on both chromosomes (*SBEIIa/b*-AB; Reg. No. GP-969, PI 670160) in the tetraploid wheat cultivar Kronos, a semidwarf durum wheat cultivar that has high yield potential and excellent pasta quality. These three double and quadruple *SBEII*-mutant lines were compared with a control sib line with no *SBEII* mutations in two field locations in California. The *SBEIIa/b*-AB line with four mutations showed dramatic increases in amylose (average 66%) and resistant starch (average 753%) relative to the control. However, the *SBEIIa/b*-AB line also showed an average 7% decrease in total starch and an 8% decrease in kernel weight. The release by the University of California–Davis of the durum wheat germplasm combining four *SBEIIa* and *SBEIIb* mutations will accelerate the deployment of these mutations in durum wheat breeding programs and the development of durum wheat varieties with increased resistant starch.

Wheat (*Triticum* ssp.) is an important cereal crop that contributed over 651 million t of food worldwide in 2012 and is a valuable source of carbohydrates, amino acids, vitamins and minerals (FAO, 2012). Durum wheat [*T. turgidum* L. subsp. *durum* (Desf.) Husn.] production has been increasing globally since the 1950s and has currently reached about 33 million t per year (Ma et al., 2013). Durum wheat is most useful for producing pasta, couscous, and flatbreads because of its unique quality aspects, including hardness, high protein content, and high gluten strength (Ma et al., 2013). The demand for food products rich in fiber is growing among consumers as a result of increased awareness of the associated health benefits, such as reduced risk of type II diabetes, obesity, cardiovascular diseases, and cancers of the colon (Yong-Cheng and Maningat, 2013; Sestili et al., 2014). High levels of amylose in the starch of wheat and other cereals are associated with increased

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resistant starch, an important component of dietary fiber. Resistant starch is defined as the undigested portion of starch that passes through the small intestine and is ultimately fermented in the large intestine by the gut microflora (Englyst et al., 1992). The portion of the starch that is resistant to enzymatic digestion is thought to originate in long amylose glucan chains that associate and form complexes that function similar to other dietary fibers. Foods with resistant starch have reduced glycemic indices, which are important in obesity and diabetes prevention. The fermentation of resistant starch in the large intestine produces short chain fatty acids that provide additional health benefits by lowering the pH of the intestinal environment, which reduces the proliferation of pathogenic bacteria and absorption of cytotoxic compounds (Sestili et al., 2014). Along with improved nutritional properties and health benefits, moderate increases in the proportion of amylose in wheat have been associated with improved qualities in food products such as improved texture in baked goods and increased firmness in pasta (Waring, 2005; Soh et al., 2006).

Starch-based foods comprise more than 50% of the human diet. Thus, improving the starch composition in food products by increasing the amount of amylose and resistant starch has the potential to provide consumers with the associated health benefits. Because of this, increasing amylose and resistant starch in wheat grain is a valuable objective for both durum and common wheat (*T. aestivum*) breeding programs. Starch branching enzymes play a major role in determining the proportion of amylose and amylopectin in the wheat grain, and downregulation or mutations of the *STARCH BRANCHING ENZYME II* (*SBEII*) genes result in increased amylose and resistant starch in the grain (Regina et al., 2006; Sestili et al., 2010; Botticella et al., 2011; Hazard et al., 2012; Slade et al., 2012). We previously obtained and described ethyl methane sulfonate (EMS) mutants for the paralogous genes *SBEIIa* and *SBEIIb* from a TILLING (targeted induced local lesions in genomes) population of the tetraploid durum wheat cultivar Kronos (Uauy et al., 2009; Hazard et al., 2012). We also identified recombinant lines that combined *SBEIIa* and *SBEIIb* mutations on a single chromosome, both in the A and B genome copies of these genes (homoeologs), but only the single *SBEIIa* mutants were characterized for amylose and resistant starch content in the previous study (Hazard et al., 2012). In this study, we register three mutant durum wheat germplasm lines developed at the University of California, Davis that carry linked *SBEIIa* and *SBEIIb* mutations in coupling in the A genome (*SBEIIa/b*-A; Reg. No. GP-968, PI 670159), in the B genome (*SBEIIa/b*-B; Reg. No. GP-970, PI 670161), and in both genomes (*SBEIIa/b*-AB; Reg. No. GP-969, PI 670160). We also determined the effect of these mutations on amylose and resistant starch content in the grain, on total starch content, and on kernel weight (KW) relative to a sib control line with no *SBEII* mutations.

Materials and Methods

Development of *SBEII* Germplasm Lines

SBEII mutants were selected from a TILLING population (Uauy et al., 2009) of the Desert durum cultivar Kronos (PI 576168), which was developed by Arizona Plant Breeders Inc. from a male sterile population (selection D03–21). A more detailed description of the identification, selection, and generation of the mutant lines is described in Hazard et al. (2012), and a summary of mutations is provided in Table 1. Mutant lines were backcrossed

twice to Kronos to reduce background mutations. In the previous study, we combined the *SBEIIa* and *SBEIIb* mutations and selected plants homozygous for the two mutations both in the A (*SBEIIa/b*-A) and in the B (*SBEIIa/b*-B) genomes (Hazard et al., 2012). In this study, we intercrossed *SBEIIa/b*-A and *SBEIIa/b*-B, self-pollinated the F^1 hybrid, and generated a new segregating population. From this segregating population we selected sib lines homozygous for *SBEIIa* and *SBEIIb* mutations only in the A genome (*SBEIIa/b*-A), only in the B genome (*SBEIIa/b*-B), in both the A and B genomes (*SBEIIa/b*-AB) and sib lines with no *SBEII* mutations to be used as controls. Selected lines were increased in the greenhouse for use in phenotyping experiments. Plants carrying the targeted mutations were selected in each generation by sequencing, using the same genome specific primers designed to screen the original TILLING population described in Uauy et al. (2009).

Phenotypic Characterization of *SBEII* Germplasm Lines

Mutant lines were grown in two field experiments and compared with wild-type sib control lines. The field experiments were grown in the University of California Experimental Field Station in Davis, CA (38°32′ N, 121°46′ W), and the University of California Research and Extension Center in Tulelake (Intermountain Research and Extension Center, 41°57′ N, 121°28′ W). In Davis, sowing occurred in December (winter planting) in a Yolo loam soil (fine-silty, mixed, superactive, nonacid, thermic Mollic Xerofluvents), and the fertilization consisted of a preplanting application of 112 kg ha^{-1} N and a top-dress application of 67 kg ha^{-1} N at tillering. In Tulelake, lines were sown in May (spring planting) in a Tulebasin mucky silty clay loam soil (fine, mixed, superactive, mesic Aquandic Endoaquolls) and were fertilized with a preplanting application of 149 kg ha^{-1} N. Each experiment was set up in a randomized complete block design with six blocks (replicates). For each block, 50 seeds per genotype were sown in 1-m rows (experimental units). Once mature, grains were harvested from each row. For the Davis experiment, KW was estimated from the weight of 100 to 1000 kernels per sample, with the exception of one sample in which 50 kernels were used due to limited seed availability. Five hundred kernels per sample were used to estimate KW in the Tulelake experiment. Whole grain flour was prepared for each sample by grinding in a UDY Cyclone Mill (UDY Corporation) using a 0.5-mm screen, and grain moisture was determined for each sample at the California Wheat Commission Milling and Baking Laboratory using AOAC Official Method 925.10 (AOAC, 2000).

Relative amylose content (amount of amylose as a percentage of total starch) was measured for 25-mg samples of whole-grain flour using the AMYLOSE/AMYLOPECTIN kit developed by Megazyme International (2011a) following the manufacturer instructions. Relative resistant starch content (amount of resistant starch as a percentage of total starch) was measured for 100-mg samples of whole-grain flour using the RESISTANT STARCH kit from Megazyme International (2011b). In this assay, both solubilized and resistant starch were calculated on a dry weight basis using percentage moisture values and following instructions provided in Megazyme International (2011b). Total starch content was measured for 100-mg samples of whole grain flour using the TOTAL STARCH kit developed by Megazyme International (2011c) following the manufacturer's instructions for the recommended KOH assay format.

To account for potential variation among assays performed at different times, complete sets of samples including all four genotypes were measured in each assay, and sets were then included as a block in the statistical analyses. Data were analyzed first combining results from both locations using a mixed model ANOVA (SAS Institute, 2011), where environment, genotype × environment, and block within environment were included as random factors. Means of the individual mutant lines were compared with the wild-type sib line using the Dunnett test. These mean comparisons are reported separately for each location to show the consistency of the results across environments. The ANOVA assumptions were tested using Shapiro–Wilk's test for normality of residuals and Levene's test for homogeneity of variances.

Characteristics

Amylose Content

The ANOVA for relative amylose content combining both locations explained 83% of the variation and showed no significant effects of environment ($P = 0.143$) or genotype × environment interactions ($P = 0.428$) but showed significant difference among genotypes ($P < 0.005$) (Table 2). Mean comparisons of mutant lines against the wild-type sib control using the Dunnett test showed no significant differences for the mutant lines carrying *SBEIIa* and *SBEIIb* mutations only in the A or B genomes (Table 3). However, the quadruple *SBEIIa/b*-AB showed significantly higher levels of amylose than the wild-type sib control in both locations ($P < 0.01$) (Table 3). The relative increase in amylose content in the *SBEIIa/b*-AB mutant lines relative to the control was 81% in Tulelake and 51% in Davis (average 66% increase).

Resistant Starch Content

The combined ANOVA for resistant starch showed significant differences among environments ($P = 0.027$) and genotypes ($P = 0.002$) but no significant genotype × environment interaction (Table 2). Approximately 91% of the variation in resistant starch is explained by the combined model (Table 2). Similar to results reported above for relative amylose content, differences in relative resistant starch between the mutants and wild-type sib control were significant only for the *SBEIIa/b*-AB quadruple mutant line (Dunnett test $P < 0.01$) and were consistent across locations (Table 3). The single-genome mutant lines showed nonsignificant differences when compared to the control (Table 3). On average, the relative resistant starch content of the *SBEIIa/b*-AB quadruple mutant was 753% higher than the control. The observed differences were higher in Davis (867% increase) than in Tulelake (640% increase) as shown by the significant effect of location (Table 2).

Total Starch Content

The combined ANOVA explained 66% of the variation in total starch content and showed a strong effect of environment ($P < 0.0001$). The effect of genotype on this trait was smaller than the effect of the environment but was still significant ($P = 0.024$). Similar trends in the variation in total starch content among genotype were observed in the two locations, resulting in a nonsignificant genotype × environment interaction (Table 2). In both locations, the *SBEIIa/b*-AB quadruple mutant showed lower values of total starch content than the

wild-type sib control, but the differences were significant only in Tulelake ($P < 0.05$, Table 3). The *SBEIIa/b*-AB mutant line showed an average 9% decrease relative to the control in Tulelake but only a 5% decrease in Davis. The *SBEIIa/b*-A and *SBEIIa/b*-B mutant lines were not significantly different from the control in both locations (Table 3).

Kernel Weight

The combined ANOVA for KW explained 63% of the variation and showed no significant differences among environments, genotypes or their interaction (Table 2). However, the differences among genotypes were close to significant levels (0.059) and when analyzed separately by location, showed significant differences in Tulelake ($P = 0.0037$) but not in Davis ($P = 0.5212$). In Tulelake, the *SBEIIa/b*-AB quadruple mutant showed 9% decrease in KW relative to the control ($P < 0.01$) (Table 3). Even though the difference was not significant in Davis, the *SBEIIa/b*-AB mutant lines also showed a 6% reduction in KW relative to the control. The single-genome mutant lines *SBEIIa/b*-A and *SBEIIa/b*-B were not significantly different from the wild-type sib control (Table 3).

Discussion

The *SBEIIa/b*-AB mutant with combined *SBEIIa/b* linked mutations in both the A and B genome copies of *SBEII* will be a useful tool for durum wheat breeding programs interested in increasing amylose and resistant starch content. To facilitate the utilization of these mutations in durum breeding programs, we selected a recurrent parent (Kronos) with high yield potential and excellent pasta quality (Jackson, 2011). Here we demonstrate that the quadruple *SBEIIa/b*-AB mutant has significantly higher levels of amylose (66%) and resistant starch (753%) than the control, while the *SBEIIa/b*-A and *SBEIIa/b*-B lines with combined mutations in only one genome did not (Table 3). The increases reported here are also significantly higher than those observed in the *SBEIIa*-AB mutants reported previously (Hazard et al., 2012) that combine mutations in both genomes but only for the *SBEIIa* gene (22% increase in amylose content and 115% increase in resistant starch). These results indicate that the *SBEIIa* and *SBEIIb* genes have redundant functions and that only simultaneous mutations in all copies of both paralogs result in large increases in amylose and resistant starch.

However, durum wheat breeders need to be aware that these dramatic increases in relative amylose and resistant starch content expected from the *SBEIIa/b*-AB mutant are also associated with reduction in total starch content (7%) and KW (8%) across both locations. The changes in KW and total starch content are likely a pleiotropic effect of the mutant *SBEII* alleles, but it is also possible that this effect results from background effects of other mutations that occurred during the original EMS mutagenesis. It is also possible that the negative impact of reductions in KW on grain yield may be compensated in some genotypes by increases in seed number. Finally, there were no significant genotype × environment interactions for the four traits considered here, suggesting that the effects of these mutations are consistent across environments (Table 2). In summary, durum wheat breeders interested in increasing resistant starch using these mutants will need to determine the negative effects of these mutations on total starch and kernel weight in their own genetic backgrounds and

environments. We are currently introgressing these four mutations into different durum genetic backgrounds to evaluate their effect on yield and pasta quality in different locations in California. In addition, we are transferring the *SBEIIa/b* linked mutations into hexaploid wheat and combining them with an available *SBEIIa*-D genome mutant, to facilitate the future utilization of these mutations in common wheat.

Availability

Seed of the three mutant lines is available from the USDA–ARS National Center for Genetic Resources Preservation (USDA-ARS National Genetic Resources Program, 2014). The seeds for *SBEIIa/b*-A, *SBEIIa/b*-B, and *SBEIIa/b*-AB were generated through a greenhouse increase of the materials harvested from the Davis field experiment and are homozygous for mutations at the *SBEIIa* and *SBEIIb* loci.

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Abbreviations

EMS	ethyl methane sulfonate
KW	kernel weight
TILLING	targeted induced local lesions in genomes

References

AOAC. Official methods of analysis of the association of official analytical chemists. 17. AOAC; Gaithersburg, MD: 2000. Official method 925.10: Solids (total) and moisture in foodstuffs (air oven method).

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(1): 156.10.1186/1471-2229-11-156 [PubMed: 22074448]

Englyst HN, Kingman SM, Cummings JH. Classification and measurement of nutritionally important starch fractions. Eur J Clin Nutr. 1992; 46(Suppl 2):S33–S50. [PubMed: 1330528]

Food and Agricultural Organization. Trends in the crop sector. FAO; Rome: 2012. FAO statistical yearbook. Part 3.

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; 52:1754–1766.10.2135/cropsci2012.02.0126 [PubMed: 26924849]

Jackson, L. Wheat cultivars for California. University of California; Davis: 2011. http://smallgrains.ucdavis.edu/cereal_files/WhtCVDescLJ11.pdf

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]

Megazyme International. Amylose/amylopectin assay procedure. K-AMYL 07/11. Megazyme; Wicklow, Ireland: 2011a. http://secure.megazyme.com/files/BOOKLET/K-AMYL_1107_DATA.pdf [accessed 16 Jan. 2014]

Megazyme International. Resistant starch assay procedure. K-RSTAR 08/11. Megazyme; Wicklow, Ireland: 2011b. http://secure.megazyme.com/files/BOOKLET/K-RSTAR_1108_DATA.pdf [accessed 16 Jan. 2014]

Megazyme International. Total starch assay procedure (amyloglucosidase α-amylase method). K-TSTA 07/11. Megazyme; Wicklow, Ireland: 2011c. http://secure.megazyme.com/files/BOOKLET/K-TSTA_1107_DATA.pdf [accessed 16 Jan. 2014]

Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, Kosar-Hashemi B, Li Z, Rahman S, Morell M. 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]

SAS Institute. SAS user's guide, version 9.2. SAS Inst; Cary, NC: 2011.

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, Jones HD, Lafiandra D. 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]

Slade AJ, McGuire C, Loeffler D, Mullenberg J, Skinner W, Fazio G, Holm A, Brandt KM, Steine MN, Goodstal JF, Knauf VC. 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

Uauy C, Paraiso F, Colasuonno P, Tran RK, Tsai H, Berardi S, Comai L, Dubcovsky J. A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. BMC Plant Biol. 2009; 9:115.10.1186/1471-2229-9-115 [PubMed: 19712486]

USDA–ARS National Genetic Resources Program. Germplasm Resources Information Network (GRIN). National Germplasm Resources Laboratory; Beltsville, MD: 2014. http://www.ars-grin.gov [accessed 18 July 2014]

Waring, S. Functionality of resistant starch in food applications. National Starch and Chemical Co; Bridgewater, NJ: 2005.

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

Table 1

Summary of *SBEIIa* and *SBEIIb* mutations. Line is the mutant number in the TILLING population.[†] Protein coordinates are based on protein sequences from the corresponding genome in *Triticum aestivum* cultivar Chinese Spring.[‡]

Gene	Genome	Line	DNA coordinates	Protein coordinates and predicted effect
SBEIIa	A	T4-2179	G401A	W220 (stop codon)
SBEIIa	B	T4-1214	G1347A	E296 (splice junction)
SBEIIb	A	T4-2574	G308A	S208 (splice junction plus stop)
SBEIIb	B	T4-764	C1290T	P283L (BLOSUM62 = −3)

[†]DNA coordinates are based on *Triticum turgidum* cultivar Kronos partial genomic sequence used for TILLING (Uauy et al., 2009).

[‡]For *SBEIIb*-B there was no complete protein available (the P283L coordinate is based on the A and D genome proteins at the same position) (Hazard et al., 2012).

Table 2

Mixed model ANOVAs for combined locations for relative amylose content, relative resistant starch content, total starch content, and kernel weight. Genotypes include *SBEIIa/b*-A, *SBEIIa/b*-B, and *SBEIIa/b*-AB mutants and wild-type sib control lines.

Source of variation	Relative amylose content	Relative resistant starch content	Total starch content	Kernel weight
Genotype[†] (*P*)	0.005	0.002	0.024	0.059
Environment[‡] (*P*)	0.143	0.027	<0.0001	0.504
Genotype × environment (*P*)	0.428	0.497	0.874	0.670
Block(environment) (*P*)	0.726	0.005	0.831	0.001
Proportion of variation explained(R^2)	0.832	0.909	0.659	0.626

[†]Error used = genotype × environment.

[‡]Error used = MS[block(environment)] + MS(genotype × environment) − MS(error).

Table 3

Effect of *SBEIIa* and *SBEIIb* mutations on relative amylose content, relative resistant starch content (RS), total starch content (TS) and kernel weight (KW) in *SBEIIa/b*-A, *SBEIIa/b*-B, and *SBEIIa/b*-AB mutants and wild-type sib control lines. Untransformed arithmetic means and *P* values from Dunnett tests are reported separately for the two locations.

Line	Amylose	*P*	RS	*P*	TS	*P*	KW	*P*
	%		%		%		mg kernel^{-1}	
Davis								
Control	26.6 ± 1.0		0.3 ± 0.1		68.3 ± 1.3		48.8 ± 1.3	
SBEIIa/b-A	27.0 ± 0.7	NS	0.6 ± 0.2	NS	68.5 ± 2.0	NS	47.1 ± 1.7	NS
SBEIIa/b-B	28.6 ± 1.1	NS	0.4 ± 0.1	NS	66.4 ± 1.2	NS	46.7 ± 1.8	NS
SBEIIa/b-AB	40.1 ± 1.3	<0.01	2.9 ± 0.4	<0.01	64.7 ± 2.8	NS	45.8 ± 1.5	NS
Tulelake								
Control	30.0 ± 1.4		0.5 ± 0.1		62.9 ± 0.8		49.7 ± 1.0	
SBEIIa/b-A	28.3 ± 1.0	NS	0.7 ± 0.1	NS	62.2 ± 1.1	NS	49.2 ± 1.3	NS
SBEIIa/b-B	29.9 ± 1.4	NS	0.7 ± 0.1	NS	60.9 ± 1.5	NS	48.0 ± 1.4	NS
SBEIIa/b-AB	54.4 ± 3.6	<0.01	3.7 ± 0.2	<0.01	57.0 ± 1.4	<0.05	45.2 ± 1.0	<0.01