RESEARCH

Induced Mutations in the *Starch Branching Enzyme II* (*SBEII*) Genes Increase Amylose and Resistant Starch Content in Durum Wheat

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ABSTRACT

Starch is the largest component of the wheat (*Triticum aestivum* L.) grain and consists of approximately 70 to 80% amylopectin and 20 to 30% amylose. Amylopectin is a highly branched, readily digested polysaccharide whereas amylose has few branches and forms complexes that resist digestion and mimic dietary fiber (resistant starch). Downregulation of the *starch branching enzyme II* (*SBEII*) gene by RNA interference (RNAi) was previously shown to increase amylose content in both hexaploid and tetraploid wheat. We generated ethyl methane sulfonate (EMS) mutants for the *SBEIIa*-A and *SBEIIa*-B homeologs in the tetraploid durum wheat variety Kronos [*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.]. Single-gene mutants showed nonsignificant increases in amylose and resistant starch content, but a double mutant combining a *SBEIIa*-A knock-out mutation with a *SBEIIa*-B splice-site mutation showed a 22% increase in amylose content ($P < 0.0001$) and a 115% increase in resistant starch content ($P < 0.0001$). In addition, we obtained mutants for the A and B genome copies of the paralogous *SBEIIb* gene, mapped them 1 to 2 cM from *SBEIIa*, and generated double *SBEIIa-SBEIIb* mutants to study the effect of the *SBEIIb* gene in the absence of *SBEIIa*. These mutants are available to those interested in increasing amylose content and resistant starch in durum wheat.

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Abbreviations: AMG, amyloglucosidase; BLOSUM, block substitution matrix for pairwise protein sequence alignments; Con A, concanavalin A; EMS, ethyl methane sulfonate; GOPOD, glucose oxidase–peroxidase; PSSM, position-specific scoring matrix; RNAi, RNA interference; SBE, starch branching enzyme; SIFT, sorting intolerant from tolerant; SS, starch synthase; TILLING, targeted induced local lesions in genomes.

Wheat provides approximately one fifth of the calories consumed by the human population and is an important source of the carbohydrates, proteins, fats, vitamins, and minerals that contribute to a healthy human diet (FAO, 2006). Since starch is the major component of the wheat kernel (~50–70% of its dry weight), any improvements in its nutritional composition have the potential to deliver benefits to a large number of people. An important component of starch nutritional value is the relative proportion of amylose (a linear chain of D-glucose molecules containing few branches) and amylopectin (a highly branched polysaccharide) (reviewed in Ball and Morell, 2003).

In wheat, amylopectin represents 70 to 80% of the starch dry weight in the grain and is readily digested by humans and other mammals. Amylose represents the other 20 to 30% of the starch dry weight and tends to form complexes that are resistant

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to digestion and produce a similar physiological effect as fiber (Ball and Morell, 2003; Botticella et al., 2011). Starch that is physically inaccessible to α-amylase digestion in the human small intestine is defined as resistant starch (Englyst and Macfarlane, 1986). In cooked foods, amylose molecules reassociate rapidly during the cooling process and form complexes that resist digestion whereas amylopectin molecules reassociate slowly and are more readily digested. Because of these characteristics, food products made from flours with higher amylose content contain more resistant starch. Amylose and resistant starch content are defined in this study as the percent amylose or resistant starch relative to the total starch in the grain.

As with fiber, increased consumption of resistant starch has been associated with reduced risk of diseases such as diabetes, obesity, heart disease, and cancers of the colon and rectum (Behall et al., 2006; Faivre and Bonithon-Kopp, 1999; Hendrich, 2010; Park et al., 2004; Yamada et al., 2005). Reduced starch digestion in the small intestine decreases the rate of glucose entering the bloodstream, which in turn reduces the demand for insulin and lowers the glycemic index of the consumed food. In addition, the starch that is not digested in the small intestine moves to the large bowel and is fermented to short chain fatty acids that play important roles in large bowel function and have been associated with increased satiety (Granfeldt et al., 2006; Willis et al., 2009).

Although normal amylose content in the wheat grain is relatively low (20–30%), it can be increased by down regulating the transcript levels of genes involved in the synthesis of amylopectin since both pathways use adenosine diphosphoglucose as substrate (Regina et al., 2006; Sestili et al., 2010). The main enzyme involved in amylose synthesis is the granule bound starch synthase (GBSSI) whereas amylopectin is produced by the concerted action of starch synthases (SSI, SSII, and SSIII), starch branching enzymes (SBEI, SBEIIa, and SBEIIb), and starch debranching enzymes (Sestili et al., 2010). The key enzyme for the addition of branching points during starch synthesis is SBEII, which is a transglycosylase enzyme (EC 2.4.1.18). Starch branching enzymes catalyze the formation of α-1,6 branches within α-glucan chains by cleaving internal α-1,4 links followed by reattachment of the cleaved glucan to another chain via an α-1,6 linkage (Ball and Morell, 2003). In cereals there are two isoforms of SBEII, designated SBEIIa and SBEIIb (Boyer and Preiss, 1978). Each isoform has unique roles in creating glucan branches of distinct lengths but also have some overlapping functions (Butardo et al., 2011). In wheat, these isoforms are encoded by genes located on the long arm of chromosomes from homeologous group 2 (Rahman et al., 2001; Regina et al., 2005).

Starch branching enzyme IIa is the major isoform in the soluble phase of the wheat endosperm and therefore was hypothesized to play a major role in determining glucan branching and the proportion of amylose and amylopectin in the wheat grain (Regina et al., 2005). Silencing of *starch branching enzyme IIa* (*SBEIIa*) by RNA interference (RNAi) in hexaploid wheat (*Triticum aestivum* L.) showed a concomitant downregulation of *SBEIIb* and resulted in starch containing up to 70% amylose whereas plants with reduced transcript levels of *SBEIIb* alone showed no significant differences in amylose content in the grain (Regina et al., 2006). Experiments in durum wheat [*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.] using RNAi to target the *SBEIIa* gene also showed large increases in amylose content (Sestili et al., 2010). In barley (*Hordeum vulgare* L.), transgenic RNAi plants with reduced levels of SBEIIb protein showed no significant differences in amylose content whereas those with reduced levels of SBEIIa protein showed intermediate amylose content. Transgenic lines with reduced levels of SBEIIa and SBEIIb showed the highest amylose content (Regina et al., 2010). Considered together, these experiments support a central role of *SBEIIa* in the determination of the amylose:amylopectin ratio in the wheat grain but also suggest that *SBEIIb* has a significant effect on amylose content in the absence of *SBEIIa*.

The contribution of the individual *SBEIIa* copies from the different wheat genomes (homeologs) to grain amylose content is not known. This information is important for wheat breeding programs and can help determine the best mutation combinations to obtain an adequate amylose:amylopectin balance to maximize the nutritional benefits of resistant starch without compromising end product quality.

In this study, we used our recently developed tetraploid wheat TILLING (targeted induced local lesions in genomes) population (Uauy et al., 2009) to identify mutations in the A and B genome copies of the two *SBEII* paralogs (*SBEIIa* and *SBEIIb*) and to generate nontransgenic durum wheat lines with increased amylose and resistant starch content. The contribution of the individual and combined *SBEIIa* homeologs to grain amylose and resistant starch content was also evaluated. Finally, we intercrossed *SBEIIa* and *SBEIIb* mutants to determine the genetic distance between these two paralogs and to combine mutations in the two paralogs in coupling. We are making all the mutants generated in this study publicly available to accelerate the development of pasta varieties with increased resistant starch content.

MATERIALS AND METHODS

Development and Characterization of *SBEII* Mutants

Detection of SBEIIa and SBEIIb TILLING Mutations and Growth Conditions

SBEII mutants were selected from a TILLING population of the Desert durum variety 'Kronos', which was developed by Arizona Grains Inc. from a male sterile population (selection D03-21). Mutations were identified in a region of the *SBEIIa*

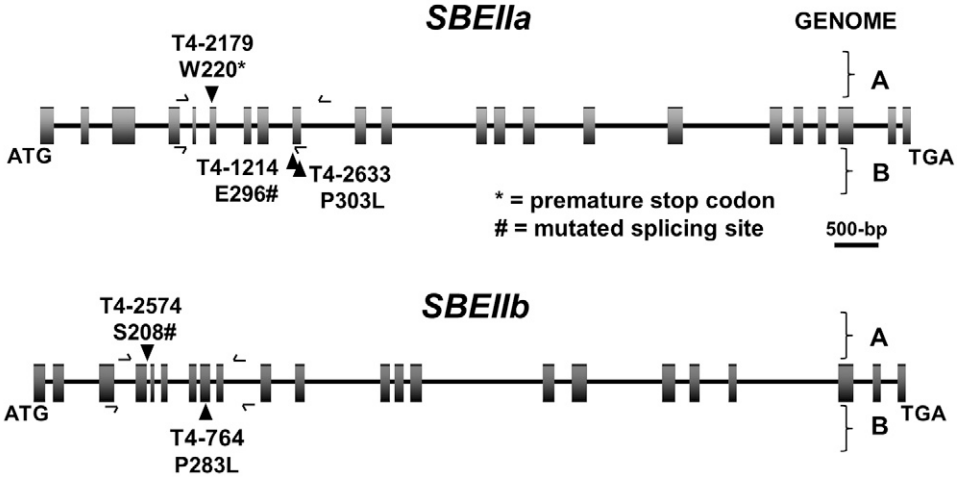

Figure 1. Structure of the *SBEIIa* and *SBEIIb* genes and location of the primers used to screen the tetraploid Kronos TILLING (targeted induced local lesions in genomes) population (⇀ ↽) and the selected mutants. T4 numbers indicate the line identification number and the numbers below the effect of the mutation on the protein. Mutation effects are described using a number for the position of the amino acid change, a letter on the left describing the original amino acid, and a letter on the right indicating the new amino acid (* indicates premature stop codon and # a deleted splicing site). Rectangles represent exons. Mutations in the A genome are indicated above the gene structure and mutations in the B genome below the gene structure.

Table 1. Selected tetraploid TILLING (targeted induced local lesions in genomes) mutants

Gene	Genome	Line	DNA†	GenBank DNA/protein	Effect
SBEIIa	A	T4-2179	G401A	GQ254774/CCD41775	W220* (stop codon)
SBEIIa	B	T4-1214	G1347A	GQ254777/CAR95900	E296# (splice junction)
SBEIIa	B	T4-2633	C1370T	GQ254777/CAR95900	P303L (BLOSUM62§ = –3)
SBEIIb	A	T4-2574	G308A	GQ254780/AK335378‡	S208# (splice junction plus stop)
SBEIIb	B	T4-764	C1290T	GQ254781/AAW80631	P283L (BLOSUM62 = –3)

†DNA coordinates based on *Triticum turgidum* cultivar Kronos partial genomic sequence used for TILLING (Uauy et al. 2009) and protein coordinates are based on complete protein sequences from the corresponding genome in *Triticum aestivum* cultivar Chinese Spring except for *SBEIIb*-B for which there is no complete protein available (the P283L coordinate is based on the A and D genome proteins that show the same position).

‡AK335378 is a DNA sequence for a complete complementary DNA (cDNA) of *SBEIIb*-A genome but has a missing "g" after position 2040, which results in a shift in the reading frame. The extra "g" is present in the corresponding cDNA GD188500.

§BLOSUM, block substitution matrix for pairwise protein sequence alignments (Henikoff, 1992).

and *SBEIIb* genes between exons 4 and 9 (Fig. 1) using genome specific primers described previously (Uauy et al., 2009). The DNA coordinates for the *SBEIIa* and *SBEIIb* mutations listed in Table 1 are determined from the first base pair of the corresponding GenBank accessions whereas protein coordinates are determined from the initial methionine. The mutation names include a letter before the coordinate representing the base or amino acid in the wild-type allele and a letter after the coordinate indicating the new base or amino acid. Mutations resulting in a premature stop codon are indicated by an asterisk (*) and those resulting in the loss of a splicing site by a hash tag (#).

The potential disruptive effect of amino acid substitutions on the protein function was evaluated using the programs SIFT (sorting intolerant from tolerant) (Ng and Henikoff, 2003) and position-specific scoring matrices (PSSMs) as reported before for the WKS1 protein (Fu et al., 2009).

For the *SBEIIa* gene we selected one mutant for the A genome (henceforth *SBEIIa*-A will be referred to as ΔA) and two for the B genome (henceforth *SBEIIa*-B will be referred to as ΔB1 and ΔB2). These mutants were backcrossed twice to Kronos to reduce background mutations and the backcross lines with mutations in different *SBEIIa* homeologs were intercrossed to combine the different mutations (Fig. 2). From the segregating progeny we selected sib lines with the following genotypes: one genotype homozygous for wild-type alleles to be used as control, one genotype for homozygous mutations in the A genome alone (ΔA), two genotypes for homozygous mutations in the B genome alone (ΔB1 and ΔB2), and two genotypes for homozygous mutations in both genomes (ΔAΔB1and ΔAΔB2) (Fig. 2). Plants carrying the targeted mutations were selected in each generation by sequencing, using the same genome specific primers designed to screen the TILLING population (Uauy et al., 2009).

Eight plants per genotype were grown in a completely randomized design under controlled conditions (greenhouse at 20–25°C and long day photoperiod). After reaching complete maturity grains were harvested from each of the eight plants per genotype (48 samples). Kernel weight was estimated from the weight of ~100 kernels per plant 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). Whole grains were then ground in a UDY Cyclone Mill (UDY Corporation) using a 0.5 mm screen.

Determination of Amylose Content

Relative amylose content was determined for 25-mg samples of whole-grain flour using the AMYLOSE/AMYLOPECTIN

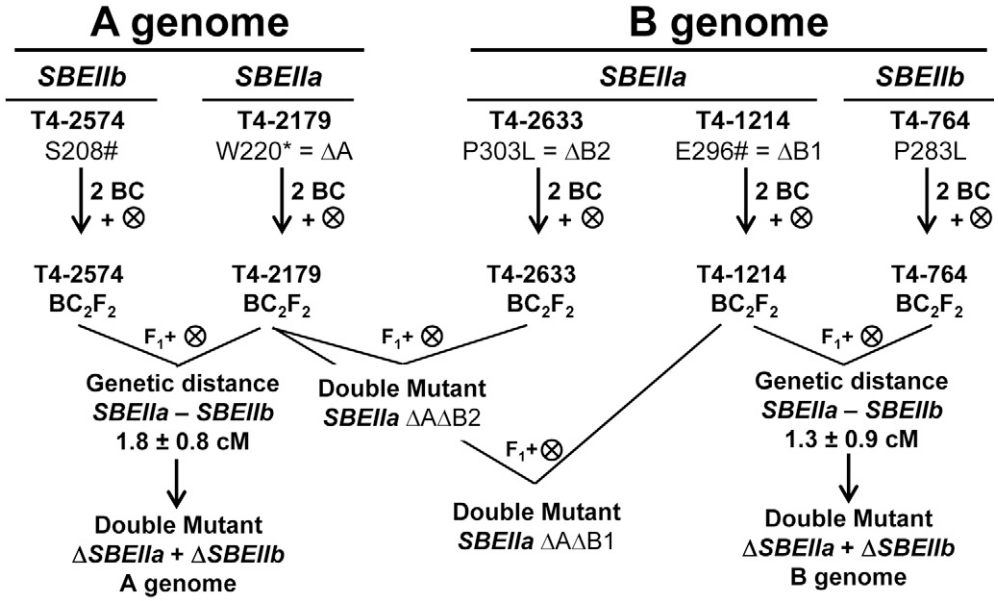

Figure 2. Crossing scheme used to reduce the background mutations and to combine the different mutations. 2 BC indicates two backcross generations to the wild-type Kronos line and ⊗ indicates self-pollination. Mutations were followed at each stage by sequencing the segregating lines.

kit developed by Megazyme International (catalog number K-AMYL), following the manufacturer's instructions. The procedure is a modification of a concanavalin A (Con A) method developed by Yun and Matheson (1990) that uses an ethanol pretreatment step to remove lipids prior to analysis (modified from Morrison and Laignelet [1983]). In this assay, the starch component in the whole-grain flour samples is dispersed by heating in dimethyl sulfoxide. Lipids are removed by precipitating the starch in ethanol and recovering the precipitated starch. The precipitated sample is dissolved in an acetate–salt solution and amylopectin is precipitated and removed from the sample by the addition of Con A followed by centrifugation. The amylose is then enzymatically hydrolyzed to D-glucose by an amyloglucosidase (AMG) and α-amylase enzyme mixture and measured colorimetrically using a glucose oxidase–peroxidase (GOPOD) reagent. The total starch is measured in a similar way using a separate aliquot of the initial acetate–salt solution. The relative amylose content in the initial starch sample is estimated as the ratio of the GOPOD reagent absorbance at 510 nm of the supernatant from the Con A precipitated sample, to that of the total starch sample.

Determination of Resistant Starch Content

Relative resistant starch content was determined for 100-mg samples of whole-grain flour using the RESISTANT STARCH kit developed by Megazyme International (catalog number K-RSTAR, AOAC Official Method 2002.02 and AACC Method 32-40 [McCleary et al, 2002]). In this assay, pancreatic α-amylase and AMG are added to whole-grain flour samples that are then incubated at 37°C with shaking for 16 h. During the incubation, the nonresistant starch is solubilized and hydrolyzed to D-glucose. The reaction is terminated by addition of ethanol and the resistant starch in the sample is recovered as a pellet after centrifugation. The resistant starch pellet is washed twice in 50% v/v ethanol and dissolved in 2 M potassium hydroxide. The solution is neutralized using an acetate buffer and the starch is quantitatively hydrolyzed to glucose using AMG. D-glucose is measured using GOPOD reagent absorbance at 510 nm, providing a measure of the resistant starch content of the sample. The nonresistant starch (solubilized starch) is determined by combining the original supernatant and washing supernatants and measuring an aliquot using GOPOD reagent absorbance at 510 nm. Both solubilized and resistant starch are calculated on a dry weight basis incorporating relative moisture values and following instructions provided in the Megazyme RESISTANT STARCH kit manual (McCleary et al., 2002).

Data Analysis

To account for potential variation among assays performed on different days, one or two complete sets of samples including all six genotypes was measured each day, and day of assay was then included as a block in the statistical analyses (randomized complete block design). Means of the individual mutant lines were compared with the wild-type sib line using a Dunnett test. The ANOVA assumptions were tested using the Shapiro-Wilk's test for normality of residuals and Levene's test for homogeneity of variances. All statistical analyses were performed using SAS 9.2 (SAS Institute, 2011).

Because the amylose determinations were more variable than the resistant starch determinations, we performed two amylose assays for each sample (two subsamples) and averaged them to increase the precision of the statistical analyses.

Scanning Electron Microscopy of Purified Starch Granules

Scanning electron micrographs were generated using purified starch from two ΔAΔB1 double mutants and two wild-type sib lines. Starch was purified from 10 mature seeds per sample using a previously described protocol (Uhlmann and Beckles, 2010). The purified starch was coated onto carbon double stick discs (Pella) mounted onto 12-mm aluminum stubs (Pella) and

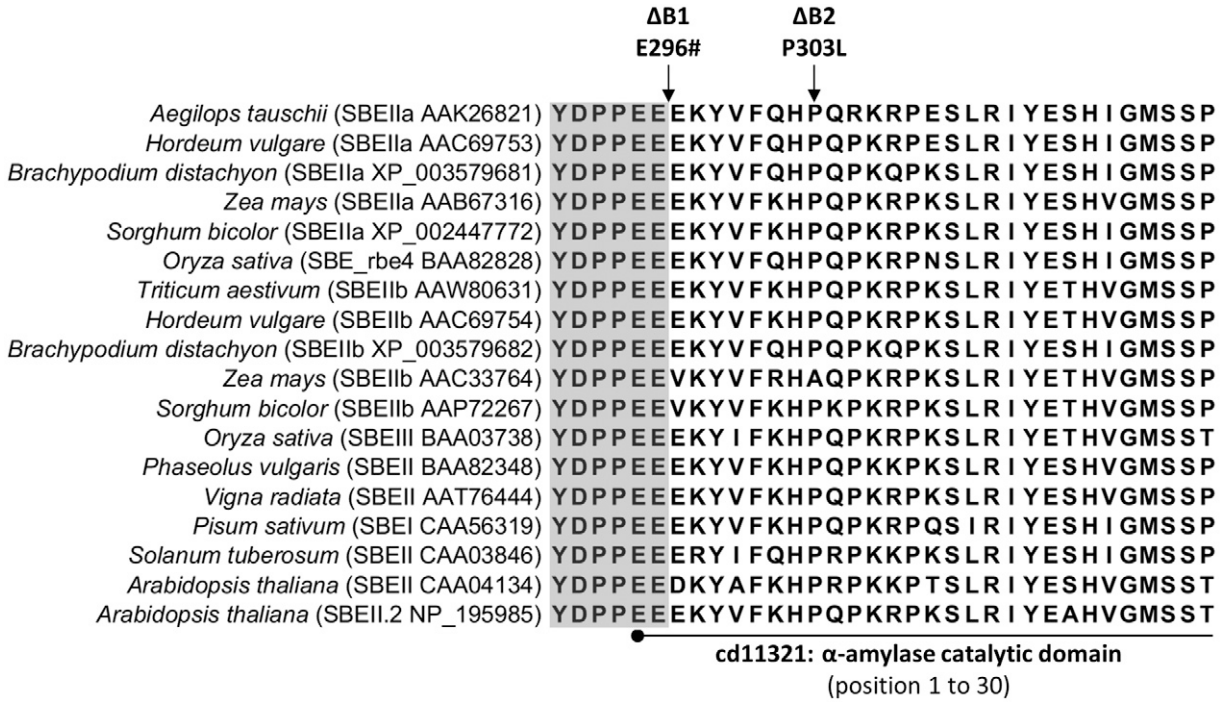

Figure 3. Selected mutations for the B genome copy of the predicted starch branching enzyme (SBEIIa) protein. The E296# mutation results in the elimination of the splicing site between exons 8 (gray) and exon 9. The P303L mutation affects a highly conserved amino acid within the catalytic domain of the starch branching enzyme (line below sequences).

sputtered with gold on a BioRad Polaron sputter coater (Model E51090). Starch granules were imaged at 500x using a FEI/ Phillips XL30 SFEG Scanning Electron Microscope.

Determination of Genetic Distances between *SBEIIa* and *SBEIIb*

Backcross two F_2 plants (BC_2F_2) homozygous for *SBEIIa* and *SBEIIb* mutations in the same genome were intercrossed (Fig. 2). The heterozygous F_1 plants were self-pollinated and the segregating plants were genotyped by sequencing using the genome specific primers designed to screen the TILLING population described previously (Uauy et al., 2009). Recombination fractions were converted to centimorgans using the Kosambi function (Kosambi, 1944) and standard deviations were calculated using standard formulas (Allard, 1956).

RESULTS

Description of the Mutants and Generation of Double *SBEIIa* Mutants

In the tetraploid wheat TILLING population (1368 lines), we detected 58 and 35 mutants for *SBEIIa* and *SBEIIb*, respectively, corresponding to an estimated mutation density of at least one mutation per 68 kb. Sequencing confirmed that all 93 mutations were G-to-A or C-to-T transitions as expected from alkylation by ethyl methane sulfonate (EMS). Out of the 93 mutations 28% were missense mutations and 5.4% were truncations.

We selected one mutant containing a mutation in the A genome copy of *SBEIIa* (*SBEIIa*-A) and two mutants containing mutations in the B genome copy of *SBEIIa* (*SBEIIa*-B) (Fig. 1; Table 1). The G-to-A mutation selected for the A genome in line T4-2179 resulted in an amino acid change from a tryptophan at position 220 into a premature stop codon (henceforth, W220* or ΔA). Since this mutation is predicted to eliminate more than 70% of the SBEIIa protein, including the complete α-amylase catalytic domain, it is expected to result in a null allele.

For the B genome copy of *SBEIIa* no premature stop codon was detected, so we selected a G-to-A mutation in line T4-1214 that resulted in the loss of the canonical splicing acceptor site AG at the end of intron 8 (henceforth E296# will be referred to as ΔB1; Fig. 1; Table 1). The first two codons of exon 9 located immediately after the mutated splicing site have potential in-frame splicing sites. If any of these putative alternative splicing sites were used by the spliceosome, the resulting protein would lose only one or two amino acids (Glu^{296} and Lys^{297}). Even under this scenario, this mutation is expected to disrupt *SBEIIa* function because these two amino acids are at the beginning of the well conserved α-amylase catalytic domain (Fig. 3). As a backup, we selected a second *SBEIIa*-B mutant, T4-2633, that carries a C-to-T mutation expected to generate a proline to leucine change in exon 9 at position 303 (P303L is referred to hereafter as ΔB2). This position is highly conserved in starch branching enzymes in plants (Fig. 3) and is located at the beginning of the $(\beta/\alpha)8$-barrel region, before the first β strand. Analysis of the PSSM for the α-amylase catalytic domain (cd11321) suggests that proline is the most common amino acid at this position whereas leucine is not found at this position in any bacterial or eukaryotic branching enzyme. This amino acid

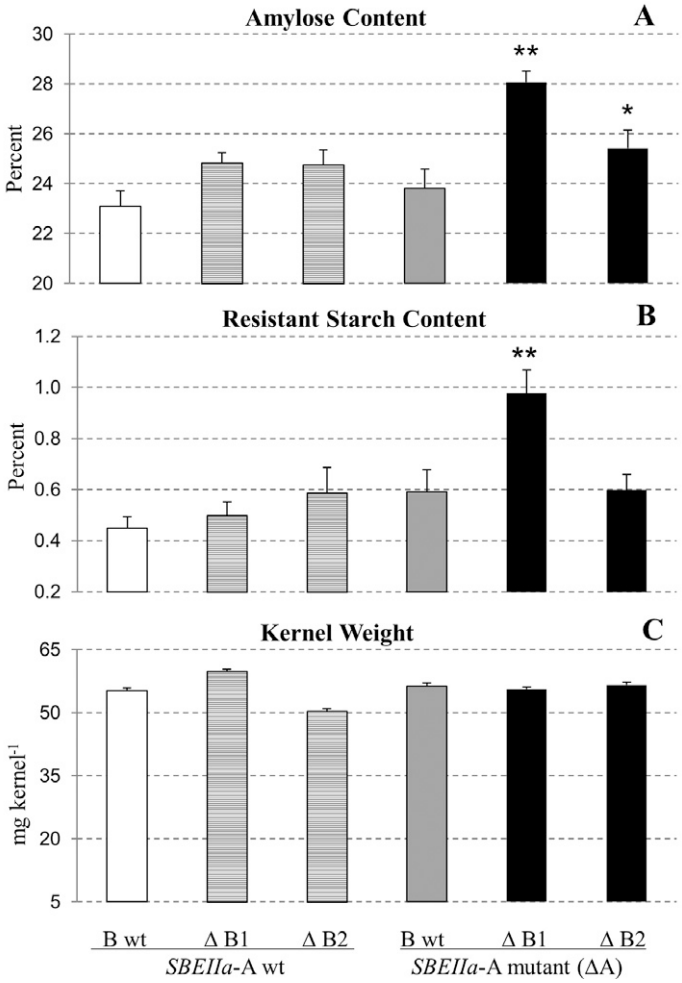

Figure 4. Characterization of durum sib lines with different combinations of *SBEIIa* alleles. A) Percent amylose content in the grain. B) Resistant starch content in the grain. C) Kernel weight. *SBEIIa* alleles: wild-type (wt) alleles from Kronos in both A and B genomes (wt, white bar), mutations in *SBEIIa*-B alone (ΔB1 = E296# and ΔB2 = P303L, bar with horizontal lines), mutations in *SBEIIa*-A alone (ΔA = W220*, solid gray bar), and combinations of the mutations in both genomes (ΔAΔB1 and ΔAΔB2, black bars). Asterisks indicate probability values in Dunnett tests of mutant lines against the wild-type sib line (*$P < 0.05$ and **$P < 0.01$).

substitution has a highly negative block substitution matrix for pairwise protein sequence alignments (BLOSUM) 62 (Henikoff, 1992) value of –3, which is indicative of a high probability of a disruptive change in the structure and/or function of the resulting protein. The potential disruptive effect of this mutation was also supported by a highly negative PSSM score (–5) and a low SIFT (Ng and Henikoff, 2003) score (0.01).

For the *SBEIIb* gene we selected one mutant for each genome (Fig. 1; Table 1). For the A genome copy (*SBEIIb*-A) we selected a G-to-A mutation identified in line T4-2574, which resulted in the loss of the GT donor splicing site following the end of exon 4. This mutation is followed immediately by an in-frame stop codon that is predicted to eliminate 75% of the SBEIIb protein. Since the eliminated portion of the protein includes the complete α-amylase catalytic domain, the truncated peptide is expected to be nonfunctional. For the B genome copy of *SBEIIb* (*SBEIIb*-B) we did not find any premature stop codon or mutated splicing site so we selected a C-to-T mutation that resulted in a proline to lysine mutation at the conserved position 283 (P283L, line T4-764; Table 1). This mutation occurs within the N-terminal "early set domain" (cd02854), which is normally associated with the α-amylase catalytic domain and has been shown to be required for branching activity (Kuriki et al., 1997). Analysis of 82 proteins from bacteria and eukaryotes suggests an almost invariable proline or serine residue at this position, with the sole exception being the marine yeast *Debaryomyces hansenii*, which has a cysteine residue at this position. The absence of a lysine residue at this position in branching enzymes characterized so far, together with the negative BLOSUM62 score (Henikoff, 1992) (–3), highly negative PSSM score (–7), and low SIFT (Ng and Henikoff, 2003) score (0.01), are all predictive of a high probability of disrupting the structure and or function of the protein.

All five selected mutants for the *SBEIIa* and *SBEIIb* genes were backcrossed for two generations to the nonmutagenized Kronos to reduce the load of background mutations and homozygous BC_2F_2 plants were selected. The *SBEIIa*-A mutant W220* was crossed with the *SBEIIb*-A mutant S208#, and the *SBEIIa*-B mutant E296# was crossed with the *SBEIIb-B* mutant P283L (Fig. 2) to determine the genetic distances between the two paralogous genes and to combine the linked mutations in coupling (see last section of results).

The two homozygous BC_2F_2 mutants containing mutations in the *SBEIIa*-B gene (ΔB1 and ΔB2) were crossed with the line homozygous for the premature stop codon mutation in the *SBEIIa-A* gene (ΔA). The double heterozygous plants were self-pollinated and lines homozygous for the A and B genome mutations were selected. Two double mutant homozygous lines were selected, one including the splice site mutation in the B genome (ΔAΔB1) and the other one including the P303L mutation (ΔAΔB2). From the same segregating population, we selected sib lines homozygous for the A genome mutation alone (ΔA), the B genome mutations alone (ΔB1 and ΔB2), and both wild-type alleles to be used as control (Fig. 2).

Effect of the Single and Double *SBEIIa* Mutations on Amylose Content

The ANOVA for amylose content showed significant differences ($P < 0.0001$) among the six sib lines included in the study. A mean comparison of each mutant against the wild-type sib line using a Dunnett test showed significant differences only for the double mutant lines ΔAΔB1 ($P < 0.01$) and ΔAΔB2 ($P < 0.05$) (Fig. 4A). The individual *SBEIIa* mutations showed only modest increases in

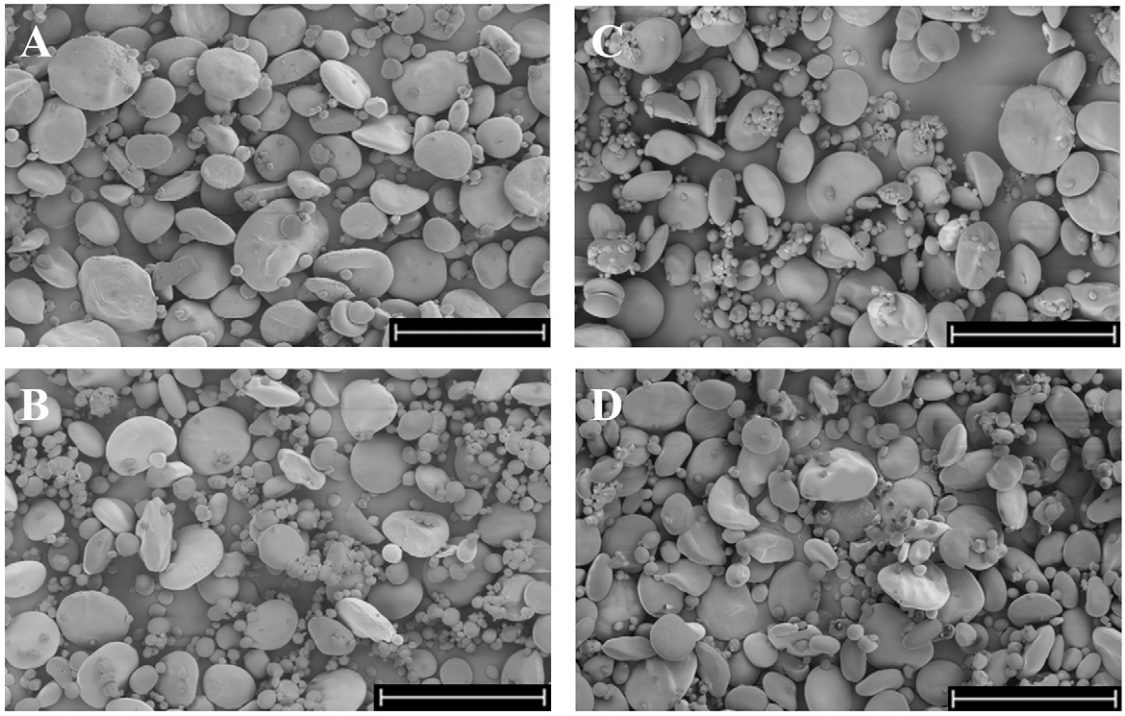

Figure 5. Scanning electron micrographs of starch granules from wild-type (A and B) and ΔAΔB1 *SBEIIa* double mutants (C and D). Bars represent 50 μm.

amylose content in the grain but none of them were significantly different from the wild-type sib line (ΔA = 3% $P = 0.87$, ΔB1 = 7.6% $P = 0.18$, and ΔB2 = 7.2% $P = 0.22$).

The increase in amylose content relative to the wild-type sib line was 22% for the ΔAΔB1 double mutant line but only 10% for the ΔAΔB2 double mutant. This suggests that the E296# splice site mutation is more effective than the P303L mutation in decreasing SBEII activity and, consequently, in increasing amylose content of the grain. However, the difference between the ΔB1 and ΔB2 mutations observed in the presence of the *SBEIIa*-A mutation was not observed in the presence of the wild-type *SBEIIa*-A allele (Fig. 4A).

Effect of the Single and Double *SBEIIa* Mutations on Resistant Starch Content

The ANOVA for resistant starch content in the grain showed significant differences ($P < 0.0001$) among the six sib lines. Only the double mutant line ΔAΔB1 showed significant increases in resistant starch relative to the wild-type sib line ($P < 0.01$; Fig. 4B).

The ΔAΔB1 double mutant line showed a 115% increase in resistant starch relative to the wild-type control ($P < 0.01$) whereas the ΔAΔB2 mutant line showed an increase of only 32% and was not significantly different than the control ($P = 0.41$). As in the amylose determinations, these results suggest that the E296# splice site mutation is more effective than the P303L mutation in increasing resistant starch content in the grain and that these differences are only manifested in the presence of the *SBEIIa*-A mutation (Fig. 4B).

The individual *SBEIIa* mutations showed smaller increases in resistant starch than the ΔAΔB1 double mutants and none of them were significantly different from the wild-type sib line (ΔA = 30% $P = 0.48$, ΔB1 = 10% $P = 0.98$, and ΔB2 = 29% $P = 0.5$).

Effect of the Single and Double *SBEIIa* Mutations on Kernel Weight and Starch Granule Shape

The overall ANOVA for kernel weight showed no significant differences among genotypes. In addition, no significant differences were detected in any of the comparisons of the mutant lines versus the control using a Dunnett test (Fig. 4C). This result suggests that the *SBEIIa* mutations had no significant impact on kernel weight and that the significant increases in amylose and resistant starch content in the ΔAΔB1 double mutant (Fig. 4A and B) were not the result of a concentration effect influenced by smaller grains (Fig. 4C).

To determine if the differences in amylose and resistant starch content in the ΔAΔB1 double mutants were correlated with differences in the morphology of the starch granules, we analyzed two double mutants carrying the ΔAΔB1 mutations (highest amylose and resistant starch content) and compared them with sib lines carrying wild-type alleles at both *SBEIIa* loci.

The scanning electron microscope images revealed no obvious morphological differences between the starch granules of the double mutant and wild-type lines (Fig. 5). The lack of morphological differences is likely related to the lower amylose content of the double mutant lines

generated in this study (28%) compared to the *SBEIIa*-RNAi transgenic lines reported before (up to 75%) (Sestili et al., 2010). In those transgenic durum lines, starch granules appeared deflated and smaller than in the nontransgenic controls (Sestili et al., 2010).

Genetic Distances between the *SBEIIa* and *SBEIIb* Mutants

Information concerning the *SBEIIa* and *SBEIIb* linkage is important to determine the feasibility of combining mutations in these two genes in coupling. To generate this information we crossed the BC_2F_2 lines homozygous for the *SBEIIa*-A and *SBEIIa*-B mutations with those homozygous for the *SBEIIb* mutation located on the same chromosome (Fig. 2). We self-pollinated the resulting BC_2F_1 lines and generated two segregating populations that were genotyped for the *SBEIIa* and *SBEIIb* mutations using sequencing. Plants carrying recombination events between *SBEIIa* and *SBEIIb* were resequenced to eliminate potential sequencing errors.

In the population segregating for *SBEIIa*-A and *SBEIIb*-A, we recovered five recombinant plants from the 140 segregating plants, which corresponds to a genetic distance of 1.8 $\pm$ 0.8 cM. In the second population segregating for *SBEIIa*-B (ΔB1) and *SBEIIb*-B we recovered two recombinant plants among a population of 80 plants, resulting in a genetic distance of 1.3 $\pm$ 0.9 cM. These two genetic distances are not significantly different from each other for the population sizes used in this study ($P = 0.65$).

Among the recombinant plants in the first population we identified three that combined the *SBEIIa*-A W220★ mutation with the *SBEIIb*-A S208# mutation in coupling on chromosome 2A. In the second population we identified one recombinant plant carrying the *SBEIIa*-B E296# mutation and the *SBEIIb*-B P283L mutation in coupling on chromosome 2B.

To see if it was possible to orient the location of these two genes we mapped them in the homeologous group 2 deletion lines (Conley et al., 2004). Both *SBEIIa* and *SBEIIb* were mapped in deletion bins 2BL2-0.36-0.50 and C-2AL1-0.85 (numbers indicate the percent length of the arm and C the centromere). These results confirmed that the two genes are physically located in the same region of the chromosome but did not provide additional information on the relative order of the two genes relative to the centromere.

DISCUSSION

On average, Americans consume approximately 8.8 kg of pasta a year (2011 survey by the Association of Pasta Manufacturers of the European Union [UN.A.F.P.A., 2011]). Therefore, the 115% increase in resistant starch observed in the ΔAΔB1 double mutant generated in this study can have a positive impact on the total amount of resistant starch consumed in the United States and in other countries. The intake of resistant starch in the United States averages 4.9 g per person per day according to the 1999 to 2002 National Health and Nutrition Examination Survey (Murphy et al., 2008). Therefore, a twofold increase in resistant starch content in all durum wheat used for pasta and consumed in the United States would result in a 1 to 2% increase in the average daily consumption of resistant starch per person.

Since natural variation in amylose content is limited in wheat (Stoddard and Sarker, 2000), the use of biotechnological approaches to expand the range of resistant starch content is an appropriate strategy. Downregulation of the starch branching enzyme *SBEIIa* by RNAi has resulted in significant increases in amylose content in the grains of both common (Regina et al., 2006) and durum wheat (Sestili et al., 2010). The advantage of the RNAi approach is that all homeologous *SBEIIa* copies are downregulated simultaneously using a single construct. In addition, the presence of 21 or more consecutive base pairs with perfect identity between the *SBEIIa* and *SBEIIb* genes within the RNAi constructs can simultaneously downregulate both genes (Regina et al., 2006). In spite of the advantages of the RNAi approach, commercialization of transgenic wheat varieties still faces prohibitive regulatory costs that have, so far, delayed the release of commercial transgenic wheat varieties.

An alternative to the transgenic approach is the combination of TILLING mutants for each of the copies of the *SBEII* genes by crossing and marker assisted selection. Although this approach is more laborious, the resulting varieties are not subject to the expensive regulations of transgenic wheat. From a research perspective, the mutagenesis approach has the additional advantage of providing information about the individual contribution of the *SBEII* homeologs to the final amylose and resistant starch content. This information is important for wheat breeders to decide which loci to introgress in their breeding programs to maximize the amount of resistant starch without compromising end-product quality.

Relative Contribution of the *SBEIIa* and *SBEIIb* Genes to Amylose Content

The expression profiles of *SBEIIa* and *SBEIIb* are different in temperate cereals such as wheat and barley than in subtropical grasses such as rice (*Oryza sativa* L.) and maize (*Zea mays* L.). In maize and rice, *SBEIIa* is predominantly expressed in the leaves whereas *SBEIIb* is predominantly expressed in the endosperm (Gao et al., 1997; Yamanouchi and Nakamura, 1992). In the soluble fraction of the wheat endosperm the amount of SBEIIa protein is two- to threefold higher than the amount of SBEIIb whereas in the endosperm starch granules SBEIIb is two- to threefold higher than SBEIIa (Regina et al., 2005). Similarly, in the barley endosperm the activities of SBEIIa and SBEIIb isoforms are roughly equivalent (Sun et al., 1998).

These differences in the relative abundance of SBEIIa and SBEIIb proteins between temperate and subtropical grasses are reflected in the differential contribution of each isoform to starch branching activity and, by extension, to the proportion of amylose and amylopectin in the grain. In maize, suppression of SBEIIb results in amylose content ranging from 50 to 90% (Garwood et al., 1976) whereas suppression of SBEIIa has no significant effect on grain amylose content (Blauth et al., 2001, 2002). In rice, lack of SBEIIb also results in significant increases in amylose content in the grain but less pronounced than those observed in maize (Nishi et al., 2001). On the contrary, the downregulation of *SBEIIb* in hexaploid wheat and barley does not result in any significant increase in amylose content whereas the simultaneous downregulation of *SBEIIa* and *SBEIIb* results in very high amylose content (Regina et al., 2006, 2010).

A recent study on tetraploid wheat (Sestili et al., 2010) claimed that the observed increases in amylose content in *SBEIIa*-RNAi transgenic plants were independent of *SBEIIb*. However, the *SBEIIa*-RNAi construct used in their study included a stretch of 25 bp at the end of exon 3 (AAATATACGAGATTGACCCAACGCT) that is identical in the *SBEIIa* and *SBEIIb* genes and therefore has the potential to downregulate the *SBEIIb* gene. The semiquantitative polymerase chain reaction method used in Sestili et al. (2010) may have lacked the necessary sensitivity to detect small differences in *SBEIIb* transcript levels between the transgenic and control plants.

This hypothesis is supported by RNAi transgenic experiments in barley, in which lines with different levels of downregulation of SBEIIa and SBEIIb proteins were identified by using antibodies specific for SBEIIa and SBEIIb (Regina et al., 2010). Relative to the wild-type barley control (28.5% amylose), the reduction of the SBEIIb protein had no significant effect on amylose content (30.5% amylose), the reduction of SBEIIa protein had a small effect (38.1% amylose), and only the simultaneous downregulation of SBEIIa and SBEIIb had a large effect on amylose content (65 to 89% amylose) (Regina et al., 2010).

Based on the previous barley results it is likely that the wheat *SBEIIb* mutations identified here will have an effect on amylose content when deployed in a genetic background that lacks functional *SBEIIa* genes. The double *SBEIIa-SBEIIb* mutant lines generated in this study (see below) will be useful to test this hypothesis.

Relative Contribution of the *SBEIIa*-A and *SBEIIa*-B Homeologs to Amylose Content

Individual mutations in one of the *SBEIIa* homeologs in tetraploid or hexaploid wheat are expected to have limited effects on amylose content due to the redundant copies present in the other genomes. In a recent study in hexaploid wheat, mutations of individual *SBEIIa* homeologs resulted in very small increases in amylose content, ranging from 2 to 4% (Botticella et al., 2011). Similarly, the small increases in amylose and resistant starch content observed in the grains of the three single mutants generated in this study relative to the wild-type sib lines were too small to be significant in the statistical tests (Fig. 4A and B). Therefore, the small differences observed in the effect of single mutations on amylose and resistant starch content might be largely affected by random variation.

Expression and protein comparisons between the *SBEIIa*-A and *SBEIIa*-B copies are also not conclusive in predicting differences in enzyme activity between these two homeologs. A recent dataset generated in a high-throughput sequencing of RNA transcripts (RNA-seq) study of developing grains in hexaploid wheat (Pellny et al., 2012) shows similar transcript levels of *SBEIIa* homeologs. In this dataset, the number of sequencing reads mapped to the *SBEIIa*-B copy was only 20 to 30% higher than the number of reads mapped to the *SBEIIa*-A and *SBEIIa*-D copies at different stages of grain development. The SBEIIa-A and SBEIIa-B proteins encoded by the two *SBEIIa* homeologs differ in 11 amino acid substitutions and two insertion–deletions, but none of them are located at previously identified functionally critical amino acid positions. In summary, with the current information it is not possible to differentiate the contribution of the two homeologous copies of the *SBEIIa* gene in tetraploid wheat to amylose or resistant starch content.

Linkage Relationship between *SBEII* Loci

The wheat genes for *SBEIIa* and *SBEIIb* are both located on the long arm of chromosome 2. The *SBEIIa* location is consistent with the known colinearity between wheat, *Brachypodium* P. Beauv., rice, and maize chromosomes. However, the wheat *SBEIIb* gene is located in a chromosome region that is not colinear to *Brachypodium*, rice, and maize (Regina et al., 2005). The non-colinear chromosome location of wheat *SBEIIb* gene seems to also be shared by barley. BLAST searches (Altschul et al., 1990) against barley flow sorted chromosomes (Deng et al., 2007) indicate that both *SBEIIa* and *SBEIIb* genes are located on chromosome arm 2HL and that the unusual *SBEIIb* location predates the divergence of the Triticeae species.

Based on the position of the *SBEIIb* gene in *Brachypodium*, rice, and maize, the wheat and barley *SBEIIb* gene was expected to map on homeologous group 6, which is colinear to rice chromosome 2 (where *SBEIIb* is located). These results indicate that in the origin of the Triticeae lineage the *SBEIIb* gene was removed from its original location and was transposed to its current location on homeologous group 2. The movement of genes and gene fragments by retroelements is a common phenomenon in the large and complex genomes of wheat and barley (Wicker et al., 2011).

Although the new location of *SBEIIb* is in the same chromosome bin as *SBEIIa*, the genetic results from this

study demonstrate that the two genes are not adjacent to each other. In the Triticeae, 1 cM corresponds on average to 1 Mb, although the relationship between genetic and physical distances varies greatly in different regions of the wheat chromosomes (Lagudah et al., 2001). The two genes also seem to be physically separated in barley since *SBEIIa* was mapped in contig 45140 and *SBEIIb* in contig 38810, suggesting that the two genes are at least 286 kb apart (Deng et al., 2007).

The genetic distance separating the *SBEIIa* and *SBEIIb* was sufficient to identify recombinant lines carrying both mutations in coupling in both the 2A and 2B chromosomes using small segregating populations. The selected 2A recombinant lines include two mutations with high probability of disrupting the two proteins. The *SBEIIa*-A W220★ mutation has a premature stop codon that is expected to truncate >70% of the protein and the *SBEIIb*-A S208# mutation results in the elimination of a splicing site followed immediately by a stop codon that truncates >75% of the protein.

We currently do not know if the mutations combined in the 2B recombinant lines are as strong as the truncation mutations combined on chromosome 2A but there is some evidence that they may also have a disruptive effect on protein function. The double mutant carrying the *SBEIIa*-B E296# mutation (ΔB1) in combination with the *SBEIIa*-A mutation (ΔA) has already shown a significant increase in amylose and resistant starch content. For this reason, this mutation was selected for recombination with the linked *SBEIIb*-B P283L mutation on chromosome 2B. The P283L mutation is located in a position that is almost invariable in other SBE proteins and is associated with very negative BLOSUM62 (Henikoff, 1992) and PSSM scores, both predictive of disruptive changes in protein function. However, additional experimental results will be required to determine the effect of *SBEIIb* on amylose and resistant starch content when deployed in combination with *SBEIIa*.

Potential Effects of Amylose Increases on Quality and Nutritional Value

In barley, the individual downregulation of the *SBEIIa* gene affects not only the proportion of amylose but also the degree of polymerization and length of the amylopectin chains (Regina et al., 2010). Since some of these modified amylopectin chains are not detected by the Megazyme AMYLOSE/AMYLOPECTIN kit, our amylose measurements may underestimate the effect of the *SBEIIa* mutations on starch composition. This may explain the relatively smaller increases in amylose content (22%) compared to those in resistant starch (115%) observed in the *SBEIIa* mutants relative to wild-type sib lines. If modified amylopectin chains are included in the resistant starch fraction they may contribute to these differences.

The low resistant starch content observed in the wild-type Kronos are consistent with values reported in other studies (McCleary and Monghan, 2002). However, measurements of the absolute value of resistant starch in wheat and other cereals is known to be affected by the inclusion of AMG, which is included in the incubations to catalyze the hydrolysis of maltodextrins, to remove the potential inhibitory effect of maltose on pancreatic α-amylase and to generate more consistent results. Since AMG can theoretically attack some components of the resistant starch, its addition can result in an underestimation of the absolute resistant starch content in the samples (McCleary and Monghan, 2002). Previous estimations of resistant starch content in durum semolina vary threefold depending on the absence (1.5%) or presence (0.5%) of AMG in the assay (McCleary and Monghan, 2002). Since the Megazyme kit uses AMG in the initial incubation step, the absolute values of resistant starch reported in this study may underestimate the actual resistant starch content in durum wheat flour. In spite of this limitation, the inclusion of AMG in the Megazyme kit does not invalidate the relative differences in resistant starch detected among genotypes, since these were consistent among replications. This procedure has been subjected to extensive inter-laboratory evaluations and has been accepted by both the AOAC International and AACC International (AOAC Official Method 2002.02 and AACC Method 32-40 [McCleary et al, 2002]).

Using this method we determined that the amylose content in the ΔAΔB1 double mutant (28%) was 22% higher than the amylose content in the wild-type sib line (23%). Although the amylose content in this line was not as high as in previously published RNAi transgenic durum wheat (Sestili et al., 2010), it was sufficient to generate a twofold increase in the amount of resistant starch in the grain. This increase may be sufficient to provide some health benefits to consumers and therefore we have initiated the introgression of the ΔA and ΔB1 mutations in our commercial tetraploid varieties by marker assisted backcrossing. Increasing the resistant starch content of wheat grains has the potential to provide nutritional and health benefits to consumers such as extended satiety and reduced glycemic index and demand for insulin (reviewed in Hendrich, 2010).

Higher amylose and resistant starch contents may also be beneficial for pasta quality. A reconstitution study in durum wheat using increasing amounts of high-amylose maize starch showed a positive correlation between amylose content and farinograph water absorption and pasta firmness (Soh et al., 2006). This study concluded that the optimum pasta quality occurred when amylose content was between 32 and 44% (Soh et al., 2006). This suggests that there is still room for increasing amylose content in the grain of durum wheat beyond the 28% found in the ΔAΔB1 double mutant lines without a significant negative impact in pasta quality.

Future Increases in Amylose Content

In spite of the fact that we combined mutations in both copies of the *SBEIIa* gene and that the increases in amylose

content were highly significant relative to the control sib line (22%, $P < 0.0001$), the 28% amylose content obtained in our double durum wheat mutant is far from the >70% amylose content observed in previous RNAi experiments in tetraploid (Sestili et al., 2010) and hexaploid wheat (Regina et al., 2006). We are currently exploring two non-mutually exclusive strategies to increase amylose and resistant starch content beyond the levels achieved in the current study.

First, we are trying to combine the *SBEIIa* and *SBEIIb* mutations. Recent RNAi transgenic experiments in barley (Regina et al., 2010) indicate that the simultaneous downregulation of these two paralogs results in higher amylose content than the downregulation of individual paralogs. To test this in wheat, we are combining the two double *SBEIIa*-*SBEIIb* mutants on chromosomes 2A and 2B (Fig. 2) to obtain lines with mutations in all four genes. We will also select lines with different combinations of mutant and wild-type *SBEIIa*-*SBEIIb* alleles to test the effect of differences in amylose and resistant starch content on pasta quality.

As a second strategy to increase amylose and resistant starch content beyond the current levels we will test additional mutations for the *SBEIIa*-B locus. It is possible that the lower amylose content found in our double mutants relative to the RNAi transgenic lines is determined by hypomorphic mutations in one of our targeted loci. The *SBEIIa*-A is an unlikely candidate since the premature stop codon identified in this line results in the lack of translation of more than 70% of the protein. However, we cannot rule out the possibility that the selected *SBEIIa*-B mutations are not complete knock-out alleles. Even though the E296# splicing site mutation seems promising based on its significant effect on amylose and resistant starch content in the double mutant lines, the first two amino acids after the splice site mutation include in-frame alternative splicing sites, which have the potential to reduce the negative impact of this mutation.

To test this hypothesis we have initiated the transfer to durum wheat of an alternative *SBEIIa*-B splice site junction mutation identified in hexaploid wheat line T6-111 after the end of exon 7 (Uauy et al., 2009). This mutation results in the translation of six additional amino acids followed by a stop codon before any potential alternative splicing site and therefore is expected to result in a nonfunctional *SBEIIa*-B allele. We have crossed T6-111 twice to Kronos to restitute the tetraploid chromosome number. The BC_1 plants will be crossed with the line carrying the W220★ premature stop codon in *SBEII*a-A to compare their levels of amylose content with our current double mutants.

In summary, we generated new durum wheat lines carrying mutations in *SBEIIa* that can be used to increase amylose content by 22% and to double the amount of resistant starch. In addition, these mutants have been useful to study the relative contribution of the different *SBEIIa* homeologs and to establish the genetic distance between *SBEIIa* and *SBEIIb*. This distance was sufficient to generate lines with *SBEIIa* and *SBEIIb* mutations in coupling in both the 2A and 2B chromosomes using small segregating populations. We plan to use these recombinants to study the relative effect of *SBEIIb* in the presence and absence of *SBEIIa*. All the mutants generated in this study are publicly available with the hope of accelerating the deployment of this beneficial trait around the world.

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References

Allard, R.W. 1956. Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia 24:235–278.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Association of Analytical Communities (AOAC). 2000. Official method 925.10. Solids (total) and moisture in foodstuffs (air oven method). In: Official methods of analysis of the association of official analytical chemists, 17th ed. AOAC International, Gaithersburg, MD.

Ball, S.G., and M.K. Morell. 2003. From bacterial glycogen to starch: Understanding the biogenesis of the plant starch granule. Annu. Rev. Plant Biol. 54:207–233. doi:10.1146/annurev.arplant.54.031902.134927

Behall, K.M., D.J. Scholfield, J.G. Hallfrisch, and H.G.M. Liljeberg-Elmstahl. 2006. Consumption of both resistant starch and beta-glucan improves postprandial plasma glucose and insulin in women. Diabetes Care 29:976–981. doi:10.2337/dc05-2012

Blauth, S.L., K.N. Kim, J. Klucinec, J.C. Shannon, D. Thompson, and M. Guiltinan. 2002. Identification of *Mutator* insertional mutants of starch-branching enzyme 1 (*sbe1*) in *Zea mays* L. Plant Mol. Biol. 48:287–297. doi:10.1023/A:1013335217744

Blauth, S.L., Y. Yao, J.D. Klucinec, J.C. Shannon, D.B. Thompson, and M.J. Guilitinan. 2001. Identification of *Mutator* insertional mutants of starch-branching enzyme 2a in corn. Plant Physiol. 125:1396–1405. doi:10.1104/pp.125.3.1396

Botticella, E., F. Sestili, A. Hernandez-Lopez, A. Phillips, and D. Lafiandra. 2011. High resolution melting analysis for the detection of EMS induced mutations in wheat *SbeIIa* genes. BMC Plant Biol. 11:156. doi:10.1186/1471-2229-11-156

Boyer, C.D., and J. Preiss. 1978. Multiple forms of (1-] 4)-alpha-D-glucan, (1-] 4)-alpha-D-glucan-6-glycosyl transferase from developing *Zea mays* L kernels. Carbohydr. Res. 61:321–334. doi:10.1016/S0008-6215(00)84492-4

Butardo, V.M., M.A. Fitzgerald, A.R. Bird, M.J. Gidley, B.M. Flanagan, O. Larroque, A.P. Resurreccion, H.K.C. Laidlaw,

S.A. Jobling, M.K. Morell, and S. Rahman. 2011. Impact of down-regulation of starch branching enzyme IIb in rice by artificial microRNA- and hairpin RNA-mediated RNA silencing. J. Exp. Bot. 62:4927–4941. doi:10.1093/jxb/err188

Conley, E.J., V. Nduati, J.L. Gonzalez-Hernandez, A. Mesfin, M. Trudeau-Spanjers, S. Chao, G.R. Lazo, D.D. Hummel, O.D. Anderson, L.L. Qi, B.S. Gill, B. Echalier, A.M. Linkiewicz, J. Dubcovsky, E.D. Akhunov, J. Dvorák, J.H. Peng, N.L.V. Lapitan, M.S. Pathan, H.T. Nguyen, X.-F. Ma, Miftahudin, J.P. Gustafson, R.A. Greene, M.E. Sorrells, K.G. Hossain, V. Kalavacharla, S.F. Kianian, D. Sidhu, M. Dilbirligi, K.S. Gill, D.W. Choi, R.D. Fenton, T.J. Close, P.E. McGuire, C.O. Qualset, and J.A. Anderson. 2004. A 2600-locus chromosome bin map of wheat homoeologous group 2 reveals interstitial gene-rich islands and colinearity with rice. Genetics 168:625–637. doi:10.1534/genetics.104.034801

Deng, W., D.C. Nickle, G.H. Learn, B. Maust, and J.I. Mullins. 2007. ViroBLAST: A stand-alone BLAST web server for flexible queries of multiple databases and user's datasets. Bioinformatics 23:2334–2336.

Englyst, H.N., and G.T. Macfarlane. 1986. Breakdown of resistant and readily digestible starch by human gut bacteria. J. Sci. Food Agric. 37:699–706. doi:10.1002/jsfa.2740370717

Faivre, J., and C. Bonithon-Kopp. 1999. Diets, fibres and colon cancer. Adv. Exp. Med. Biol. 472:199–206.

FAO. 2006. FAO statistical yearbook 2005–2006. FAO, Rome, Italy. http://www.fao.org/docrep/009/a0490m/a0490m00.HTM#Contents_en (accessed 3 May 2012).

Fu, D., C. Uauy, A. Distelfeld, A. Blechl, L. Epstein, X. Chen, H. Sela, T. Fahima, and J. Dubcovsky. 2009. A kinase-START gene confers temperature-dependent resistance to wheat stripe rust. Science 323:1357–1360. doi:10.1126/science.1166289

Gao, M., D.K. Fisher, K.N. Kim, J.C. Shannon, and M.J. Guiltinan. 1997. Independent genetic control of maize starch-branching enzymes IIa and IIb – Isolation and characterization of a Sbe2a cDNA. Plant Physiol. 114:69–78. doi:10.1104/pp.114.1.69

Garwood, D.L., J.C. Shannon, and R.G. Creech. 1976. Starches of endosperms possessing different alleles at amylose-extender locus in *Zea mays* L. Cereal Chem. 53:355–364.

Granfeldt, Y., X. Wu, and I. Bjorck. 2006. Determination of glycaemic index; Some methodological aspects related to the analysis of carbohydrate load and characteristics of the previous evening meal. Eur. J. Clin. Nutr. 60:104–112. doi:10.1038/sj.ejcn.1602273

Hendrich, S. 2010. Battling obesity with resistant starch. Food Technol. 64:22–30.

Henikoff, J.G. 1992. Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. USA 89:10915–10919.

Kosambi, D.D. 1944. The estimation of map distances from recombination values. Ann. Eugen. 12:172–175. doi:10.1111/j.1469-1809.1943.tb02321.x

Kuriki, T., D.C. Stewart, and J. Preiss. 1997. Construction of chimeric enzymes out of maize endosperm branching enzymes I and II: Activity and properties. J. Biol. Chem. 272:28999–29004. doi:10.1074/jbc.272.46.28999

Lagudah, E., J. Dubcovsky, and W. Powell. 2001. Wheat genomics. Plant Physiol. Biochem. 39:335–344. doi:10.1016/S0981-9428(00)01233-X

McCleary, B.V., and D.A. Monghan. 2002. Measurement of resistant starch. J. AOAC Int. 85:665–675.

McCleary, B.V., M. McNally, and P. Rossiter. 2002. Measurement of resistant starch. J. AOAC International. 85:103-1111.

Morrison, W.R., and B. Laignet. 1983. An improved colorimetric procedure for determining apparent and total amylose in cereal and other starches. J. Cereal Sci. 1:9–20.

Murphy, M.M., J.S. Douglass, and A. Birkett. 2008. Resistant starch intakes in the United States. J. Am. Diet. Assoc. 108:67–78. doi:10.1016/j.jada.2007.10.012

Ng, P.C., and S. Henikoff. 2003. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res. 31:3812–3814. doi:10.1093/nar/gkg509

Nishi, A., Y. Nakamura, N. Tanaka, and H. Satoh. 2001. Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. Plant Physiol. 127:459–472. doi:10.1104/pp.010127

Park, O.J., N.E. Kang, M.J. Chang, and W.K. Kim. 2004. Resistant starch supplementation influences blood lipid concentrations and glucose control in overweight subjects. J. Nutr. Sci. Vitaminol. (Tokyo) 50:93–99. doi:10.3177/jnsv.50.93

Pellny, T.K., A. Lovegrove, J. Freeman, P. Tosi, C.G. Love, J.P. Knox, P.R. Shewry, and R.A.C. Mitchell. 2012. Cell walls of developing wheat starchy endosperm: Comparison of composition and RNA-seq transcriptome. Plant Physiol. 158:612–627. doi:10.1104/pp.111.189191

Rahman, S., A. Regina, Z.Y. Li, Y. Mukai, M. Yamamoto, B. Kosar-Hashemi, S. Abrahams, and M.K. Morell. 2001. Comparison of starch-branching enzyme genes reveals evolutionary relationships among isoforms. Characterization of a gene for starch-branching enzyme IIa from the wheat D genome donor *Aegilops tauschii*. Plant Physiol. 125:1314–1324. doi:10.1104/pp.125.3.1314

Regina, A., A. Bird, D. Topping, S. Bowden, J. Freeman, T. Barsby, B. Kosar-Hashemi, Z.Y. Li, S. Rahman, and M. Morell. 2006. High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proc. Natl. Acad. Sci. USA 103:3546–3551. doi:10.1073/pnas.0510737103

Regina, A., B. Kosar-Hashemi, Z.Y. Li, A. Pedler, Y. Mukai, M. Yamamoto, K. Gale, P. Sharp, M.K. Morell, and S. Rahman. 2005. Starch branching enzyme IIb in wheat is expressed at low levels in the endosperm compared to other cereals and encoded at a non-syntenic locus. Planta 222:899–909. doi:10.1007/s00425-005-0032-z

Regina, A., B. Kosar-Hashemi, S. Ling, Z.Y. Li, S. Rahman, and M. Morell. 2010. Control of starch branching in barley defined through differential RNAi suppression of starch branching enzyme IIa and IIb. J. Exp. Bot. 61:1469–1482. doi:10.1093/jxb/erq011

SAS Institute. 2011. SAS user's guide, version 9.2, SAS Institute, Inc., Cary, NC.

Sestili, F., M. Janni, A. Doherty, E. Botticella, R. D'Ovidio, S. Masci, H.D. Jones, and D. Lafiandra. 2010. Increasing the amylose content of durum wheat through silencing of the SBEIIa genes. BMC Plant Biol. 10:144. doi:10.1186/1471-2229-10-144

Soh, H.N., M.J. Sissons, and M.A. Turner. 2006. Effect of starch granule size distribution and elevated amylose content on durum dough rheology and spaghetti cooking quality. Cereal Chem. 83:513–519. doi:10.1094/CC-83-0513

Stoddard, F.L., and R. Sarker. 2000. Characterization of starch in *Aegilops* species. Cereal Chem. 77:445–447. doi:10.1094/CCHEM.2000.77.4.445

Sun, C.X., P. Sathish, S. Ahlandsberg, and C. Jansson. 1998. The two genes encoding starch-branching enzymes IIa and IIb are differentially expressed in barley. Plant Physiol. 118:37–49.

doi:10.1104/pp.118.1.37

Uauy, C., F. Paraiso, P. Colasuonno, R.K. Tran, H. Tsai, S. Berardi, L. Comai, and J. Dubcovsky. 2009. A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. BMC Plant Biol. 9:115–128. doi:10.1186/1471-2229-9-115

Uhlmann, N.K., and D.M. Beckles. 2010. Storage products and transcriptional analysis of the endosperm of cultivated wheat and two wild wheat species. J. Appl. Genet. 51:431–447. doi:10.1007/BF03208873

Union des Associations de Fabricants de Pates Alimentaires de L'U.E (UN.A.F.P.A.). 2011. Survey on pasta consumption (updated to October 2011). UN.A.F.P.A., Rome, Italy. http://www.pasta-unafpa.org/ingstatistics4.htm (accessed 5 May 2012).

Wicker, T., K.F.X. Mayer, H. Gundlach, M. Martis, B. Steuernagel, U. Scholz, H. Simkova, M. Kubalakova, F. Choulet, S. Taudien, M. Platzer, C. Feuillet, T. Fahima, H. Budak, J. Dolezel, B. Keller, and N. Stein. 2011. Frequent gene movement and pseudogene evolution is common to the large and complex genomes of wheat, barley, and their relatives. Plant Cell 23:1706–1718. doi:10.1105/tpc.111.086629

Willis, H.J., A.L. Eldridge, J. Beiselgel, W. Thomas, and J.L. Slavin. 2009. Greater satiety response with resistant starch and corn bran in human subjects. Nutr. Res. 29:100–105. doi:10.1016/j.nutres.2009.01.004

Yamada, Y., S. Hosoya, S. Nishimura, T. Tanaka, Y. Kajimoto, A. Nishimura, and O. Kajimoto. 2005. Effect of bread containing resistant starch on postprandial blood glucose levels in humans. Biosci. Biotechnol. Biochem. 69:559–566. doi:10.1271/bbb.69.559

Yamanouchi, H., and Y. Nakamura. 1992. Organ specificity of isoforms of starch branching enzyme (Q-Enzyme) in rice. Plant Cell Physiol. 33:985–991.

Yun, S.H., and N.K. Matheson. 1990. Estimation of amylose content of starches after precipitation of amylopectin by concanavalin-A. Starch/Staerke 42:302–305.