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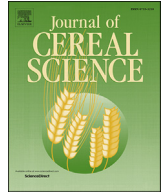Dubcovsky, Jorge

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# Combined mutations in five wheat *STARCH BRANCHING ENZYME II* genes improve resistant starch but affect grain yield and bread-making quality

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## ABSTRACT

Increases in the proportion of amylose in the starch of wheat grains result in higher levels of resistant starch, a fermentable dietary fiber associated with human health benefits. The objective of this study was to assess the effect of combined mutations in five *STARCH BRANCHING ENZYME II* (*SBEII*) genes on starch composition, grain yield and bread-making quality in two hexaploid wheat varieties. Significantly higher amylose (~60%) and resistant starch content (10-fold) was detected in the *SBEII* mutants than in the wild-type controls. Mutant lines showed a significant decrease in total starch (6%), kernel weight (3%) and total grain yield (6%). Effects of the mutations in bread-making quality included increases in grain hardness, starch damage, water absorption and flour protein content; and reductions in flour extraction, farinograph development and stability times, starch viscosity, and loaf volume. Several traits showed significant interactions between genotypes, varieties, and environments, suggesting that some of the negative impacts of the combined *SBEII* mutations can be ameliorated by adequate selection of genetic background and growing location. The deployment of wheat varieties with increased resistant starch will likely require economic incentives to compensate growers and millers for the significant reductions detected in grain and flour yields.

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## 1. Introduction

Wheat (*Triticum* spp.) is a major source of carbohydrates for human nutrition, representing nearly 20% of the ingestion of calories worldwide (FAOSTAT, 2015). The starch in the grain endosperm accounts for most of this intake and comprises two different types of polysaccharides. Amylose is a polymer of α-D-(1—4) linked D-glucose molecules with limited branching (20—30% of the grain starch), whereas amylopectin consists of chains of D-glucose that are highly branched through α-D-(1—6) linkages (70—80% of the grain starch) (Sharma et al., 2008).

The linear helical chains of amylose form complexes that limit access and digestion by amylases. Thus, high levels of amylose in the starch are associated with increased levels of resistant starch (RS), which is defined as the starch that resists digestion in the small intestine of healthy human individuals. Resistant starch acts as a prebiotic dietary fiber, and plays a beneficial role in human digestive physiology (Sharma et al., 2008). Given the importance of wheat in human nutrition, higher levels of RS in wheat products have the potential to deliver health benefits to a considerable fraction of the human population (Regina et al., 2006; Hazard et al., 2015). With increased awareness of the impact of diet on human health, many consumers are showing a growing interest in functional foods (Homayouni et al., 2014).

As RS reaches the colon, it is fermented by gut bacteria and yields short-chain fatty acids, in particular butyrate (Raigond et al., 2015). These substances are a major energy source for colonocytes and can thus improve mucosal integrity. Short-chain fatty acids also improve the lumen environment, making it less conducive to the formation of cancerous tumors (Chapman, 2003). As a result, long-term consumption of RS has the potential to prevent colorectal

cancer, the third most common cause of cancer-related mortality worldwide (Amini et al., 2016).

Resistant starch may also be implicated in the prevention of type 2 diabetes by an increase in insulin sensitivity (Robertson et al., 2005). Foods containing RS have lower glycemic index and rate of digestion, which leads to sustained and lower levels of glucose release into the bloodstream (Fuentes-Zaragoza et al., 2010; Wong and Louie, 2016). RS has also been reported to promote satiety and increase gut hormones that are effective in reducing energy intake, thus increased levels of RS in wheat products can contribute to reduce the occurrence of obesity (Keenan et al., 2006; Willis et al., 2009).

Down regulation of genes involved in amylopectin biosynthesis has been a useful strategy to increase the amount of amylose in the wheat starch (Regina et al., 2006; Sestili et al., 2010; Slade et al., 2012; Hazard et al., 2015; Schönhofen et al., 2016). We previously demonstrated that down regulation of five STARCH BRANCHING ENZYME II (SBEII) genes in common wheat led to an increase in the amount of grain amylose (63%) and resistant starch (1057%) (Schönhofen et al., 2016). However, a significant decrease in total starch (7.8%) and altered starch viscosity parameters suggested that further studies were necessary to understand the impact of the combined SBEII mutations on grain yield and bread-making quality.

The aim of this study was to quantify the impacts of the SBEII mutant alleles on common wheat grain yield and grain yield components as well as on grain, flour, starch and bread quality properties. The findings presented in this study provide valuable information for the deployment of common wheat varieties and products with increased resistant starch.

## 2. Materials and methods

### 2.1. Materials

The development of a hard red common wheat line carrying five loss-of-function mutations in the STARCH BRANCHING ENZYME II (SBEII) genes was previously described (Schönhofen et al., 2016). Mutant alleles of SBEIIa and SBEIIb genes in the A/B genomes and SBEIIa of the D genome (sbeIIa/b-AB, sbeIIa-D) were backcrossed five times (>98% identity) into the recurrent hard red spring variety Lassik (henceforth, Lassik SBEII quintuple mutant). This line has been deposited in the National Small Grain Collection (NSGC) under accession number PI 675647. For the present study, the same five mutations were backcrossed five times into Patwin-515HP, a hard white spring common wheat variety widely grown in California, carrying stripe rust resistance genes Yr5 and Yr15 and the high grain protein content gene GPC-B1 (PVP No. 201600390). The resulting mutant line is >98% identical to the recurrent parent, and these two lines will be referred to hereafter as Patwin control and Patwin SBEII mutant for simplicity.

### 2.2. Experimental procedures

#### 2.2.1. Growth conditions

The Lassik and Patwin SBEII quintuple mutants were evaluated side by side their respective recurrent parents (wild-type alleles at all SBEII loci) in two locations in the 2015–2016 growing season. The first location was at the UC Experimental Field Station in Davis, CA (38° 32″ N, 121° 46″ W) in the Sacramento Valley. The second location was at the West Side Research and Extension Center (WSREC) in Five Points, CA (36° 20″ N, 120° 6″ W) in the San Joaquin Valley.

In the Sacramento Valley, plots were sown on November 7, 2015 at a density of 300 seeds/$m^2$ in a Yolo loam soil (fine-silty, mixed, superactive, nonacid, thermic Mollic Xerofluvents). Pre-

planting fertilization consisted of 24 kg $ha^{-1}$ N followed by 24 kg $ha^{-1}$ N at tillering and irrigation consisted of two flood irrigations. In the San Joaquin Valley, seeds were sown on November 18, 2015 at 250 seeds/$m^2$ in a Panoche soil (fine-loamy, mixed, superactive, thermic Typic Haplocambid). The fertilization regime included four applications of 26 kg $ha^{-1}$ N (pre-planting, tillering, boot and flowering stages) and irrigation consisted of four sprinkler and four flood irrigations. The experimental units consisted of plots measuring 3.7 $m^2$ in the Sacramento Valley and 9.3 $m^2$ in the San Joaquin Valley.

#### 2.2.2. Yield and yield components

Spike density was estimated by selecting two 1 $m^2$ areas in each plot at random and counting the number of spikes before harvest. The number of spikelets per spike and the number of grains per spike were assessed by collecting ten spikes within each plot at random. Both locations were harvested in June 2016 and grain production per plot was determined and adjusted for plot length. Kernel weight was estimated based on a sample of 1000 grains.

#### 2.2.3. Quality evaluations

Grain evaluation, milling and quality determinations of flour and bread were performed at the California Wheat Commission Milling and Baking Lab (http://www.californiawheat.org/milling/). The analyses were conducted according to the standard American Association for Cereal Chemist International approved methods (AACCI Intl., Approved methods of analysis, 11th ed. AACC Intl., St. Paul) and are summarized in Supplementary Table 1.

Grain analyses included test weight, moisture, ash, protein, and kernel hardness. Wheat grains were tempered overnight to reach 14.5% moisture and were milled using AACCI Intl. approved method 26–21.02 in a Brabender Quadrumat Senior Mill. Total extraction and percent of white flour were recorded. Flour evaluations included moisture, ash, protein, wet gluten and gluten index, falling number and solvent retention capacity (SRC). Dough properties were assessed using a Brabender farinograph, and baking tests were performed using AACCI Intl. approved method 10–10.03. Two loaves per sample were produced using 200 g of flour and the water amount determined in the farinograph. Ascorbic acid, sugar and yeast solutions were added uniformly to all samples. After mixing to full development, dough fermentation and proofing time consisted of 90 and 42 min, respectively. Samples were baked for 23 min at 218 °C and were then removed from the oven and left to cool down to room temperature for 1 h. Loaf weights and volumes were recorded and crumb color determined using a Minolta Chroma Meter CR-310. Loaf symmetry, crumb structure and texture were scored using AACCI Intl. method 10–12.01 as a guideline. Bread blending tests were conducted by mixing flours from wild-type and mutant Patwin plants at 3:1, 1:1 and 1:3 ratios.

#### 2.2.4. Starch properties

Pasting curves for each sample were generated using a Rapid Visco Analyzer (RVA, AACCI Intl. Method 76–21.01, Supplementary Table 1). Relative amylose content, total starch, damaged starch and resistant starch were evaluated using kits developed by Megazyme International following the manufacturer's protocols (Supplementary Table 1). Relative amylose content (as a percent of total starch) was determined for 25 mg samples of white flour using the AMYLOSE/AMYLOPECTIN kit. Total starch content was evaluated in samples of 100 mg of white flour using the TOTAL STARCH kit (KOH format). Analyses of damaged starch in 100 mg white flour were conducted using the STARCH DAMAGE kit. Finally, resistant starch content was determined on samples of 100 mg white flour and 100 mg of ground bread crumb using the RESISTANT STARCH kit. For the bread crumbs, samples were freeze-dried using a

lyophilizer two days after baking, and were kept at 4 °C in closed Falconer tubes until the analyses were completed. Moisture content was determined for all flour and bread crumb samples to calculate values on a dry basis.

### 2.2.5. Data analysis

Statistical differences in yield and yield components, grain, flour, starch and bread quality attributes between the quintuple mutants and the wild-type *SBEII* alleles were estimated using a 3-way factorial design including genotype (mutant and wild-type), variety (Lassik and Patwin), and environments (Sacramento Valley and San Joaquin Valley). All two-way and three way interactions were included in the model. The two locations were treated as fixed effects in the combined analyses and blocks were considered random and nested within location. Within each location, the experiment was arranged in a split-plot randomized complete block design with six blocks. The varieties Lassik and Patwin were randomized in the main plots, followed by randomization of the mutant and wild-type genotypes within each main plot. As expected for this design, the significances of the differences between varieties were tested using the Variety * Block(Location) error term, and between wild-type and *SBEII* quintuple mutants using the residual error. ANOVA assumptions of normality of residues and homogeneity of variances were tested using Shapiro−Wilk's and Levene's tests, respectively. All statistical analyses were performed using SAS version 9.4 (SAS Institute, 2015; Supplementary Table 1).

## 3. Results and discussion

### 3.1. Yield components and grain yield

A significant reduction of 2.8% in kernel weight ($P = 0.015$) and 5.8% in grain yield ($P = 0.015$) were detected in the *SBEII* mutants relative to their recurrent parents Lassik and Patwin, in the 3-way factorial analyses (Table 1). However, when the differences in kernel weight were analyzed separately for each variety, only the difference in Patwin was statistically significant ($-5\%$, $P < 0.001$). A differential response among varieties was supported by a

significant Genotype * Variety interaction ($P = 0.037$).

The larger reduction in kernel weight observed in Patwin *SBEII* quintuple mutants was compensated by a 12.6% increase in the number of spikes per m$^2$ ($P = 0.0056$), resulting in smaller reductions in total grain yield in the mutant lines in Patwin than in Lassik. A significant Genotype * Location interaction ($P = 0.008$) in grain yield was attributed to larger differences between genotypes in the San Joaquin Valley ($P = 0.004$, 11.2% reduction) than in the Sacramento Valley (non-significant differences). Variations in the number of spikelets per spike and grains per spike were not statistically significant among genotypes and were not included in Table 1.

The reductions in grain yield and kernel weight observed in the hexaploid lines carrying the five *SBEII* mutant alleles relative to the wild-type sib-lines is consistent with the reduced grain yield (15.4%) and kernel weight (5.2%) previously reported for tetraploid wheat lines carrying four *SBEII* mutations (Hazard et al., 2015). The reductions in grain yield may be associated with negative effects of the *SBEII* mutations both in grains (see reduction in total starch in Section 3.4.3) and leaves. In maize, SBEIIa was shown to be required for proper branching of amylopectin and formation of uniform starch granules in the chloroplasts. The absence of this protein led to irregular starch granules that could not be properly degraded during the dark phase of the diurnal cycle, failing to provide the necessary carbon for metabolism and growth at night (Yandeau-Nelson et al., 2011). It would be interesting to investigate if a similar effect occurs in the *SBEII* mutant lines in wheat.

### 3.2. Grain quality

#### 3.2.1. Protein

Differences in grain protein content between mutant and wild-type lines were not significant in the combined factorial analysis ($P = 0.077$, Table 1), but were significant for the variety Lassik in the Sacramento Valley ($P = 0.007$). Lassik had a significantly lower grain protein content than Patwin ($P = 0.0004$), and this may have contributed to the larger differences in grain protein content between genotypes observed in this variety relative to Patwin. The

**Table 1**

Comparison of grain yield components, grain attributes and flour yield between quintuple *SBEII* mutant lines (*sbeII*) and their respective wild-type control lines. Untransformed means are presented for the combined analysis and for the individual varieties and locations. *P* values for the 3-way factorial ANOVA are presented at the bottom of each trait. Protein is reported on a 12% moisture basis and ash on a 14% moisture basis.

| Location | Genotype | Kernel weight | Grain yield | Spikes per area | Protein | Hardness | Test weight | Flour extraction rate |
|---|---|---|---|---|---|---|---|---|
| | | mg kernel | kg ha$^{-1}$ | no. spikes/m$^2$ | % | SKCS | kg/hl | % |
| All Locations | Lassik wild-type | 38.3 ± 0.5 | 9472.5 ± 531.2 | 685.7 ± 22.5 | 12.0 ± 0.3 | 64.5 ± 0.9 | 77.9 ± 1.0 | 70.2 ± 0.4 |
| | Lassik *sbeII* | 38.1 ± 0.5 | 8515.0 ± 413.2 | 631.1 ± 18.1 | 12.6 ± 0.4 | 79.7 ± 1.2 | 73.0 ± 0.8 | 60.9 ± 0.6 |
| | Patwin wild-type | 38.0 ± 0.7 | 9832.7 ± 399.5 | 590.3 ± 9.6 | 13.0 ± 0.5 | 70.8 ± 3.3 | 77.5 ± 0.6 | 68.6 ± 0.5 |
| | Patwin *sbeII* | 36.1 ± 0.6 | 9661.4 ± 290.2 | 664.7 ± 18.6 | 13.2 ± 0.6 | 89.5 ± 2.1 | 73.5 ± 0.5 | 48.4 ± 1.7 |
| Sacramento Valley | Lassik wild-type | 37.7 ± 0.8 | 7996.5 ± 369.0 | 679.4 ± 24.9 | 12.5 ± 0.3 | 63.8 ± 1.7 | 75.0 ± 1.0 | 69.1 ± 0.5 |
| | Lassik *sbeII* | 37.2 ± 0.7 | 7569.8 ± 197.0 | 643.0 ± 15.8 | 13.8 ± 0.1 | 76.4 ± 1.0 | 70.5 ± 0.2 | 62.4 ± 0.4 |
| | Patwin wild-type | 38.3 ± 0.8 | 9325.4 ± 240.7 | 603.9 ± 13.1 | 14.6 ± 0.4 | 60.8 ± 1.8 | 75.7 ± 0.5 | 69.7 ± 0.5 |
| | Patwin *sbeII* | 36.7 ± 0.5 | 9877.1 ± 264.5 | 678.8 ± 21.4 | 15.0 ± 0.1 | 82.6 ± 0.8 | 72.0 ± 0.1 | 53.6 ± 1.2 |
| San Joaquin Valley | Lassik wild-type | 38.9 ± 0.3 | 10948.4 ± 483.7 | 692.0 ± 39.9 | 11.4 ± 0.4 | 65.2 ± 0.8 | 80.8 ± 0.1 | 71.2 ± 0.3 |
| | Lassik *sbeII* | 39.1 ± 0.7 | 9460.2 ± 595.9 | 619.1 ± 33.6 | 11.5 ± 0.5 | 83.0 ± 0.6 | 75.6 ± 0.2 | 59.4 ± 0.6 |
| | Patwin wild-type | 37.6 ± 1.2 | 10340.1 ± 735.7 | 576.7 ± 12.8 | 11.5 ± 0.3 | 80.7 ± 1.9 | 79.4 ± 0.3 | 67.4 ± 0.7 |
| | Patwin *sbeII* | 35.5 ± 0.9 | 9445.6 ± 531.1 | 650.6 ± 31.4 | 11.6 ± 0.5 | 96.4 ± 0.7 | 75.1 ± 0.4 | 43.1 ± 0.9 |
| Source of Variation | | | | | | | | |
| Genotype† (*P*) | | 0.0147 | 0.0155 | 0.5 | 0.077 | <0.0001 | <0.0001 | <0.0001 |
| Location ‡ (*P*) | | 0.7 | 0.022 | 0.5 | <0.0001 | 0.0271 | 0.2 | <0.0001 |
| Variety § (*P*) | | 0.1 | 0.029 | 0.2 | 0.0004 | <0.0001 | 0.9 | <0.0001 |
| Variety x Genotype † (*P*) | | 0.037 | 0.080 | 0.0002 | 0.4 | <0.0001 | <0.0001 | <0.0001 |
| Location x Genotype † (*P*) | | 0.9 | 0.008 | 0.5 | 0.1 | 0.8 | 0.4 | <0.0001 |
| Location x Variety x Genotype † (*P*) | | 0.5 | 0.7 | 0.5 | 0.4 | 0.001 | 0.4 | 0.2 |
| Block(Location) § (*P*) | | 0.3 | 0.054 | 0.5 | 0.012 | 0.7054 | 0.5 | 0.2 |
| Variation Explained ($R^2$) | | 0.83 | 0.90 | 0.79 | 0.89 | 0.98 | 0.96 | 0.99 |

Error used: † = MS(Error), ‡ = MS[block(location)], § = MS[Variety*Block(Loc)].

known negative correlation between yield and grain protein content may have contributed to the significantly lower grain protein content ($P < 0.0001$) observed in the San Joaquin Valley (higher grain yield) than in the Sacramento Valley (lower grain yield).

### 3.2.2. Kernel hardness

Grain hardness increased significantly in the *SBEII* mutants (25%, $P < 0.0001$) relative to the wild-type controls (Table 1). This trait also showed significant two-way (Genotype * Variety) and three-way interactions (Genotype * Variety * Environment).

Kernel hardness is determined mainly by the interaction between puroindoline proteins located in the surface of the starch granules and the endosperm's protein matrix (Beecher et al., 2002). Therefore, the altered morphology of the starch granules in the *SBEII* mutants (Slade et al., 2012) or transgenic RNAi lines (Sestili et al., 2010) might affect the association between the starch granules and the protein matrix of the endosperm. Since kernel hardness, kernel weight and grain protein content tend to be positively correlated (Giroux et al., 2000), the effect of the *SBEII* mutations on kernel size and/or protein content can also contribute to the observed differences in kernel hardness. Significant Genotype * Variety interactions were observed for all three parameters (Table 1). Kernel hardness is positively associated with increased starch damage during milling, and negatively correlated with flour yield (Martin et al., 2001). Thus, the observed increase in kernel hardness might have contributed to the higher content of flour ash and reduced flour yield in the *SBEII* mutant lines.

### 3.2.3. Test weight

The *SBEII* quintuple mutants showed a significant decrease in test weight in the 3-way factorial analysis (5.7%, $P < 0.0001$) and a significant Genotype * Variety interaction (Table 1). This significant interaction was the result of a larger effect of the *SBEII* mutations on test weight in Lassik than in Patwin, even though their respective control lines exhibited similar values. A significant reduction in test weight was also observed in a tetraploid wheat variety with combined mutations in four *SBEII* genes (Hazard et al., 2015). A positive correlation has been previously reported between kernel starch content and test weight in durum wheat (El-Khayat et al., 2006). Therefore, the reduced total starch content observed in the *SBEII* mutant lines (Hazard et al., 2015 and Section 3.4.3. in this study) may have contributed to the reduced test weight.

### 3.2.4. Flour extraction rate

Flour extraction rate decreased 21.3% in the *SBEII* quintuple mutants relative to the isogenic control lines ($P < 0.001$) (Table 1). These reductions were more noticeable in Patwin (29.4%, $P < 0.001$) than in Lassik (13.2%, $P < 0.001$), and in the Sacramento Valley (16.4%, $P < 0.001$) than in the San Joaquin Valley (8.5%, $P < 0.001$). These differences were reflected in highly significant Genotype * Variety and Genotype * Environment interactions for this trait (Table 1).

These significant interactions suggest that the choice of genetic background and growing location can reduce the adverse effect of the mutations on flour extraction rate. This can have a significant economic impact since wheat flour milling profitability is largely influenced by milling efficiency. As indicated before, the increase in grain hardness observed for the *SBEII* mutant lines may have contributed to the decrease in flour yield. This is also apparent in the stronger reduction in flour yield found for Patwin, as this line has harder grains than Lassik.

As only one standardized milling protocol was used in this study, it would be worth testing the potential of different milling protocols to minimize the negative impact of the combined *SBEII* mutations on flour yield, while keeping acceptable levels of ash content. In addition, breeding for increased test weight can also attenuate the detrimental impact of the *SBEII* mutations on flour yield, as this trait is positively correlated with flour yield, is highly heritable and is easy to measure.

## 3.3. Flour and dough quality

### 3.3.1. Flour protein

Protein concentration in the flour was 9.5% higher in the lines carrying the *SBEII* mutant alleles than in those carrying the wild-type alleles ($P = 0.0001$, Table 2). A similar increase (11.9%) was observed in the semolina extracted from a durum line with mutations in four *SBEII* genes (Hazard et al., 2015). Durum wheat lines without active STARCH SYNTHASE IIA (SSIIa), another key enzyme in the amylopectin biosynthesis, showed similar effects as the durum lines without SBEII activity. SSIIa null durum mutants showed significant increases in grain protein associated with smaller grains, reduced amounts of total starch and increases in amylose content in two independent studies (Hogg et al., 2015; Botticella et al., 2016). The significantly lower kernel weights detected in both tetraploid and hexaploid wheat varieties can contribute indirectly to an increase in protein concentration. To separate the concentration effect, we analyzed the differences between genotypes in total protein content (protein % * kernel weight). The reduced differences between genotypes observed for total protein content (protein % * kernel weight, 5.9%, $P = 0.021$) than for protein concentration is consistent with a partial contribution of kernel size to the observed differences in flour protein content.

Interestingly, the differences in protein content in the flour (9.5%) were higher than those observed in the grain (3.2%, Table 1). The differences were particularly noticeable in the San Joaquin Valley, where protein content in the *SBEII* mutants increased by less than 1% in the grain and more than 14% in the flour relative to the control lines (Table 2). Larger differences between genotypes were observed in the San Joaquin Valley for both flour protein content (Table 2) and flour extraction rate (Table 1). Since milling is the only process between grain and flour, it would be interesting to investigate if the larger differences in extraction rate between genotypes contribute to the larger differences in protein content observed in the flour relative to the grain. Experiments controlling extraction rates will be required to test this possibility.

Since high protein content is beneficial for both pasta and bread-making quality, and also contributes to the nutritional value of wheat, the increased protein content on the *SBEII* mutants can be considered a positive pleiotropic effect.

### 3.3.2. Flour ash

The positive effect of the *SBEII* mutations on flour protein content was partially offset by parallel increases in ash content (22.7%, $P < 0.0001$, Table 2). Significant increases in ash content (~40%) were also detected in a tetraploid wheat carrying loss-of-function mutations in all four *SBEII* genes relative to the isogenic control (Hazard et al., 2015). These results suggest that the higher levels of ash content may be a problem common to high-amylose wheat varieties.

The increases in ash content in the *SBEII* mutants were more pronounced in the San Joaquin Valley than in the Sacramento Valley, resulting in a significant Genotype * Location interaction ($P = 0.007$). This trend parallels the larger reduction in flour yield observed in the San Joaquin Valley, and suggests that the effect of the *SBEII* mutations on flour yield may be also associated with the increases in ash content. Flour ash is also positively correlated with grain hardness (Martin et al., 2001) and therefore, the harder grains of the *SBEII* mutants may have also contributed to the higher ash content.

**Table 2**

Comparison of flour and dough quality characteristics between quintuple *SBEII* mutant lines (*sbeII*) and their respective wild-type control lines. Untransformed means are presented for the combined analysis and for the individual varieties and locations. *P* values for the 3-way factorial ANOVA are presented at the bottom of each trait. Protein and ash are reported on a 14% moisture basis. MTI = mixing tolerance index.

| Location | Genotype | Protein | Ash | Wet gluten | Gluten index | Falling number | Farinograph | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Absorption | Develop. time | Stability | MTI |
| | | % | % | % | % | sec | % | min | min | BU |
| All Locations | Lassik wild-type | 10.5 ± 0.5 | 0.35 ± 0.01 | 31.8 ± 1.3 | 78.5 ± 2.7 | 326.5 ± 8.1 | 64.4 ± 0.3 | 4.5 ± 0.4 | 8.2 ± 1.1 | 26.5 ± 2.2 |
| | Lassik *sbeII* | 11.5 ± 0.3 | 0.42 ± 0.01 | 33.8 ± 1.2 | 80.6 ± 2.3 | 467.5 ± 7.2 | 82.4 ± 0.3 | 5.2 ± 0.1 | 7.8 ± 0.6 | 32.5 ± 2.4 |
| | Patwin wild-type | 11.5 ± 0.5 | 0.40 ± 0.01 | 34.3 ± 1.7 | 85.8 ± 3.2 | 390.8 ± 13.6 | 66.4 ± 0.5 | 10.4 ± 2.1 | 20.4 ± 2.1 | 15.1 ± 1.5 |
| | Patwin *sbeII* | 12.6 ± 0.4 | 0.50 ± 0.01 | 36.7 ± 1.8 | 79.3 ± 3.6 | 403.5 ± 11.5 | 89.8 ± 0.5 | 6.9 ± 0.6 | 10.5 ± 0.9 | 32.2 ± 2.9 |
| Sacramento Valley | Lassik wild-type | 11.9 ± 0.5 | 0.38 ± 0.01 | 35.2 ± 1.1 | 73.7 ± 2.4 | 318.2 ± 13.2 | 64.9 ± 0.6 | 5.3 ± 0.7 | 9.5 ± 1.9 | 22.5 ± 2.8 |
| | Lassik *sbeII* | 12.5 ± 0.1 | 0.44 ± 0.01 | 37.3 ± 0.3 | 73.9 ± 1.5 | 466.2 ± 7.4 | 82.2 ± 0.2 | 5.1 ± 0.2 | 9.5 ± 0.7 | 25.5 ± 0.6 |
| | Patwin wild-type | 13.1 ± 0.4 | 0.42 ± 0.01 | 39.2 ± 1.5 | 75.7 ± 2.0 | 356.7 ± 17.1 | 67.5 ± 0.8 | 11.4 ± 2.5 | 16.8 ± 1.5 | 17.0 ± 2.6 |
| | Patwin *sbeII* | 13.8 ± 0.2 | 0.48 ± 0.01 | 42.1 ± 0.9 | 68.6 ± 2.2 | 384.8 ± 14.4 | 90.9 ± 0.8 | 5.7 ± 0.4 | 8.1 ± 0.5 | 40.3 ± 2.3 |
| San Joaquin Valley | Lassik wild-type | 9.1 ± 0.2 | 0.33 ± 0.01 | 28.5 ± 1.5 | 83.2 ± 4.2 | 334.8 ± 9.2 | 64.0 ± 0.2 | 3.6 ± 0.3 | 7.0 ± 1.0 | 30.5 ± 2.7 |
| | Lassik *sbeII* | 10.5 ± 0.4 | 0.41 ± 0.01 | 30.3 ± 1.0 | 87.4 ± 1.6 | 468.9 ± 13.1 | 82.6 ± 0.6 | 5.3 ± 0.2 | 6.2 ± 0.3 | 39.5 ± 2.1 |
| | Patwin wild-type | 9.9 ± 0.3 | 0.39 ± 0.01 | 29.5 ± 1.2 | 95.9 ± 0.6 | 424.8 ± 7.5 | 65.3 ± 0.4 | 9.3 ± 3.5 | 24.0 ± 3.5 | 13.2 ± 1.5 |
| | Patwin *sbeII* | 11.3 ± 0.3 | 0.53 ± 0.01 | 31.3 ± 1.3 | 90.1 ± 2.6 | 422.2 ± 15.2 | 88.7 ± 0.4 | 8.2 ± 1.0 | 13.0 ± 1.0 | 24.0 ± 2.0 |
| Source of Variation | | | | | | | | | | |
| Genotype† (*P*) | | 0.0001 | <0.0001 | 0.0039 | 0.24 | <0.0001 | <0.0001 | 0.017 | 0.0007 | <0.0001 |
| Location ‡ (*P*) | | <0.0001 | 0.069 | <0.0001 | <0.0001 | 0.0098 | 0.014 | 0.024 | 0.081 | 0.7 |
| Variety § (*P*) | | 0.0009 | <0.0001 | 0.0068 | 0.067 | 1.0 | <0.0001 | 0.010 | <0.0001 | 0.0065 |
| V x G † (*P*) | | 0.9 | 0.2 | 0.8 | 0.0265 | <0.0001 | <0.0001 | 0.0721 | 0.0015 | 0.0018 |
| L x G † (*P*) | | 0.097 | 0.0072 | 0.6 | 0.5 | 0.2 | 0.4 | 0.1 | 0.6 | 0.3 |
| L x V x G † (*P*) | | 0.7 | 0.090 | 0.8 | 0.7 | 0.6 | 0.4 | 0.6 | 0.8 | 0.0071 |
| B(L) § (*P*) | | 0.3 | 0.020 | 0.2 | 0.5 | 0.5 | 0.2 | 0.3 | 0.9 | 0.7 |
| $R^2$ | | 0.92 | 0.91 | 0.92 | 0.85 | 0.91 | 0.99 | 0.65 | 0.82 | 0.89 |

Error used: † = MS(Error), ‡ = MS[block(location)], § = MS[Variety*Block(Loc)].

### 3.3.3. Wet gluten and gluten index

Wet gluten increased significantly in the lines carrying the *SBEII* mutations relative to the lines carrying the wild-type alleles (6.7%, *P* = 0.0039, Table 2). However, these differences were no longer significant when flour protein content was included as a co-variable (*P* = 0.47). This result suggested that the increases in wet gluten were likely mediated by the increase in flour protein content observed for the *SBEII* mutant lines.

Whereas wet gluten is directly affected by the amount of protein, Gluten Index is mainly associated with the strength of the gluten (higher values indicate stronger gluten). Although no statistically differences between genotypes were detected in the 3-way factorial analysis, a significant interaction was observed between genotype and variety (*P* = 0.027, Table 2). Surprisingly, the two varieties showed opposite effects of the *SBEII* mutations on gluten index. Whereas a slight increase (non-significant) was observed in Lassik *SBEII* mutant, Patwin *SBEII* mutant line presented a significant reduction of 7.6% compared to the wild-type (*P* < 0.0098).

Although we still do not have a clear explanation for this difference, it is tempting to speculate that it might relate to the sizeable decrease in flour extraction rate observed in Patwin *SBEII* mutants (29.4%, Table 1). A qualitative protein gradient exists from the center to the outer layer of the wheat kernel endosperm (Tosi et al., 2011), which may have contributed to the reduced gluten index observed in the line with a larger reduction in flour yield (Patwin).

### 3.3.4. Falling number (FN)

Falling number increased 21.4% in the *SBEII* mutant lines (*P* < 0.0001, Table 2) relative to their respective wild-type controls. However, this increase was not uniform across varieties, resulting in a highly significant Variety * Genotype interaction (*P* < 0.0001). The increase in FN was significant in Lassik (43.2%, *P* < 0.0001), but not in Patwin (3.2%, *P* = 0.29). A highly significant increase in FN was also observed in a tetraploid line carrying mutations in all four *SBEII* genes (Hazard et al., 2015).

The higher FN found for the *SBEII* mutant lines parallels the decreased susceptibility of the starch to breakdown under high temperature (95 °C) and mechanical shearing conditions used in the rapid visco analyzer test (Section 3.4.1.). It also indicates decreased effectiveness of the amylases present in the flour to degrade high-amylose starch. Since this may impair the release of fermentable sugars and yeast production of $CO_2$ in the dough (Mares and Mrva, 2008), additional α-amylase enzymes may be necessary to stimulate starch degradation in high-resistant starch wheat bread recipes (see Section 3.5.2. on loaf volume).

### 3.3.5. Farinograph

The farinograph test, which characterizes the rheological behavior of the dough, showed significant differences between lines with contrasting *SBEII* alleles (Table 2). Lines with the mutant *SBEII* alleles showed a highly significant (*P* < 0.001) increase in water absorption and mixing tolerance index (MTI), and a significant decrease in development time and stability. All four parameters showed higher differences between mutant and wild-type *SBEII* alleles in Patwin than in Lassik, resulting in highly significant (*P* < 0.005) Variety * Genotype interaction for water absorption, MTI and stability and a marginally non-significant interaction for dough development (*P* = 0.07). The higher water absorption of flours obtained from the *SBEII* mutant lines is likely associated with their higher levels of starch damage (31.7%, Section 3.4.4) and flour protein content (9.5%, Section 3.3.1.). Damaged starch granules have a higher capacity to absorb water than undamaged starch granules (Manley, 2000). Higher water absorption is desirable for bread products, but undesirable for cookies and cakes because it is associated with longer baking time (and higher energy costs) to evaporate the additional water.

Although farinograph stability is usually associated with gluten strength, inconsistent results were observed in this study between stability and gluten index, which is an indirect measure of gluten strength. Flours of the *SBEII* mutant lines showed reduced stability relative to the wild-type controls for both varieties, whereas gluten index showed contrasting effects between varieties (Table 2). These

results indicate that factors other than gluten strength are contributing to the observed differences between these two traits.

In summary, the large effect of the combined *SBEII* mutations on the different farinograph parameters suggests that bakers and food technicians will need to adjust their bakery formulations for wheat products with increased resistant starch.

### 3.3.6. Solvent retention capacity (SRC)

Flours from the lines with combined *SBEII* mutations showed significantly higher retention values than those from the control lines for all four solvents (water, 5% w/w lactic acid in water, 5% w/w Na$_2$CO$_3$ in water, and 50% w/w sucrose in water, Supplementary Table 2). Except for the lactic acid solvent, the differences between the lines with mutant and wild type *SBEII* alleles were larger in Patwin than in Lassik.

The water solvent can hydrate and swell the three main flour functional components (glutenins, damaged starch and pentosan sugars) and provides similar information to the farinograph water absorption described above. The results from the lactic acid solution emphasize the swelling of the glutenin network and acts as predictor of gluten quantity, quality and functionality. The higher lactic acid retention values observed for the *SBEII* mutant flours likely reflect the higher protein and wet gluten content present in the flours of the mutant lines (Table 2). High values of lactic acid retention are generally associated with beneficial effects on yeast-leavened products, as gluten proteins form a network that retains CO$_2$ produced during fermentation, giving the dough strength and elasticity.

The sodium carbonate solvent has a highly alkaline pH, which easily solvates damaged starch granules, exaggerating their swelling and allowing a distinction from the undamaged starch. The higher retention values of the *SBEII* mutant lines in this solvent correlate well with their increased levels of starch damage (Supplementary Table 2). In yeast-leavened products, some starch

damage is beneficial to obtain fermentable sugars produced by amylase hydrolysis. In the production of cookies, however, starch damage can elongate baking time, increase dough stiffness and decrease cookie diameter (León et al., 2006). Finally, the sucrose solvent emphasizes the swelling of pentosan molecules found in the cell wall. These non-starch polysaccharides have the ability to bind large quantities of water and were reported to affect bread characteristics (Denli and Ercan, 2001).

### 3.4. Starch properties

#### 3.4.1. Rapid visco analyses (RVA)

The flours from lines carrying combined *SBEII* mutations showed significantly lower peak viscosity and final viscosity; and significantly higher break down, setback and pasting temperature values than the lines carrying the wild type alleles (Table 3, Fig. 1). Following the trend observed in other starch properties, the RVA parameters showed larger changes between genotypes in Patwin than in Lassik. However, the Variety * Genotype interactions were only significant for peak viscosity, breakdown and setback parameters. Similar reductions in starch swelling and viscosity were obtained by Yamamori et al. (2006) for a *SSIIa*-deficient common wheat and by Hazard et al. (2015) in a durum wheat with loss-of-function mutations in all four *SBEIIa* and *SBEIIb* genes.

The lower RVA peak values observed in the *SBEII* mutant lines indicate reduced swelling of the starch granules, which is likely associated with the higher amylose content found in these lines (Table 3). Amylose is known to suppress swelling and to maintain granule integrity (Sasaki, 2005). Breakdown values are generally negatively correlated with peak viscosity, and the same trend is observed in this study. The lower breakdown values of the mutant lines indicate an improved ability of their starch granules to withstand heat and shear stress. This result likely indicates useful increases in heating and mixing tolerances of the flours with

**Table 3**
Comparison of starch and RVA pasting curve between quintuple *SBEII* mutant lines (*sbeII*) and their respective wild-type control lines. Untransformed means are presented for the combined analysis and for the individual varieties and locations. *P* values for the 3-way factorial ANOVA are presented at the bottom of each trait. RS = resistant starch; RVU = rapid visco units.

| Location | Genotype | Amylose | RS in flour | RS in crumb | Total starch | Starch damage | RVA Peak | Breakdown | Setback | Final viscosity | Pasting temperature |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % starch | g/100 g | g/100 g | % | 'as is' | RVU | RVU | RVU | RVU | °C |
| All Locations | Lassik wild-type | 30.7 ± 0.4 | 0.4 ± 0.04 | 2.1 ± 0.1 | 75.7 ± 0.3 | 5.4 ± 0.2 | 217.3 ± 14.0 | 61.3 ± 2.4 | 92.4 ± 0.8 | 248.4 ± 11.7 | 71.2 ± 3.8 |
| | Lassik *sbeII* | 45.2 ± 0.7 | 3.9 ± 0.2 | 6.2 ± 0.1 | 71.5 ± 0.4 | 7.5 ± 0.3 | 79.5 ± 2.5 | 8.3 ± 0.6 | 71.8 ± 3.8 | 143.0 ± 2.5 | 78.6 ± 3.8 |
| | Patwin wild-type | 29.3 ± 0.4 | 0.5 ± 0.04 | 1.2 ± 0.05 | 73.3 ± 0.6 | 6.9 ± 0.4 | 259.8 ± 10.9 | 100.1 ± 4.4 | 80.9 ± 1.8 | 240.6 ± 14.0 | 64.3 ± 1.5 |
| | Patwin *sbeII* | 50.5 ± 0.9 | 6.0 ± 0.3 | 6.8 ± 0.1 | 68.4 ± 0.4 | 8.7 ± 0.5 | 66.4 ± 2.5 | 6.8 ± 0.8 | 46.0 ± 2.2 | 105.6 ± 3.1 | 82.7 ± 2.8 |
| Sacramento Valley | Lassik wild-type | 31.2 ± 0.7 | 0.5 ± 0.1 | 2.0 ± 0.1 | 75.4 ± 0.4 | 4.9 ± 0.1 | 209.9 ± 19.0 | 57.2 ± 2.7 | 92.3 ± 1.3 | 245.0 ± 15.8 | 79.5 ± 4.5 |
| | Lassik *sbeII* | 44.3 ± 1.1 | 4.6 ± 0.3 | 6.0 ± 0.2 | 71.0 ± 0.6 | 6.6 ± 0.1 | 81.2 ± 4.0 | 7.7 ± 0.9 | 68.6 ± 6.3 | 142.1 ± 3.4 | 79.7 ± 6.8 |
| | Patwin wild-type | 29.2 ± 0.6 | 0.6 ± 0.1 | 1.2 ± 0.1 | 71.8 ± 0.6 | 5.7 ± 0.2 | 247.1 ± 14.0 | 95.9 ± 7.0 | 76.4 ± 1.8 | 227.7 ± 18.8 | 65.6 ± 1.5 |
| | Patwin *sbeII* | 49.2 ± 1.2 | 6.8 ± 0.4 | 6.8 ± 0.1 | 68.2 ± 0.3 | 7.3 ± 0.3 | 67.4 ± 3.3 | 7.0 ± 5.5 | 46.8 ± 3.8 | 107.2 ± 5.5 | 83.9 ± 4.3 |
| San Joaquin Valley | Lassik wild-type | 30.2 ± 0.3 | 0.3 ± 0.03 | 2.1 ± 0.04 | 76.1 ± 0.5 | 5.9 ± 0.2 | 224.7 ± 22.0 | 65.4 ± 18.6 | 92.5 ± 0.9 | 251.9 ± 18.6 | 62.8 ± 3.8 |
| | Lassik *sbeII* | 46.0 ± 0.9 | 3.2 ± 0.2 | 6.4 ± 0.1 | 72.0 ± 0.5 | 8.4 ± 0.3 | 77.8 ± 3.3 | 8.9 ± 3.9 | 74.9 ± 4.5 | 143.8 ± 3.9 | 77.6 ± 4.0 |
| | Patwin wild-type | 29.3 ± 0.4 | 0.4 ± 0.04 | 1.2 ± 0.05 | 74.9 ± 0.4 | 8.0 ± 0.1 | 272.4 ± 16.2 | 104.4 ± 5.2 | 85.4 ± 1.7 | 253.5 ± 21.2 | 63.1 ± 2.6 |
| | Patwin *sbeII* | 51.8 ± 1.2 | 5.2 ± 0.2 | 6.8 ± 0.09 | 68.5 ± 0.7 | 10.0 ± 0.4 | 65.5 ± 4.1 | 6.6 ± 1.3 | 45.1 ± 2.7 | 104.0 ± 3.4 | 81.5 ± 3.8 |
| Source of Variation | | | | | | | | | | | |
| Genotype† (P) | | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | 0.0003 |
| Location ‡ (P) | | 0.3 | 0.0014 | 0.3 | 0.0105 | <0.0001 | 0.4 | 0.0930 | 0.2 | 0.4 | 0.0265 |
| Variety § (P) | | 0.0108 | <0.0001 | 0.1 | <0.0001 | <0.0001 | 0.1 | <0.0001 | <0.0001 | 0.0465 | 0.7 |
| V x G † (P) | | <0.0001 | <0.0001 | <0.0001 | 0.3 | 0.4 | 0.004 | <0.0001 | 0.0073 | 0.1 | 0.0774 |
| L x G † (P) | | 0.0262 | 0.0002 | 0.2 | 0.1 | 0.0793 | 0.2 | 0.1 | 0.6 | 0.4 | 0.2 |
| L x V x G † (P) | | 0.9 | 0.7 | 0.4 | 0.0618 | 0.5 | 0.8 | 0.8 | 0.0929 | 0.5 | 0.2 |
| B(L) § (P) | | 0.2 | 0.0257 | 0.0479 | 0.2 | 0.2 | 0.4 | 0.6 | 0.1 | 0.6 | 0.9 |
| $R^2$ | | 0.98 | 0.98 | 0.99 | 0.92 | 0.95 | 0.95 | 0.98 | 0.92 | 0.90 | 0.72 |

Error used: † = MS(Error), ‡ = MS[block(location)], § = MS[Variety*Block(Loc)].
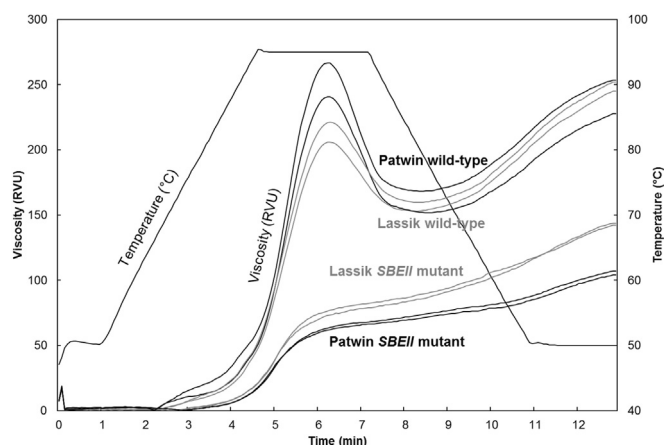
**Fig. 1.** Rapid visco analyzer (RVA) viscogram of wild-type controls and quintuple *SBEII* mutant lines in Patwin (black) and Lassik (grey). Viscosity curves (RVU) are the averages across replications within each location. The temperature profile (°C) indicates the conditions of the RVA analyses. RVU = rapid visco units.

increased amylose and resistant starch content. The lower setback values found in the *SBEII* mutant lines indicate reduced retrogradation, which is the conversion of amylose and amylopectin molecules to a more crystalline structure after heating and cooling in water. Since retrogradation is directly related to bread staling or aging (Wang et al., 2015), the reduced setback values found in the flours from the *SBEII* mutants indicate potential favorable effects on the shelf life of bread products with increased resistant starch.

The significant reduction in final viscosity detected in the *SBEII* mutant lines indicates a reduced capacity of the starch from the mutant lines to make a viscous paste after heating and cooling. This decrease will have either a positive or negative effect, depending on the cooking method used and the desired attributes of the final product. Finally, the increase in pasting temperature observed in the mutant lines suggests that higher cooking temperatures might be optimum for products with high levels of amylose and resistant starch. However, additional studies will be required to determine the effect of these higher temperatures on the thermal stability of other ingredients present in the formula.

### 3.4.2. Amylose and resistant starch

The *SBEII* quintuple mutants showed highly significant ($P < 0.0001$) increases in amylose content (~60%) and resistant starch in the flour (~1000%) (Table 3). Significant Genotype * Variety interactions ($P < 0.0001$) were detected for both parameters as a result of larger differences between genotypes in Patwin than in Lassik. Significant interactions were also detected between Genotype * Location, suggesting that the effect of the *SBEII* mutations is also modulated by the environment.

The levels of grain amylose content detected in this study (45–50%) are similar to the 44–50% levels reported by Schönhofen et al. (2016) and the 56% level reported by Slade et al. (2012) in hexaploid wheat. Similar levels of amylose content were reported in *SBEIIa/b* mutants in tetraploid wheat (40–54%, Hazard et al., 2014, 2015; 47% Slade et al., 2012; 53% Sestili et al., 2015). The higher amylose level (82%) reported by Regina et al. (2015) in hexaploid wheat may be the result of the different methods used to determine amylose content, or by the different mutations used in their study (*sbeIIa*-ABD + *sbeIIb*-AD). The hexaploid mutant lines used in the current study have a wild type copy of the *SbeIIb* gene in the D genome and an amino acid change (P283L) in the B genome that may have some residual activity (Hazard et al., 2014). The content of resistant starch in the wheat grains is largely determined

by the amylose to amylopectin ratio, as these polysaccharides exhibit different structural and physiological characteristics. Whereas the linear helical chains of amylose limit the action of intestinal amylases, amylopectin provides multiple sites where the enzymes can initiate hydrolysis (Ahuja et al., 2013). The levels of RS are very sensitive to variations in methodology. For example, in Slade et al. (2012) these levels varied from 5.4 to 11.2 g/100 g in hexaploid wheat and from 4.7 to 6.2 g/100 g in tetraploid wheat when they changed the lot of pancreatic alpha-amylase (PAA) used in their Megazyme assays. Since these differences also affected the RS content in the wild-type control plants, the percent increase in RS in the mutants relative to the controls was very similar between enzyme lots (1141–1251%). These values are similar to the ones found in this study (875–1100%) and in previously published studies in hexaploid (947-1057%, Schönhofen et al., 2016 and 1078%, Regina et al., 2015) and tetraploid wheat (640–917%, Hazard et al., 2014, 2015; 393–581%, Slade et al., 2012; 689% Sestili et al., 2015).

Even though RS values in the bread crumb were higher than those in the flour (Table 3), the percent increases in the *SBEII* mutants compared to the control lines was smaller (293% in the bread crumb *vs.* 1000% in the flour). This was caused by relatively higher increases in the crumb of the control lines than in the crumb of the mutant lines. Differences in RS between genotypes were larger in Patwin than in Lassik for both flour and crumb values. RS content in the crumb seems to have been influenced not only by the amylose and resistant starch present in the flour but also by the extent of retrogradation. For instance, the RVA setback values for the *SBEII* mutant in Lassik were larger than the values in Patwin and this was associated with a larger increase in RS content in the crumb relative to the flour in Lassik (59%) than in Patwin (13.3%). Modifications in the ingredients ratio, baking time and storage conditions can potentially result in further increases in RS content in the bread crumb (Amaral et al., 2016).

### 3.4.3. Total starch

Total starch content decreased 6.1% in the *SBEII* mutant lines ($P < 0.0001$) compared to the wild-types (Table 3). Similar results were reported by Slade et al. (2012), who found a 5% reduction in total starch in a *SBEIIa*-mutated common wheat line, and Hazard et al. (2015), who observed a 5.7% decrease in this component in *SBEIIa/b* durum wheat mutants. The reductions in total starch likely contributed to the observed reductions in kernel size and the increases in flour protein (Table 2). In addition, the relative decrease in amylopectin and starch branching in *SBEII* mutant lines leads to a decrease in the number of non-reducing ends that are available for enzymatic elongation, which could reduce the amount of starch formed (Ahuja et al., 2013).

### 3.4.4. Starch damage

Starch damage increased 31.71% in the *SBEII* mutants relative to the isogenic wild-type controls and the differences were consistent across varieties and locations (Table 3). Starch damage likely contributed to the 31.65% increase in farinograph water absorption detected in the flours of the mutant lines relative to the controls, as damaged granules absorb considerably more water than intact starch.

### 3.5. Bread-making quality

### 3.5.1. Bake absorption and loaf weight

Bake absorption increased 28.3% in *SBEII* mutant lines relative to the wild-type controls ($P < 0.0001$), with larger increases detected for mutants in Patwin (31.6%) than in Lassik (24.8%) (Table 4). This correlates well with the water absorption differences observed in

**Table 4**
Comparison of baking quality characteristics between quintuple *SBEII* mutant lines (*sbeII*) and their respective wild-type control lines. Untransformed means are presented for the combined analysis and for the individual varieties and locations. *P* values for the 3-way factorial ANOVA are presented at the bottom of each trait.

| Location | Genotype | Bake absorption | Loaf weight | Loaf volume | Color L (black-white) | Color b (yellow - blue) |
|---|---|---|---|---|---|---|
| | | ml | g | cm$^3$ | | |
| All Locations | Lassik wild-type | 144.9 ± 0.6 | 142.6 ± 0.4 | 801.9 ± 40.2 | 78.1 ± 0.9 | 10.2 ± 0.2 |
| | Lassik *sbeII* | 180.8 ± 0.6 | 158.9 ± 0.8 | 669.2 ± 28.2 | 76.7 ± 0.6 | 13.2 ± 0.3 |
| | Patwin wild-type | 148.9 ± 1.1 | 142.9 ± 0.5 | 872.7 ± 23.2 | 77.8 ± 0.8 | 10.6 ± 0.2 |
| | Patwin *sbeII* | 196.0 ± 1.0 | 166.4 ± 0.8 | 566.5 ± 34.8 | 77.1 ± 0.6 | 15.2 ± 0.4 |
| Sacramento Valley | Lassik wild-type | 145.8 ± 1.1 | 141.9 ± 0.6 | 883.3 ± 14.7 | 77.6 ± 1.4 | 10.5 ± 0.3 |
| | Lassik *sbeII* | 180.4 ± 0.3 | 156.7 ± 0.7 | 752.9 ± 16.4 | 77.4 ± 0.9 | 12.7 ± 0.3 |
| | Patwin wild-type | 150.9 ± 1.7 | 143.5 ± 0.9 | 944.6 ± 12.7 | 77.2 ± 1.5 | 10.4 ± 0.3 |
| | Patwin *sbeII* | 197.8 ± 1.5 | 164.5 ± 0.6 | 679.2 ± 11.8 | 78.0 ± 0.9 | 14.0 ± 0.3 |
| San Joaquin Valley | Lassik wild-type | 143.9 ± 0.4 | 143.3 ± 0.5 | 720.4 ± 65.2 | 78.6 ± 1.1 | 10.0 ± 0.2 |
| | Lassik *sbeII* | 181.2 ± 1.2 | 161.1 ± 0.8 | 585.4 ± 20.8 | 76.1 ± 0.6 | 13.6 ± 0.3 |
| | Patwin wild-type | 146.8 ± 0.9 | 142.3 ± 0.5 | 800.8 ± 12.0 | 78.4 ± 0.7 | 10.8 ± 0.3 |
| | Patwin *sbeII* | 194.3 ± 1.0 | 168.3 ± 1.1 | 453.8 ± 10.7 | 76.4 ± 0.6 | 16.3 ± 0.3 |
| Source of Variation | | | | | | |
| Genotype† (*P*) | | <0.0001 | <0.0001 | <0.0001 | 0.2 | <0.0001 |
| Location ‡ (*P*) | | 0.0258 | 0.0005 | <0.0001 | 0.9 | 0.0135 |
| Variety § (*P*) | | <0.0001 | <0.0001 | 0.4 | 1.0 | 0.0001 |
| Variety X Genotype † (*P*) | | <0.0001 | <0.0001 | 0.0002 | 0.7 | <0.0001 |
| Genotype X Location † (*P*) | | 0.3 | 0.002 | 0.3 | 0.2 | <0.0001 |
| Location X Variety X Genotype † (*P*) | | 0.6 | 0.4 | 0.3 | 1.0 | 0.4 |
| Block(Location) § (*P*) | | 0.2 | 0.7 | 0.7 | 0.4 | 0.1 |
| Variation Explained (*R*$^2$) | | 0.99 | 0.99 | 0.93 | 0.46 | 0.98 |

Error used: † = MS(Error), ‡ = MS[block(location)], § = MS[Variety*Block(Loc)].

the farinograph, and it is likely that both are the result of increased flour protein content, damaged starch and pentosan sugars, as reported previously. High bake absorption is a desirable attribute for bakers, as it increases the amount of dough produced from the same quantity of flour, increasing bread yield and profitability. As a result of higher water content, loaf weight increased 13.9% in the quintuple *SBEII* mutant lines relative to the wild-types ($P < 0.0001$), with larger differences in Patwin (16.4%) than in Lassik (11.4%).

### 3.5.2. Loaf volume

Loaf volume showed an opposite trend to the one observed for loaf weight (Table 4). Loaf volume decreased 26.2% in *SBEII* mutants in comparison with wild-type lines ($P < 0.0001$), with larger reductions observed in Patwin (35.1%, $P < 0.0001$) than in Lassik (16.5%, $P < 0.0001$) (Fig. 2). Van Hung et al. (2005) also reported a

significant decrease in loaf volume using 50% of high amylose wheat flour from a line with no functional *SSIIa*. The high falling number values observed in flours from the *SBEII* mutant lines (Table 2) suggested a low α-amylase enzymatic activity. Since this could lead to reduced production of $CO_2$ and dough volume, additional falling number and baking tests were performed with the addition of 5% fungal α-amylase (only for samples collected in the Sacramento Valley). This addition resulted in FN values similar to those observed in flours from the respective wild-type lines (data not shown). The baking test carried out with the same additive resulted in doughs with better texture and handling, but the loaf volumes did not improve. Loaf volumes for *SBEII* mutant samples including the 5% fungal α-amylase (Lassik 743 ± 14.5 cm$^3$ and Patwin 659 ± 7.8 cm$^3$) were not significantly different ($P = 0.29$) from those obtained without the extra α-amylase (Table 4,
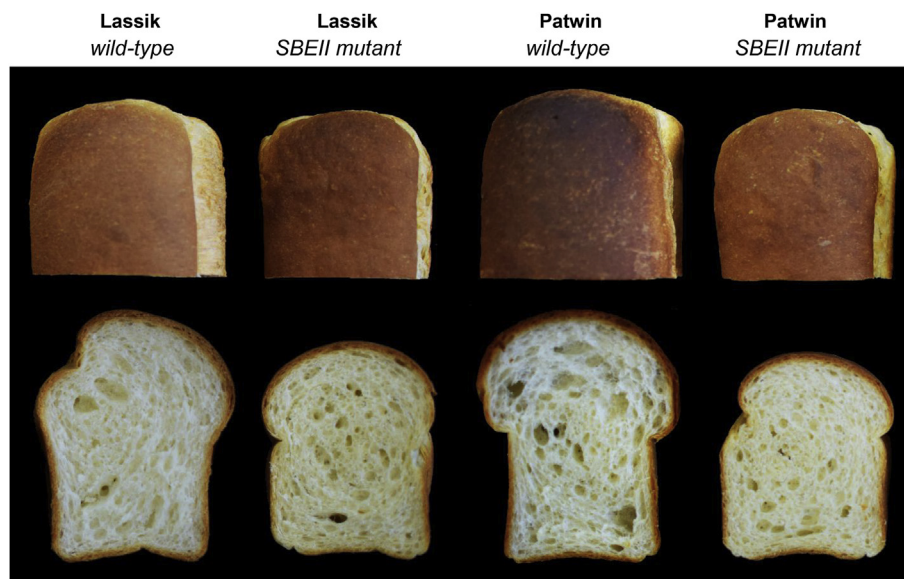


**Fig. 2.** Loaves of wild-type controls and quintuple *SBEII* mutant lines baked using AACCI approved method 10—10.03.

Sacramento Valley). A longer proofing time or the addition of fermentable sugars might be necessary for the proper development of the doughs from wheats with very high levels of RS.

In a blending test using different proportions of flour from Patwin mutant and wild-type lines (3:1, 1:1 and 1:3 ratios), increases in the proportion of flour from the *SBEII* mutant lines resulted in proportional increases in RS and decreases in loaf volume (Supplementary Table 3). The crumb structure and texture showed acceptable scores even when the mixture consisted of one part of flour from the wild-type line to three parts from the mutant (data not shown). However, when the flour was 100% from the *SBEII* mutant lines, structure was scored as coarse, texture as very coarse with thick cell walls, and symmetry as having irregular shape and limited oven spring.

### 3.5.3. Crumb color

Crumbs of the breads obtained using flours from the *SBEII* mutants appeared yellower than the wild-type controls, and this was validated by a 36.5% increase in color b values in the mutant lines ($P < 0.0001$, Table 4). Higher differences in color b values between *SBEII* mutants and wild-type lines were observed for Patwin (43.4%) than for Lassik (29.4%); and for lines grown in the San Joaquin Valley relative to those grown in the Sacramento Valley. No significant differences between genotypes were detected for the L color-opponent dimension (Table 4), indicating that both genotypes had similar crumb lightness.

Differences between genotypes in b-color (Table 4) and amylose content (Table 3) showed similar profiles across varieties and locations, suggesting a possible connection between the two traits. In the Patwin blending experiment described in Supplementary Table 3, the b color values were proportional to the relative fraction of flour from the mutant lines in the blend.

## 4. Conclusions

Common wheat lines combining mutations in five out of the six *SBEII* genes present in hexaploid wheat showed significant effects on most of the agronomic and quality parameters tested in this study. These results indicate that breeders, growers, millers and bakers will need to adapt their protocols to the different properties of the *SBEII* mutant lines. To minimize the negative impact of the *SBEII* mutations on grain size, test weight and total grain yield, breeders will have to test different genetic backgrounds and growers will have to find optimum environments and growing conditions. Millers will have to adjust their milling protocols to increase flour extraction rates and reduce flour ash in the mutant lines. Finally, bakers will need to adapt their baking formulas to the higher water absorption and altered starch properties of the *SBEII* mutant lines. As shown in this study, bakers have the flexibility of blending high-RS and regular flours to identify the levels of RS that can produce a positive impact on health while maintaining quality. It is likely that the production of high-RS wheat varieties will require economic incentives to compensate growers and millers for the lower grain and flour yield of the mutant lines. High-RS wheat varieties will also have to be segregated from the rest of the crop to preserve their higher value and to avoid mixing flours with contrasting quality characteristics.

Although only two varieties and two locations were used in this study, highly significant Genotype * Variety and Genotype * Location interactions were detected for several traits. These results indicate that selection of the appropriate genetic backgrounds and environments can minimize the negative agronomic and quality effects associated with the *SBEII* mutations. The results presented in this study provide a guide to start the deployment of these valuable mutations in commercial common wheat varieties.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2017.03.028.

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