



# Mutation of the maize *sbe1a* and *ae* genes alters morphology and physical behavior of *wx*-type endosperm starch granules

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**Abstract**—In maize, three isoforms of starch-branching enzyme, SBEI, SBEIIa, and SBEIIb, are encoded by the *Sbe1a*, *Sbe2a*, and *Amylose extender* (*Ae*) genes, respectively. The objective of this research was to explore the effects of null mutations in the *Sbe1a* and *Ae* genes alone and in combination in *wx* background on kernel characteristics and on the morphology and physical behavior of endosperm starch granules. Differences in kernel morphology and weight, starch accumulation, starch granule size and size distribution, starch microstructure, and thermal properties were observed between the *ae wx* and *sbe1a ae wx* plants but not between the *sbe1a wx* mutants when compared to *wx*. Starch from *sbe1a ae wx* plants exhibited a larger granule size with a wider gelatinization temperature range and a lower endotherm enthalpy than *ae wx*. Microscopy shows weaker iodine staining in *sbe1a ae wx* starch granules. X-ray diffraction revealed A-type crystallinity in *wx* and *sbe1a wx* starches and B-type in *sbe1a ae wx* and *ae wx*. This study suggests that, while the SBEIIb isoform plays a dominant role in maize endosperm starch synthesis, SBEI also plays a role, which is only observable in the presence of the *ae* mutation.

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**Keywords:** Maize; Mutation; Branching enzyme; Starch; Morphology; Physical behavior

## 1. Introduction

In maize (*Zea mays* L.), multiple starch-branching enzyme (SBE) isoforms, SBEI, SBEIIa, and SBEIIb, catalyze the formation of  $\alpha$ -(1 $\rightarrow$ 6)-glucan linkages in the biosynthesis of amylopectin, the branched macromolecules of starch.<sup>1</sup> These isoforms are coded by three genes, *Sbe1a*, *Sbe2a*, and *Ae* (*Sbe2b*), each of which exhibit differential expression patterns in both tissue-specific and temporal dimensions.<sup>2–9</sup> The highly branched amylopectin molecule is composed of thousands of linear  $\alpha$ -(1 $\rightarrow$ 4)-D-glucan unit chains and 4–5.5%  $\alpha$ -(1 $\rightarrow$ 6)-glucosidic branch points with average chain length of 18–25 anhydro-glucose units.<sup>10</sup> According to the cluster model proposed by Robin et al.<sup>11</sup> and modified by Hizukuri,<sup>12</sup> the amylopectin molecule con-

sists of three types of unit chains, referred to as A, B and C chains. Unit chains are arranged in clusters, where short A and B 1 chains are located within individual clusters, whereas long B 2 and B 3 chains connect 2 and 3 clusters.

In vitro bioassays with individual SBE isoforms purified from maize endosperm using amylose as a substrate<sup>13–15</sup> indicated that SBEI predominantly transferred less branched, longer chains with degree of polymerization (DP) greater than 10, whereas SBEII preferentially transferred highly branched, shorter glucan chains with DP 3–9. By introducing the *Ae* (*Sbe2b*) gene into a SBEIIb-defective *ae* mutant in rice, Tanaka et al.<sup>16</sup> found that SBEIIb activity positively correlated with the proportion of the short chains with DP  $\leq$  13 of amylopectin but negatively correlated with onset gelatinization temperature ( $T_o$ ) of endosperm starch in independent transgenic lines. Antisense suppression of multiple forms of SBEII in potato tuber starch also

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caused few short chains (DP 6–23) and more long chains (DP 23–60) of amylopectin.<sup>17</sup>

The mutation of the Waxy (*Wx*) gene results in the absence of functional granule-bound starch synthase, leading to a starch with entirely amylopectin and lacking amylose (*wx* mutant).<sup>18,19</sup> Satoh et al.<sup>20</sup> distinguished the structural differences between wild-type and *sbel* mutants in rice using APTS-capillary electrophoresis. They found that the proportions of amylopectin chains with  $DP \geq 37$  and  $12 \leq DP \leq 21$  decreased, and chains with  $DP \leq 10$  and  $24 \leq DP \leq 34$  increased in *sbel* rice endosperm in both wild-type and *wx* backgrounds. Based on the above findings, Satoh and co-workers proposed that *Ae* plays a distinct role in forming short A chains, whereas *Sbel* mainly relates to B chain formation.<sup>20–22</sup> However, in maize endosperm, no distinguishable starch structural changes were found within *sbela* mutant endosperms as compared to those of the wild-type,<sup>23</sup> regardless of *wx* status. However, significant structural alterations in endosperm amylopectin were observed between *ae wx* and *sbela ae wx*.<sup>24</sup> By introduction of *sbe* mutants into the *wx* background, the double or triple homozygous *sbe*- and *ae*-containing mutants contain amylopectin only, which is suitable for branching architecture analysis<sup>24</sup> without the interference of amylose molecules in starch and for revealing the relationship between amylopectin molecular structure and the physical behavior of amylopectin.

By analysis of the *sbela* and *ae* mutations in the *wx* background, Yao et al.<sup>24</sup> reported that, while no difference in amylopectin structure was observed in the *sbela* mutation alone when compared to W64A wild-type controls, the double homozygous *sbela ae* mutant exhibited increased branching compared to the *ae* mutant alone. No pleiotropic effects of the *sbela* and *ae* mutations on the expression of the SBEIIa, nor on the expression of any of the major soluble starch synthase isoforms or starch-debranching enzymes, were observed. A reciprocal inhibition between the *sbela* and *ae* genes was proposed to explain the structural changes. The present study was undertaken to further investigate the effects of *sbela* and *ae* mutations alone and in combina-

tion on kernel characteristics and on the morphology and physical behavior of maize endosperm starch granules.

## 2. Results and discussion

### 2.1. Kernel phenotype and starch accumulation in kernels

The kernels of *wx* and *sbela wx* types appear identical, with a yellowish, smooth surface. In contrast, *sbela ae wx* and *ae wx* kernels are both shrunken and brown in color, with *sbela ae wx* kernels being darker than *ae wx* kernels. The kernel weight, endosperm mass (expressed as a percentage of the total kernel weight), and starch content (the percentage of starch in endosperm) were significantly reduced in both *ae wx* and *sbela ae wx* types as compared to those of the *wx* and *sbela wx* type (Table 1), suggesting that these reductions are caused by the interaction of *sbela* and *ae* mutations and not by the *sbela* mutation alone. However, the kernel weight and the percentage of starch in the endosperm in the *sbela ae wx* mutant were not statistically different from those in the *ae wx* mutant.

### 2.2. Starch granule size and size distribution

The size of starch granules and size distributions were also measured for the various genotypes. As seen in Table 1 and Figure 1, respectively, starch granules isolated from the *wx* and *sbela wx* kernels had similar granule size distributions (generally smaller than 25  $\mu\text{m}$  in diameter) with mean diameters of about 11  $\mu\text{m}$  (Table 1). Granules from *ae wx* starch were much smaller, with a mean diameter of 5.9  $\mu\text{m}$ . Granules from *sbela ae wx* starch (7.3  $\mu\text{m}$ ) were significantly smaller than those from *wx* and *sbela* types, but larger than those from the *ae wx* genotype. This difference could be at least partly due to the smaller number of very small granules in *sbela ae wx* starch. Boyer et al.<sup>25</sup> previously reported an increased frequency of medium and small starch granules in *ae*-containing maize mutants.

**Table 1.** Physical properties of combinations of *sbela* and *ae* mutations in a *wx* background<sup>a</sup>

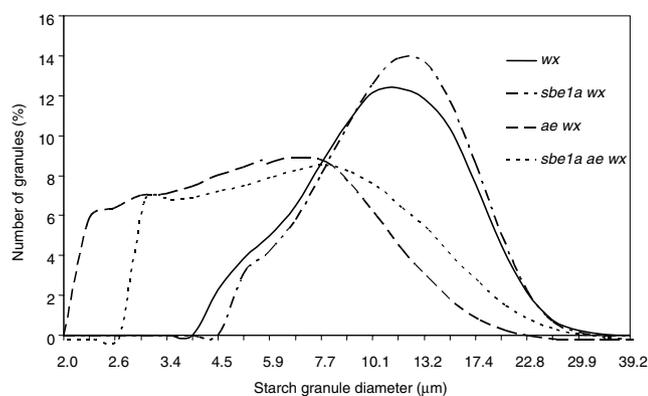
Genotype	Kernel weight (g)	Endosperm in kernel (%)	Starch in endosperm (%)	Granule size <sup>b</sup> ( $\mu\text{m}$ )	Thermal analysis of starch <sup>c</sup>			
					$T_o$ ( $^{\circ}\text{C}$ )	$T_p$ ( $^{\circ}\text{C}$ )	$T_c - T_o$ ( $^{\circ}\text{C}$ )	$\Delta H$ (J/g)
<i>wx</i>	0.200a <sup>d</sup>	81.8a	78.3a	10.8a	64.7a	71.2a	15.5a	17.3a
<i>sbela wx</i>	0.193a	79.8a	78.6a	11.1a	63.0b	71.1a	15.4a	18.1a
<i>ae wx</i>	0.124b	75.0b	64.5b	5.9b	62.8b	77.6b	36.1b	17.4a
<i>sbela ae wx</i>	0.118b	66.1c	65.0b	7.3c	60.5c	78.0b	34.8b	13.3b

<sup>a</sup> Analysis is based on dry-weight basis.

<sup>b</sup> Starch granule size is expressed as the number-average granule diameter.

<sup>c</sup> Thermal properties of starch are indicated as onset gelatinization temperature ( $T_o$ ), peak gelatinization temperature ( $T_p$ ), gelatinization temperature range ( $T_c - T_o$ ), and gelatinization enthalpy ( $\Delta H$ ).

<sup>d</sup> Means with the same letters (a–c) within the same columns are not significantly different at  $\alpha = 0.05$ .



**Figure 1.** Starch granule size distributions in *ae* and *sbela* mutant maize. Endosperm starch was purified, and approximately 40 mg was analyzed using a laser-scattering particle-size distribution analyzer. Size measurements in  $\mu\text{m}$  are presented as a function of the percentage of granules within a moving window of up to 40  $\mu\text{m}$ .

### 2.3. Microscopy of starch granules

Consistent with the size measurements, when observed by bright-field microscopy, the granule sizes of the *ae wx* and *sbela ae wx* genotypes appeared smaller than those of *wx* and *sbela wx* (Fig. 2). The granules in the *wx* and *sbela wx* genotypes were oval or angular in shape and more uniform in size, whereas some large granules in *ae wx* and *sbela ae wx* genotypes were oval, and most of them were irregular in shape (Figs. 2 and 3). When viewed under polarized light (Fig. 2, right-hand panels), all *wx* and *sbela wx* starch granules show birefringence in the form of the typical maltese crosses, indicating a symmetrical radial molecular orientation in the granules. However, many of the *ae wx* and *sbela ae wx* starch granules show a much weaker maltese cross (as indicated by arrows in Fig. 2), suggesting diminished radial order of the starch granules. Additionally, irregular granules with irregular maltese crosses were more frequently observed in *ae wx* than *sbela ae wx* starch granules, indicating that the overall granule organization was altered due to *sbela* and *ae* mutations. After staining with  $\text{I}_2/\text{KI}$ , the starch granules of the *wx* and *sbela wx* starch were light orange, but the granules of *ae wx* starch were orange-brown and those of *sbela ae wx* were mostly a lighter orange-brown (Fig. 3), indicating the diversity of iodine binding ability of amylopectin among starches due to the differences in amylopectin structure. Under polarized light, the orange color intensity of *sbela ae wx* granules was much weaker than that of *ae wx* granules (Fig. 3, right-hand panels). This result suggests that, in the presence of the *ae* gene, the *sbela* gene either influences the length of the linear regions in amylopectin structure or the accessibility of these linear regions when in granule form. Yao et al.<sup>24</sup> showed an increase in amylopectin branching in *sbela ae wx*

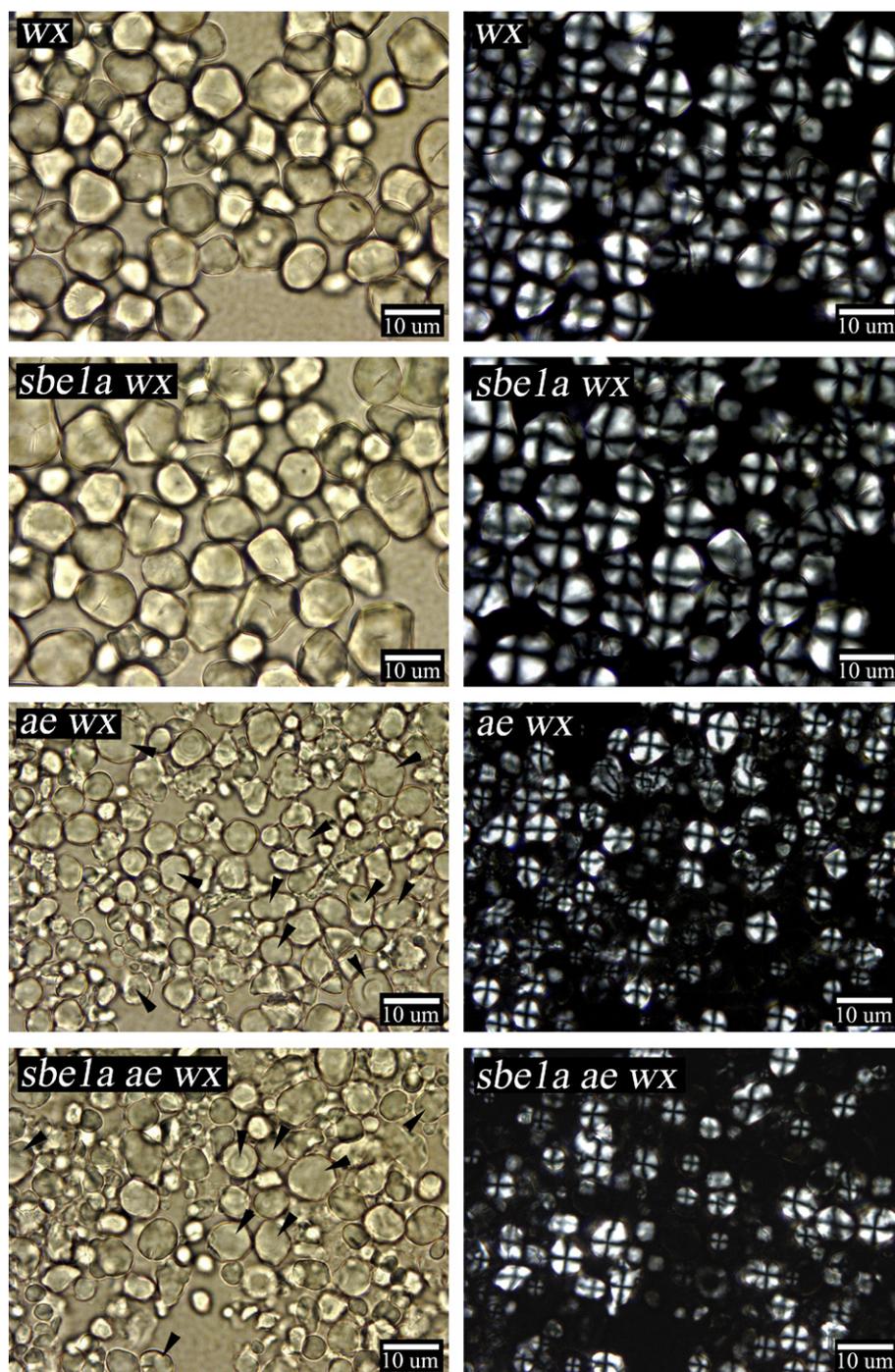
starch as compared to the *ae wx* starch. Thus, the observed granule morphological and microscopic differences might be attributed to amylopectin structural changes at the molecular level (such as branch chain length, relationship among branch points, and molecular size).

### 2.4. Microstructure

The microstructure of starch granules was viewed by DIC microscopy after partial acid hydrolysis. The micrographs of starch granules hydrolyzed by 2.2 N HCl for 12 h and 48 h are shown in Figure 4. Symmetrical ring structures were extended from the central region toward the edges in most starch granules of *wx* and *sbela wx*, but were evident only in the central region of granules of *sbela ae wx* and *ae wx* at 12 h hydrolysis (Fig. 4, left-hand panels). It appears that *ae wx* and *sbela ae wx* granules tend to have rings only toward the center of the granules, even at 48 h hydrolysis (Fig. 4, right-hand panels). There seems to be a fundamental difference in the nature of the *ae*-containing starches in the organization of the outer portion of the granules. Compared with *ae wx* starch granules, there was some suggestion of radial structure for some *sbela ae wx* granules.

### 2.5. Thermal properties

The thermal behaviors of the starches isolated from various genotypes were determined and are presented in Table 1 and Figure 5. The gelatinization temperature range of *wx* (15.5 °C) and *sbela wx* (15.4 °C) starches was narrower than for *ae wx* (36.1 °C) and *sbela ae wx* (34.8 °C) starches. The onset gelatinization temperature (60.5 °C) of *sbela ae wx* starch was significantly lower than for the other three starches (64.7 °C, 63.0 °C, 62.8 °C for *wx*, *sbela wx*, and *ae wx*, respectively), and the enthalpy (13.3 J/g) of *sbela ae wx* starch was smaller as well (Table 1 and Fig. 5). The structural diversity noted above may contribute to differences in thermal properties between *sbela ae wx* and *ae wx* starches. A lower onset temperature and smaller enthalpy would be consistent with shorter double-helical segments of external chains and a smaller portion of the amylopectin in the form of double helices, as suggested by shorter average branch-chain length and lower ratio of A and B 1 chains to B 2 and B 3 chains of amylopectin in *sbela ae wx* than in *ae wx* starch.<sup>24</sup> The greater gelatinization temperature range ( $T_c - T_o$ ) of the *ae wx* and *sbela ae wx* starches could indicate greater structural heterogeneity among crystallites in these two starches, or it could indicate greater heterogeneity in the population of granules. Greater heterogeneity of granule size is apparent from the light micrographs (Figs. 2 and 3).

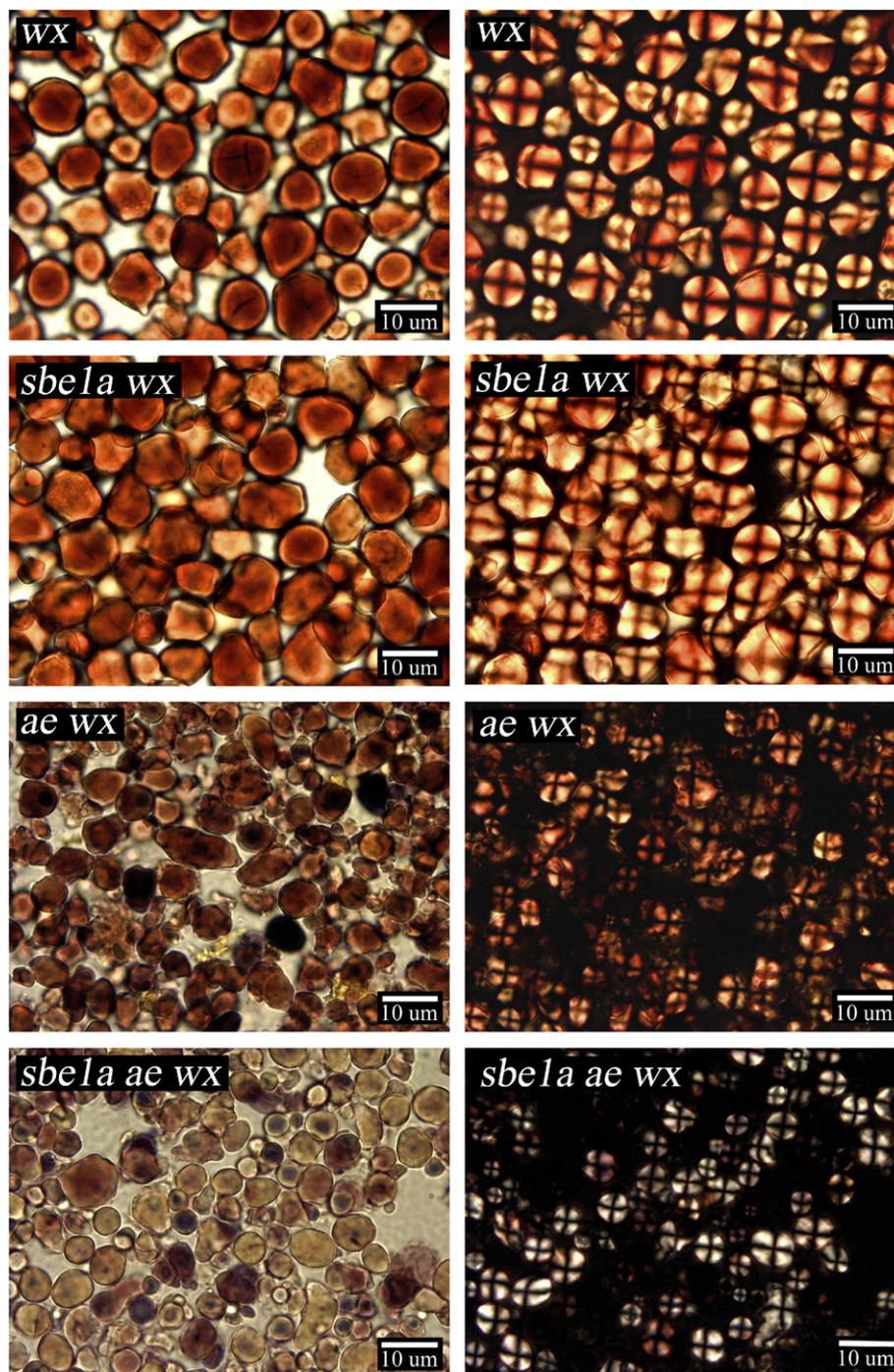


**Figure 2.** Bright-field (left) and polarized-light (right) micrographs of native starch granules for *wx*, *sbel1 wx*, *ae wx* and *sbel1 ae wx* mutant maize. For each genotype as indicated on top left of each panel, starch granules observed under bright-field light (left) were the same field as those under polarized light (right). Bars on the bottom right of each panel indicate 10  $\mu\text{m}$ , and magnifications are equivalent in all images. Arrows indicate granules with weaker maltese crosses as observed under polarized light (right).

## 2.6. X-ray diffraction studies

Figure 6 shows the X-ray diffraction patterns of the starches. The *wx* and *sbel1 wx* starches show typical A-type crystalline patterns, with strong peaks at 15.3°, 17.1°, and 23.3°, whereas *sbel1 ae wx* and *ae wx* types

show typical B-type crystalline patterns, with strong peaks at 5.6° and 17.2° and medium intensity peaks at 15°, 22.2°, and 24.0°. There is a major effect of the *ae* gene, but little or no effect of the *sbel1* gene, regardless of the presence of the *ae* gene. Small differences in extent of crystallinity would be difficult to observe.



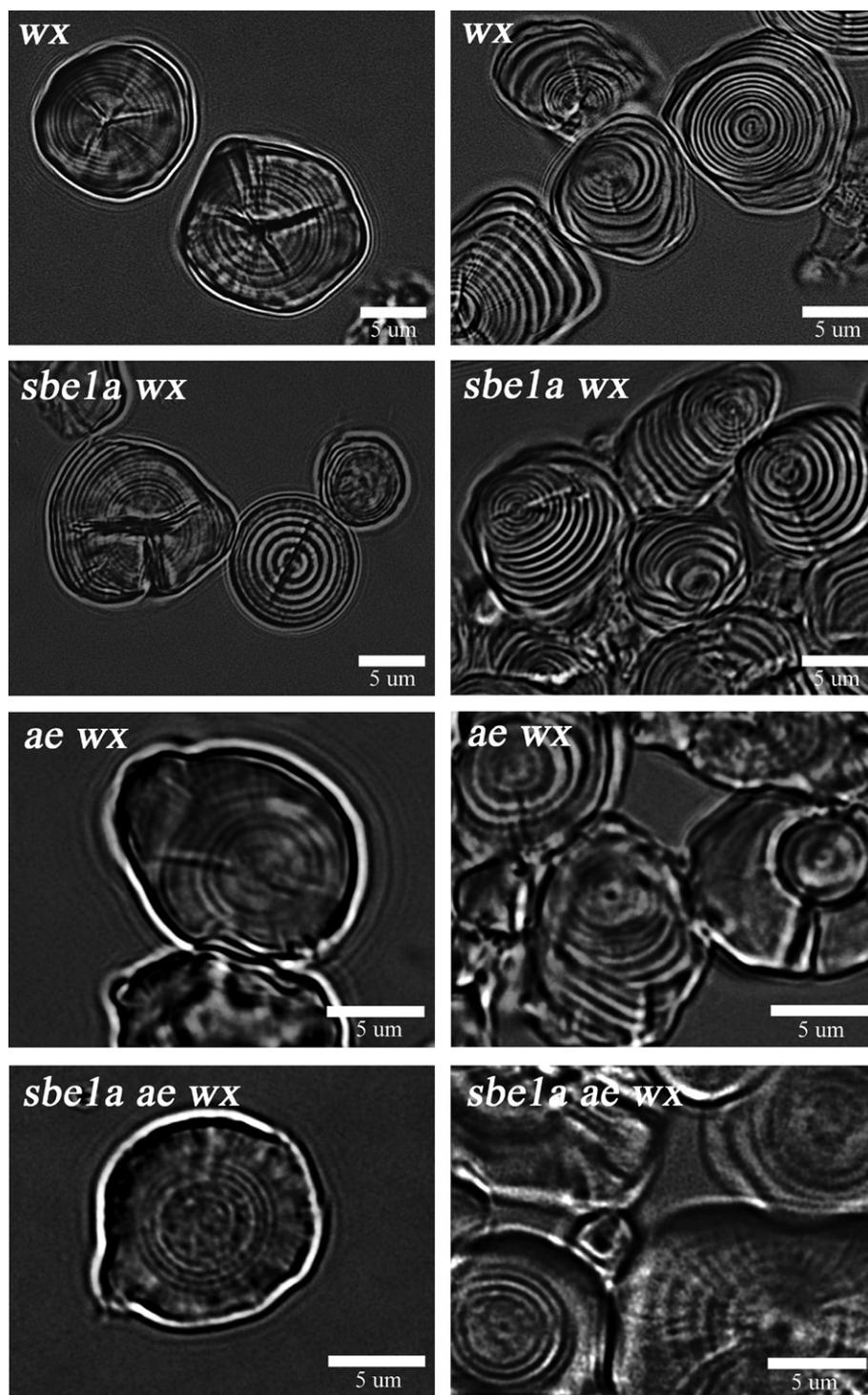
**Figure 3.** Bright-field (left) and polarized-light micrographs (right) with I<sub>2</sub>/KI staining of starch granules from the maize genotypes used in this study. The starch granule field in left panel was the same as in right panel for each genotype. Bars on the bottom right of each panel indicate 10 µm and magnifications are equivalent in all images.

## 2.7. Conclusions

The *sbela ae* and the *ae* genotypes differed at several different structure levels, such as in granule morphology, birefringence, growth rings, crystallinity, and molecular structure. However, we did not observe an effect of *sbela* in the absence of *ae*. It is possible that more pre-

cise physical and chemical analytical techniques would allow effects to be observed.

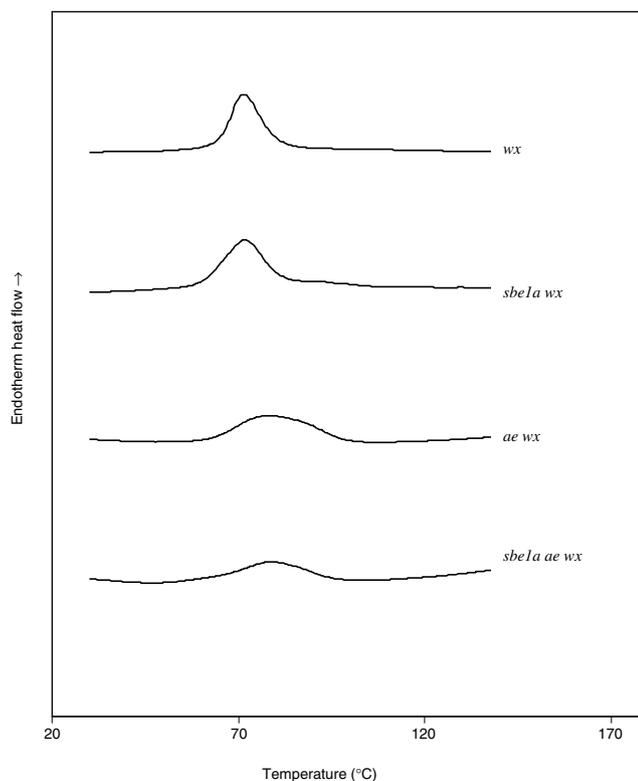
The *ae* mutation strongly affects the morphological and physical behavior of endosperm starches of *wx* mutant maize; for many observations, an additional effect of the *sbela* mutation is evident only in the presence of the *ae* mutation. This indicates that the



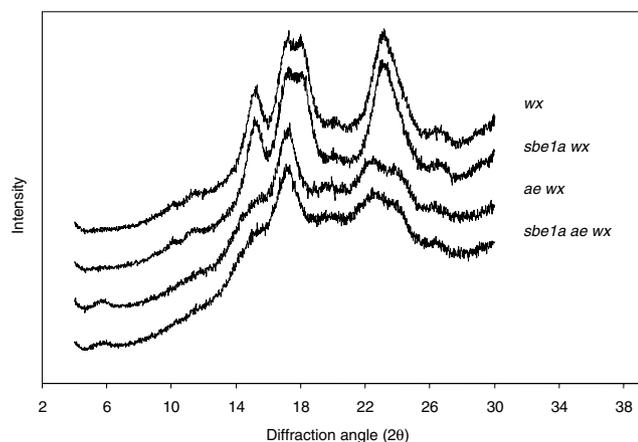
**Figure 4.** Laser differential interference contrast micrographs of starch granules after partial acid hydrolysis. Purified endosperm starch granules were hydrolyzed by 2.2 N HCl for 12 h (left) and 48 h (right). Bars on lower right of each panel indicate 5 µm.

SBEIIb protein (encoded by the *Ae* gene) plays a dominant role in determination of the starch-branching pattern in maize, but also reveals that the SBEI isoform plays a more subtle role in determining starch structure. We suggest that these differences in structure may affect

the nature of the starch granules and the kernel composition. This outcome is consistent with the effect of these mutations on amylopectin structure, as we have previously reported.<sup>24</sup> The novel effects on starch properties and structure we have observed in the double mutant



**Figure 5.** DSC thermograms for starches isolated from the various genotypes used in this study. Samples at 30% solids (w/w, db) were scanned from 5 °C to 180 °C at 10 °C/min.



**Figure 6.** X-ray diffraction patterns of *wx*, *sbel1 wx*, *ae wx*, and *sbel1 ae wx* mutant maize starches. Samples were scanned from 4° to 30° 2θ at step size of 0.02° with a counting time of 2 s.

*sbel1 ae* suggests that either interactions between the two SBE isoforms might exist or SBE isoforms possibly have some overlapping substrate specificity, and that the presence of SBEIIb can compensate for the lack of SBEI. Further studies are now underway to test this hypothesis.

### 3. Experimental

#### 3.1. Germplasm and plant growth

Homozygous mutants of *wx*, *sbel1 wx*, *ae wx*, and *sbel1 ae wx* in the W64A inbred line were field grown in the summer of 2004 at Pennsylvania State University Agricultural Experiment Station. Starches were from the same genetic material as that described by Yao et al.<sup>24</sup> Ears were harvested at physiological maturity (approximately 50 days after pollination), oven dried at 35 °C for 3 days and stored at room temperature. Kernels pooled from two or three ears in each genotype were used for endosperm starch analysis.

#### 3.2. Analysis of kernel starch content

Two kernel samples (5 g each collected from individual ears) of each genotype were soaked in a steeping solution (0.02 M NaOAc, 0.01 M HgCl<sub>2</sub>, pH 6.5) at 40 °C for 48 h. After rinsing with deionized water, the pericarp and embryo of kernels were removed with a scalpel. The endosperm was ground with a small amount of 80% EtOH for 5 min using a mortar and pestle. The suspension of endosperm was air dried in a fume hood for 24 h and ground again for moisture and starch analyses. Moisture content was measured by AACC Method 44-15A.<sup>26</sup> Starch content was determined using a total starch analysis assay kit (Megazyme International Ltd., Ireland) and expressed on a dry basis. Analyses were in triplicate.

#### 3.3. Starch isolation

Two kernel samples (25 g each) from each genotype were used for starch isolation according to the method of Boyer et al.<sup>25</sup> with minor modification as described by Yao et al.<sup>27</sup> The kernels were treated in a steeping solution and prepared as described above. The endosperm was homogenized in a Waring blender with 100 mL of 0.02 M NaOAc for 3 min and then passed through 105-μm mesh nylon bolting cloth, and rinsed with 50 mL 0.02 M NaOAc. This step was repeated twice for maximum release of starch granules from endosperm cells. The purification of starch granules in suspension followed the procedure of Boyer et al.<sup>25</sup>

#### 3.4. Granule size and size distribution

Granule size and size distribution of starches from each genotype were determined in duplicate using a Horiba laser-scattering particle-size distribution analyzer LA-920 (Malvern Instruments Inc., UK). Approximately 40 mg of starch was transferred into distilled water in the dispersion tank of the instrument with circulation

via ultrasound for 2 min. Granule size was expressed as number-average diameter automatically calculated from instrument software.

### 3.5. Bright-field and polarized-light microscopy of starch granules

Bright-field and polarized-light microscopy of starch granules were carried out using a light microscope (BX50, Olympus, Melville, NY) equipped with digital camera (GATAN, Bioscan, Warrendale, PA). Samples were prepared and examined with and without iodine staining (0.4% I<sub>2</sub>/0.6% KI solution) according to the procedure of Evans et al.<sup>28</sup>

### 3.6. Differential interference contrast microscopy

Differential interference contrast (DIC) microscopy of partial acid-hydrolyzed starches, treated at room temperature with 2.2 N HCl for 12 h and 48 h, was performed following the method of Li et al.<sup>29</sup>

### 3.7. Differential scanning calorimetry of purified starch

Thermal analysis was performed using a differential scanning calorimeter (DSC-7, Perkin–Elmer Corp., Norwalk, CT) as described by Klucinec and Thompson.<sup>30</sup> Indium was used as a calibration standard, and a sealed, empty stainless steel pan was used as reference. Starch samples (approximately 17 mg) were weighed into a stainless steel pan (Perkin–Elmer), brought to 30% solids (w/w, db) by adding deionized water, sealed, and stored at room temperature for 24 h. Samples were heated from 5 °C to 180 °C at 10 °C/min. Samples were analyzed in triplicate. Gelatinization temperature parameters (onset,  $T_o$ ; peak,  $T_p$ ; conclusion,  $T_c$ ; temperature range,  $T_c - T_o$ ) and endotherm enthalpy ( $\Delta H$ ) were calculated using thermal analysis software (7 Series, Perkin–Elmer).

### 3.8. X-ray diffraction studies of purified starch

X-ray diffraction patterns of purified starches from the mutant maize were examined using a Philips X-ray diffractometer (X'Pert-MRD, Philips Electronic, NV) operated at 45 mA and 40 kV. Samples containing about 10% moisture were scanned from 4° to 30° 2 $\theta$  at a step size of 0.02° with a counting time of 2 s. Each sample was analyzed in duplicate.

### 3.9. Statistical analyses

Data reported are means of at least duplicate determinations. Analysis of variance (ANOVA) and least significant difference (LSD) were performed by SAS System

Version 9.1.3 (SAS Institute, Cary, NC). Level of significance used was at  $\alpha = 0.05$ .

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