Modification of starch composition, structure and properties through editing of TaSBEIIa in both winter and spring wheat varieties by CRISPR/Cas9

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Introduction
Diet-related noninfectious chronic diseases, such as diabetes, coronary heart disease, and certain colon and rectum cancers, are major causes of morbidity and mortality in both developed and developing countries (Chen et al., 2012; Regina et al., 2006). It is estimated that about 600 million people may suffer from diabetes in the year of 2035 (Unwin et al., 2013). Resistant starch (RS), as a major component of dietary health fibre, refers to any starch or starch products that are not digested and absorbed in the stomach or small intestine, resulting in decreased blood sugar level after human consumption (Asp, 1992). Cereals high in RS have the potential to lower the risk of those serious noninfectious diseases (Regina et al., 2006). A cereal grain with high-amylose content (AC) is the source of RS (Jiang et al., 2010). A positive relationship between RS and AC has been well established (Lee et al., 2013; Morell et al., 2003; Rahman et al., 2007; Topping et al., 2010; Yamamori et al., 2006; Yang et al., 2006). Besides, modified amylopectin (AP) structure with longer amylopectin chain lengths also contributes to RS (Regina et al., 2012). Although functional properties of a high RS diet are gaining acceptance as a desirable diet for some consumers, cereal crops that are high in RS are not widely available (Zhu et al., 2012). Thus, there is an increasing need to develop cereal crops high in RS to meet rapidly growing challenges in nutrition for public health at population level (Chen et al., 2012; Li and Gilbert, 2016; Regina et al., 2006).

Common wheat (Triticum aestivum L., 2n = 6x = 42, AABBDD) is a major staple crop consumed by more than 30% of the world population. It is the main source of cereal-based processed products, such as bread, cookies, pasta and noodles. Wheat consumption is increasing worldwide along with increasing affluence. In general, starch contains two major glucose polymers, amylose and amylopectin, which differ in the degree of polymerization (DP) of glucan chains and in the frequency of branches. In wheat endosperm, starch consists of approximately 70-80% amylopectin and 20-30% amylose. Modification of the starch composition of wheat by increasing its RS content presents...
an opportunity for a potentially large-scale improvement in public health. Studies over past decades have established that down-regulation of the two different isoforms of starch-branching enzyme (SBE) II (TaSBEIIa and TaSBEIIb) through plant-mediated RNA interference (RNAi) increased wheat amylose content. Suppression of TaSBEIIb expression alone had no effect on amylose content; however, suppression of both TaSBEIIa and TaSBEIIb expression resulted in starch containing >70% amylose (Regina et al., 2006), implying TaSBEIIa plays a more profound role in determining the starch property of wheat. Traditional mutagenesis methods, such as TILLING, have been implemented in generation of TaSBEII mutants with increased AC and RS contents in wheat (Hazard et al., 2012; Regina et al., 2015; Slade et al., 2012). However, TILLING generally produces many random mutations in wheat genome and is labour-intensive and time-consuming to identify the desired mutations. In addition, RNAi construct may silence both TaSBEIIa and TaSBEIIb simultaneously because of the high similarity of these two genes (Regina et al., 2006). To date, the roles of TaSBEIIa homologs and their effects on starch composition, structure, physicochemical and nutritional properties as well as end-use quality in a sole genetic background remains to be elucidated in order to provide defined information on development of high-amyllose wheat and end-use of these products.

CRISPR/Cas9, as a simple, versatile, robust and cost-effective system for genome manipulation, has recently become the widely used tool for generation of sequence-specific targeted mutagenesis for both functional genomics and crop improvement (Ma et al., 2015). The hexaploid nature of common wheat makes generation of targeted mutagenesis in genes, which usually have three homologs located in each of the three subgenomes, very challenging. So far, only a few ergonomically important traits in wheat have been successfully improved through CRISPR/Cas9 such as increased powdery mildew resistance (Wang et al., 2014), grain size and weight (Wang et al., 2019; Wang et al., 2018; Zhang et al., 2019; Zhang et al., 2018), protein content (Zhang et al., 2018), decreased gliadin content (Sánchez-León et al., 2018), haploid induction (Liu et al., 2020), male sterility (Okada et al., 2019) and improved tolerance to pre-harvest sprouting (Abe et al., 2019). To date, increasing AC and RS contents in wheat through manipulating key genes involved in starch biosynthesis by genome editing has not been documented yet.

Increasing RS in wheat will have a profound impact on global population health. We envisioned that manipulating TaSBEIIa genes through genome editing could be an alternative way to modify the starch composition and structure of wheat to increase its AC and RS contents for human health benefits. Here, we report the creation of transgene-free high-amylose wheat plants in both winter wheat cv ZM and spring wheat cv Bobwhite through CRISPR/Cas9-mediated targeted mutagenesis of TaSBEIIa. We successfully generated transgene-free high AC and RS wheat through genome editing for the first time. We further dissected the roles of three homeologs of TaSBEIIa in starch composition, amylose content, fine structure of amylopectin, as well as physicochemical and nutritional properties of starch in a sole genetic background. Moreover, we evaluated the effects of different TaSBEIIa mutants on end-use qualities including bread- and biscuit-baking qualities. Our results provide fundamental information for improving RS content in wheat as well as other cereal crops for global population health benefits.

**Results**

CRISPR/Cas9-mediated targeted mutagenesis of TaSBEIIa in wheat

We designed two guide RNAs (gRNAs) for targeting the second exon of TaSBEIIa-A (TaSBEIIa homolog in A subgenome), TaSBEIIa-B (TaSBEIIa homolog in B subgenome) and TaSBEIIa-D (TaSBEIIa homolog in D subgenome), respectively, based on the conserved sequences among these three homeologs of hexaploid wheat (GenBank Accession No. HE591389.1, FM865435.1 and AF338431.1) and the sequences from our donor materials, a modern winter wheat variety cv Zhangmai 7698 (ZM) and a model spring wheat cv Bobwhite (Figure 1a). ZM is a hard white wheat variety with high gluten content and good bread-baking quality and widely grown with over 6.67 million hectares each year in central part of China, whereas Bobwhite is a model spring wheat variety. The gRNA1 contained a restriction site Ddel that was used for screening mutants using PCR-based restriction enzyme (PCR/RE) digestion assay (Figure 1a). We placed each gRNA cassette driven by a TaU6 promoter into the binary vector pCXUN-Cas9, respectively (Figure 1b). We then transformed the CRISPR/Cas9 vectors into immature embryos of ZM and Bobwhite by particle bombardment, respectively. We recovered the regenerated plants after two rounds of hygromycin selection on induction media and one round of regeneration on regeneration media. For ZM, we identified five plants with edited alleles from 13 transgenic plants for gRNA1 (Figure 1c, e) and two edited plants from three transgenic plants for gRNA2 (Figure 1d, f). For Bobwhite, of three transgenic lines obtained, we detected one edited line with chimeric mutations including a wild type (WT), a deletion of 15 bp and a deletion of 1 bp in TaSBEIIa-A, heterozygous mutations in TaSBEIIa-B (WT, an insertion of 11 bp) and TaSBEIIa-D (WT, a deletion of 14 bp) (data not shown).

We further investigated if off-target effects occurred in these mutant lines. Based on the predictions of the WheatCrispr (https://crispr.bioinfo.nrc.ca/WheatCrispr/), we identified several potential off-target sites of these two gRNAs. We then used

![Figure 1](https://example.com/figure1.png)  
**Figure 1** Targeted mutagenesis of TaSBEIIa and immunodetection of TaSBEIIa in different mutant lines. (a) The structure of TaSBEIIa. The SNPs among TaSBEIIa homologs are shown as asterisks. The detail underneath shows partial sequences of TaSBEIIa and its homologs, as well as the target sites for the two gRNAs (gRNA1 and gRNA2) targeting exon 2 of TaSBEIIa on the long arm of chromosome 2. Target sites 1 and 2 are underlined, respectively. PAM sites (5'-NGG-3') are highlighted in red. Dde I restriction enzyme site is highlighted in light blue. (b) Schematic presentation of the T-DNA structure in a CRISPR/Cas9 construct. (c) and (d) detection of mutations in TaSBEIIa-gRNA1 and TaSBEIIa-gRNA2 via PCR/RE assay in T0 generation. The PCR products of TaSBEIIa-gRNA1 and TaSBEIIa-gRNA2 mutant lines are resistant to Dde I and T7 Endonuclease I (T7EI) digestion, respectively. (e) and (f) sequencing results of the TaSBEIIa-gRNA1 and TaSBEIIa-gRNA2 mutant lines, the PAM motifs are highlighted in red, target sequences are underlined, insertions are highlighted in purple, dashes indicate deletions. (g) Immunodetection of TaSBEIIa extracted from 15 dpa endosperms of ZM sbella mutant lines. Arrows indicate the expected three TaSBEIIa isoforms from three different subgenomes, respectively. M, DL2000 DNA ladder; WT, wild type.
Gene editing of TaSBEIIa in wheat by CRISPR/Cas9

(a) TGCA*TTTGGGCGCCGTCCCTGGTGTCGGGTAGG

(b) TaSBEIIa-A (2AL)
TaSBEIIa-B (2BL)
TaSBEIIa-C (2DL)

(c) gRNA1 target
TaSBEIIa-A
gRNA2 target

(d) SBEIIa-A
SBEIIa-B
SBEIIa-C

(e) WT-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
WT-B
TGCA*TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B017-1-A
TGCA*TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B017-1-B
TGCA*TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B017-1-D
TGCA*TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B13-C14-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B13-C14-B
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B13-C14-D
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B62-16-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B86-38-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B86-38-B
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B86-38-D
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B49-S70-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B49-S70-B
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B49-S70-D
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG

(f) WT-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
WT-B
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
WT-D
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B028-8-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B041-S103-A
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B041-S103-B
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG
B041-S103-D
TGCA**TTTGGGCGCCGTCCCTGGTGTCGGGTAGG

(g) A1, B1, C1, D1, E1, F1, G1

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Inheritance and stability of the mutations and generation of transgene-free wheat TaSBEIIa mutant lines

To investigate whether the mutants generated through CRISPR/Cas9-mediated editing could be transmitted to the next generation, we self-pollinated ZM T0 mutant plants, and further genotyped individual T1 progenies using genomic-specific primers as listed in Table S1. We randomly selected 5 to 33 T1 progenies derived from each T0 plant for further genotyping analysis (Table 1). All of the mutations detected in ZM T0 plants were transmitted to the T1 generation without occurrence of new mutations. For mutations that were homozygous in the T0 generation, the transmission rates were 100%, and those that were heterozygous in the T0 generation segregated in a Mendelian fashion (homozygous/heterozygous/WT = 1:2:1) in the T1 generation (Table 1). For mutations that were heterozygous in T0, the transmission rates were 100%, and those that were homozygous in the T0 generation, the transmission rates were 100%, and those mutations that were heterozygous in the T0 generation segregated in a Mendelian fashion (homozygous/heterozygous/WT = 1:2:1) in the T1 generation (Table 1). For mutations that were heterozygous in T0, the transmission rates were 100%, and those mutations that were homozygous in the T0 generation segregated in a Mendelian fashion (homozygous/heterozygous/WT = 1:2:1) in the T1 generation (Table 1).

Table 1 Transmission and segregation of CRISPR/Cas9-mediated target mutations and transgenes from T0 to T1 generation

| Plant ID | Genotype of TaSBEIIa homoelog | Mutation detected (bp) | No. of tested plants | WT plants | Heterozygous | Homozygous | Mutation transmission(%) | Cas9/gRNA/ hptII |
|----------|--------------------------------|------------------------|----------------------|-----------|-------------|------------|-------------------------|-----------------|
| B017-1   | aa                            | −15, −42               | 16                   | 0 (AA)    | 0 (Aa)      | 16 (aa)    | 100                     | 16+: 0–         |
|          | bb                            | −5/+3                  | 0 (BB)  0 (Bb)       | 16 (bb)   | 100         |
|          | Dd                            | −40/wt                 | 0 (DD)  3 (Dd)       | 13 (dd)   | 100         |
| B13-14   | aa                            | −2, −6                 | 5                    | 0 (AA)    | 0 (Aa)      | 5 (aa)     | 100                     | 3+: 2–          |
|          | bb                            | −7/−20                 | 0 (BB)  0 (Bb)       | 5 (bb)    | 100         |
|          | Dd                            | +1/wt                  | 0 (DD)  1 (Dd)       | 4 (dd)    | 100         |
| B62-16   | AA                            | wt                     | 19                   | 19 (AA)   | 0 (Aa)      | 0 (aa)     | 13+: 6–                 | 73: 7:4         |
|          | BB                            | wt                     | 19 (BB)  0 (Bb)      | 0 (bb)    | 100         |
|          | Dd                            | −12/wt                 | 5 (DD)  11 (Dd)      | 3 (dd)    | 73.7        |
| B86-68   | aa                            | −6/−8                  | 9                    | 0 (AA)    | 0 (Aa)      | 9 (aa)     | 100                     | 5+: 4–          |
|          | bb                            | −1                     | 0 (BB)  0 (Bb)       | 9 (bb)    | 100         |
|          | Dd                            | −3/wt                  | 0 (DD)  5 (Dd)       | 3 (dd)    | 88.9        |
|          |                                |                        |                      |           |             |            |                        |                 |
| B49-570  | Aa                            | +1/wt                  | 19                   | 2 (AA)    | 17 (aa)     | 89.5       | 15+: 4–                 | 68.4: 4:1:3:6   |
|          | Bb                            | −6/wt                  | 6 (BB)  11 (Bb)      | 2 (bb)    | 84.7        |
|          | Dd                            | −1, −14, wt            | 1 (DD)  2 (Dd)       | 16 (dd)   | 94.7        |
| B028-8   | AA                            | wt                     | 12                   | 12 (AA)   | 0 (Aa)      | 0 (aa)     | 7+: 5–                  | 100             |
|          | BB                            | wt                     | 12 (BB)  0 (Bb)      | 0 (bb)    | 100         |
|          | dd                            | −7                     | 0 (DD)  0 (Dd)       | 12 (dd)   | 100         |
| B041-5103| aa                            | −4, −13                | 33                   | 0 (AA)    | 33 (aa)     | 100        | 25+: 8–                 | 100             |
|          | bb                            | −4, −10                | 0 (BB)  0 (Bb)       | 33 (bb)   | 100         |
|          | dd                            | −41                    | 0 (DD)  0 (Dd)       | 33 (dd)   | 100         |

*indicates deletion of the indicated number of nucleotides; ‘+’ indicates insertion of indicated number of nucleotides; ‘−’−’ indicates simultaneous deletion and insertion of the indicated number of nucleotide at the same site; ‘−’−’ indicates multiple types of deletions occurring in different mutation events at the same target site.

Table 1. Transmission and segregation of CRISPR/Cas9-mediated target mutations and transgenes from T0 to T1 generation

aBased on the number of plants carrying the observed mutation over the total number of plants tested.

bSegregation of the heterozygous lines conforms to a Mendelian 1:2:1 ratio according to the $\chi^2$ test ($P > 0.5$). For transgene analysis, ‘+’ represents that Cas9 gRNA/cassette and hptII were present in these mutant lines, we performed PCR amplification using the primer sets designed to specifically amplify Cas9 gRNA cassette and hptII sequences, respectively (Figure 1 and Table 1). We successfully recovered Cas9 gRNA cassette and hptII transgene-free plants from the T1 progenies of seven T0 mutants (Figure S1).

Following segregation, for ZM, we obtained two transgene-free T1 lines AAbbDD (B86-8-47) and aabBD (B86-8-48) derived from T0 line B86-8, one T1 line AABbd (B028-8-230) from T0 line B028-8, one T1 line AABbd (B13-14-56) from T0 line B13-14, one T1 line aabBdd (B49-70-107) from T0 line B49-70 and one T1 line aabbbd (B41-103-48) from T0 line B41-103. The a, b and d in these genotypes correspond to the null mutations in A, B and D genome-specific PCR and Sanger sequencing to determine the potential off-target effect. As shown in Table S2, no mutations were detected at the tested putative off-target loci in wheat genome. However, the segregation patterns of lines B86-E8 and B49-570 did not fit a Mendelian ratio, probably due to aberrant gamete or seed formation (Table 1). For one chimeric line we obtained in Bobwhite, following segregation in T2 generation, we got two homozygous lines with one line having a WT TaSBEIIa-A, a 11-bp insertion in TaSBEIIa-B, and a 14-bp deletion in TaSBEIIa-D, and another triple-null line having a 1-bp deletion in TaSBEIIa-A, a 11-bp insertion in TaSBEIIa-B, and a 14-bp deletion in TaSBEIIa-D, respectively (Table S3).
homoeologs of TaSBEIIa, respectively, in which the detected mutations caused frame shifts in the encoding sequences. The genotypes of these mutant lines were listed in Table S3. The generation of different transgene-free mutant lines of ZM and Bobwhite enabled us to evaluate the effects of single, double or triple-null alleles of three homoeologs of TaSBEIIa from A, B and D subgenomes, respectively, on starch composition and properties in a sole genetic background.

Identification of TaSBEIIa isoforms
To further confirm the presence or absence of different isoforms of TaSBEIIa in the obtained different mutant lines harbouring different null alleles, the 15 days post-anthesis (DPA) endosperms from ZM WT control (AABBDD), and six transgene-free T2 homozygous mutant lines with genotypes of AAbbDD, AAbbDd, aabbDD, aaBBdd, AAbbdd and aabbdd, were analysed for TaSBEIIa protein expression using an affinity gel electrophoresis system containing a carbohydrate substrate, β-limit dextrin, for separation of TaSBEIIa isoforms, followed by immunoblotting with anti-TaSBEIIa serum. This separation system could detect isoforms of three homoeologs from three wheat subgenomes and allow for the simultaneous scoring for the presence or absence of the TaSBEIIa protein from each subgenome, respectively (Regina et al., 2015). As expected, according to the band pattern in the 1-D affinity gel, the WT control (AABBDD) had three bands, the AAbbdd line had only one band, whereas the aabbdd had no bands at all (Figure 1g). However, the aabbDD double mutant line showed similar two bands as those of aabbDD, whereas AAbbDD and AAbbDd mutant lines showed similar three bands as these of WT control. This phenomenon is probably due to the fact that both TaSBEIIa-B and TaSBEIIa-D may each produce two distinct proteins with similar size at mature stage of seed development due to missplicing (http://plants.ensembl.org/Triticum_aestivum/Gene/Summary?db=core;g=TraesCS2B02G309500 or TraesCS2D02G290800).

Field performance of different ZM and Bobwhite TasbeIIa mutant lines
Field performances of these mutant lines were evaluated under the same conditions. No visible phenotypic differences, for example plant height and tiller number, were observed between ZM mutant lines and WT plants (Figure 2a). However, decreased plant height and tiller number were observed in Bobwhite double- and triple-null lines in comparison with WT control (Figure 2b). Furthermore, the length and width of grains from both ZM and Bobwhite TasbeIIa mutant lines were slightly lower than those of WT plants, resulting in decreased 1,000-grain weight across different mutant lines, especially the aabbdd triple-null lines (Table S4, Figure 2c, d). We also found that the number of spikelet and grain number per main spike from both ZM and Bobwhite mutant lines was slightly lower than those of WT plants (Table S4).

The dynamic accumulation patterns and morphologies of starch granules in the endosperms of mutant lines
The dynamic accumulation patterns of starch granules in the grain endosperm during grain development were further examined by light microscopy (Leica DMS5000B, Leica Microsystems). The morphological features of starch granules in developing grains at different stages, namely 6, 12, 18 and 24 days post-anthesis (DPA) from WT plants, partial-null and triple-null mutant lines were as shown in Figure 3a, b. The starch granules from ZM and Bobwhite WT plants grew rapidly from 6 to 24 DPA and yielded granules with diameter more than 10 µm which were classified as A-type granules (>10 µm in diameter) at 12 DPA (Figure 3a, b). Compared with the starch granules of WT plants, the shapes of starch granules from different mutant lines showed irregular morphologies with their diameters being much smaller, ranging from 1 to 10 µm. In particular, among these mutant lines, the aabbdd triple-null lines had the most significant changes and its starch granules appeared slenderest and most distorted (Figure 3a, b). It also appeared that knock out of TaSBEIIa-D may have more sound effect on sizes and morphologies of starch granules than that of TaSBEIIa-B (Figure 3a). We also noticed that AABBdd and aabbdd genotypes had more profound effects on starch granules in Bobwhite than in ZM (Figure 3a, b), indicating that the effects of null alleles of TaSBEIIa homoeologs on starch granules are affected by wheat genetic backgrounds.

Furthermore, scanning electron microscopy was used to determine morphological changes of the starch granules in mature endosperms of these mutant lines. As indicated in Figure 3c, d, the starch granules of WT plants showed large A-type (>10 µm in diameter), medium B-type (3–10 µm in diameter) and smaller C-type (<3 µm in diameter) granules that were smooth, spherical to ellipsoidal in shape, typical of a WT plants wheat starch, whereas starches from all mutant lines had distorted granules with varying intensities compared to these of WT plants. In particular, changes in starch granule morphology were more pronounced in aabbdd triple-null lines than those in single or double mutant lines (Figure 3c, d). Especially, the starch granules in aabbdd endosperms were highly irregular in shape, and a large proportion of A-type granules appeared to be sickle-shaped, whereas the endosperm starch granules from other mutant lines had relatively smooth spherical-to-ellipsoidal A-, B- and C-type granules (Figure 3c, d). It is worth noting that starch granules in the endosperm of Bobwhite aabbdd triple-null line possessed more profound changes with severely distorted granules in comparison to ZM aabbdd triple-null line (Figure 3c, d).

Moreover, size distribution analyses of the starch granules indicated that there were two peaks in the curve of the starch granule volume percentage vs the granule diameter as shown in Figure 3e, f. The percentage of A-type and B-type starch granules increased in ZM and Bobwhite aabbdd triple-null lines in comparison to WT plants, whereas C-type starch granules (<3 µm) decreased correspondingly (Figure 3e, f, Table S5). These results were in consistent with the morphological changes of the starch granules in mature endosperms (Figure 3c, d). As for the diameter of starch granules, both cultivars showed similar trend (Figure 3e, f, Table S5). Although the peak value of A-type starch granules in aabbdd triple lines was higher in WT control, which indicated a higher percentage of A-type granules, the curve of volume percentage vs granule diameter of the aabbdd triple lines shifted to the left in reference to the WT control (Figure 3e, f). As a result, the mean diameters of A-type decreased significantly in aabbdd triple-null lines (Table S5).

Impact of sbella mutants on starch composition and structures
The total starch contents were measured as a percentage of wholemeal flour (WF) and fine flour (FF), respectively. Compared to WT control, the total starch contents decreased slightly in the wholemeal and fine flours of aabbdd triple-null lines (Table 2). In contrast, the amylose contents of fine flours increased significantly in ZM aabbdd mutant line (45.4% vs 23.4%) and Bobwhite
mutant lines (47.1% vs 25.3%) in comparison with those of WT plants controls (Student’s t-test, **P < 0.01) (Table 2). Similarly, the amylose content of wholemeal flour increased significantly in ZM aabbdd mutant line (38.0% vs 22.6%) and Bobwhite mutant lines (38.7% vs 22.8%) compared with those of WT controls (Student’s t-test, **P < 0.01) (Table 2). When calculated as a percentage of total starch, the amylose contents of ZM triple-null line were 65.4% and 62.9% in fine flour and wholemeal flour, whereas these of Bobwhite were 69.7% and 61.8%, respectively (Table 2). The ratios of amylose/amyllopectin (AC/AP) in

wholemeal and fine flour also increased significantly in ZM and Bobwhite aabbdd triple-null lines (Student’s t-test, **P < 0.01), respectively (Table 2). Moreover, analyses of the molecular size distribution of debranched starches demonstrated that all mutant lines displayed altered chain length distributions (CLDs) of debranched starches. Especially, the ZM aabbdd triple-null line showed significantly increased proportion of shorter chain amyllopectin with degree of polymerization (DP) of 6–8, decreased proportion of DP 9–17 and an increased proportion of larger chains of >18 DP in comparison to the WT control (Figure 4a, c).
While the ratio of DP 7 short chains increased to 0.5%, that of DP 12 short chains decreased 2.0% in ZM aabbdd triple-null lines (Figure 4c). For Bobwhite, the aabbdd triple-null lines showed significantly increased proportion of shorter chain amylopectin with DP 6-8, decreased proportion of DP 9-13 and an increased proportion of larger chains of DP 15-24 in comparison to WT control (Figure 4b, d). While the ratio of DP 7 short chains increased to 1.0%, that of DP 12 short chains decreased 2.5% in Bobwhite aabbdd triple-null lines (Figure 4d). Different profiles of DP between ZM and Bobwhite triple-null lines further suggest the effects of different genetic backgrounds of winter and spring wheat on amylopectin structures.
Table 2 Starch compositions of different ZM and Bobwhite mutant lines

| Properties   | AA8BDD (WT plants) | AAbbdd (898-847) | AABbbd (828-830) | aibbDD (886-848) | aibbDD (813-1456) | AAbbDD (849-70107) | aibbbd (841-10348) | AA8BDD (WT plants) | AAbbdd (C2-53-1-C5-2-17) | aibbbd (C2-53-1-C5-2-18) |
|--------------|---------------------|------------------|------------------|-------------------|------------------|-------------------|-------------------|-------------------|---------------------|---------------------|
| TS (% of WF) | 70.1 ± 0.7          | 69.6 ± 0.4       | 69.2 ± 0.3       | 70.7 ± 1.6        | 67.9 ± 0.6*      | 70.8 ± 0.8        | 60.4 ± 0.8**      | 70.9 ± 1.2         | 66.5 ± 0.6**        | 62.6 ± 0.8**         |
| TS (% of FF) | 76.5 ± 0.3          | 77.9 ± 0.2**     | 77.4 ± 0.4*      | 75.6 ± 0.4        | 73.9 ± 0.4**     | 75.5 ± 0.5*       | 69.5 ± 0.5**      | 77.4 ± 0.5         | –                   | 67.6 ± 0.3**         |
| AC (% of WF) | 22.6 ± 0.1          | 22.6 ± 0.2       | 24.5 ± 0.5**     | 23.9 ± 0.6*       | 24.1 ± 0.4**     | 24.9 ± 0.3**      | 38.0 ± 0.4**      | 22.8 ± 0.2         | 29.6 ± 0.9**        | 38.7 ± 0.7**         |
| AC (% of FF) | 23.4 ± 1.3          | 24.9 ± 0.7       | 24.3 ± 0.8       | 26.0 ± 0.7        | 28.3 ± 0.3*      | 24.3 ± 0.4        | 45.4 ± 0.3**      | 25.3 ± 0.7         | –                   | 47.1 ± 0.4**         |
| AC/TS (% in WF) | 32.3 ± 0.4       | 32.5 ± 0.2       | 35.4 ± 0.9**     | 33.9 ± 1.0        | 35.5 ± 0.7**     | 36.3 ± 0.7**      | 62.9 ± 1.1**      | 32.1 ± 0.4         | 44.4 ± 1.4**        | 61.8 ± 1.7**         |
| AC/TS (% in FF) | 30.6 ± 1.7       | 31.9 ± 0.9       | 31.5 ± 1.1       | 34.4 ± 1.1*       | 38.3 ± 0.6**     | 32.2 ± 0.6        | 65.4 ± 0.2**      | 32.7 ± 1.0         | –                   | 69.7 ± 0.9**         |
| AC/AP (ratio of WF) | 0.3 ± 0.0       | 0.3 ± 0.0        | 0.3 ± 0.0        | 0.3 ± 0.0         | 0.3 ± 0.0        | 0.3 ± 0.0         | 0.6 ± 0.0         | 0.3 ± 0.0         | 0.4 ± 0.0           | 0.6 ± 0.1**         |
| AC/AP (ratio of FF) | 0.3 ± 0.0       | 0.3 ± 0.0        | 0.3 ± 0.0        | 0.4 ± 0.1         | 0.4 ± 0.0        | 0.3 ± 0.0         | 0.8 ± 0.0         | 0.3 ± 0.1         | –                   | 0.9 ± 0.0**         |
| RS (% of WF) | 1.2 ± 0.0          | 1.2 ± 0.1        | 1.1 ± 0.1        | 1.1 ± 0.1         | 1.2 ± 0.2        | 1.2 ± 0.1         | 6.6 ± 0.3**       | 1.1 ± 0.1         | 1.3 ± 0.1*          | 8.7 ± 0.3**         |
| RS/TS (% in WF) | 1.8 ± 0.1       | 1.7 ± 0.2        | 1.7 ± 0.1        | 1.7 ± 0.1         | 1.7 ± 0.2        | 1.8 ± 0.1         | 11.0 ± 0.4**      | 1.7 ± 0.1         | 2.3 ± 0.1**         | 15.1 ± 0.5**        |

Values reported are means ± SD. The phenotype data of each genotype were compared to that of genotype AA8BDD using the two-tailed Student's t-test; *significant at P < 0.05, **significant at P < 0.01. TS, total starch; WF, wholemeal flour; FF, fine flour; AC, amylose content; AP, amylopectin; RS, resistant starch. “-”, not determined.
The structure, physiochemical and nutritional properties of the starches from wholemeal flours of different ZM and Bobwhite sbeIIa mutant lines. (a) and (b) chain length distribution (CLD) profiles of debranched starch from different sbeIIa mutant lines compared with WT control of ZM and Bobwhite. (c) and (d) differences were calculated by subtracting chain length distributions of isoamylase-debranched AAABBDD from ZM and Bobwhite sbeIIa mutant lines, respectively. (e) and (f) rapid viscosity profiles of wheat flours from different ZM and Bobwhite sbeIIa mutant lines. PV: peak viscosity; HV: hot viscosity; FV: final viscosity. (g) and (k) resistant starch contents as a percentage of wholemeal flours from different ZM and Bobwhite mutant lines. (h) and (l) resistant starch contents as a percentage of total starch in wholemeal flours from different ZM and Bobwhite mutant lines. (i) and (m) contents of soluble pentosans as a percentage of retrogradation wheat flours from ZM and Bobwhite mutant lines. (j) and (n) protein contents in the flours of different ZM and Bobwhite mutant lines. (o) and (p) contents of reducing sugar in the flours of ZM and Bobwhite mutant lines.

Figure 4

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Physicochemical properties of starch from mutant lines

The changes of starch composition, granule morphology, and fine structure of amylopectin may lead to the alternations of physicochemical properties of starch. In ZM, the wholemeals from aabbdd triple-null line failed to paste at the temperature profile that was used for rapid visco analyser (RVA) analysis and demonstrated significantly decreased values of several viscosity parameters such as the peak viscosity (PV), hot viscosity (HV), breakdown value (BDV, PV-HV), final viscosity (FV) and setback value (SBV, FV-HV), whereas these values of other mutant lines were comparable to those of WT plants (Figure 4e). In Bobwhite, both the flours from AAbbdd double mutant line and aabbdd triple-null line failed to paste at the temperature profile for RVA analysis of WT control, demonstrating significantly decreased values of these viscosity parameters (Figure 4f). We further analysed the thermal properties of starch from different mutant lines by differential scanning calorimetry (DSC). The peak temperature indicates the transition induced by melting amylopectin crystals, whereas the end temperature represents the transition of the dissociation of the amylose-lipid complex (Regina et al., 2010). As indicated in Table S6, the onset, peak and end gelatinization temperatures for the starch of aabbdd triple-null lines significantly increased, but the gelatinization enthalpy of thermo-gelatinization significantly decreased in comparison with those of ZM and Bobwhite WT plants (Student’s t-test, **P < 0.01), which was the typical characteristics of high-amylose cereals in consistent with the previous report (Regina et al., 2010).

Nutritional properties of starch derived from the mutant lines

The RS contents of ZM aabbdd triple-null lines were around 6.6% and 11.0%, whereas that of Bobwhite were around 8.7% and 15.1%, significantly higher than those of WT controls, which were around 1.2% and 1.8% in ZM, and 1.1% and 1.7% in Bobwhite, respectively (Figure 4g, k, Table 2), when measured as a percentage of wholemeal flour and total starch following the protocol described by the AOAC Method 2002.02 (McCleary et al., 2002). Consequently, the retrograded resistant starch (RSIII) also increased significantly (Figure 4h, I, Figure S2). The contents of RSIII from the fine flours of triple-null lines reached at 8.0% in ZM and 10.0% in Bobwhite, respectively (Figure S2). At the same time, the content of soluble pentosan, which is considered as one of the major components of healthy dietary fibre, when measured as a percentage of wholemeal flour, were also significantly higher in the grains of aabbdd triple-null lines in comparison to both ZM and Bobwhite WT controls (respectively Figure 4i, m). We also found the protein contents significantly increased in the flour of ZM and Bobwhite mutant lines compared to WT controls (Figure 4j, n). Furthermore, after α-amylase and amyloglucosidase treatment, the contents of reducing sugar, which are positively and linearly correlated to glycemic index (GI) (Regina et al., 2006), were much lower in both ZM and Bobwhite mutant lines compared to these of WT controls. In particular, both ZM and Bobwhite aabbdd triple-null lines had significantly decreased reducing sugar level after digestion (Figure 4o, p). These results indicated that the mutant lines, especially the aabbdd triple-null lines, generated in this study had improved potential health benefits to humans.

The end-use quality of the edited mutant lines

We then tested the end-use quality of different mutant lines for making bread and cookies in order to provide basic information for end-use of the partial-null and triple-null lines in food processing industry. As we mentioned earlier, ZM is a high gluten content, hard wheat variety generally used for making bread or steam bread. The appearance quality of breads made of the flour from different ZM mutants containing different null alleles was as shown in Figure 5a. Whereas significant changes were observed in aabbdd triple-null line, no significant difference existed between single/double null lines and WT plants. These were further confirmed by colour, volume and texture profile analysis (TPA), all of which are the key indexes for evaluation of bread-baking quality. We observed that the parameters of both crust and crumb colour were significantly different in bread made of aabbdd triple-null lines (Figure 5b). Namely, much higher L* value, lower a* and b* values were observed in aabbdd triple-null line, indicating the appearance of bread made by high-amylose flour was much brighter (Figure 5b). The volume of bread made of flour from aabbdd triple-null line decreased from 27.0% to 30.8% in comparison with either other mutant lines or WT controls. The hardness of bread made of aabbdd line increased dramatically, reaching around 4-time higher than that of the other lines. On the contrary, the springiness of bread made of aabbdd triple-null line decreased, indicating its texture was less spongy (Figure 5b).

We also tested the baking quality of Bobwhite mutant lines in making cookies. As indicated in Figure 5c, the aabbdd triple-null line showed a brighter biscuit-baking appearance quality as well. It had less volume, brighter colour, higher hardness and lower sensory score (Figure 5d). The diameter and thickness of biscuit made by aabbdd decreased by 1.7% and 11.2%, respectively, compared to these of WT control. The colour parameter of L* increased while the parameters of both a* and b* decreased in aabbdd line, indicating that the biscuit made by aabbdd triple-null line showed a brighter appearance (Figure 5d). All three TPA attributes including hardness, crispness and chewiness increased significantly in aabbdd triple-null line in comparison with WT control. In terms of sensory evaluation, aabbdd triple-null line showed the lowest score, which could be ascribed to its less softening, rough taste and heterogeneous structure. No significant difference was observed between partial-null lines and WT control.

Discussion

Modification of the starch composition, structure and properties through gene editing of TaSBEIa in both winter and spring wheat varieties

Given the fact that wheat is a major staple crop and is the main source of cereal-based processed products, modification of the starch composition in wheat to increase its RS content presents an opportunity for a potentially large-scale improvement in public health. However, due to the emphasis on processing quality in breeding practice in the past decades, in which high amylopectin wheat are generally recognized to be related to good end-use quality, amylose and RS contents in modern wheat varieties remain very low. Besides, functional redundancy of genes in polyploidy species like wheat makes a forward genetics approach to select a desired phenotype very challenging, time-consuming and in some cases impossible. Here, we modified the starch

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Figure 5 The appearance and qualities of bread and biscuits prepared using fine flours from different mutant lines containing different partial-null or triple-null sbeIIa alleles. (a) The appearance quality of breads prepared using the fine flour from different ZM mutants containing different sbeIIa null alleles. (b) Bread-baking quality of the fine flour from different ZM mutants containing different sbeIIa null alleles. (c) The appearance quality of biscuits prepared using the fine flour from different Bobwhite mutants containing different sbeIIa null alleles. (d) Biscuit-baking quality of the fine flour from different ZM mutants containing different sbeIIa null alleles. L* represented whiteness (value 100) or blackness (value 0), a* represents red (+100) or green (−100), and b* represents yellow (+100) or blue (−100). Note: a. Different small letters in the same column of each number are significantly different at the 0.05 probability level (P < 0.05). b. D and T represented diameter and thickness, respectively. c. Six parts were comprised in sensory evaluation. 1 stood for the clearness of pattern (1 to 10 points), 2 for the shape of biscuits (1 to 10 points), 3 for stickness (1 to 20 points), 4 for crispness (1 to 20 points), 5 for flavour (1 to 20 points), 6 for texture (1 to 20 points). Total score was the sum of 1 to 6.
structure and properties by targeted mutagenesis of TaSBEIIa through CRISPR/Cas9 in both winter wheat ZM and spring wheat Bobwhite, respectively (Figure 1). Following segregation, we successfully achieved six transgene-free, homozygous partial-null and triple-null lines including AAbbDD, AABBdd, aabbDD, AAbbdd, aabbDd and aabbbdd in ZM, and two mutant lines AAbbdd and aabbbdd in Bobwhite (Table 1 and Table S3), which would otherwise be cumbersome, time-consuming or not feasible through either plant-mediated RNAi (Regina et al., 2006) or TILLING in the polyplody wheat genome (Regina et al., 2015; Slade et al., 2012). Considering that chemical treatment or physical radiation-induced mutations might be accompanied by undesired and uncharacterized mutations in the whole genome (Asaoka et al., 1986; Yano et al., 1985), as well as the long regulatory process of transgenic stable food crops, we here demonstrated again the merits of targeted mutagenesis through CRISPR/Cas9 in wheat improvement. In particular, a series of mutant lines generated in this study enabled us to fully assess the effects of partial-null and triple-null alleles on starch in a sole genetic background both in winter and spring wheat varieties for the first time (Figures 1–5). Our aforementioned analyses revealed that the aabbbdd triple-null lines of both ZM and Bobwhite had pronounced effects on starch composition, amylopectin structure, physicochemical and nutritional properties of starch, as well as end-use quality, whereas either no or marginal such effects of single or dual null lines were detected. Our results provide basic information in breeding for high-amylose wheat as well as other cereal crops with increased health benefits through manipulating SBEIIa function.

Effects of aabbbdd triple-null alleles on starch composition, structure, physicochemical and nutritional properties, and end-use qualities

The amylose content of ZM and Bobwhite aabbbdd triple-null lines increased significantly to 65.4% and 69.7% of total starch in the fine flour, and 62.9% and 61.8% of total starch in the wholemeal flour, respectively (Table 2). The amylose contents of ZM and Bobwhite aabbbdd triple-null lines generated in this study are comparable or even higher than these of previous reports. For example, through TILLING and combining with selected TaSBEIIa null alleles from each subgenome in wheat by conventional breeding, the amylose contents could increase to 47-55% of total starch (Slade et al., 2012). Similarly, the amylose content of a TaSBEIIa triple-null line, CS10-C12 (A2B2nD2), which was generated by TILLING combined with conventional breeding, was 67.4%, compared to 27.2% of its one progenitor, a spring wheat variety cv Sunstate (Regina et al., 2015). Besides, a highest amylose content of 72.4% was recorded in an RNAi-SBEIIa wheat (Asaoka et al., 1985), as well as the long regulatory process of transgenic stable food crops, we here demonstrated again the merits of targeted mutagenesis through CRISPR/Cas9 in wheat improvement. In particular, a series of mutant lines generated in this study enabled us to fully assess the effects of partial-null and triple-null alleles on starch in a sole genetic background both in winter and spring wheat varieties for the first time (Figures 1–5). Our aforementioned analyses revealed that the aabbbdd triple-null lines of both ZM and Bobwhite had pronounced effects on starch composition, amylopectin structure, physicochemical and nutritional properties of starch, as well as end-use quality, whereas either no or marginal such effects of single or dual null lines were detected. Our results provide basic information in breeding for high-amylose wheat as well as other cereal crops with increased health benefits through manipulating SBEIIa function.

In conclusion, we modified the starch composition, structure and physicochemical properties by targeted mutagenesis of TaSBEIIa through CRISPR/Cas9 in both modern winter wheat variety ZM and model spring wheat variety Bobwhite, respectively, and successfully generated transgene-free high-amylose

In conclusion, we modified the starch composition, structure and physicochemical properties by targeted mutagenesis of TaSBEIIa through CRISPR/Cas9 in both modern winter wheat variety ZM and model spring wheat variety Bobwhite, respectively, and successfully generated transgene-free high-amylose
wheat with significantly increased RS contents. Our in-depth investigation revealed that the aabbdd triple-null lines of both ZM and Bobwhite had pronounced effects on starch composition, structure, physicochemical and nutritional properties of starch, as well as end-use quality, whereas either no or marginal such effects of single or dual null lines were detected. Besides, the partial-null and triple-null alleles of TaSBEIIa in winter wheat and spring wheat with different genetic backgrounds have different effects on starch morphologies, amylopectin structures and RS content. Furthermore, although the aabbdd triple-null lines incur a slightly yield penalty and side-effect on end-use quality, however, given its significantly improved RS and nutritional values for health benefits, the potential application of high RS wheat could be redeemed by using the flours of the triple-null lines as additives in food processing industry or for making cookies. Our findings provide fundamental information for improving RS content and nutritional properties in wheat and likely other cereal crops through genome editing, as well as end-use and multiple breeding applications of high-amylose products for global population health benefits.

**Experimental procedures**

**Construction of the CRISPR/Cas9-related vectors**

The constructs used in this study were based on the vector pCXUN-Cas9 in which the codon-optimized Cas9 was driven by the ubiquitin gene promoter of maize (Zea mays L.) (Sun et al., 2016). The backbone of pCXUN-Cas9 contains a hygromycin resistant gene (hptII) for callus selection. The gRNAs expression cassette which is driven by a wheat U6 promoter was introduced into pCXUN-Cas9 with HindIII, by using pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China). All primer sets used in this study were as listed in Table S1.

**Wheat transformation**

The plasmid of pCXUN-Cas9-gRNA1 or pCXUN-Cas9-gRNA2 was transformed into immature embryos by particle bombardment, respectively, followed the protocol described previously (Altpeter et al., 1996). The embryos derived from Cultivar ZM or Bobwhite were put on resting medium for one week and then were selected on medium containing 15 and 30 mg/L hygromycin for two weeks, respectively. Then the vigorously grown calli were transferred to regeneration media to generate green plants.

**Molecular characterization of different mutant lines**

Wheat genomic DNA from approximately 0.2 g of leaf tissue was extracted using a DNA Quick Plant System (Tiangen, Beijing, China). PCR amplification was performed using EASY Taq polymerase (TransGen Biotech, Beijing, China) and 50 ng of genomic DNA as a template. The primer sets SBE-C2-AF1/SBE-C2-AR1, SBE-C2-BF1/SBE-C2-BR1 and SBE-C2-DF1/SBE-C2-DR1 were designed to flank the designated target sites. The PCR products amplified for gRNA1 and gRNA2 were digested with Ddel and T7 Endonuclease I (7TEI), respectively. Then the undigested band with Ddel was recovered and directly sequenced to screen for the plants with mutations in TaSBEIIa. And the PCR products which can be digested by 7TEI were also sequenced to screen for the plants with mutations in TaSBEIIa. The sequence chromatograms were analysed by a web-based tool (http://dsdecode.scgene.com/ ) to check the genotype and zygosity of the tested plants (Liu et al., 2015). PCR products were also cloned into the TA cloning vector P-easy (TransGen Biotech, Beijing, China), and 10 positive colonies for each sample were sequenced. Any plant carrying deletions or insertions not causing coding frame shift were excluded from further analyses.

To investigate off-target effects, we selected 3, and 2 potential off-target sites based on the prediction of the WheatCrispr (https://crispr.bioinfo.nrc.ca/WheatCrispr/), for the targets of gRNA1 and gRNA2, respectively (Table S2). Site-specific genomic PCR and Sanger sequencing was used to determine the off-target effects. The primer sets were as listed in Table S1.

**Affinity gel electrophoresis and immunoblotting**

The endogenous levels of SBEIIa proteins in different mutant lines were confirmed by western blotting with samples of 15 DPA grains, by using the previously described method (Regina et al., 2015). The polyclonal antibody was produced in rabbits by using the N-terminal sequences of wheat SBEIIa as described by Rahman et al. (2001).

**Starch composition and molecular structure**

Grains from different stages of development (6, 12, 18 and 24 DPA) in ZM and Bobwhite mutant lines were collected and used to quantify starch granule sizes and morphology by light microscopy (Leica DMS0008, Leica Microsystems) at each stage, according to the previously described protocol (Chen et al., 2014). Mature seeds were harvested and dried at 37°C for at least 3 d. Thousand-grain weights (g) of selected lines were weighed in triplicate. Grain dimensions were measured with a vernier caliper.

The granular morphology of starch was examined by scanning electron microscopy (SEM). Starch was extracted and purified following the protocol described (Regina et al., 2006). Samples were examined and photographed by FEI Quanta 450 (FEI Company, Hillsboro, OR,USA). To determine the chain length distributions of starch, flourosephore assisted carbohydrate electrophoresis (FACE) was carried out to analyse the chain distribution pattern of isoamylase-debranched starches using PA800 plus pharmaceutical analysis system (Carbohydrate labelling and analysis, Beckman Coulter, America, http://www.beckmancoulter.com/) as previously described (O’Shea et al., 1998).

**Total starch, amylose and resistant starch from flours of different mutant lines**

The total starch, amylose and resistant starch content of the fine flour and wholemeal flours were measured with the starch assay kits Megazyme K-STAR, K-AMYL and K-RSTAR, respectively (Megazyme, Wicklow, Ireland, http://www.megazyme.com) following the described protocol.

**Starch granule isolation and particle size analysis**

Starch granules were extracted according to the protocol described in a previous report (Yamamori and Quynh, 2000). The size distribution of starch granules was determined by a Saturn DigiSizer S200 laser-diffraction analyser (Micromeritics, America) using the previous method (Zhang et al., 2010).

**Physiochemical properties of starch from different mutant lines**

To determine the pasting properties of wheat flour, the sample was measured by Rapid Visco Analyzer (RVA Techmaster, Newport Scientific, Narrabeen, Australia), according to the China National Standard (GB/T 24853-2010). For the gelatinization temperature analysis, the flours were analysed by a differential
scanning calorimeter (DSC1 STArE system, METTLER TOLEDO, Switzerland) following the described protocols.

Pentosan, protein and reducing sugar level
Soluble pentosan content was examined by orcinol hydrochloric acid method (Butardo et al., 2011). Crude Protein content in wheat flour samples from SBElia mutant lines was measured by Flow Injection Analyzer (SEAL AA3, Germany). The amount of soluble reducing sugars was measured according to the protocol described previously with minor modification (Göni et al., 1997). The amount and concentration of glucose released was determined using a glucose oxidase-peroxidase solution following the protocol of Megazyme K-GLUC (Mega- zyme, Wicklow, Ireland).

Bread- and biscuit-baking quality
The quality of bread was evaluated by volume, colour and texture analysis according to previous report (Zhong et al., 2018). Bread was prepared according to the China National Standard (GB/T 14611-2008).

The quality of biscuits was evaluated in terms of volume, colour, sensory evaluation and texture analysis according to previous report (Zhong et al., 2016). Biscuits were prepared in parallel for each replicate under laboratory conditions. Twelve of the 20 biscuits from each replicate were stored intact for sensory evaluation. The other eight biscuits were measured for thickness and diameter by a vernier caliper and then were measured for colour and subjected to a puncture test to evaluate the hardness, crispness and chewiness using a TA-XT plus Texture Analyzer equipped with a cylinder probe P2 (Stable Micro System, Godalming, UK).

Statistical analyses
Significance of various properties of different mutant lines was analysed by one-way variance (ANOVA) (SPSS version 13.0; SPSS Inc., Chicago, IL), and the Student’s t-test was used to examine the differences between the two groups of data. Results with a corresponding probability value of $P < 0.05$ and $P < 0.01$ were considered to be statistically significant and very significant, respectively. For each treatment, the standard deviation of the mean (SD) was calculated based on at least three biological replicates. For the $\chi^2$ test, $P > 0.05$ were considered to be very good agreement with expected segregation ratio.

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Conflict of interests
The authors submitted a patent application based on the results reported in this paper.

Author’s contributions
L. X. and Y. M. conceived and designed the experiments; J. L., G. J., Y. S., C. J., Y. Z. and L. Y. performed the experiments; J. L., G. J., Y. S. and Y. Z. analysed the data; L. X. and J. L. wrote the manuscript; L. X. and Y. M revised the manuscript. All authors read and approved the final manuscript.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Isolation of transgene-free T1 plants from B49-S70 and B041-S103 mutant lines
Figure S2. Retrogradation resistant starch (RSIII) contents of retrogradation flour from ZM and Bobwhite WT control and triple-null lines
Table S1. Primers used in this study
Table S2. Analysis of potential off-target effects
Table S3. Genotypes of different transgene-free homozygous T1 mutant lines
Table S4. Field performance and major yield components of different mutant lines
Table S5. Volume percentage of different size granules isolated from grains of different mutant lines containing different null alleles
Table S6. Physiochemical properties of starch from different mutant lines