Creating Amylose-Free Barley Cultivars with High Soluble Sugar Content By Genome Editing

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Abstract

Background

Amylose biosynthesis is strictly associated with granule-bound starch synthase I (GBSSI) encoded by the *Waxy* gene. Waxy barley has extensive prospects for application in functional food development and the brewing industry; however, amylose-free waxy barleys are relatively scarce in nature.

Results

Here we created new alleles of the *Waxy* gene using CRISPR/Cas9 genome editing. Mutagenesis of single bases in these novel alleles caused absence of intact waxy protein in grain of the edited line. Consequently, B-type granules disappeared. The amylose and amylopectin contents of the edited line were zero and 31.73%, while those in the wild type (WT) were 33.50% and 39.00%, respectively. The absence of waxy protein led to increase in soluble sugar content to 37.30% compared with only 10.0% in the WT. Typical soluble sugars, sucrose and β-glucan, were 39.16% and 35.40% higher in the edited line than in the WT, respectively. Transcriptome analysis identified differences between the edited line and the WT that could partly explain the reduction in amylose and amylopectin contents and the increase in soluble sugar, sucrose and β-glucan contents.

Conclusions

The barley cultivar with novel alleles of the *Waxy* gene contained zero amylose, lower amylopectin, and higher soluble sugar, sucrose and β-glucan than the wild type. This new cultivar provides a good germplasm resource for improving the quality of barley.

Background

Barley (*Hordeum vulgare* L.) is an ancient cereal crop that currently ranks fourth in production of grain crops worldwide after maize, wheat and rice. Approximately two-thirds of barley is used for feed, one-third is used for brewing and only a tiny fraction (2%) is used for food directly [1]. Barley is increasingly becoming a desirable food grain for human diets because of potential health benefits such as lowering glycemic load related to dietary fiber, high soluble fiber (β-glucan) content and nutritional value [2]. Moreover, barley can be produced at high altitudes and in deserts with extreme climates, where wheat and most other cereal crops cannot grow; thus, barley is always a staple food source in harsh landscapes.

Starch is one of the main components (60–70%) of cereal grains and usually contains about 25% amylose and 75% amylopectin. Amylose is essentially a linear molecule composed of glucosyl units connected by α-(1-4)-linkages together with a few branches. Amylopectin is larger than amylose and has many branches formed by α-(1,6)-bonds in addition to the α-(1,4)-linkages in the linear chains [3–6].

Barley can be divided into waxy barley and non-waxy barley according to its amylose content. Non-waxy barley starch contains 20–35% amylose, while waxy barley starch contains virtually no or low amounts of amylose. The content and ratio of amylose and amylopectin determine starch properties and characteristics and have a considerable influence on the processing, malting and food quality of barley grain [6]. Like other waxy crops, waxy barley has many merits, such as high swelling power, pasting viscosity, freeze-thaw stability, breakdown value and thickening properties, and low pasting temperature and setback value [7–9]. Furthermore, previous research has shown that waxy barley contains more β-glucan compared with non-waxy barley. Barley β-glucan is a soluble dietary fiber regarded as an important functional ingredient for lowering blood cholesterol, reducing glycemic response, regulating intestinal flora and maintaining weight [10, 11]. Starch is usually stored in large, lens-shaped A-type granules (diameter 10-35 μm) and small, spherical B-type granules (diameter less than 2–7 μm) in barley endosperm. The small starch granules comprise 6–30% of the total starch mass of barley. Waxy barley contains fewer small B-granules by weight than high-amylose barley [6, 12]. During beer production, small B-granules in barley grains are protected by a heterogeneous matrix (proteins and cell walls), allowing them to more easily escape degradation and cause technical problems [12]. Therefore, waxy barley has extensive prospects for application in the functional food processing and brewing industries.

Starch biosynthesis involves a series of coordinated enzymes. Amylopectin biosynthesis requires ADP glucose pyrophosphorylase, soluble starch synthase (SS), starch branching enzyme (BE) and starch debranching enzyme [13]. Amylose biosynthesis is strictly associated with granule-bound starch synthase I (GBSSI) encoded by the *Waxy* gene [14–16]. The barley *Waxy* gene is located on the chromosome 7H (17,089,965-17,094,192) reverse strand, contains 12 exons and 11 introns, and encodes the 60-KDa GBSSI protein (waxy protein). Variations in the barley *Waxy* gene or its regulatory sequences, such as point, insertion and deletion mutations, influence the function or catalytic activity of GBSSI protein and further affect amylose biosynthesis [5, 7, 13, 16, 17]. An approximately 400 bp deletion in the 5′ untranslated region of the barley *Waxy* gene, including the transcriptional start site, TATA box and exon 1, significantly reduces the expression level of *Waxy* and the amylose content of grain [13, 17–19]. The C2453-to-T mutation in *Waxy* stops peptide chain elongation of GBSSI, thus inhibiting amylose biosynthesis in barley [18]. Amylose-free barley ‘CDC Alamo’ has normal *Waxy* transcription and GBSSI protein levels, but significantly lower GBSSI activity; three single-nucleotide polymorphism (SNP) mutations in the *Waxy* gene of ‘CDC Alamo’ decrease GBSSI activity by 90% [5]. Recently, a G3935-to-T mutation in the *Waxy* gene resulting in substitution of Trp for the 513th Gly was identified in amylose-free barley ‘CDC Fibar’ and Z999 [13, 19]. Only four naturally occurring alleles of the *Waxy* gene are related to waxy barley, making them relatively scarce.

The recent emergence of CRISPR/Cas9 genome editing technology offers the potential to obtain novel genotypes in a much faster manner than traditional approaches, significantly accelerating crop breeding [20–24]. Here, we generated a novel waxy barley cultivar using the CRISPR/Cas9 system. The edited line carrying a biallelic mutation possessed ultra-low amylose, higher soluble sugar, fewer small B-granules and increased β-glucan content in grains compared with the wild type (WT).

Results

RNA-guided Cas9-induced genome editing of Waxy in barley

In barley grain, amylose biosynthesis is strictly associated with GBSSI encoded by the *Waxy* gene. We employed CRISPR/Cas9 technology to knock-out the *Waxy* gene of barley cultivar 'Golden Promise'. Two target sites were designed for exon 1 of the *Waxy* gene (Figure 1A) and assembled into a single vector using Golden Gate ligation, generating the construct pLGYE001-wx (Figure 1A). pLGYE001-wx was then transformed into immature barley embryos via *Agrobacterium*-mediated transformation. A total of 15 plantlets were regenerated from 355 transformed calli, and nine independent transgenic plants were identified using a PAT/bar test strip resistance kit and PCR amplification (Figure S1A and B).

Targeted mutagenesis in transgenic plants was examined by amplification of the exon 1 region of the *Waxy* gene and DNA sequencing; the resulting sequences were aligned against the WT DNA sequence. We identified two edited plants, wx13 and wx14, with biallelic mutation at the T1 site among nine transgenic plants. No plants edited at the T2 site were identified among the transformation events. Line wx13 harbored two types of 1-bp (-G and -T) deletion, while line wx14 had a 1-bp (-G) deletion and a 1-bp (+A) insertion at the T1 site (Figure 1B). The mutagenesis caused frameshifts, and premature termination codons appeared at 124 bp and 412 bp, respectively. This meant that the *wx13-1*, *wx13-2* and *wx14-1* alleles only encoded 41 amino acids while the *wx14-2* allele encoded 137 amino acids, and all lost the functional domains of the starch synthase catalytic domain and glycosyl transferases group 1 (Figure S2). Two allelic genes were simultaneously edited in wx13 and wx14 lines, meaning that T0 plants could be used for phenotype analysis. For further evaluation of translation of the *Waxy* gene, we extracted total GBSSI proteins from grains of WT, wx13 and wx14 plants. SDS-PAGE was employed to determinate GBSSI protein content. The 60-KDa GBSSI protein was detected in the WT, but it was absent in wx13 and wx14, consistent with amino acid sequence analysis of the new *Waxy* alleles (Figure 1C).

Phenotypic analysis of modified lines

We employed the I_2/KI stain method to differentiate waxy and non-waxy endosperms in barley. The endosperm of WT grains displayed the characteristic blue coloration after the I_2/KI stain, while endosperms from the mutants stained brownish red, indicating that the WT was non-waxy barley while wx13 and wx14 contained very low amounts of amylose or were amylose-free (Figure 2A). Starch granules were separated for further microscopic observation. As in the endosperm observation, starch granules of the WT were blue, while those in wx13 and wx14 were brownish red. Interestingly, the starch granules of the WT consisted of A-type and lots of B-type granules, whereas the starch granules of the mutants were mainly A-type and rarely B-type granules (Figure 2A). Because wx13 and wx14 had similar endosperm and starch granule phenotypes, only line wx14 was chosen for further analysis. Starch granules were also observed at different developmental stages (8, 16, 24 and 32 dpa). Only A-type granules were detected at 8 and 16 dpa stages, while abundant accumulation of B-type granules appeared at 24 and 32 dpa stages in WT endosperms; in contrast, the endosperms of wx14 contained

very few B-type granules throughout the developmental process (Figure S3). We performed scanning electron microscopy to identify structural differences between the WT and wx14. Compared with the large lenticular A-type granules and a mass of small spherical B-type granules of the WT endosperm, A-type granules in the endosperm of wx14 were relatively shriveled and irregular, and the quantity of B-type granules was decreased (Figure 2B). This result was consistent with the microscopic observation of starch granules.

Although the I_2/KI stain could differentiate the mutant and WT based on relative contents of amylose and amylopectin, we used chemical methods to measure the absolute contents of amylose, amylopectin, soluble sugar, sucrose, β-glucan and protein more specifically. The amylose content of the WT was 33.50%, but that of the wx14 mutant was reduced to zero. The content of amylopectin was relatively stable in both the WT and wx14 (39.00% in WT and 31.73% in wx14). Line wx14 had a relatively low starch content. The protein content of wx14 was close to that of the WT (10.88% in WT, 11.94% in wx14). The soluble sugar content of wx14 was 37.3% while that of the WT was 10.2%. The amount of soluble sugar in wx14 was increased by almost as much as the amount of amylose was reduced. The content of two typical soluble sugars, sucrose and β-glucan, in wx14 was 39.16% and 35.40% higher than that in the WT, respectively (Figure 3A and Table S2). Although the components of the grain in wx14 had undergone substantial changes compared with the WT, plant height, number of tillers, spike length and grains per spike were unaffected. However, the thousand-grain weight of the wx14 mutant was 10.42% lower than that of the WT (Figure 3B and Table S3), which was related to grain phenotype.

Transcriptome differences between the WT and wx14 mutant

High-throughput RNA sequencing (RNA-Seq) has emerged as a powerful and cost-efficient tool for transcriptome analysis and transcript profiling in various plant species [25-27]. RNA-Seq has a large advantage in providing relatively comprehensive information on the nucleotide sequences of all genes expressed in the transcriptome. We chose RNA-Seq to identify transcript differences between the WT and wx14. First, we measured the transcript abundance of the *Waxy* gene using qRT-PCR to choose the right time period for transcriptome analysis. The expression level of the *Waxy* gene peaked at 16 dpa in both the WT and wx14. Its expression level was always higher in the WT than in wx14, except at 32 dpa. *Waxy* gene expression level fluctuated more in the WT than in wx14 (Figure S4). The SNP modification of the *Waxy* gene in wx14 should have had little impact on qRT-PCR; however, the expression level of *Waxy* gene was changed in wx14 compared with the WT. This result implied that expression of the *Waxy* gene was positively regulated by its product. Loss of *Waxy* gene function did not produce amylose in wx14; however, the low content of amylose may resulte in decreased expression level of the *Waxy* gene.

We collected grains of WT and wx14 at 16 dpa to perform transcriptome analysis. Based on FPKM value, 1,204 differentially expressed unigenes (DEGs) were identified, with 386 down-regulated and 818 up-regulated in wx14 compared with the WT (Figure S5). Of these, 526 DEGs could be categorized into cellular process, environmental information processing, genetic information processing, metabolism, and organismal systems functions, with almost all of the DEGs associated with metabolism. Within the

metabolism category, 95 and 80 DEGs were enriched in biosynthesis of other secondary metabolites and carbohydrate metabolism, respectively (Figure S6); 36 structural genes in starch and sucrose metabolism showed different expression levels. Similar to the results of qRT-PCR, expression levels of the *Waxy* gene were consistent with the lower content of amylose in wx14 compared with the WT. A block in amylose biosynthesis promoted the biosynthesis of amylopectin and other polysaccharides sharing the same substrate, D-glucose. Expression levels of genes encoding two glucose-1-phosphate adenylyltransferases, two starch synthases and two 1,4-alpha-glucan branching enzymes, related to amylopectin biosynthesis, were higher in wx14 than in the WT, and their log2FoldChange values were 2.05–3.08. Genes encoding five glucan endo-1,3-beta-glucosidases had different expression levels between the two genotypes, with four up-regulated in wx14. The block in amylose biosynthesis also reduced the release of D-glucose from sucrose. The gene encoding the related enzyme 4-alpha-glucosidase had low expression level in wx14 (Figure 4 and Table S4). The low expression level of *alpha-glucosidase* resulted in high content of sucrose. The genes encoding beta-amylase and alpha-amylase showed lower expression because of the low starch content in wx14.

Discussion

In this study, we created a new barley cultivar through genome editing of the *Waxy* gene and evaluated its chemical and transcriptome characteristics.

Natural variation in the *Waxy* gene, inducing low amylose content in barley, is relatively rare in barley. Until now, only four alleles have been reported despite hundreds of cultivars possessing the waxy phenotype. All alleles encode partial or whole GBSS protein [13, 19]. We edited the *Waxy* gene using CRISPR/Cas9 technology to produce complete silencing of *Waxy* gene expression. Our new alleles only produced 24 amino acids, which did not contain the functional domain of GBSS protein. Complete silencing of *Waxy* gene expression offers advantages for studying the function of the *Waxy* gene compared with previous research. Using CRISPR/Cas9 technology, edited lines should have the same genetic background as the WT except for the *Waxy* gene, giving reliable conclusions about the function of the *Waxy* gene without the influence of other genes. Previous research has usually compared the characteristics of different cultivars or near-isogenic lines to draw conclusions, which cannot thoroughly remove the influences of background differences.

Compared with 33.50% amylose in the WT, edited lines had zero amylose. The soluble sugar content of modified lines was raised to 37.30%, while the WT only possessed 10.20%. Amylose and soluble sugars should be the compounds with the largest changes induced by loss of *Waxy* gene function. Although amylopectin, sucrose, β-glucan and protein also showed different accumulation in grains of the two genotypes, the range of variation was below 8%. In contrast, amylose content varied by 33.50%, while soluble sugar varied by 27.10%. It is highly possible that the lost amylose in the edited lines was partially turned into soluble sugar, accompanied by up-regulation of the structural genes for biosynthesis of other polysaccharides. Even though the structural genes for amylopectin biosynthesis showed high expression levels in the mutant lines, the amylopectin content decreased to 31.73% from 39.00% in the WT. This can

be explained by amylopectin being produced from amylose via a branching enzyme. The high expression levels of structural genes in amylopectin biosynthesis could not make up for the blockage in amylose biosynthesis. Glycometabolism is primary metabolism, which most likely influences secondary metabolism; however, we did not perform chemical measurements. The transcriptome revealed disturbances in secondary metabolism. Morphological changes were reflected by the thousand-grain weight and starch granules. Previous research demonstrated that waxy barley contains fewer small B-granules by weight than high-amylose barley [6, 12]. We were unable to detect small B-granules in our edited lines, while A-granules were similar to those in the WT. This was caused by complete loss of GBSS protein function, producing zero amylose content.

Our new barley cultivar should be useful in actual production. β-Glucan is a soluble dietary fiber and regarded as an important functional ingredient for lowering blood cholesterol, reducing glycemic response, regulating intestinal flora and maintaining weight [10, 11]. After modifying the *Waxy* gene, the new barley cultivar showed 35.40% higher β-glucan content than the WT. Moreover, the soluble sugar content reached 37.30% in the edited line. In some parts of East Asia, immature barley grains are used to produce sweet, fermented grains. The high soluble sugar content of the new cultivar will enhance flavor. Our new barley cultivar possessed very different chemical compounds from currently available cultivars, providing more choice in food production.

Conclusions

Four novel alleles of the *Waxy* gene, which caused complete silence of the *Waxy* gene, were created via CRISPR/Cas9-mediated genome editing. A barley cultivar with novel alleles of the *Waxy* gene showed fewer B-type granules and contained zero amylose, lower amylopectin, and higher soluble sugar, sucrose and β-glucan than the wild type, while its agronomic traits were similar, except for thousand-grain weight. Transcriptome and qRT-PCR analysis confirmed that blockage of *Waxy* gene expression reduced the expression levels of structural genes related to starch and increases sucrose and β-glucan biosynthesis. This new cultivar provides a good germplasm resource for improving the quality of barley.

Materials And Methods

Plant materials

The non-waxy spring barley (*Hordeum vulgare* L.) ‘Golden Promise’ was presented by Wendy A. Harwood from Crop Transformation Group, Department of Crop Genetics, John Innes Centre, Norwich, UK. No permission was required in collecting this barley. The barley was grown in a greenhouse under controlled conditions with 18°C during 16 h of light and 15°C during 8 h of darkness. When immature embryos were 1.5 to 2 mm in diameter, immature grains were collected from the center of spikes. Immature embryos were used for *Agrobacterium*-mediated transformation.

SgRNA design and plasmid construction

Exon 1 of the *Waxy* gene in 'Golden Promise' was cloned and sequenced using the specific primers wxF/R (Table S1). Two target sites located in exon 1 of the *Waxy* gene were selected using the online software CRISPR-GE (http://skl.scau.edu.cn/home/). The CRISPR/Cas9 system consisting of PMED and pLGYE001 was a gift from the Crop Research Institute, Shandong Academy of Agricultural Sciences. A construct containing two sgRNAs driven by TaU3 was constructed according to previously published protocols [28, 29]. In brief, four primers, T1F, T1F0, T2R and T2R0 (Table S1), containing target sequences were synthesized, and the T1-gRNA-Ter-TaU3-T2 cassette was amplified using plasmid PMED as template in a 50 μL reaction (Sangon Biotech, Shanghai, China). This cassette was then digested using *Bsa*I and ligated to pLGYE001 (NEbiolabs, Beijing, China). The recombinant vector pLGYE001-wx was transferred into *Agrobacterium* EHA105 competent cells (Huayueyang, Beijing, China).

Agrobacterium-mediated transformation of barley

Agrobacterium-mediated transformation of barley (*Hordeum vulgare* L.) 'Golden Promise' was performed as described [30]. In brief, immature embryos were separated from sterile grains and co-cultivated with *Agrobacterium* EHA105 harboring the CRISPR/Cas9 vector pLGYE001-wx at 24°C for 3 days in the dark. Embryos were subsequently transferred to selective medium containing 5 mg/L glufosinate ammonium (PPT) (Coolaber, Beijing, China) and cultured at 24°C for 6 to 8 weeks in the dark. Proliferating calli were transferred to transition medium containing 3 mg/L PPT and cultured at 24°C under 16 h low light/8 h dark photoperiod. Small plantlets were transferred to regeneration medium containing 3 mg/L PPT and cultured at 24° under 16 h light/8 h dark photoperiod for 2 weeks. Putative transgenic plants were then transferred to soil and grown to maturity in the greenhouse under controlled conditions of 18°C during 16 h light and 15°C during 8 h darkness.

Detection of transgenic plants and mutations

Transgenic plants were identified using a PAT/bar test strip resistance kit for Bar protein (Youlong, Shanghai, China). Genomic DNA was isolated from leaves of putative transgenic plants using a TaKaRa MineBEAT Plant Genomic DNA Extraction Kit (TaKaRa, Beijing, China). For detection of T-DNA insertions, PCR with primers ubiF/R (Table S1) was performed using 100 ng of DNA in a 20 μL reaction (Sangon Biotech, Shanghai, China). For detection of mutations in transgenic plants, the specific PCR primers wxF/R were used for PCR amplification. Amplified DNA fragments of target genes were ligated into pGEM-Teasy Vector (Promega, Shanghai, China), and 20 positive clones were sequenced. Nucleotide sequencing results were analyzed using the AlignX program (Invitrogen, California, USA).

SDS-PAGE analysis of GBSSI protein

GBSSI protein was extracted according to previous reports with some modifications [13, 31]. Fine powder was scraped from the endosperm of the grain, then homogenized in washing extraction buffer (55 mmol/L Tris-HCl, pH 6.8; 23 g/L SDS; 5% (v/v) β-mercaptoethanol). The mixture was incubated at 4°C overnight and centrifuged at 9000 g for 10 min. The supernatant was discarded, and the sediment was washed twice with distilled water and finally dried overnight. GBSSI protein was released from the starch

by boiling (10 mg) in 400 μL of protein extraction buffer (containing 62 mmol/L Tris-HCl, pH 6.8; 23 g/L SDS; 5% (v/v) β-mercaptoethanol; 10% (v/v) glycerol; 0.05 g/L bromophenol blue) for 5 min and centrifuged at 9000 g for 10 min. Protein supernatant (20 μL) was separated using SDS-PAGE gels (Sangon Biotech, Shanghai, China) for 3 h at 50 mA. SDS-PAGE gels were stained using a solution of 0.05% (w/v) Coomassie Blue R-250, 5% (v/v) ethanol and 12% (w/v) acetic acid for 3 h and then immersed in 10% (w/v) acetic acid overnight to remove excess stain.

Iodine staining and scanning electron microscopy of endosperm

Grains of WT and edited lines were randomly selected. Transverse sections of grains were stained using 0.01% (w/v) I_2–0.1% (w/v) KI solution. Stained starch granules were examined and images were captured using an Olympus BX53 microscope (Olympus Corp, Tokyo, Japan). The transverse sections of dried grain were attached to metallic stubs using carbon stickers and sputter-coated with gold for 30 s. Images were observed and captured using an SU8100 scanning electron microscope (Hitachi, Tokyo, Japan).

Expression analysis using qRT-PCR

Total RNA was extracted from developing grains of WT and edited lines at 8, 16, 24 and 32 days post-anthesis (dpa) using an RNAprep Pure Plant Kit (Tiangen, Beijing, China). The cDNAs were synthesized using a PrimeScript™M RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan). The gene-specific qPCR primers wxQF/R (Table S1) were designed according to the barley 'Golden Promise' *Waxy* mRNA sequence. The housekeeping gene *α-Tublin* was co-amplified as a control for normalizing cDNA templates. qRT-PCR was performed in a 7500 Fast Real-Time PCR System (ABI, Carlsbad, USA) using TB Green™ *Premix Ex Taq*™ II (TaKaRa, Tokyo, Japan). Every sample was analyzed with three replicates.

Transcriptome analysis

Total RNA of immature grains of the WT and edited line at 16 dpa was used for transcriptome analysis. Six libraries, with three biological replicates, were prepared and sequenced using an Illumina HiSeq 2000 system (Novogene, China). Raw reads were processed to obtain clean reads by removing low quality reads, and assembly of the clean reads was performed using Trinity. Functional annotation was performed by comparing all unigenes with the following databases: NCBI nonredundant protein sequences (Nr), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO). Unigene expression levels were calculated using fragments per kb per million reads (FPKM) values. Unigenes with differential expression levels between the WT and edited line were analyzed using a chi-square test with IDEG6 software [32]. The false discovery rate method was employed to determine the threshold P-value at a false discovery rate ≤ 0.001, and the absolute value of $|\log_2 \text{ratio}| \geq 2$ was used as the threshold to determine the significance of differential expression levels of unigenes. Significantly enriched GO and KEGG terms were obtained from the set of differentially abundant unigenes using the hypergeometric test.

Determination of grain quality and agronomic traits

T1 lines were generated from edited plants grown in the greenhouse under controlled conditions of 18°C during 16 h light and 15°C during 8 h darkness. Analysis of the agricultural traits of WT and edited lines was performed using 20 plants per T1 line. Plant height, number of tillers, spike length and grains per spike were recorded. Six repetitions of thousand-grain weight for the WT and edited lines were also recorded. Amylose/amylopectin, soluble sugar, sucrose, β-glucan and protein contents of grains were determined, respectively, via the dual-wavelength iodine binding method [33], phenol-sulfuric acid colorimetry method, high-performance liquid chromatography [34], streamlined enzymatic method [35] and the Kjeldahl method [36]. Every sample was analyzed three times. The statistical software PASW Statistics 18 (IBM SPSS, Chicago, USA) was used for data analysis.

Declarations

Ethics approval and consent to participate

No permission was required in collecting the 'Golden Promise'.

Availability of data and materials

The transcriptomic data has been successfully uploaded to CNGBdb (https://db.cngb.org/), Submission ID: sub027210; BioProject ID: CNP0002515. All data generated or analyzed during this study are included within the article and its additional files.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

J.L. and B.L. conceived and designed the experiments. Y.L., Y.J., F.T., X. G., D.C. and Y.Z. performed the experiments and analyzed the data. G.L. provided the CRISPR/Cas9 system. B.L. and Y.L. wrote the draft manuscript. All authors have read and agreed to the published version of the manuscript.

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Figures

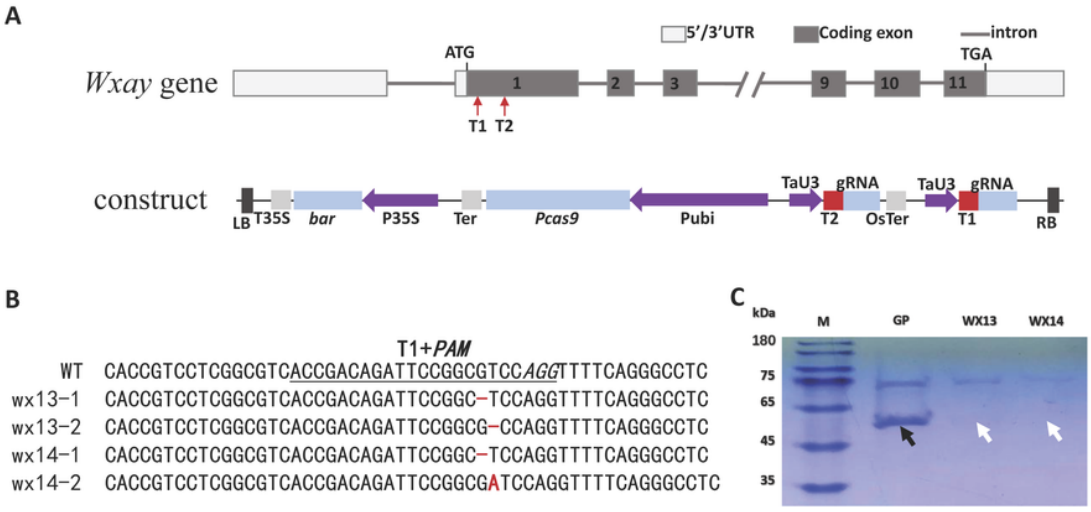

Figure 1

(A) Schematic diagram of target sites in the *Waxy* gene and the genome editing vector. (B) Mutation types in transgenic plants revealed by DNA sequencing. (C) SDS-PAGE analysis of GBSSI protein. T1 and T2 are located in coding exon 1; PCas9: *Cas9* gene modified with plant-optimized codons; TaU3: snRNA gene promoter from wheat; gRNA: guide RNA; T: target site; black arrow: GBSSI protein; white arrow: deletion of GBSSI protein.

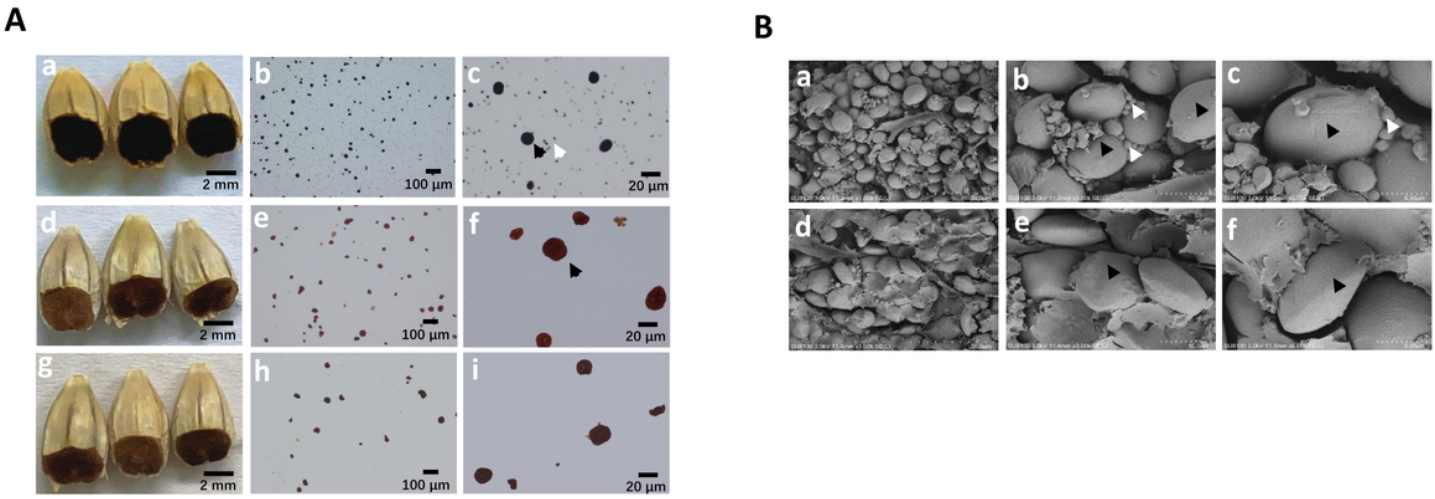

Figure 2

Identification of endosperm and starch granules in wild-type and mutant grains. (A) Endosperm and starch granules stained with I_2/KI. (a-c) Wild type; (d-f) wx13; (g-i) wx14. (B) Scanning electron microscopy images of endosperm ultrastructure and starch granules. (a-c) Wild type; (d-f) wx14. Black arrow: A-type granules; white arrow: B-type granules.

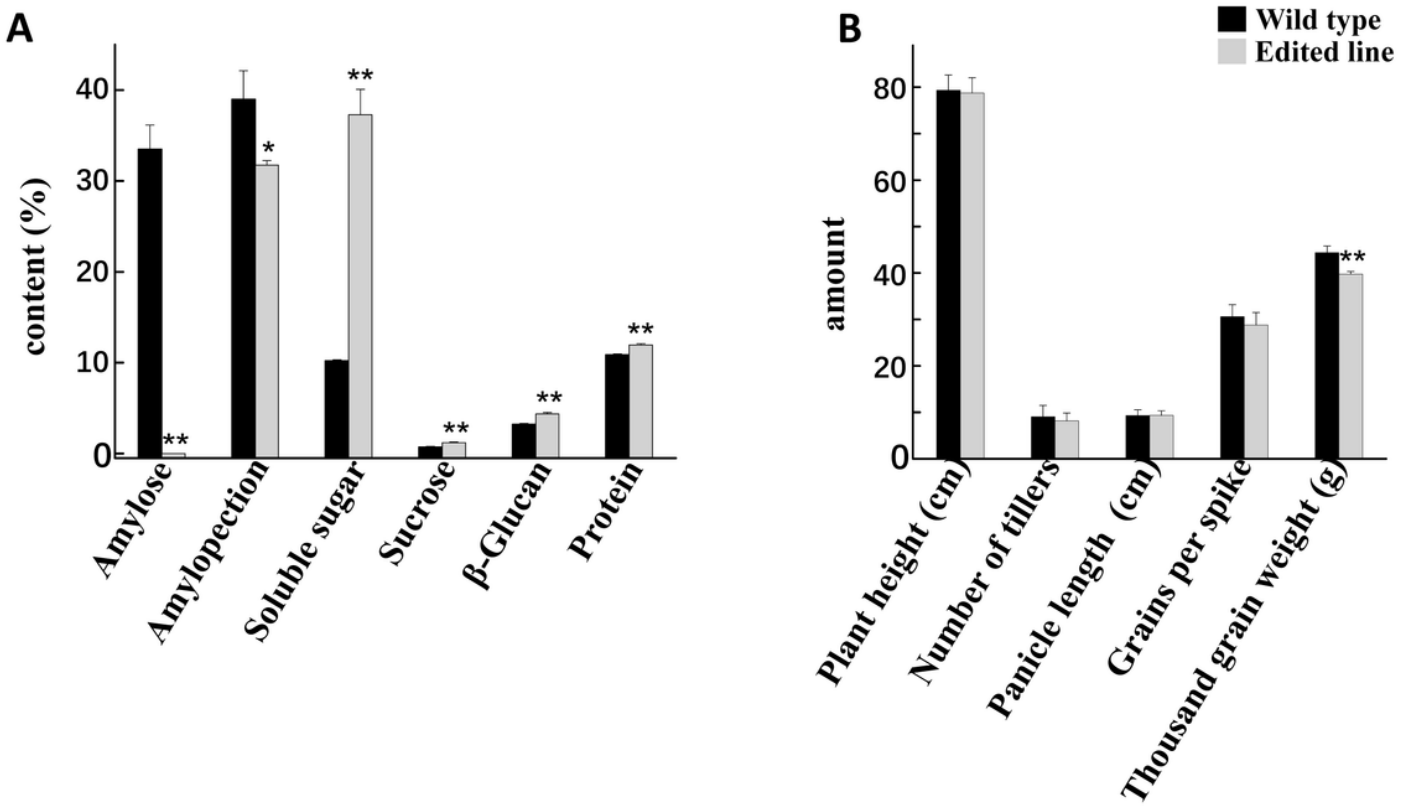

Figure 3

Grain compounds and agronomic traits between the wild type and wx14. (A) Content of amylose, amylopectin, soluble sugar, sucrose, β-glucan and protein. (B) Plant height, number of tillers, panicle length, grains per spike and thousand-grain weight. $^{*}P < 0.05$; $^{**}P < 0.01$.

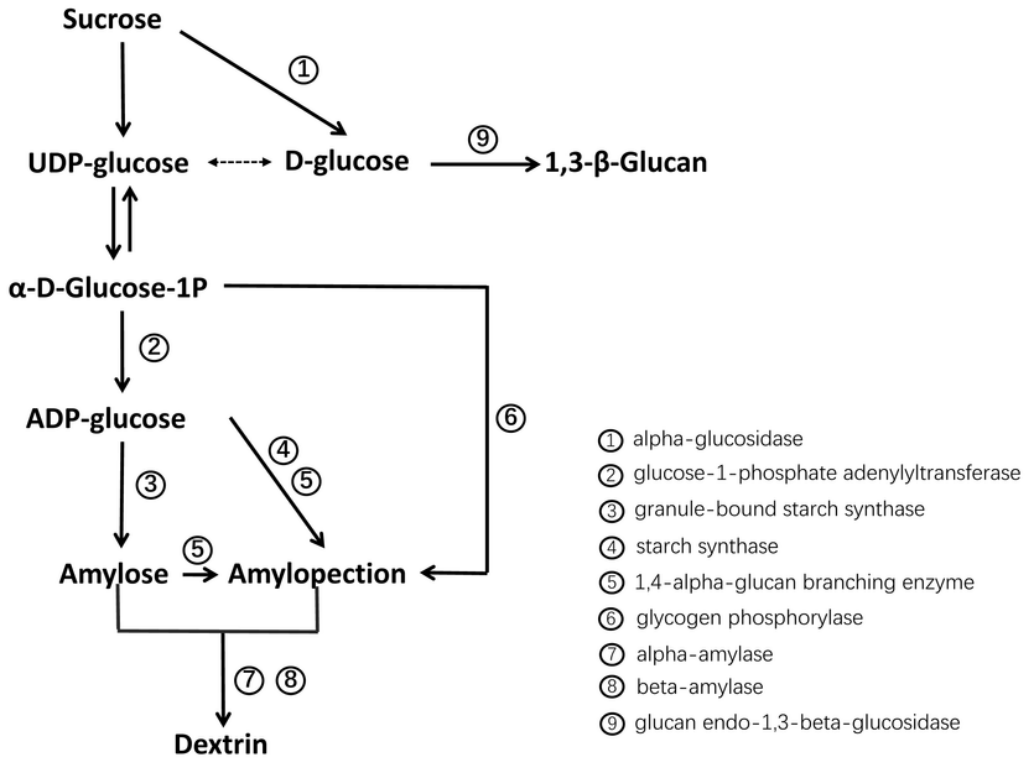

Figure 4

Structural genes in starch and sucrose metabolism with different expression levels between the wild type and wx14. Red numbers: up-regulated genes in wx14; green numbers: down-regulated genes in wx14.

Supplementary Files

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- SupportingInformation.docx