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The impact of GBSSI inactivation on starch structure and functionality in EMS-induced mutant lines of wheat

Sujon Kumar[1], Yulong Li[1], Jia Zheng[2], Jing Liu[1], Qiang Xu[1], Yazhou Zhang[1], Huaping Tang[1], Pengfei Qi[1], Mei Deng[1], Jian Ma[1], Guoyue Chen[1], Yuming Wei[1], Youliang Zheng[1] and Qiantao Jiang[1*]

Abstract

Background Starch, a major component of wheat (*Triticum aestivum* L.) grain, plays a crucial role in determining processing quality. Granule-bound starch synthase I (GBSSI), the enzyme primarily responsible for elongating α-1,4-glucan chains into linear amylose molecules, is a key determinant of starch quality. In this study, a mutant population of the wheat cultivar SM126, a high-quality variety form Sichuan, China, was generated using ethyl methanesulfonate (EMS) mutagenesis. This research investigates the effects of GBSSI inactivation on starch structure and functionality.

Results A waxy mutant (Wx-Abd) was identified by screening an M4 seed library with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of grain endosperm flour. DNA sequencing revealed a single nucleotide polymorphism (SNP) in the fourth exon, causing a premature stop codon and inactivation of the Wx-Abd allele. In previous work, the Wx-abD mutant was identified in the M2 generation, and crossing the M2-31 line with the M4-6165 line produced four distinct Wx protein subunits in the SM126 background. Comparisons between the Wx-abd line and the wild-type SM126 (Wx-AbD) showed significant differences in starch properties. The Wx-abd line exhibited reduced Wx gene expression, a distinct surface depression on starch granules, and a higher proportion of B-type starch granules. Notably, it exhibited significantly lower amylose content (7.02%) compared to SM126 (22.32%), along with a reduction in total starch content. Additionally, the Wx-abd line showed a higher gelatinization temperature.

Conclusion Inactivation of GBSSI in the Wx-abd line resulted in altered starch structure, particularly a decrease in amylose content and changes in granule morphology. These findings suggest that the Wx-abd line represents a valuable genetic resource for wheat breeding programs focused on improving starch quality for food production, with its high agronomic performance making it suitable for further breeding applications.

Keywords Waxy wheat, GBSSI, Gene expression, Starch properties, Thermodynamic properties

*Correspondence:
Qiantao Jiang
qiantaojiang@sicau.edu.cn
[1]State Key Laboratory of Crop Gene Exploration and Utilization in Southwest China, Triticeae Research Institute, Sichuan Agricultural University, Chengdu 611130, Sichuan, China
[2]Key Laboratory of Wuliangye-flavor Liquor Solid-state Fermentation, China National Light Industry, Wuliangye Group Co., Ltd., Yibin 644007, China

Background

Wheat is an essential staple crop that contributes to approximately 20% of global caloric intake, primarily through carbohydrates, and serves as a significant source of minerals, vitamins, and lipids [1]. Starch, the primary component of wheat grains, accounts for approximately 70% of the grain endosperm's dry weight. This polysaccharide comprises two main polymers: amylose, which constitutes 20–30% of total starch, and amylopectin, which makes up 70–80% [2, 3]. Amylose is a linear polysaccharide of D-glucose units connected by α-1,4-glycosidic bonds, forming a helical structure [4]. In contrast, amylopectin is a branched polysaccharide with a main chain of α-1,4-linked glucose units and branches formed by α-1,6-glycosidic bonds, resembling a tree-like structure [5]. The amylose-to-amylopectin ratio profoundly influences the quality of wheat-based products, affecting their physiochemical, functional, and structural attributes [6, 7].

Starch biosynthesis is a complex process regulated by enzymes encoding multiple isoforms responsible for amylopectin and amylose synthesis. Amylopectin synthesis involves three key enzymes: starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (DBE) [8, 9]. Amylose synthesis is primarily driven by granule-bound starch synthase I (GBSSI), also known as the waxy protein [10, 11]. In common wheat, GBSSI is encoded by three homoeologous loci: Wx-A1, Wx-B1, and Wx-D1, located at 7AS, 4AL/7BS, and 7DS, respectively [12, 13]. Wheat is classified into waxy, partial waxy, and non-waxy types based on GBSSI activity [14]. Waxy wheat lacks GBSSI, resulting in nearly zero amylose content, while partial waxy wheat has reduced GBSSI activity, leading to intermediate amylose levels [15]. These genetic variations significantly affect starch properties, making them invaluable for diverse applications [16, 17].

GBSSI, SS, and glycogen synthase (GS) belong to the GT5 (GT-B) glucosyltransferase family, which shares analogous structural properties [18–20]. These enzymes facilitate the transfer of glucosyl units from donor substrates to acceptor molecules, thereby altering enzyme activity [21, 22]. Research on GS structure has identified key amino acid residues in GBSSI that affect its functionality, providing insights into the regulation of amylose synthesis in waxy proteins [23]. Waxy wheat, valued for its unique starch properties, was first developed by crossing the Japanese cultivars Kanto 107 (Wx-A1 null) and K79 (Wx-B1 null) with the Chinese cultivar BaiHuo (Wx-D1 null), resulting in the first fully waxy hexaploid wheat [12, 24, 25]. Compared to earlier research on waxy wheat, our study highlights variations such as enhanced characterization of starch properties and the unique phenotypic outcomes observed in different mutant lines. The Wx-D1 locus exhibits limited variation, with only seven null variants identified across diverse geographic regions [26]. Among the three Wx loci, Wx-D1 shows the lowest frequency of null mutations compared to Wx-A1 and Wx-B1, and its null allele is particularly rare in common wheat [27]. Therefore, the development of induced Wx-D1 null mutants is crucial for studying and enhancing genetic resources for wheat improvement.

Chemical mutagenesis, particularly with ethyl methanesulfonate (EMS), has been extensively used in genetic research and crop breeding for over 70 years [28]. EMS efficiently induces C-to-T transitions, generating C/G-to-T/A substitutions with a high frequency (99%) and significantly broadening genetic variation [29, 30]. Random fluctuations in gene frequencies, along with mutations, immigration, and selection, can lead to diverse genetic changes [31]. Previous studies have developed SBEIIa mutants through EMS mutagenesis in tetraploid and common wheat, resulting in increased amylose content [32]. A waxy mutant, Wx-abd, was developed from EMS-mutagenized common wheat *cv.* Gao 8901, exhibiting reduced Wx gene expression and decreased starch content [33]. This distinct reduction in total GBSSI activity leads to alterations in starch composition, subsequently influencing the end-use functionality of wheat flour in diverse food applications [33]. In earlier work, we produced an EMS-mutagenized hexaploid wheat *cv.* SM126 [34]. In the current study, we identified a Wx-D1 null mutant from the M4 population. Our objective was to explore the molecular mechanisms underlying this newly identified mutation and its effects on starch's quantitative and qualitative properties, including content, morphology, physicochemical characteristics, and functionality.

Methods

Plant materials

An EMS-mutagenized population of the Chinese wheat cultivar Shumai126 (cv. SM126, Wx-B1 null) was developed to induce mutations [34]. A specific mutant (M4-6165) was identified from the M4 generation that lacked the Wx-D1 protein (GenBank accession: PV484716), as determined by SDS-PAGE. Previously, we identified another mutant (M2-31) that lacked Wx-A1 protein. To generate a Wx-null mutant, M2-31 was crossed with the M4-6165 mutant, producing four lines: Wx-abD (lacking Wx-A1 protein), Wx-Abd (lacking Wx-D1 protein), Wx-abd (lacking Wx-A1, Wx-D1, and Wx-B1 proteins), and Wx-AbB (lacking Wx-B1 protein). Fifteen plants from each mutant line were grown in a controlled greenhouse environment (light/dark: 16/8 h, temperatures: 24/20°C, illuminance: 350 $\mu E \cdot m^{2} \cdot s^{-1}$). Each plant produced multiple spikes, and seeds from each plant were harvested and stored separately.

Electrophoresis of waxy proteins

SDS-PAGE was performed following a previously published protocol [35]. Waxy proteins were extracted from endosperm flour (half-seeds) mixed with 700 µL of protein extraction buffer I (55 mM Tris-HCl, 2.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% glycerol, pH 6.8). The homogenate was centrifuged at 4 °C and 120,000 × g for 5 min [36]. Samples were washed twice with 800 µL of ddH_2O and acetone to remove salts and lipids. Protein extraction buffer II (55 mM Tris-HCl, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% (w/v) bromophenol blue, 1 mg flour/10 µL) was then added. The samples were boiled for 5 min and centrifuged again to remove insoluble material. Proteins were analyzed via SDS-PAGE on a 12% acrylamide gel. GBSSI proteins were visualized by silver staining, using the Thermo unstained protein marker as a molecular weight reference [37, 38].

Cloning of the waxy gene

Genomic DNA was extracted from 2–3-week-old leaves of the mutant (M4-6165) and native SM126 plants using the CTAB method [39] and stored at -80 °C. Due to the high sequence similarity among Wx alleles in the A, B, and D genomes, a segmented cloning approach was required for the 2.8 kb Wx-D1 open reading frame (ORF). Specific primers were designed based on the Wx-A1, Wx-B1, and Wx-D1 sequences from Ensembl Plants (https://plants.ensembl.org/index.html), using DNAMAN software (Table 1). High-fidelity PCR amplification was performed using LA Taq polymerase with GC buffer (TaKaRa, Dalian, China) to minimize errors in the amplified DNA sequence. PCR reactions were conducted in 50 µL volumes using previously established temperature and cycling conditions [34]. The amplified fragments were cloned into the pBM23 vector (BioMed, San Diego, CA, USA) and transformed into *E. coli* (DH5α) competent cells. Sanger sequencing of three biological replicates was conducted by TsingKe Biotech (Beijing, China).

Quantitative real-time PCR (qRT-PCR)

Relative expression levels of the three Wx protein subunits (Wx-abD, Wx-Abd, and Wx-abd) in SM126 lines were compared using quantitative real-time PCR (qRT-PCR). Subunit-specific primers (Table 1), designed based on each subunit's cDNA, were used to amplify target genes in qRT-PCR reactions. Reactions were conducted using ChamQ™ Universal SYBR® qPCR Master Mix on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The resulting qRT-PCR data were analyzed using CFX Manager software (Bio-Rad). Relative expression levels of each Wx protein subunit in SM126 were calculated using the $2-\Delta\Delta Ct$ method [40]. Expression levels were normalized to those of the reference genes *actin* and *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH).

KASP marker development for Wx gene subunit

To target the mutant single nucleotide polymorphism (SNP) in the Wx-D1 gene, a Kompetitive Allele Specific

Table 1 Primer sequences for the study

Primers	Sequence (5′–3′)	Description
TaWx-D1F	CACTACACTGCTCTCCTGCG	Wx-Abd amplification
TaWx-D1R	AGCTTGAACTAGTAGGCGTGC	
TaWx-D2F	CCCTACCCGCGACTTTAACG	
TaWx-D2R	CTTCACGGTGGGTGGCGA	
SM126-Wx-A-F	GAAGGTGACCAAGTTCATGCTGGAATGCGCTACGGAACGG	Allele-specific primers for TaWx-abD
SM126-Wx-A-H	GAAGGTCGGAGTCAACGGATTGGAATGCGCTACGGAACGA	
SM126-Wx-A-G	AAGAAGGACTGAAGGAGGATGC	
SM126-Wx-D-F	GAAGGTGACCAAGTTCATGCTCGGCAGGCTGGAGGAGC	Allele-specific primers for TaWx-Abd
SM126-Wx-D-H	GAAGGTCGGAGTCAACGGATTCGGCAGGCTGGAGGAGT	
SM126-Wx-D-G	GAAGGTCGGAGTCAACGGATTCGGCAGGCTGGAGGAGT	
TaWx-A1-qRT-F	GAAACCGCACCGATTCGA	Detection expression of Wx-abD
TaWx-A1-qRT-R	CCAGGCGTCCTTGTACTGGTC	
TaWx-D1-qRT-F	CTGGAGAAGGTCCGGGG	Detection expression of Wx-Abd
TaWx-D1-qRT-R	TCGGCACTTCCAGCGCC	
TaWx-1-qRT-F	GTACAAGGACGCCTGGGACA	Detection expression of Wx-abd
TaWx-1-qRT-R	CGCGCTTGTAGCAGTGGAAG	
TaWx-A1b-F	GAAGGTGACCAAGTTCATGCTCAGTGGCGCAAGCTTACGG	Allele-specific primers for Wx-abD
TaWx-A1b-H	GAAGGTCGGAGTCAACGGATTGCAGGAAGAAACATTTATAAGAAGGCGT	
TaWx-A1b-G	GCGCCCTGGAGCAAGACT	
TaWx-D1b-F	GAAGGTGACCAAGTTCATGCTAGAAGCACGTCCTCCCAGTTCTT	Allele-specific primers for Wx-Abd
TaWx-D1b-H	GAAGGTCGGAGTCAACGGATTGGCGGTCATCGACTTACCTTCCA	
TaWx-D1b-G	AGAGTTAAGACTACAATGGTGCTCTTGT	

PCR (KASP) molecular marker was developed (Table 1). We designed a high-throughput, genome-specific KASP assay using the PolyMarker platform (http://www.polymarker.info). Sequence homology analysis on the Ensembl Plant website (http://plants.ensembl.org) confirmed the marker's specificity [41]. Reactions were performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) following established protocols [34, 42]. The marker's efficacy was validated using M5 generation plants.

Granule-Bound starch synthase I (GBSSI) activity assay

GBSSI enzymatic activity was analyzed using a previously described method [43]. Grain samples were harvested from four Wx protein subunit lines in SM126, 15 days post-anthesis (DPA). A commercial GBSSI Enzyme Activity Assay Kit (No. BC3295, Solarbio Science and Technology, Beijing, China) was used according to the manufacturer's protocol. Each assay was performed in triplicate to account for experimental variability.

Extraction and purification of starch

Starch was extracted from mature wheat grains using a CsCl-modified version of a previously established protocol [44]. M5 generation seeds were soaked in water overnight, ground into a fine slurry, and aliquoted into 2.0 mL centrifuge tubes. Each sample was layered over 1 mL of 80% (w/v) CsCl and centrifuged at 3,500 × g for 5 min. The resulting pellets were washed three times with 1 mL of washing buffer (55 mM Tris-HCl, pH 6.8; 2.3% (w/v) SDS; 1% DTT; 10% glycerol) at 3,500 × g for 5 min. Starch granules were then washed twice with acetone and air-dried. The extracted starch powder was used to analyze granule function, physicochemical properties, and morphology. All analyses were performed in triplicate.

Determination starch, protein, and granule morphology

Using the total starch and amylose assay kit (Megazyme, Bray, Ireland), starch and amylose contents were quantified in four SM126 lines, each expressing a distinct Wx protein subunit, according to the manufacturer's protocols. Protein content was determined using an automated Kjeldahl analyzer (Foss Analytical Kjeltec™ 8400 series, Beijing, China). All values represent the average of three independent replicates. Purified starch from the four distinct Wx protein subunit was analyzed using a particle size analyzer (MasterSizer 3000E, Malvern Instruments). The starch granule volume per unit volume and the percentage of B- and A-type granules were calculated [45]. The microstructure of starch granules was analyzed using a scanning electron microscope (SEM; Zeiss Scanning Electron Microscope 300 Series, Germany). Mature grains and purified starch samples were mounted on aluminum stubs, and granule morphology was observed in triplicate.

Starch gelatinizing properties

The thermal behavior of gelatinized starch was analyzed using a differential scanning calorimeter (DSC 2920, TA Instruments, New Castle, DE, USA). Accurately weighed samples (10 ± 0.5 mg) of native starch were transferred to Tzero aluminum pans (TA Instruments, Waters LLC, USA) in triplicate. Distilled deionized water (ddH_2O) was added in a 1:2 (w/w) ratio and mixed thoroughly. To avoid bubbles, the mixture was gently blown upon. The sealed pans were equilibrated at room temperature for 2 hours before being placed in the DSC instrument. The analysis was conducted over a temperature range of 40–100 °C at a constant heating rate of 10 °C/min. An empty Tzero aluminum pan served as the reference. Thermodynamic parameters, including enthalpy change (ΔH), onset temperature (To), peak temperature (Tp), and final temperature (Tc), were determined using the Universal Analysis 2000 v4.7A software package (TA Instruments, Waters LLC, USA).

Statistical analysis

A minimum of three independent replicates was performed for each test. All data are reported as mean ± standard deviation (SD). Analysis of variance (ANOVA), followed by Tukey's HSD post hoc test was conducted using SPSS v20 (SPSS Inc., Chicago, IL, USA). For comparisons between the four groups (Wx-abD, Wx-Abd, Wx-abd, and Wx-AbD), two-tailed Student's t-tests were performed. *P*-values < 0.01 were considered statistically significant for both t-tests and ANOVA.

Results

Identification of Wx protein in the mutant library

A library of 25,000 M4-mutagenized *cv.* SM126 lines was screened for Wx protein variants using SDS-PAGE. Three seeds per line were randomly selected for analysis, and a deletion mutant lacking the Wx-Abd subunit was identified in the M4-6165 line (Fig. 1a). Common wheat contains three Wx protein subunits (Wx-A1, Wx-D1, Wx-B1), with molecular weights of approximately 60 kDa. The molecular weight hierarchy is Wx-A1 > Wx-D1 > Wx-B1. The common wheat *cv.* SM126, a naturally occurring variety, lacks the Wx-B1 subunit and expresses only Wx-A1 and Wx-D1.

Molecular mechanisms of Wx-D1 gene Silencing

To explore the molecular mechanism of Wx-D1 gene silencing in M4-6165, version 2.0 of the Chinese Spring Genome database was utilized (https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies). The Wx gene sequence of the A, B, and D genomes of common wheat was downloaded for comparison, and two pairs of specific primers for Wx-D1 amplification were designed with an overlap of approximately 1,000 bp. Sequence analysis

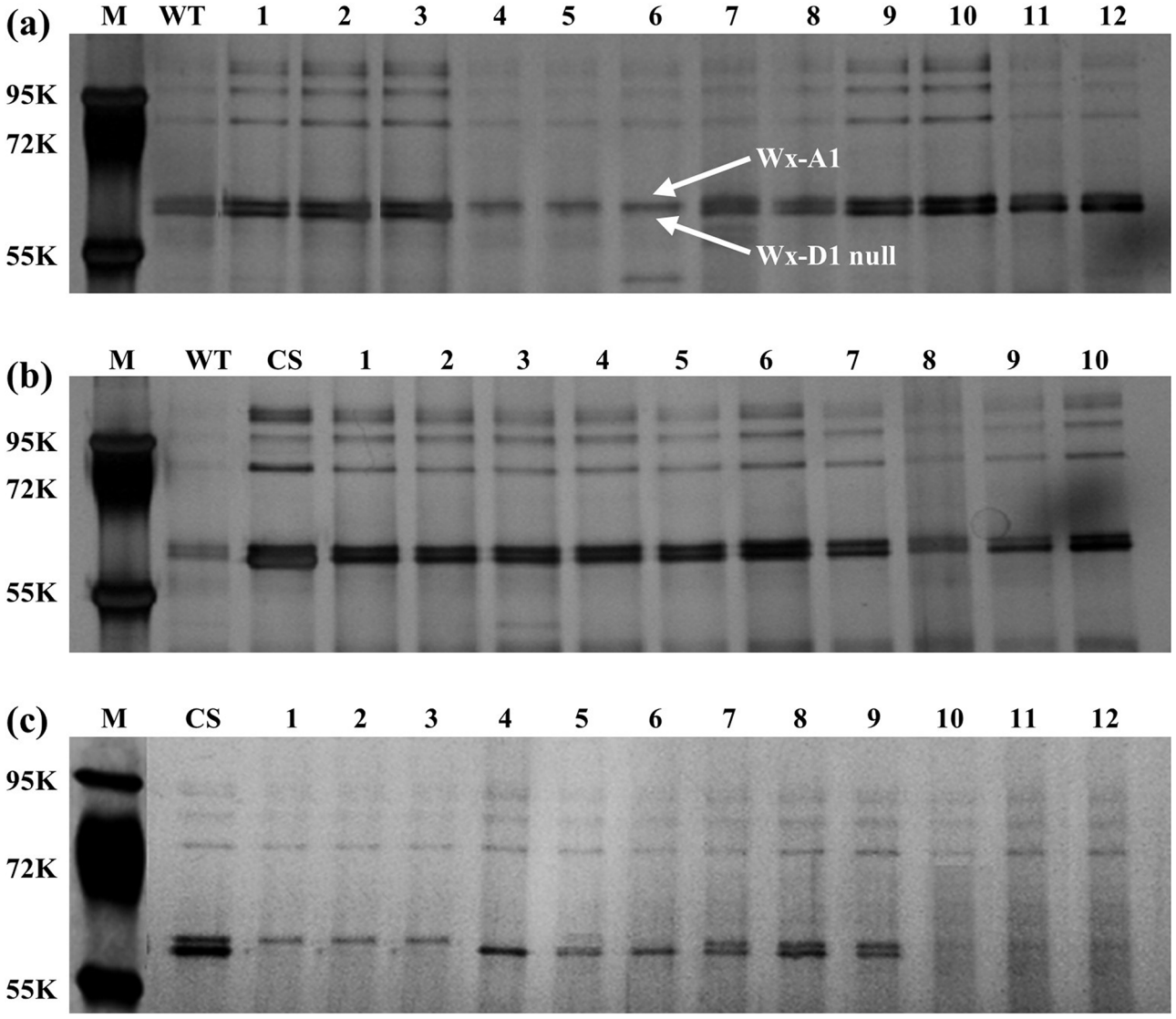

Fig. 1 SDS-PAGE analysis of waxy proteins in SM126 and its derivatives. (**a**) Identification of Wx-D1 protein subunit deletion mutant. White arrows indicate the present and absence of waxy proteins in the M4-6165 mutant. (M: Protein Marker; WT: Wild-type (SM126); Lanes 1–3: M4-6475 line; Lanes 4–6: M4-6165 line; Lanes 7–9: M4-6436 line; Lanes 10–12: M2-6166 line). (**b**) Hybridization of the F1 generation from the M4-6165 × M2-31 cross. (M: Protein Marker; WT: Wild-type (SM126); CS: Chinese Spring; Lanes 1–10: F1 hybrids of M4-6165 × M2-31). (**c**) Detection of different Wx protein subunits in SM126. (CS: Chinese Spring; Lanes 1–3: Lack of Wx-D1 protein (Wx-abD); Lanes 4–6: Lack of Wx-A1 protein (Wx-Abd); Lanes 7–9: Lack of Wx-B1 protein (Wx-AbD); Lanes 10–12: Lack of all Wx protein subunits (Wx-abd)

showed that the Wx-D1 allele of SM126 was Wx-D1a. In contrast, the Wx-D1 allele of M4-6165 underwent a C/T SNP substitution in exon 4 (C3210T), resulting in a codon change from CAG to the stop codon TAG. This premature termination prevented the expression of the Wx-D1 protein (Fig. 2).

Generating novel Wx protein subunit combinations

In this study, M4-6165 served as the paternal parent, while the Wx-A1 protein subunit deletion mutant M2-31 (previously identified) was used as the maternal parent of SM126. Crossing these two lines generated 20 F1 generation hybrids. To determine the composition of Wx protein subunits in the F1 generation, SDS-PAGE analysis was performed, using Chinese Spring (CS) as a control. M4-6165 had a Wx genotype of AAbbdd, and M2-31 had a genotype of aabbDD. Consequently, the F1 generation, with a Wx genotype of AabbDd, expressed only Wx-A1 and Wx-D1 protein subunits, confirming successful hybridization (Fig. 1b). The F1 generation was self-crossed in a greenhouse to obtain an F2 segregating population. SDS-PAGE analysis of the F2 isolated population revealed four distinct combinations of SM126 Wx protein subunits (Fig. 1c).

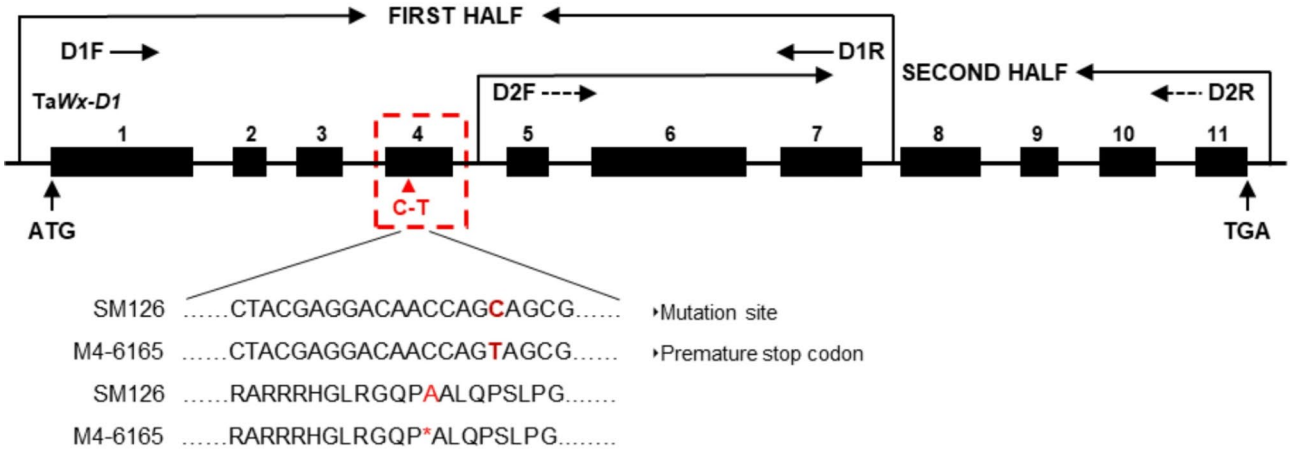

Fig. 2 Analysis of Wx-D1 gene sequences in wheat parental SM126 and mutant M4-6165 lines. The black boxes represent exon regions, and the lines represent introns. The regions targeted by the PCR primers are indicated by black arrows. The first amplified region spans from exon 1 to intron 5, and the second region spans from exon 5 to exon 11. The SNP in exon 4 and the associated amino acid variation are highlighted in red

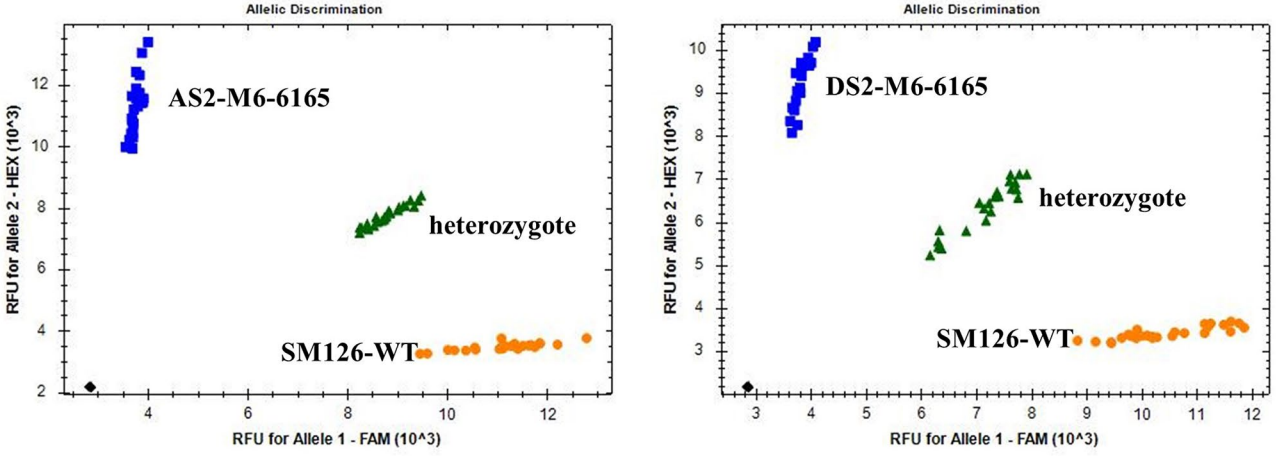

Fig. 3 Identification of a KASP (Kompetitive Allele-Specific PCR) marker for four different Wx protein subunits in SM126.Genotyping of the M6 population, M4-6165, and M2-31 lines was performed using fluorescence signals for discrimination. The two clusters represent the genotypes of homozygous M4-6165 mutants and homozygous SM126 wild-type. Mutants M2-31 and M4-6165 (HEX-tagged, Wx-A1/Wx-D1 silenced) formed the heterozygous group

Validation of the KASP marker

A Kompetitive Allele Specific PCR (KASP) marker was developed to verify genetic stability. Using SM126 wild-type, M2-31, and M4-6165 as controls, three distinct fluorescence scatter groups were observed (Fig. 3). SM126 (native Wx-B1 null) expressed FAM-tagged alleles (Wx-A1/Wx-D1), while M2-31 and M4-6165 expressed HEX-tagged alleles (Wx-A1/Wx-D1 silencing), and a heterozygous group was also identified. These findings, validated by SDS-PAGE data, confirmed the genetic stability of the four mutations [46].

Regulation of Wx gene expression

The expression patterns of the three SM126 lines, each containing a different Wx protein subunit, were compared using qRT-PCR in developing endosperm at 15 days after anthesis (DAA). The analysis showed that the Wx-AbD genotype consistently exhibited the highest expression levels for all three genes, while expression levels in the Wx-abD and Wx-Abd genotypes were ($P < 0.01$) significantly reduced (Fig. 4a-c). This decreased expression in mutant genotypes led to altered starch composition and properties, which influenced grain texture, cooking quality, and nutritional value.

Enzymatic activity assays

GBSSI enzyme activity (in U/g) was measured in immature endosperm from four different wheat genotypes (Wx-AbD, Wx-abD, Wx-Abd, and Wx-abd) form SM126 lines to assess the effect of mutations on GBSSI activity. Enzyme activity was highest in Wx-AbD (6 U/g) and decreased significantly ($P < 0.01$) in Wx-abD (4 U/g), Wx-Abd (2.5 U/g), and Wx-abd (0.5 U/g) genotypes. The reduced enzyme activity in the Wx-abD, Wx-Abd, and

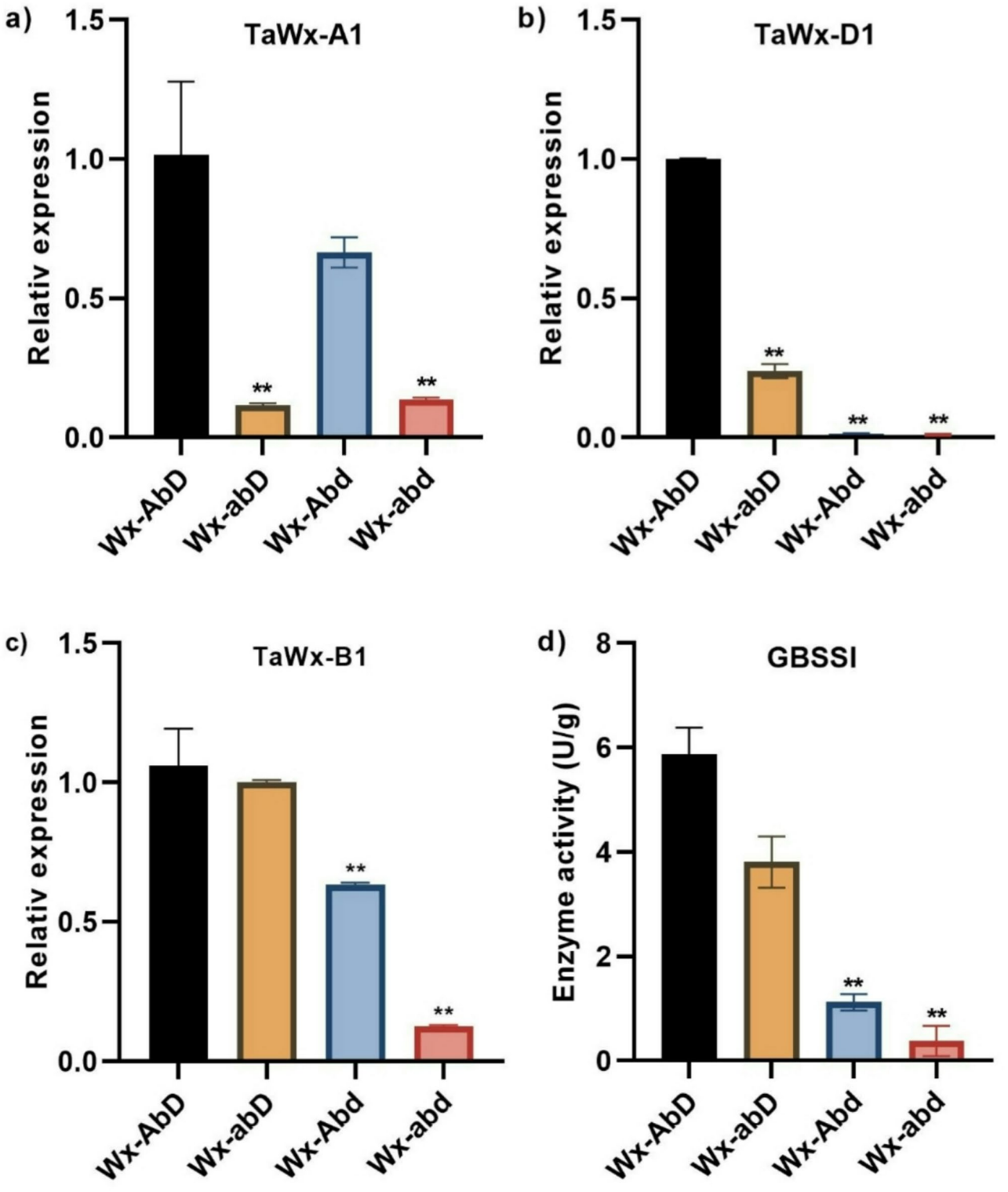

Fig. 4 Analysis of the expression levels (**a-c**) and (**d**) GBSSI enzyme activity of wheat Wx protein subunits in SM126 lines (Wx-abD, Wx-Abd, and Wx-AbD). GBSSI activity and expression levels were measured in endosperms 15 days after anthesis (DAA). Asterisks indicate statistically significant differences between the Wx-AbD and mutant lines, as determined by Student's t-test (**$P < 0.01$)

Wx-abd genotypes likely contributed to alterations in starch composition and properties (Fig. 4d).

Grain morphology and composition analysis

Grains from four Wx protein subunit combinations of SM126 were randomly selected and measured using a scanning device. Statistical analysis (Table 2) revealed no significant differences in grain area, length, and width among the Wx-AbD, Wx-Abd, and Wx-abd. Wx-abD exhibited the smallest values, consistent with its lower 1,000-grain weight. Specifically, grain areas for Wx-AbD and Wx-abd were 20.99 mm^2 and 19.49 mm^2, lengths were 7.01 mm and 6.71 mm, and widths were 3.80 mm and 3.71 mm, respectively. Overall, no significant morphological differences were observed among the four Wx protein subunit combinations of SM126 (Fig. 5). Amylose content varied significantly ($P < 0.01$) ranging from 7.02 to 22.32%, with Wx-AbD showing the highest and Wx-abd the lowest levels. Total starch content ranged from 44.79 to 61.47%, correlating inversely with the Wx-abd genotype, In contrast, protein content differences were not significant across the genotypes (Fig. 6).

Starch granule characterization

Starch granules play a crucial role in determining starch properties. The microstructure of starch granules in mutant and wild-type genotypes revealed the effects of different Wx gene combinations on granule morphology (Fig. 7). Both A- and B-type granules were present in the four waxy mutants. A-type granules were spherical or round cake-shaped across all four mutants, while B-type granules were more prevalent in Wx-abD, Wx-Abd, and Wx-abd than in Wx-AbD. No significant differences were observed in the size and shape of A-type granules among Wx-AbD, Wx-abD, and Wx-Abd. However, A-type granules decreased in Wx-abd and exhibited distinct surface depressions, indicating that deletion of the Wx protein subunit affected granule composition and structure.

Thermodynamic properties of starch

The thermodynamic properties of starch were assessed using differential scanning calorimetry (DSC) (Table 3). Significant genotypic differences ($P < 0.01$) were observed in onset temperature (To), peak temperature (Tp), completion temperature (Tc), and gelatinization enthalpy (ΔH). The Wx-abD genotype exhibited the lowest onset temperature (61.0 ± 0.07 °C), indicating that its starch granules swelled at lower temperatures compared to other genotypes. In contrast, Wx-abd showed the highest peak temperature (67.29 ± 0.04 °C) and gelatinization enthalpy (6.91 ± 0.23 J/g), suggesting that its starch granules required more energy for complete gelatinization. The Wx-AbD and Wx-Abd genotypes displayed intermediate thermodynamic properties, with To, Tp, Tc, and ΔH values falling between those of Wx-abD and Wx-abd. These findings highlight the significant influence of Wx genes on starch properties, which in turn affect the quality of wheat-based products.

Discussion

Chemical mutagenesis artificially induces genetic mutations in crops and has been extensively utilized in crop functional genomics and breeding [9]. The limited availability of natural variations in many crops makes artificial mutagenesis an important tool for creating diverse populations with altered traits [47]. Ethyl methanesulfonate (EMS) mutagenesis can induce mutations across the genome in a random manner [48]. Mutation frequencies vary across species, with different ranges observed in maize and rice (1–500 kb) [49, 50], barley (1–1000 kb) [51], tetraploid wheat (1/40 kb), and hexaploid wheat (1–25 kb) [52]. Hexaploid wheat exhibits the highest mutation frequencies, likely due to its heterohexaploid nature and large genome size, which allow the generation of numerous mutation sites following EMS mutagenesis. Using EMS mutagenesis, we identified the M4-6165 mutant, which lacks the Wx-D1 (Wx-Abd) protein from a 25,000-mutant library. To investigate the molecular mechanism underlying Wx-Abd gene silencing in M4-6165, we performed gene cloning and subsequent analyses, which revealed a C/T SNP substitution in the 4th exon. In the EMS-mutagenized population, the mutant M2-31 was previously identified [34]. This mutant exhibits a deletion of the Wx-A1 (Wx-abD) protein. To investigate the molecular basis of this deletion, we discovered a G/A SNP substitution at position 2168 bp from the start codon, located within the cleavage site of the 8th intron. Further analysis of the Wx-A1

Table 2 Physicochemical and morphological characteristics of grains from different Wx protein subunits in SM126

Accession	Total starch (%)	Amylose content (%)	Protein content (%)	Grain area (mm^2)	Grain length (mm)	Grain width (mm)	1000-grain weight (gm)
Wx-AbD	61.47 ± 0.07	22.32 ± 0.38	12.90 ± 0.11	20.99 ± 0.21	7.01 ± 0.20	3.80 ± 0.007	55.25 ± 0.73
Wx-abD	54.52 ± 0.15	19.52 ± 0.91	13.86 ± 0.24	18.38 ± 0.17	6.59 ± 0.12	3.53 ± 0.009	44.95 ± 0.88**
Wx-Abd	57.70 ± 0.26	18.91 ± 0.04	14.05 ± 0.01	19.11 ± 0.10	6.72 ± 0.16	3.61 ± 0.007	51.07 ± 0.72
Wx-abd	44.79 ± 0.024**	7.02 ± 0.16**	12.29 ± 0.09	19.49 ± 0.33	6.71 ± 0.22	3.71 ± 0.008	51.43 ± 1.07

The mean ± SD was calculated from three replications of each sample

**Asterisks indicate the significance with $P < 0.01$

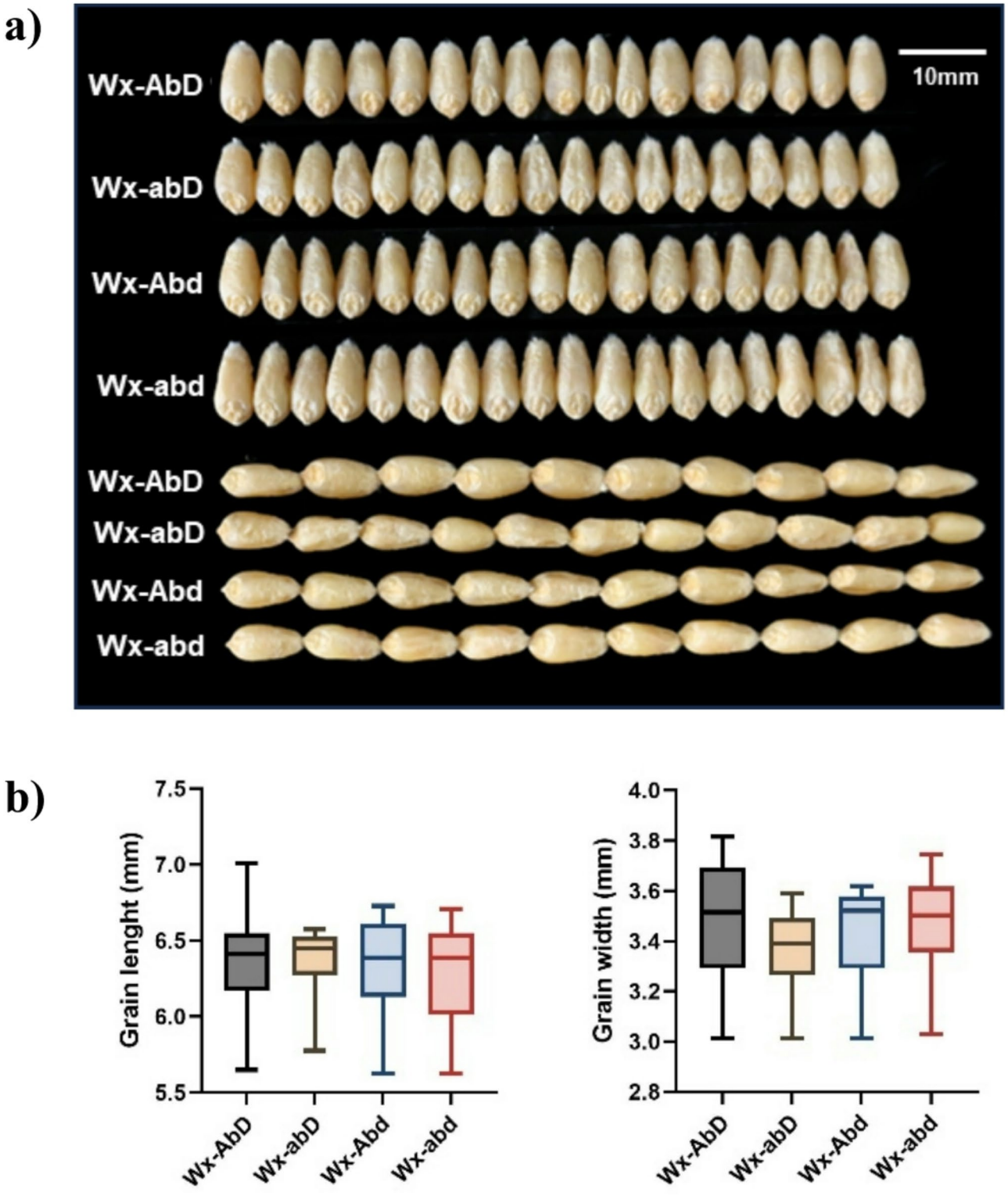

Fig. 5 Investigation of the effects of different Wx protein subunits on grain morphology. Grain length and width (a, b) were measured in four SM126 lines. For each line, 50 randomly selected seeds were measured three times. Statistical significance was determined using a threshold of $P < 0.01$

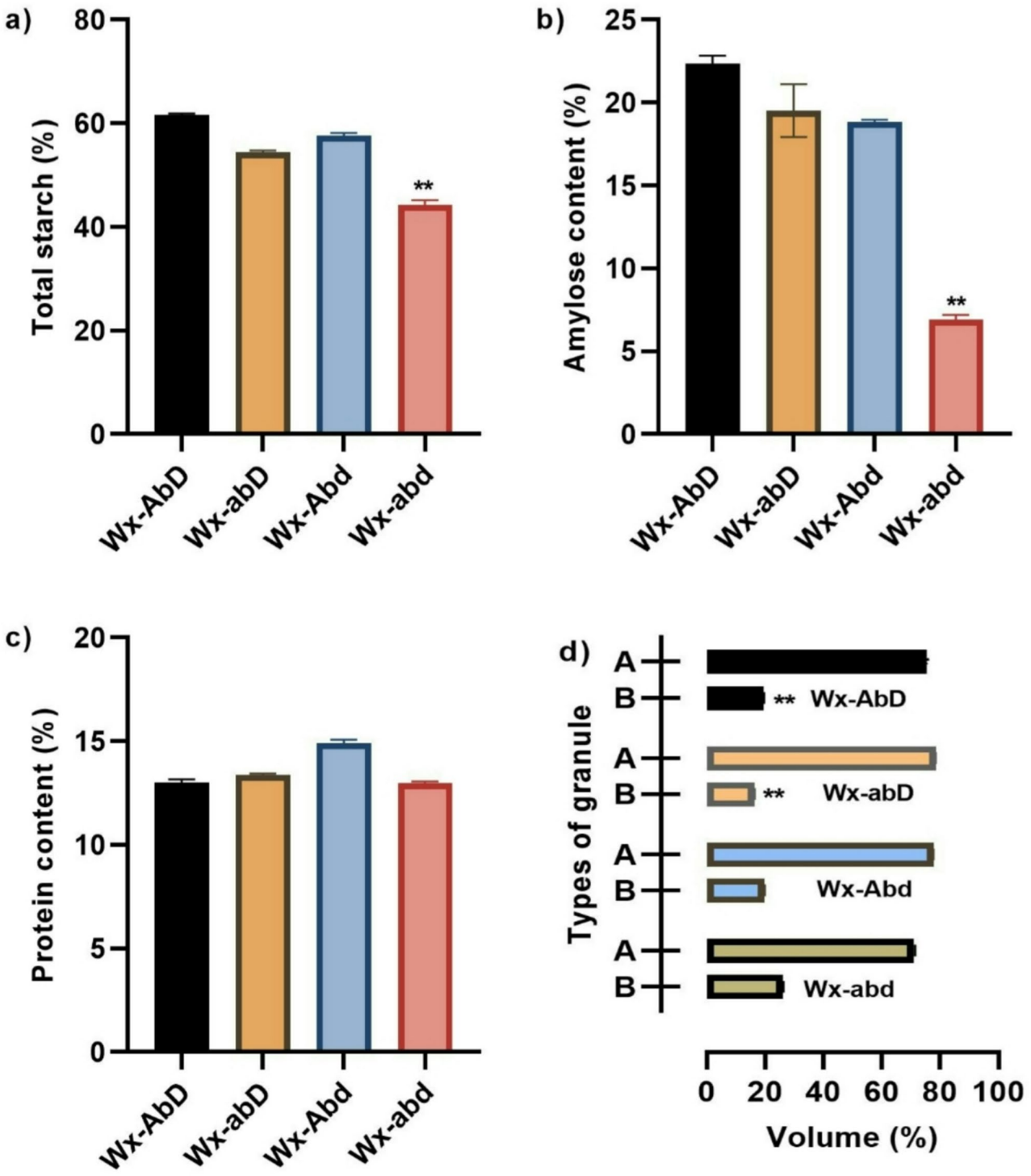

Fig. 6 Analysis of total starch (**a**), amylose content (**b**), protein content (**c**), and granule types (**d**) in the four different Wx protein subunits of SM126 lines. Significant differences were observed among the various Wx proteins ($P < 0.01$), except for protein content, where no significant variations were found. The data presented are the means of three replicates

transcript sequence in M2-31 revealed 52 misclipped cDNAs, categorized into five types.

Previous studies on Wx genes have shown that allelic variation is highest in Wx-A1, followed by Wx-B1, and Wx-D1 [26]. Inactivation of Wx genes typically results from insertions or deletions, ranging from single base pairs to multiple base pairs or even larger DNA fragments [34, 53]. In our previous studies, we identified two wheat

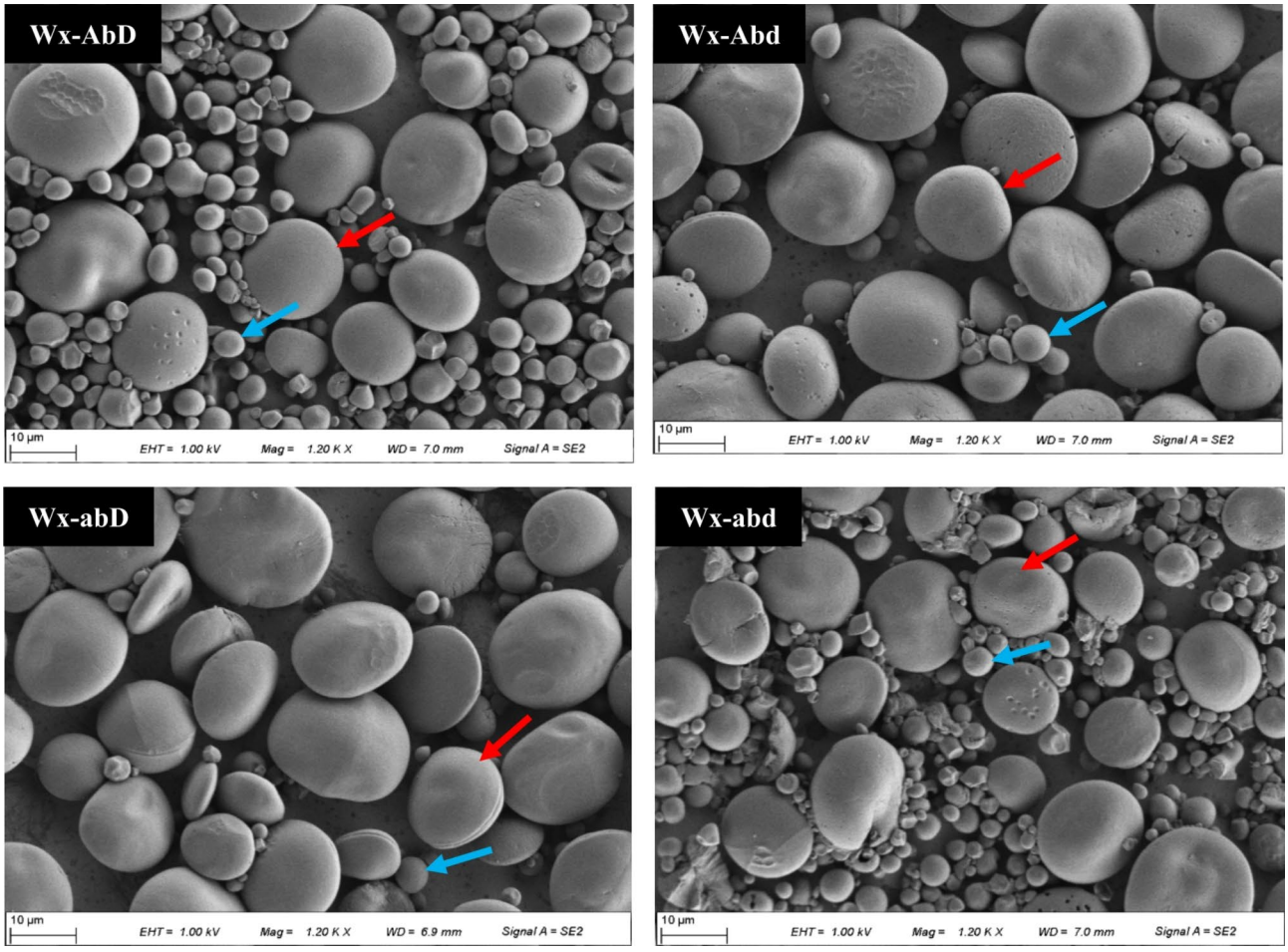

Fig. 7 Starch granule structure in the four different Wx protein subunits of SM126 lines analyzed using Scanning Electron Microscopy (SEM). The red arrow indicates A-type starch granules, and the blue arrow indicates B-type starch granules

Table 3 Thermodynamic characterization of starch from different Wx protein subunits in SM126

Accession	Onset temperature	Peak temperature	Completion temperature	Gelatinization enthalpy
	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
Wx-AbD	62.73 ± 0.17	65.54 ± 0.25	76.73 ± 0.18	4.94 ± 0.07
Wx-abD	60.52 ± 0.11**	65.29 ± 0.40	74.09 ± 0.40**	4.69 ± 0.23**
Wx-Abd	63.04 ± 0.07	65.71 ± 0.05	75.42 ± 0.18	3.91 ± 0.07
Wx-abd	63.02 ± 0.28	67.29 ± 0.04**	75.79 ± 0.40	6.91 ± 0.23**

DSC measured 1:2 (w/w db) starch: water ratio

Each sample was measured in three replications and expressed as the mean ± SD

** Highly significant difference shows the significance with $P < 0.01$

protein deletion mutants (Wx-A1 and Wx-B1) in the EMS-mutagenized M2 generation of the tetraploid wheat (*Triticum turgidum* L.) variety "Jianyang Dwarf Lanmai" [54]. Genetic analysis revealed specific mutations in both mutants: a single G/A SNP at position 1086 bp in the Wx-A1 gene of M2-219 and two G/A SNPs at positions 207 bp and 1122 bp in the Wx-B1 gene of M2-504. These findings highlight the random and unpredictable nature of mutations induced by EMS in wheat. Currently, there are a total of 42 Wx variant types in common wheat, with 19, 16, and 7 types for Wx-A1, Wx-B1, and Wx-D1, respectively. Previous research on Wx-D1 variants in common wheat has yielded diverse results. A study of 1,960 varieties identified the Wx-D1b variant exclusively in the Chinese endemic wheat variety "White Heat" [55]. In contrast, screening of 324 European common wheat varieties revealed the absence of any Wx-D1 variants [56]. Further investigation of 420 Spanish common wheat varieties detected only the Wx-D1g variant [57]. These findings underscore the significant geographical

and varietal variation in the distribution of Wx-D1 variants within common wheat. Therefore, the Wx-D1 deletion mutation in M4-6165 is crucial for creating waxy wheat in this study and contributes to the enrichment of wheat germplasm resources for genetic and breeding purposes. Among the Wx homologs, the Wx-B1 subunit exhibits the strongest amylose synthesis capability (Wx-B1 > Wx-D1 > Wx-A1). Our study analyzed starch in four different Wx protein subunit combinations. The total starch and amylose content were significantly reduced in the Wx-abd mutant compared to other haplotypes, with reductions of 27% and 69%, respectively (Fig. 6a-b; Table 2). This severe reduction in amylose content aligns with diminished GBSSI activity, indicating a critical role for Wx genes in amylose biosynthesis. Interestingly, protein content showed non significant variation across Wx-AbD (Fig. 6c), suggesting that Wx gene mutations primarily affect starch biosynthesis without impacting protein accumulation. This distinction reflects the independent regulatory pathways for starch and protein synthesis in wheat grains, consistent with previous findings [25, 55, 58]. However, the observed amylose content of Wx-abd (7.02%) was higher than expected based on earlier reports (0.5–5%). This discrepancy may be explained by the presence of extra-long chains within amylopectin, which can interfere with accurate amylose content measurements.

The waxy protein is the sole enzyme responsible for amylose synthesis and plays a crucial role in amylose synthesis [37]. Amylose content significantly influences wheat starch characteristics, such as gelatinization temperature and peak viscosity. Low-amylose wheat typically exhibits desirable properties, including lower gelatinization temperatures, improved freeze-thaw stability, and enhanced water-holding capacity [59]. These characteristics contribute to superior cooking and baking performance, as well as extended shelf life in food products [60]. Previous research has shown that wheat flour with specific rheological properties, sus as high peak viscosity, low gelatinization temperature, and high setback value, is optimal for producing high-quality white noodles [61, 62]. In this study, we investigated the influence of waxy proteins on starch structure and function. By analyzing mutant genotypes, we established a direct correlation between variations in waxy protein expression and changes in amylose content [54]. Low amylose content contributes to a bulkier texture, enhancing noodle toughness and reducing breakage. Additionally, its enhanced freeze-thaw stability, lower gelatinization temperature, and superior water-holding capacity make low-amylose wheat suitable for diverse applications, including frozen foods, processed products, baked goods, and specialized formulations sus as gluten-free or low-glycemic foods [38, 60].

Since amylose and amylopectin content influence the size and shape of starch granules, which are key factors of starch behavior and end-use quality [63], the study concluded that waxy proteins play a crucial role in starch quality. This finding aligns with previous research indicating that waxy wheat typically has smaller starch granules [64, 65]. Starch composition and behavior are strongly dependent on the relative volumes of A- and B-type granules. In this study, we observed that grain morphological traits and starch granule composition were also affected by mutations in the Wx genes. The Wx-abD mutant displayed reduced grain area and 1000-grain weight compared to the wild-type, with significant reductions in starch granule size, particularly for A-type granules (Fig. 6d). These results highlight the interconnectedness between starch composition and grain development. Although the Wx-abd mutant exhibited reduced starch content, its grain size and weight remained relatively stable, suggesting compensatory mechanisms or alternative pathways contributing to grain development, consistent with previous findings [66]. The Wx-AbD mutant genotype showed a lower frequency of A-type granules, likely due to their association with higher amylose content (Fig. 6d). Conversely, Wx-AbD and Wx-abd mutants showed an increased number of B-type granules. Furthermore, we observed reduced diversity in the shapes and sizes of granule types in the Wx-abd mutant, consistent with prior findings [67].

GBSSI, a key enzyme and the predominant protein in starch granules [68], belongs to the GT5 family of glycosyltransferases [69]. In our study, we found significant ($P < 0.01$) variation in GBSSI enzyme activity across different genotypes. The expression levels of the genes showed significant reductions in the Wx-abD, Wx-Abd, and Wx-abd mutants compared to the wild-type Wx-AbD (Fig. 4a-c). Among these, Wx-abd exhibited the lowest gene expression levels, which was consistent with a marked reduction in GBSSI enzyme activity (Fig. 4d). These findings suggest a direct correlation between reduced Wx gene expression and GBSSI activity, which is crucial factor for amylose synthesis. The observed variation in Wx gene expression may result from differences in regulatory elements or post-transcriptional modifications. Although our study did not pinpoint the exact mechanisms, future investigation could help elucidate the regulatory basis for these differences. These variations in enzyme activity have potential implications for starch composition and grain development [70]. As the thermal properties of starch are inherently linked to its composition and morphology, the gelatinization of waxy wheat requires a higher energy input than that of normal starch [71, 72]. Gelatinization enthalpy (ΔH), a measure of amylopectin crystallinity, exhibits a negative correlation with amylose content [72]. In accordance with

previous research, the Wx-abd mutant, characterized by reduced amylose, exhibited a precocious gelatinization onset, a diminished peak temperature, and an elevated gelatinization enthalpy [54]. A key limitation of using thermodynamic parameters to assess gelatinization is the requirement for large quantities of seed for starch extraction, making the process complex, time-consuming, and unsuitable for high-throughput screening of early-generation materials. It is well-established that amylose content influences gelatinization behavior, with a direct relationship observed between amylose content and the peak gelatinization temperature (Tp) of starch [73].

Conclusion

The present study provides evidence of significant differences ($P < 0.01$) among four Wx protein subunits combinations in SM126 lines, resulting from the manipulation of the waxy gene. It also evaluates the effects of the absence waxy proteins on the structural, compositional, and functional properties of starch. Inactivation of the waxy gene influenced starch characteristics, including microstructure, and subsequent thermodynamic properties, leading to the development of new starch types with distinct traits. Waxy hexaploid wheat can enhance the shelf life of bread, a valued commodity in the bread-making industry, while partial waxy wheat is advantageous for producing high-quality noodles. Therefore, these findings have potential applications for improving wheat end-use quality.

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Author contributions
S K: Validation, Formal analysis, Investigation, Data curation, Writing-original draft, Visualization. Y L: Methodology, Resources, Software. J L: Methodology, Resources. J Z: Writing– review & editing, Funding acquisition. Q X, Y Z: Writing– review & editing. H T: Resources, Supervision. P Q, M D, J M and G C: Visualization, Writing - review & editing. Y W, Y Z: Supervision, Funding acquisition. Q J: Conceptualization, Supervision, Funding acquisition, Project administration, Writing - review & editing. All authors read and approved the final manuscript.

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Data availability
The datasets generated and/or analyzed during this study are available in the TsingKe Biotech repository (https://www.tsingke.com.cn). The GBSSI (Wx-D1) gene sequence data presented in this study are deposited in the NCBI repository under accession number PV484716.

Declarations

Ethics approval and consent to participate
The authors declare that all studies comply with relevant institutional, national, and international guidelines and legislation for plant ethics in the methods section.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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