Carbohydrate Polymers 368 (2025) 124105

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Modification of starch traits in commercial wheat through *TaWaxy* gene editing

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ARTICLE INFO

Keywords:
Genome editing
Amylose content
Waxy property
Flour processing quality
Starch
Wheat

ABSTRACT

Amylose content (AC) is a key determinant of wheat quality, and the *TaWaxy* gene determined amylose synthesis with a dose-dependent effect on AC. In this study, the *TaWOX5* gene, which significantly enhances wheat transformation efficiency, was combined with CRISPR/SpCas9 system to generate *TaWaxy* mutants in a commercial winter wheat Jimai 22. Seven transgene-free mutant types were produced, compared to only three transgene-free mutants in the spring wheat variety Ningchun 4. The *TaWaxy* mutants from the two varieties showed decreased ACs ranging from 0 to 19.05 %. Results demonstrated that the Waxy-B1 protein has the most significant effect on amylose synthesis. The mutants with *TaWaxy-abd, TaWaxy-ab*, and *TaWaxy-bd* alleles showed waxy wheat trait. Interestingly, the *TaWaxy-b* mutant from Jimai 22 exhibited a waxy trait, unlike the *TaWaxy-b* mutant from Ningchun 4. Transmission electron microscope and scanning electron microscopy showed increased B-type starch granules in mutant grains. The mutants displayed varying effects on bread, cake, cookie, and noodle quality. All mutants showed decreased quality in bread and cake production, while *TaWaxy-ad-JM* and *TaWaxy-b-NC* mutants showed improved noodle and cookie quality. The generated mutants provide optimized optimized amylose content, enhancing noodle and biscuit quality as a practical alternative to blending.

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide. As consumer awareness of health and food quality continues to grow, there is an increasing demand for high-quality wheat flour products (Zi et al., 2018). Grain hardness, gluten quality, and starch composition in the endosperm are major determinants of end-product quality in wheat (Ram & Mishra, 2008). Starch is the main component of wheat grain, making up 60–75 % of the dry seed mass. It typically consists of two forms of polymer: amylopectin and amylose (Liu et al., 2021). Amylopectin is made up mainly of long chains of (1–4)-linked D-glucopyranosyl units, with occasional (1–6) branching linkages, resulting in clusters about 9–10 nm long. In contrast, amylose is a relatively linear molecule consisting of (1–4)-linked D-glucopyranosyl units (Chen et al., 2016). The structure and content of amylose and amylopectin in starch granules determine the pasting, gelation, and retrogradation properties of starch, which directly affect product quality and stability of wheat flour (Abdel-Aal et al., 2002). Granule-bound starch synthase (GBSSI, EC 2.4.1.21), also known as waxy protein, is encoded by the *Waxy* gene. The GBSSI is the key enzyme in amylose synthesis (Murai et al., 1999). In wheat, GBSSI is encoded by three homeoalleles (*TaWaxy-A1*, *TaWaxy-B1* and *TaWaxy-D1*), which are located on chromosomes 7AS, 4AL (translocated from 7BS), and 7DS, respectively (Yamamori et al., 1994).

Although natural mutations in the *Waxy* genes have been reported (Nakamura et al., 1995; Sano, 1984; Shure et al., 1983), breeding wheat varieties with these mutations using traditional methods is time-consuming and challenging due to the close linkage of undesirable traits. Fortunately, the advent of simple and efficient CRISPR/Cas9 technology has enabled significant progress in modifying the waxy

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https://doi.org/10.1016/j.carbpol.2025.124105
Received 24 April 2025; Received in revised form 14 July 2025; Accepted 21 July 2025
Available online 22 July 2025

quality of plants. For examples, the *OsWaxy* gene was edited using CRISPR/Cas9 for producing glutinous rice (Ma et al., 2015), and the mutations in the *Waxy* gene were further introduced into two elite japonica rice varieties, reducing amylose contents (ACs) (Zhang, Zhang, Botella and Zhu, 2018). Despite the knockout strategy that disables the *Waxy* gene results in a non-functional allele, various quantitative changes in the *Waxy* gene remain important. In rice, two targets in the promoter region (the CAAT-box) were edited and produced novel soft rice with AC ranging from 17.8 % to 19.6 %, compared to 24.6 % in the wild type (Zeng et al., 2020). Similarly, a novel *Waxy* allele was created by editing the region near the TATA box of the *Waxy* promoter, resulting in a stable and moderate decrease in AC (Huang et al., 2020). In addition, altering intron splicing patterns by targeting the 5' UTR splicing site produced rice materials with a AC ranging from 9.8 % to 11.5 % (Zeng et al., 2020). A series of novel *Waxy* allele mutants with ACs ranging from 0.3 % to 29.43 % were generated through base editing in rice (Huang et al., 2021; Xu et al., 2021). The CRISPR/Cas has also been used to generate the waxy corn (Dong et al., 2019; Gao et al., 2020; Qi et al., 2020), waxy cassava (Bull et al., 2018), and waxy sweet potato (Wang et al., 2019).

However, only a few studies have investigated the mutation of the *Waxy* genes in wheat using CRISPR/Cas9 (Liu et al., 2020; Zhang et al., 2018, Zhang et al., 2021). In our previous research, *TaWaxy* variants were obtained in a widely cultivated spring wheat variety Ningchun 4, but their functions have not yet been analyzed (Liu et al., 2020). The waxy wheat with low ACs (3.6 %) was created in another spring wheat variety Fielder, but in which the *TaWaxy-B1* allele was deficient (Sato et al., 2021; Zhang et al., 2021), leading to an un-optimal agronomic performance. Critically, fine-tuning amylose content to moderate levels—rather than achieving a fully waxy phenotype—holds great promise for improving the end-use quality of wheat for various food products. Yet, this strategy remains underexplored. Given that hexaploid wheat possesses three homoeologous *Waxy* genes, it is plausible that partial knockouts involving one or two loci could yield intermediate amylose levels, striking a desirable balance between processing quality and nutritional value. However, the feasibility and outcomes of such a targeted gene-editing approach have not been thoroughly investigated. Therefore, expanding the genetic toolkit by generating a broader spectrum of *TaWaxy*-edited lines in high-yielding, commercially relevant wheat varieties remains a critical objective. This approach offers a promising path to optimize AC for specific end-use qualities while preserving agronomic performance—a key challenge in modern wheat breeding.

Gene editing relies on transgenic technology platforms, yet genotype dependency has long been a major bottleneck in plant genetic transformation. This limitation significantly hinders the application of transgenic and genome editing technologies for the genetic improvement of elite cultivars. In maize, CRISPR/Cas9 system combined with the application of morphogenic genes, such as *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*), expanded the transformable genotypes and facilitated the development of the waxy mutants in the inbred line PH184C (Gao et al., 2020). *TaWOX5* significantly promotes the formation of embryogenic calli and plant regeneration in wheat. When introduced into the transformation-friendly cultivar Fielder, transformation efficiencies reached 75–96 %. Remarkably, even in recalcitrant varieties such as Jimai 22, efficiencies ranged from 17 % to 82 %, representing a substantial improvement in wheat genetic transformation and an expansion of the applicable genotype range. In addition, *TaWOX5* has been shown to enhance transformation efficiency in other cereal crops, including barley, maize, *Triticum monococcum*, rye, and hexaploid triticale. Furthermore, overexpression of *TaWOX5* did not interfere with normal plant development, highlighting its potential as a robust and versatile tool for cereal crop transformation (Wang et al., 2022).

Although waxy wheat (AC $\approx$ 0 %) is commonly used in flour blending to improve noodle texture, the direct relationship between AC levels and end-use quality traits remains unclear. In this study, we generated a series of *TaWaxy* mutants in Jimai 22 and Ningchun 4 using *TaWOX5* as a transformation enhancer, resulting in lines with ACs ranging from 0 % to 19.05 %. This allowed us to systematically investigate the influence of varying amylose levels on flour properties and processing quality. Our ultimate goal is to determine whether moderately low-AC wheat lines can be directly used in specific products—such as noodles—without blending, thereby simplifying flour formulation and enhancing product consistency.

2. Materials and methods

2.1. Plant materials

A commercial planted spring wheat variety Ningchun 4 and a largely cultivated winter wheat variety Jimai 22 in China were used in this study, which were obtained from the National Crop Germplasm Bank of the Chinese Academy of Agricultural Sciences (CAAS). The homozygous *TaWaxy*-edited mutants generated from Ningchun 4 were achieved in our previous study (Liu et al., 2020).

2.2. Construction of vectors for gene editing and wheat transformation

The vector pWMB110-SpCas9-TaWOX5-TaU3 used in this study for editing the *TaWaxy* genes in Jimai 22 was detailed described by Wang et al. (2022). The target sgRNAs, gW296 (GGCGGCCTCGGCGACGTCCTCGG) and gW830 (AAGACCAAGGAGAAGATCTATGG), were constructed onto the pWMB110-SpCas9-TaWOX5-TaU3 system following the methods outlined by Liu et al. (2020). The genome editing vector was then introduced into immature wheat embryos via *Agrobacterium*-mediated transformation, as described by Wang et al. (2022).

2.3. Detection of TaWaxy-edited mutant plants

Genomic DNA was extracted from the candidate mutants using the NuClean PlantGen DNA Kit (CWBIO, CW0531M). The allele *TaWaxy* genes on different homoeologous chromosomes were amplified using their specific primers (Table S1) (Liu et al., 2020). The PCR products were digested and separated on a 2 % agarose gel, among which the products corresponding to mutation types differing from the wild type were then sequenced.

2.4. Phenotype investigating and iodine staining

Grain characteristics including grain length, grain width, and thousand-grain weight (TKW) were measured using a scaled camera-assisted phenotyping system (Wanshen Detection Technology Co., Ltd., Hangzhou, China). Iodine potassium iodine reagent (0.1 % I_2 / 1 % KI) was dropped on the surface of the half grain part without embryo, and photos were taken three minutes later (Nakamura et al., 1995).

2.5. Amylose content and viscosity determination

Total starch content in wheat grains was determined following the method described by Shi et al. (2018). Briefly, 0.1 g of sample was extracted with 80 % ethanol at 70 °C for 2 h, followed by centrifugation at 12,000 rpm. The supernatant was discarded, and the residue was cooled before adding 2 mL of KOH. After vortexing for 20 min, 8 mL of sodium acetate buffer was added and mixed thoroughly. The mixture was then treated with 0.1 mL of amyloglucosidase and incubated at 50 °C for 30 min with continuous agitation. A 0.1 mL aliquot of the resulting solution was diluted to 1 mL with distilled water to prepare the test solution. For colorimetric analysis, 0.1 mL of the test solution was combined with 3 mL of GOPOD reagent, incubated at 50 °C for 20 min, and the absorbance was measured at 510 nm. AC was measured using the iodine binding method as described by Garg and Jana (2011).

Specifically, 0.01 g of sample was homogenized with 100 μL of anhydrous ethanol and 900 μL of sodium hydroxide, followed by heating in a boiling water bath for 10 min and cooling to room temperature. To prepare the stock solution, 9 mL of distilled water was added to the cooled mixture with thorough mixing. For color development, 500 μL of the stock solution was mixed with 100 μL of acetic acid and 200 μL of iodine solution, and the final volume was adjusted to 10 mL with distilled water. After incubation at room temperature for 10 min, absorbance was measured spectrophotometrically at 620 nm. Amylopectin content was calculated by subtracting the amylose content from the total starch content. The pasting viscosity of wheat flour was assessed using a Rapid Visco Analyzer (RVA-Super4, Perten, Sweden). Precisely 3.5 g of flour (adjusted to 14 % moisture content) was weighed into the test canister and mixed with 25 mL of distilled water. The RVA program consisted of an initial high-speed mixing phase (960 rpm for 10 s at 50 °C), followed by a continuous low-speed stirring phase (160 rpm). The temperature profile included a heating ramp (12 °C/min to 95 °C), a holding phase (2.5 min at 95 °C), a cooling ramp (12 °C/min back to 50 °C), and a final holding period (2 min at 50 °C). Viscosity changes were recorded in real-time using Thermocline for Windows software. All starch content and RVA measurements were conducted with technical support from Sanshu Biotech Co., Ltd. (Shanghai, China).

2.6. Observation of starch granules by transmission electron microscope and scanning electron microscopy

The developing wheat grain samples at 12, 16, 20, 24, and 28 days post anthesis (DPA) were collected from the edited mutants and their wild types for transmission electron microscope (TEM) observation. Each seed invaded with Gluta (Biorigin, Beijing, China) extract solution is cut into 2–3 slices of 1 mm in thickness in the middle part with a sterile blade, which were then quickly cut into 1 mm^3 cubes and vacuumized with a vacuum pump. The next steps included sample dehydration, ultra-thin section preparation, and so on. Observation by TEM was carried out using an H7500 TEM (Hitachi, Tokyo, Japan). Consequently, the morphology and size distribution of starch in the grains were observed under a scanning electron microscopy (SEM) (Zeiss Merlin Compact, Oberkochen, Germany) (Barrera et al., 2013). Further, the morphology of starch granules was observed with the SEM at 500–10000 times magnification. At least five fields of view from different regions were captured for each observation. For quantitative analysis, all SEM and TEM images were analyzed using ImageJ software (version 1.54 g) to evaluate the dynamic changes and size distribution of starch granules (Thilagashanthi et al., 2021; Zhang & Wang, 2023).

2.7. Detection of off-target mutations via sanger sequencing

The potential off-target sites of sgRNAs homology of *TaWaxy* genes were searched in CRISPR-cereal (http://crispr.hzau.edu.cn/CRISPR-Cereal/index.php). Then, potential off-target regions were amplified and sequenced in the edited T_0 plants.

2.8. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from seeds collected approximately 20 days after flowering using an RNA extraction kit (TransGen Biotech, Beijing, China). First-strand cDNA synthesis was performed with the HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co., Ltd). Gene-specific primers targeting the three *TaWaxy* homoeologs were designed (Table S1). RT-qPCR analysis was conducted to evaluate the relative expression levels of these genes in three wheat varieties (Fielder, Ningchun 4, and Jimai 22) and in *TaWaxy*-edited Jimai 22 mutants. The reactions were performed using the Taq Pro Universal SYBR qPCR Master Mix on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Paisley, UK). Relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with normalization to the endogenous reference gene *TaACTIN* (Li et al., 2025).

2.9. Analysis of flour processing quality

The developed *TaWaxy*-edited mutants with different genetic backgrounds were evaluated for bread-making, cake-making, cookie-making, and noodle-processing features. Firstly, the rheological properties of flour doughs in the mutants and wild types were tested in accordance with the National Standards of China GB/T 14614–2019. The bread-making procedure was performed by the bread-baking test of wheat flour-straight dough method according to the National Standards of China GB/T 14611–2008. Bread size and loaf score were calculated according to the description in a publication (Cai et al., 2022). For the processing analysis of noodles and cakes, the preparation and evaluation methods respectively referred to the Chinese national standards GB/T 17320–2013 and GB/T 24303–2009. These experiments were carried out at the Wheat Quality Laboratory at Institute of Agricultural Product Quality and Safety, Heilongjiang Academy of Agricultural Sciences, China. Cookie-making experiments were conducted at Institute of Agricultural Sciences for Lixiahe Region in Jiangsu, China. The quality of cookies was evaluated according to the standards of the American Association of Cereal Chemists (AACC) AACC10–50D and AACC10–52, in which the key quality parameters included cookie diameter (D), thickness (T), spread ratio (D/T), and texture.

2.10. Statistical analysis

The results of physicochemical parameters and processing quality of mutant and wild type wheat flour are expressed as the mean ± standard deviation of triplicate experiments. Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to a 5 % level of significance using SPSS 24.0 Statistical Software Program (IBM, Armonk, USA).

3. Results

3.1. Detection of TaWaxy gene mutations in the T_0 generation derived from Ningchun 4 and Jimai 22

In our previous study, a total of 87 T_0 mutants with mutations in the *TaWaxy* genes were obtained in Ningchun 4 *via* knocking-out approach using the CRISPR vector (pWMB110-SpCas9-TaU3) with two sgRNAs (gW296 and gW830) (Liu et al., 2020). To obtain the similar mutants from an extremely recalcitrant wheat variety Jimai 22 in plant regeneration and transformation, which was the most widely cultivated during the past ten years in China, the regeneration gene *TaWOX5* was incorporated into the editing vector pWMB110-SpCas9-TaU3 (Fig. 1A), on which the two sgRNAs (gW296 and gW830) were used for knocking-out the *TaWaxy* genes (Fig. 1B). In T_0 generation in Jimai 22, a total of 75 transgenic plants were obtained, and 56 mutants were confirmed by the polymerase chain reaction - restriction enzyme analysis (PCR-RE), resulting in a mutation efficiency of 74.67 %.

The mutation types and efficiencies of the two targets sites in T_0 generation derived from both wheat varieties included biallelic mutations, heterozygous mutations, and large fragment deletions (LFDs) (Fig. 1C, D). The mutation efficiency for editing *TaWaxy-7A* was higher compared to *TaWaxy-4A*, while *TaWaxy-7D* allele showed the lowest mutation efficiency. Among the two sgRNA target sites, gW830 exhibited higher mutation efficiency than gW296. Additionally, LFDs were observed between the two target sites in the mutant plants with an efficiency range of 10.67 % to 16.09 % (Table S2). We also detected the re-insertion of reverse sequence fragments at the target sites (Fig. 1C). In the mutants generated from Ningchun 4 and Jimai 22, the mutation efficiency for the three homologous genes was higher than that for either the double or single genes (Table S3). Seven potential off-target sites with 2 or 3 mismatches were predicted for the two *TaWaxy* targets

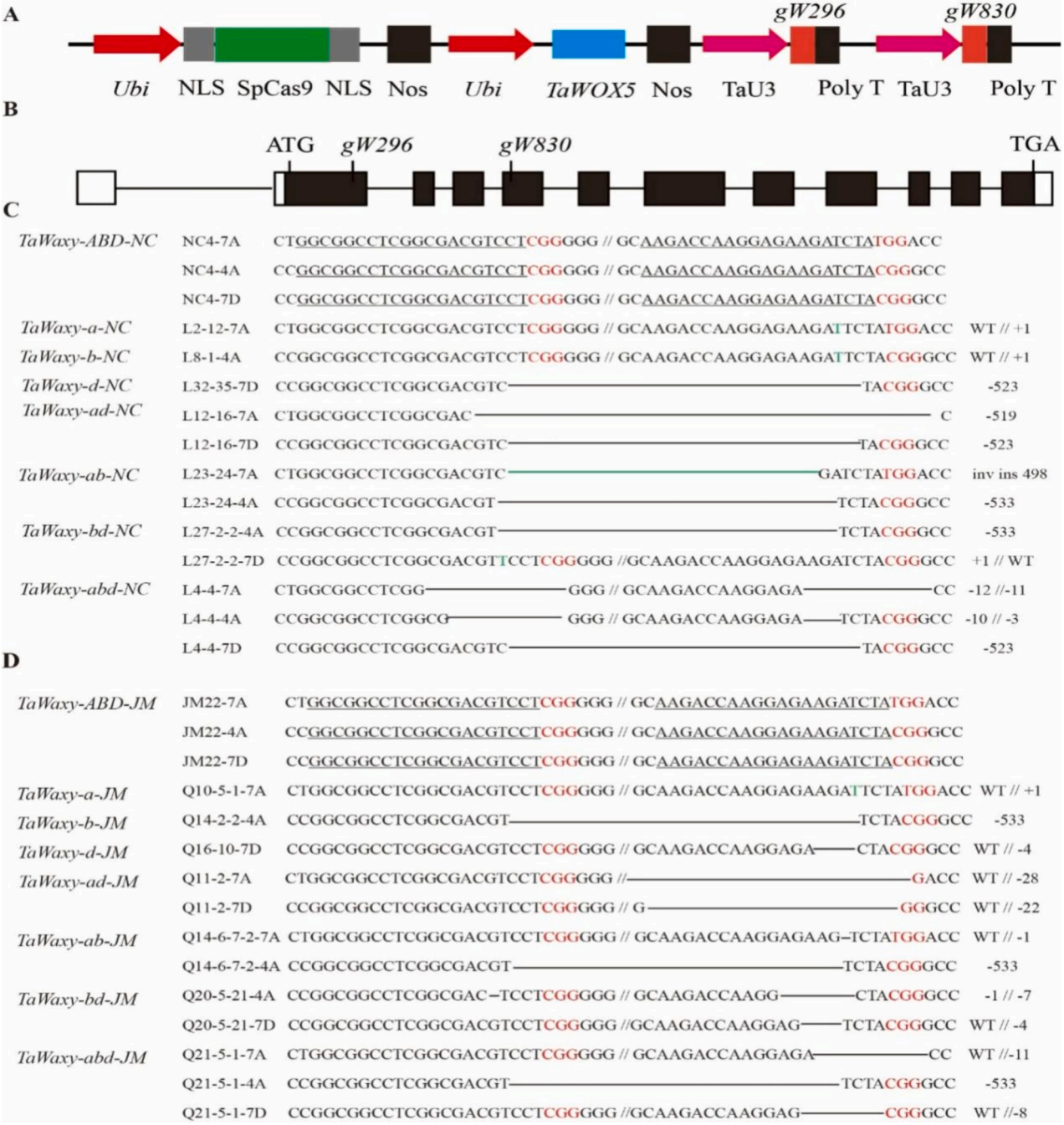

Fig. 1. Targeted mutagenesis of wheat *TaWaxy* genes by CRISPR/SpCas9 system. A: Schematic diagram of the CRISPR/SpCas9 system used for editing the *TaWaxy* genes. B: Structure of *TaWaxy* genes showing the target sites of two sgRNAs (gW296 and gW830). C, D: Mutations and sequences alterations of *TaWaxy* in Ningchun 4 and Jimai 22 after CRISPR/SpCas9 editing. The mutation types showed earlier correspondence to the gW296 target site, while those showed later correspondence to the gW830 target site. The "inv ins" indicates inverted insertion of nucleotide sequences. Red letters represent the PAM sequences, black underlines denote the sgRNAs sequences, green letters indicate inserted nucleotide, solid green lines and black lines represent inverted insertions and deletion, respectively.

(Table S4), but no mutations were detected at these sites.

3.2. Selection of TaWaxy-edited homozygous and transgenic-free mutants in T_1-T_3 generations with genetic backgrounds of Ningchun 4 and Jimai 22

The seeds from T_0 edited mutants with various mutation types derived from Ningchun 4 and Jimai 22 were planted for three generations, and PCR-RE was used to detect mutations in the *TaWaxy* genes in each generation. The PCR and *bar* strip assay were employed to identify transgenic-free plants in the three continuous generations. Finally, seven types of homozygous mutants with *TaWaxy*-edited were identified from Ningchun 4 and Jimai 22 genetic background (Table S5). All the seven mutant types derived from Jimai 22 were transgenic-free, while only three mutant types from Ningchun 4 (*TaWaxy-a, TaWaxy-b and TaWaxy-abd*) were transgenic-free (Table S5). The seven different mutant types from Ningchun 4 and Jimai 22 were used for subsequent experiments, namely L2–12 (*TaWaxy*-a-NC), L8–1 (*TaWaxy*-b-NC), L32–35 (*TaWaxy*-d-NC), L23–24 (*TaWaxy*-ab-NC), L27–2-2 (*TaWaxy*-bd-NC), L12–16 (*TaWaxy*-ad-NC), L4–4 (*TaWaxy*-abd-NC), Q10–5-1 (*TaWaxy*-a-JM), Q14–2-2 (*TaWaxy*-b-JM), Q16–10 (*TaWaxy*-d-JM), Q14–6–7-2 (*TaWaxy*-ab-JM), Q20–5-21 (*TaWaxy*-bd-JM), Q11–2 (*TaWaxy*-ad-JM), and Q21–5-1 (*TaWaxy*-abd-JM) (Table S5).

3.3. Agronomic traits of different mutant types from Ningchun 4 and Jimai 22

The mutants were largely indistinguishable from the wild types Ningchun 4 and Jimai 22 in terms of perimeter, diameter, grain length, and grain width (Fig. S1; Table S6). However, a significant difference was observed in TKW (Table S6). In Ningchun 4 background (44.84 ± 0.26 g), the TKW of *TaWaxy-a* (44.10 ± 0.54 g) and *TaWaxy-b* (45.88 ± 0.54 g) mutants was slightly increased or showed little difference, while the TKW of other mutants was significantly reduced (37.71–41.16 g). In Jimai 22 background (46.58 ± 0.61 g), the TKW of *TaWaxy-a* (49.47 ± 0.56 g) and *TaWaxy-d* (47.14 ± 0.55 g) mutants showed a slight increase, and the value of other mutants exhibited a significant reduction (40.10 to 45.15 g).

3.4. Dynamic change of starch granules in TaWaxy-edited mutants during grain development

Wheat grain starch contains two types of starch granules: A-type (diameter > 10 μm) and B-type (diameter 0–10 μm). In this study, the dynamic changes in starch granules were observed in *TaWaxy-abd-NC* mutant and wild type Ningchun 4 from 12 to 28 DPA by TEM. The results showed that B-type starch granules formed after 12 DPA, and the *TaWaxy-abd-NC* mutant (57.33–90.60 %) had more B-type starch granules than its wild type (24.76–85.29 %) (Fig. S2, Table S7). However, the constrictions in the mutants tended to be more unevenly distributed and fewer than that in Ningchun 4. It is clearly observed that the differences in starch granules between Ningchun 4 and the *TaWaxy-abd-NC* mutant were more pronounced at 28 DPA.

The starch granules in the seven different mutant types and their wild type Jimai 22 observed at 28 DPA by TEM showed that the number of B-type starch granules was increased in all the mutant types (83.27–96.15 %), and the distribution of constrictions in the mutants was more uneven than it in the wild type (Fig. 2A, Table S8). The images by SEM revealed a significant increase in the number of B-type starch granules across all the seven mutant types (63.75–74.86 %) in comparison with their wild type Jimai 22 (57.38 ± 1.69 %) at 28 DPA (Fig. 2B, Table S8), especially for mutants *TaWaxy-b-JM*, *TaWaxy-ab-JM*, *TaWaxy-bd-JM*, and *TaWaxy-abd -JM*, which was consistent with the results by TEM observation.

3.5. Investigation of starch content and viscosity characteristic in TaWaxy-edited mutants

Starch granule staining was normally employed to assess waxy protein existing or deficiency in cereal grains (Nakamura et al., 1995). When stained with an I_2-KI solution, the endosperms of the mutant types *TaWaxy-abd*, *TaWaxy-ab*, and *TaWaxy-bd* with the genetic backgrounds of the two commercial varieties (Ningchun 4 and Jimai 22) turned red-brown, indicating a significant decrease in AC. Interestingly, the endosperm of *TaWaxy-b-JM* also turned red-brown, while *TaWaxy-b-NC* turned blue. The endosperms of the other mutants and the wild types turned blue (Fig. S3). Moreover, SDS-PAGE was used to detect waxy proteins in Ningchun 4 and its corresponding mutants. Although the molecular weights of different waxy proteins are relatively similar and therefore difficult to distinguish, the expected absence of specific waxy proteins was still observable in the respective mutants. These protein-level changes were consistent with the DNA genotyping results, further confirming the accuracy of the mutations (Fig. S4).

Compared to the wild types (18.61 % - 20.18 %), the AC in all of the *TaWaxy* mutants was significantly reduced (Fig. 3A, Table S9). The AC in *TaWaxy-abd*, *TaWaxy-ab*, and *TaWaxy-bd* mutants was less than 0.4 % in both genetic background. In Ningchun 4 genetic background (20.18 ± 0.23 %), the AC of the mutants decreased in the following order, ranging from 44.95 % to 5.60 %: *TaWaxy-a-NC*, *TaWaxy-d-NC*, *TaWaxy-b-NC*, and *TaWaxy-ad-NC*. Similarly, in the Jimai 22 background (18.61 ± 0.17 %), AC also followed a descending trend, ranging from 44.65 % to 26.70 %: *TaWaxy-d-JM*, *TaWaxy-a-JM*, *TaWaxy-ad-JM*, and *TaWaxy-b-JM* (Fig. 3A, Table S9). However, the ranking pattern was slightly different from that observed in Ningchun 4. The content of amylopectin

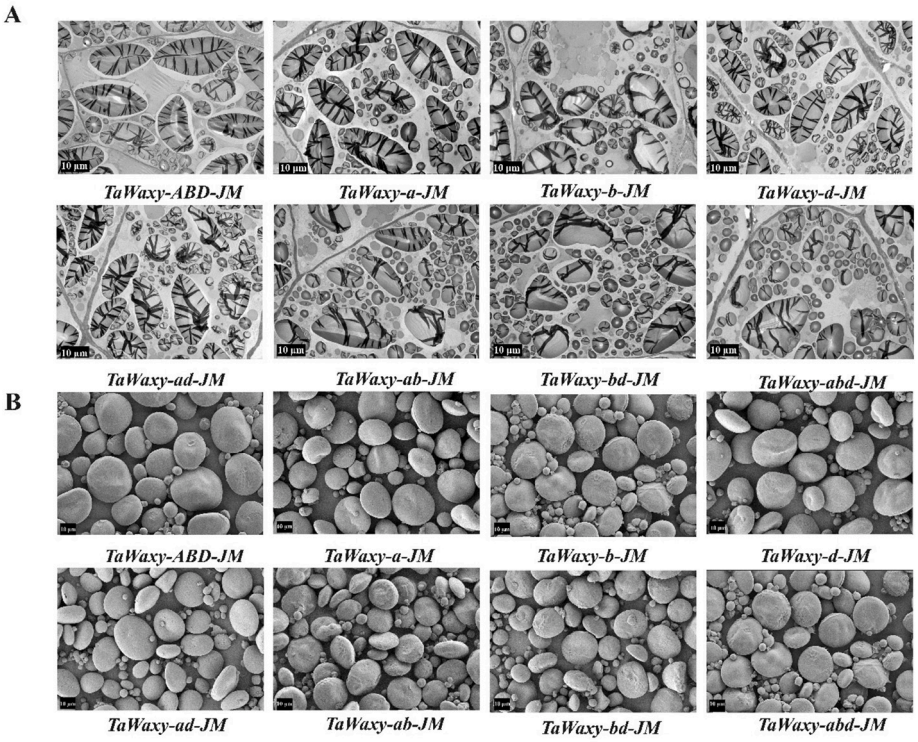

Fig. 2. Observation of starch granules in *TaWaxy* mutants and wild type grains using TEM and SEM. A, B: Starch granule morphology of Jimai 22 and its corresponding *TaWaxy* mutants, observed by TEM and SEM (scale bars = 10 μm).

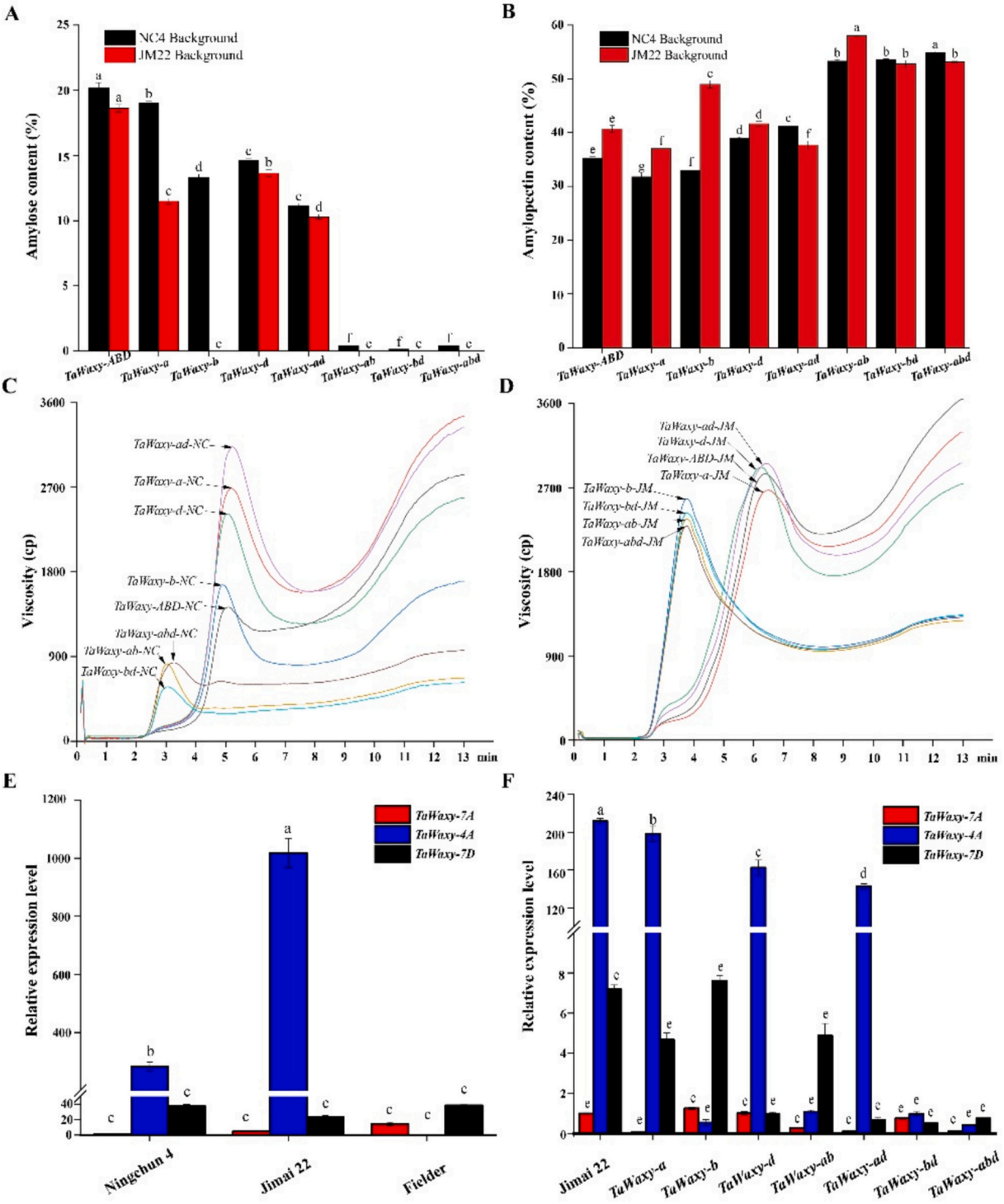

Fig. 3. Characterization of starch content, physicochemical properties and quantitative *TaWaxy* expression in *TaWaxy* mutants and their wild types. A, B: Amylose and amylopectin contents in the grains of *TaWaxy*-edited mutants and their corresponding wild types, Ningchun 4 and Jimai 22. Different lowercase letters indicate statistically significant differences ($p < 0.05$, Student's *t*-test). Error bars represent standard deviation (SD, $n = 3$). C, D: RVA profiles of Ningchun 4, Jimai 22, and their respective *TaWaxy*-edited lines. E, F: Comparative analysis of *TaWaxy* gene expression in three wheat cultivars (Ningchun 4, Jimai 22, and Fielder) and CRISPR-edited Jimai 22 mutants.

in *TaWaxy-ab*, *TaWaxy-bd*, and *TaWaxy-abd* mutants with both genetic backgrounds was significantly increased (Fig. 3B). The total starch content was decreased in all the mutants (Table S9).

RVA analysis—a technique used to measure the pasting characteristics and viscosity, as well as to evaluate starch quality and its eating and cooking quality—revealed significant differences in gelatinization properties among different mutants (Fig. 3C, D; Table S10). The mutants (*TaWaxy-abd*, *TaWaxy-ab*, and *TaWaxy-bd*) which exhibited significantly reduced AC, showed lower peak viscosities, shorter peak times, and lower pasting temperature, suggesting that excessively low AC negatively impacts flour quality. However, the *TaWaxy-ad* mutants in both genetic background displayed the highest peak viscosity and peak time, which may indicate that this mutation could improve the flour quality of wheat.

3.6. Expression analysis of TaWaxy genes in different wheat cultivars and mutants

Using RT-qPCR analysis, we investigated the expression profiles of *TaWaxy* genes in the endosperm at 28 DPA across three wheat cultivars (Ningchun 4, Jimai 22, and Fielder) and CRISPR-edited mutants derived from Jimai 22. The results revealed significant allelic differences in gene expression among the tested genotypes. Notably, in Fielder, the absence of the *TaWaxy-B1* locus resulted in no detectable expression. In contrast, *TaWaxy-4 A* showed significantly higher expression in Jimai 22 (1017.68 ± 50.03), approximately 3.6 times that observed in Ningchun 4 (282.94 ± 15.44) (Fig. 3E). In Jimai 22 and Ninghcun 4, the expression level of *TaWaxy-4 A* was significantly higher than that of *TaWaxy-7 A* and *TaWaxy-7D*. *TaWaxy-7 A* consistently showed the lowest expression across all cultivars, with expression levels decreasing in the order: Fielder > Jimai 22 > Ningchun 4. Compared with wild-type Jimai 22, all corresponding knockout mutants displayed sharply reduced or nearly undetectable expression levels of the targeted *TaWaxy* genes (Fig. 3F).

3.7. End-use quality analysis of different TaWaxy-edited mutant types

The end-use quality of the *TaWaxy*-edited mutants and their corresponding wild types Ningchun 4 and Jimai 22 was evaluated. Due to

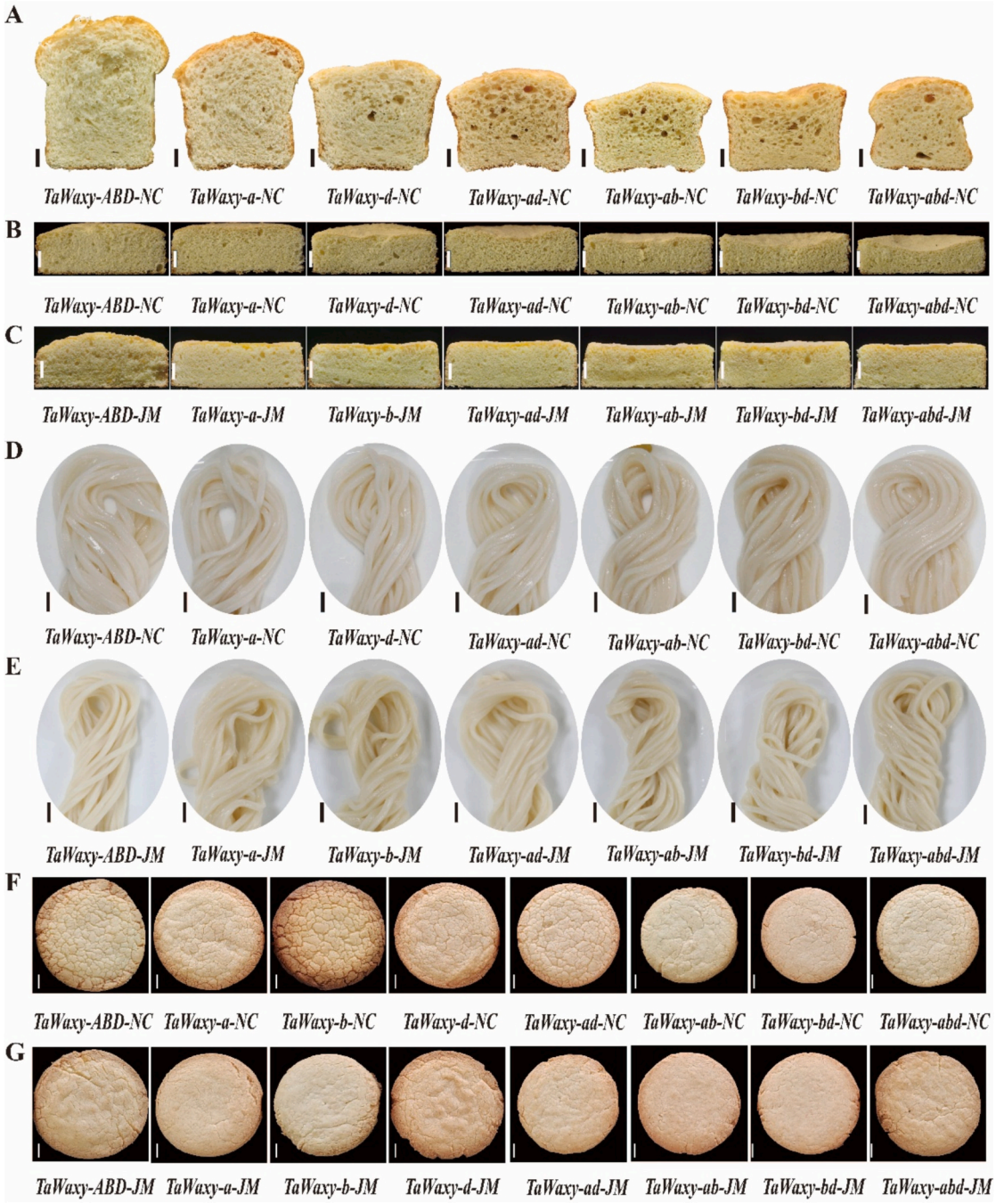

Fig. 4. Processing quality analysis of *TaWaxy*-edited mutants and their corresponding wild types, Ningchun 4 and Jimai 22. A, B, D, F: Bread, cakes, noodles, and cookies prepared using *TaWaxy-edited-NC* mutants and their wild type Ningchun 4, respectively (Scale bar = 1 cm). C, E, G: Cakes, noodles, and cookies prepared using *TaWaxy-edited-JM* mutants and their wild type Jimai 22. (Scale bar = 1 cm).

limited seed availability, only the cookie quality of the *TaWaxy-b-NC* and *TaWaxy-d-JM* mutants was tested. The rheological properties of the dough showed that the mutants of Ningchun 4 had significantly lower farinograph quality number (31.3–51.7 mm), dough development time (2.5–3.2 min), and stabilization time (0.9–3.4 min), but the water absorption rate of flour was significantly increased (64.6–74.2 %), and the degree of softening was also significantly increased (186.0–223.3 FU) except that for *TaWaxy-a-NC* (130.7 ± 2.6 mm) compared with Ningchun 4 (120.3 ± 3.2 mm) (Fig. S5; Table S11). The bread-processing quality test revealed that both the bread volume (375.00–573.33 ml) and scores (28.67–51.67) for the mutants derived from Ningchun 4 (750.00 ± 5.77 ml, 76.00 ± 0.58) were significantly reduced (Fig. 4A; Table S12). In the cake-processing quality test, all mutants with the two genetic backgrounds exhibited smaller volumes (393.33–536.67 ml) and lower scores (31.33–75.00) compared to their respective wild types (25.3 ± 0.3, 13.3 ± 0.3) (Fig. 4B, C; Table S13). For noodle-processing quality, some key quality parameters such as viscoelasticity (19.7–24.3), smoothness (10.3–11.3), and taste were significantly lower in the *TaWaxy-edited-NC* mutants compared to their wild-type Ningchun 4. However, the smoothness of noodles was notably improved in the *TaWaxy-edited-JM* (12.7–13.4) mutants compared to their wild type Jimai 22 (11.8 ± 0.2). Additionally, the viscoelasticity and total noodle scores were enhanced in the *TaWaxy-ad-JM* mutant relative to Jimai 22 (23.8 ± 0.2, 79.1 ± 0.1) (Fig. 4D, E; Table S14). The cookie-processing quality analysis showed that the cookie diameter in most *TaWaxy*-edited mutants was increased, in which the highest diameter observed was 80.15 mm in the *TaWaxy-b-NC* mutant, compared to 78.46 mm in wild type Ningchun 4 (Fig. 4F, G; Table S15). These results suggest that silencing wheat *TaWaxy* genes led to a significant decrease in end-use quality, emphasizing the crucial role of the *TaWaxy* genes in determining flour end-use quality, particularly for bread and cake processing.

4. Discussion

4.1. TaWOX5-mediated development of new germplasm in commercial wheat varieties

It is well known that wheat transformation mediated by *Agrobacterium* has strong genotype-dependency. The *WOX5* transcription factor is known to regulate stem cell homeostasis in plants and is also closely associated with organ regeneration processes (Zhai & Xu, 2021). *TaWOX5*, a wheat homolog, has shown promise in enhancing transformation efficiency across various *Triticeae* species, including *Triticum monococcum*, barley, rye, and triticale, as well as in maize (Chang et al., 2024; Wang et al., 2022). Jimai 22, the most widely cultivated wheat variety in China, with an annual planting area exceeding two million hectares, presents a significant challenge for transformation due to its poor quality callus derived from immature embryos. The *TaWOX5* gene can significantly improve transformation efficiency of Jimai 22 (Wang et al., 2022). In this study, the *TaWOX5*-CRISPR-Cas9 vector was used to generate *TaWaxy* gene mutants in Jimai 22. This vector enables direct genome editing in commercial wheat varieties, facilitating the development of new wheat germplasm.

4.2. The role of TaWaxy in determining amylose content and its underlying mechanism

The *TaWaxy* gene plays a crucial role in determining wheat starch quality and processing characteristics. Numerous studies have consistently demonstrated a dose-dependent effect of the *TaWaxy* gene on AC (Guzmán & Alvarez, 2016). Specifically, the TaWaxy-B1 protein has the most pronounced effect on amylose synthesis (Wickramasinghe & Miura, 2003), while the absence of the TaWaxy-A1 or TaWaxy-D1 protein does not always lead to a significant reduction in AC (Kim et al., 2003). Our study further validated these findings by using *TaWaxy* gene mutants in both genetic backgrounds Jimai 22 and Ningchun 4 generated *via* genome editing technology. In particular, the absence of TaWaxy-B1 protein in both Ningchun 4 and Jimai 22 resulted in a significant decrease in AC, and the *TaWaxy-b-JM* showed amylose levels approaching 0 %. On the other hand, the lack of TaWaxy-A1 or TaWaxy-D1 protein caused a relatively smaller decrease in AC. However, the impact of TaWaxy-B1 protein varied considerably among different wheat varieties. A previous study reported that the AC in Fielder, a variety that naturally lacks TaWaxy-B1 protein, was 17.2 % (Zhang et al., 2021). In contrast, in this study, the AC in the *TaWaxy-b-NC* mutant was 13.3 %, while it in the *TaWaxy-b-JM* mutant was nearly 0 % (Table S9). Those findings underscore that the contribution of the TaWaxy-B1 protein to AC differs across varieties, suggesting that AC is also influenced by the genetic background, which is consistent with previous research (Zhang et al., 2005).

Previous studies have demonstrated a significant positive correlation between AC and GBSSI activity (Zi et al., 2018). In EMS-induced mutants with high amylose content, the expression levels of key starch synthesis genes, including GBSSI and its homologs, were found to be higher than those in the parental lines (Mishra et al., 2016). To further investigate the functional divergence of *TaWaxy-B1* across different wheat cultivars, we analyzed the expression profiles of the three *TaWaxy* homoeologs. The results showed that *TaWaxy-4 A* exhibited significantly higher expression in Jimai 22 (1017.68 ± 50.03), approximately 3.6 times that observed in Ningchun 4 (282.94 ± 15.44) (Fig. 3E), while Fielder naturally lacks this gene. In both Jimai 22 and Ningchun 4, *TaWaxy-4 A* was the most dominantly expressed homoeolog, surpassing *TaWaxy-7 A* and *TaWaxy-7D*. Functional analysis of *TaWaxy* knockout lines provided further insight into its role in regulating AC. In Jimai 22-derived mutants such as *TaWaxy-b-JM*, *TaWaxy-abd-JM*, *TaWaxy-ab-JM*, and *TaWaxy-bd-JM*, silencing of *TaWaxy-4 A* resulted in a dramatic reduction in total *TaWaxy* transcript levels, leading to nearly complete depletion of amylose and a fully waxy phenotype. These results strongly support a positive regulatory relationship between *TaWaxy* expression—especially *TaWaxy-4 A*—and amylose synthesis. In Ningchun 4, the expression level of *TaWaxy-7D* was higher than that in Jimai 22, whereas *TaWaxy-4 A* expression was lower. As a result, the knockout of *TaWaxy-4 A* in the *TaWaxy-b-NC* mutant did not cause a complete loss of AC, and the grain did not exhibit a fully waxy phenotype. Only when additional homoeologs were knocked out (as in *TaWaxy-abd-NC*, *TaWaxy-ab-NC*, and *TaWaxy-bd-NC*) did the mutants exhibit a fully waxy endosperm. These findings underscore that the overall expression levels and functional redundancy among *TaWaxy* homoeologs collectively determine AC, in alignment with previous reports.

4.3. Genetic regulation of starch granule morphogenesis and its integration with GBSSI function

Wheat seeds are characterized by a bimodal distribution of starch granules, consisting of large, lenticular A-type granules (>10 μm) and smaller, spherical B-type granules (<10 μm) (Wei et al., 2010). The development of these granules is tightly controlled by a network of genes that orchestrate the timing, size, and number of granules. For example, *B-GRANULE CONTENT 1* (*BGC1*) selectively suppresses A-type granule initiation during early endosperm development and promotes B-type granule formation at mid-developmental stages (Chia et al., 2020). This dual regulatory role is reminiscent of the phenotype observed in *STARCH SYNTHASE 4* (*SS4*) mutants, where loss of *SS4* similarly alters granule morphology (Hawkins et al., 2021). In addition to granule morphology regulators, protein–protein interactions also play critical roles in starch biosynthesis. *Protein Targeting To Starch 1* (*PTST1*) interacts directly with *GBSSI* through its coiled-coil domain, facilitating its correct localization and function during amylose synthesis (Sharma et al., 2022). Meanwhile, plastidial α-glucan phosphorylase modulates B-granule size and abundance *via* interaction with BGC1 (Kamble et al., 2023). Temporal regulation is further mediated by *MYOSIN-RESEMBLING CHLOROPLAST PROTEIN* (*MRC*), whose deficiency leads to

premature initiation of B-type granules (Chen, Chen, et al., 2024). Starch biosynthesis is also subject to transcriptional regulation by complex gene networks. For instance, *TabHLH95* and *TaNF-YB1* cooperatively regulate the expression of key biosynthetic genes, while the *TaDL-TaB3-TaNF-YB1* complex activates the expression of *TaSUS2* and *TaAGPL2*, both essential for starch precursor supply (Liu et al., 2023, 2025). In parallel, *NF-Y* trimeric complexes modulate starch synthesis indirectly by repressing *TaNAC019*, a known negative regulator (Chen, Zhao, et al., 2024).

Together, these findings suggest that the synthesis and deposition of amylose in wheat is not only governed by *TaWaxy* (*GBSSI*) expression but also intricately coordinated with granule morphogenesis, protein interaction networks, and multilayered transcriptional controls. The differential expression and interaction of *TaWaxy* homoeologs among cultivars directly impacts AC and starch granule traits, ultimately influencing end-use quality in wheat.

4.4. Effects of TaWaxy gene mutants on end-use quality and their potential application value

Noodles, an integral part of Chinese culinary heritage, account for approximately 40 % of total wheat consumption in the country (Liu et al., 2001). Despite the cultural and economic significance, scientific research on noodle quality lags notably behind of bread quality. The relationship between wheat quality traits, particularly starch characteristics and noodle quality remains insufficiently explored. Generally, the wheat varieties with low AC are preferred to be cultivated for noodle making purpose (Yamamori et al., 1992). In this study, quality assessments for bread and cake making revealed a decrease of all quality parameters in the *TaWaxy*-edited mutants. However, the results from noodle-processing test exhibited a significant variation across the different materials. Ningchun 4, a dominant spring wheat variety in northwest China, is particularly well-suited for noodle production and achieved the highest comprehensive noodle quality score in this study (Table S14). Yet, all the *TaWaxy*-edited mutants from Ningchun 4 showed a decline trend in noodle quality scores. In contrast, Jimai 22, a variety that typically scores moderately in noodle quality, exhibited better noodle quality when paired with the *TaWaxy-ad-JM* mutant. These findings emphasize that AC plays a key role in noodle quality. However, noodle quality is a complex and multifactorial trait. The development of new wheat germplasm specifically optimized for noodle production cannot be solely achieved by modulating AC. Other factors must also be considered in breeding programs aimed at improving noodle quality.

It is widely recognized that the wheat varieties with an AC of less than 1 % is classified as waxy wheat. The potential applications for waxy wheat are extensive, given its unique properties. Waxy wheat flour is normally served as a foundational material for modified starchy foods. By blending fully waxy wheat flour with the flour containing varying levels of amylose (ranging from 0 % to 30 %), a diverse range of products can be produced to meet the demands of both food and non-food industries. Notably, waxy starch significantly improves the freshness and shelf life of flour-based products such as bread, due to its superior water-holding capacity and slower retrogradation and staling rates (Graybosch, 1998). This makes it especially valuable for the production of refrigerated and frozen foods. Moreover, the addition of waxy wheat flour to common wheat flour resulted in a reduction of the total acidity and ester, and fusel oil content in the brewed white liquor, while its flavor is enhanced (Zhao et al., 2013). Since the first waxy wheat was successfully developed by Nakamura et al. (1995), previous efforts to breed waxy wheat have not been focused on main commercial wheat varieties. This study, in contrast, aims to fill this gap by utilizing gene editing technique to create new waxy wheat germplasm based on the application of the most widely cultivated winter and spring wheat varieties Jimai 22 and Ningchun 4 in China. In addition, this research has led to the development of two novel mutants, *TaWaxy-ad-JM* and *TaWaxy-b-NC*, in which the quality of noodles and biscuits was dramatically improved, respectively. This advancement enriches the wheat germplasm pool and provides new options for both consumers and industries.

5. Conclusion

In this study, we successfully generated seven distinct TaWaxy mutant types in the commercial wheat variety Jimai 22 using the *TaWOX5*-mediated CRISPR/SpCas9 system. Additional *TaWaxy* mutants were developed in Ningchun 4, with amylose contents (ACs) ranging from 0 to 19.05 %. All Jimai 22 mutants were confirmed to be transgene-free, while three transgene-free types were obtained in Ningchun 4. Functional analysis revealed that *TaWaxy-B1* plays a predominant role in amylose synthesis, while the loss of *TaWaxy-A1* or *TaWaxy-D1* had variable effects on AC. Notably, *TaWaxy-abd*, *TaWaxy-ab*, and *TaWaxy-bd* mutants showed typical waxy wheat traits. Processing quality evaluation demonstrated that the *TaWaxy-ad-JM* mutant significantly improved noodle-making quality, whereas the *TaWaxy-b-NC* mutant was better suited for cookie production. Overall, our findings provide a toolkit of gene-edited wheat germplasms with tailored amylose levels, offering practical breeding resources for product-specific wheat improvement.

CRediT authorship contribution statement

Yuliang Qiu: Writing – original draft, Investigation, Data curation, Conceptualization. **Xi Li:** Validation, Investigation, Data curation. **Ming Fan:** Resources, Investigation, Formal analysis. **Huali Tang:** Methodology, Investigation. **Shuangxi Zhang:** Validation, Project administration. **Weihong Huang:** Methodology, Formal analysis. **Zhiyang Han:** Data curation. **Surong Wang:** Validation, Investigation. **Hao Peng:** Validation, Formal analysis. **Yonggui Xiao:** Project administration, Conceptualization. **Xingguo Ye:** Writing – review & editing, Supervision. **Ke Wang:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

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.

Acknowledgements

This research was supported by the grants from the Biological Breeding-National Science and Technology Major Project (2024ZD04077), and the Science and Technology Department of Ningxia in China (2022BBF02039).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2025.124105.

Data availability

Data will be made available on request.

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