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Creating of novel *Wx* allelic variations significantly altering *Wx* expression and rice eating and cooking quality

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ABSTRACT

Granule-bound starch synthase I (GBSSI) encoding gene *Waxy* (*Wx*), which largely regulates the amylose content of rice grains, is a master module determining rice eating and cooking quality (ECQ). Fine-tuning amylose level of grains is an ideal strategy to improve rice quality. Through fine editing of Wx^a promoter and 5′UTR by CRISPR/Cas9 system, we created 14 types of novel *Wx* allelic variations, of which MT7 and MT13 were able to alter *Wx* expression and amylose content of grains. MT7 showed fragment deletion and base insertions in CAAT-boxes, hardly detectable expression levels of GBSSI mRNA and protein, and generated 5.87% amylose in grains. MT13 had fragment deletions in the A-box and the TATA-box, low expression levels of GBSSI mRNA and protein, and generated 9.61% amylose in grains. Besides of the amylose content, MT7 and MT13 significantly reduced protein content and increased lipid content of grains compared with Wx^a. A comparison of MT7, MT13 and other allelic lines demonstrated the importance of base insertion around the second CAAT-box and 31bp-deletion following the second TATA-box in modulating *Wx* expression. Thus, our study generated two novel *Wx* allelic variations which significantly alter *Wx* expression and amylose content of rice grains, providing not only new germplasms for soft rice breeding, but also insights into candidate *cis* elements of *Wx*.

1. Introduction

Rice (*Oryza sativa*) is grown in over 100 countries and regions worldwide for feeding over 50% of the global population, and is a unique cereal consumed primarily as intact starch endosperms (kernels), which is mainly composed of amylose and amylopectin (Park et al., 2019; Preiss and Sivak, 1998). The amylose content (AC), which is about 20% in conventional rice, is arguably the most important quality indicator of rice, particularly with respect to eating and cooking qualities (ECQ) (Juliano, 1998). Based on the AC, rice cultivars are commercially classified into five categories: glutinous (0–5%), very low (5–12%), low (13–20%), intermediate (21–25%), and high amylose (≥26%) (Juliano, 1998; Pandey et al., 2012). In general, after cooking, high AC rice exhibits a firm and separated texture, whereas low AC rice is characterized by elastic, glossy and sticky grains (Li et al., 2016; Zhang et al., 2013). Different countries and regions have different cultural favors for rice categories. In East Asia countries like China, Japan and Korea, rice with low AC and sticky and soft texture are more favored by consumers, while individuals from India and Bangladesh prefer firm and separate rice with high AC (Custodio et al., 2019; Misra et al., 2019). So, cultivating rice varieties with appropriate ACs is a major objective for breeding.

The amylose synthesis in endosperm is determined by the granule-bound starch synthase I (GBSSI) encoded by gene *Waxy* (*Wx*) (Sano, 1984; Wang et al., 1995). The diversity of AC in rice is mainly due to the allelic variations of *Wx* (Tian et al., 2009; Traore et al., 2011; Zhang et al., 2017). To date, at least ten natural *Wx* alleles including Wx^{lv}, Wx^a, Wx^{in}, Wx^b, $Wx^{op/hp}$, Wx^{mw}, Wx^{la}, Wx^{mq}, Wx^{mp}, and *wx* have been identified in rice cultivars (Cai et al., 1998; Larkin and Park, 2003; Liu et al., 2009; Mikami et al., 2008; Sato et al., 2002; Wanchana et al., 2003; Yang et al., 2013; Zhang et al., 2019a, 2021; Zhou et al., 2021). The higher the level of *Wx* alleles expression and GBSSI enzyme activity the rice cultivar shows, the higher AC it has (Kumar and Khush, 1986; Liu et al., 2014). It is expected that mild regulation of AC can be implemented by fine-tuning of *Wx* expression to improve rice ECQ. Recent studies have

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demonstrated that finely tuned *Wx* expression is also crucial for head rice yield (Deng et al., 2022; Zhu et al., 2024).

Because of the all-rounder in grain quality, *Wx* has been considered as a master module for rice quality improvement programs (Deng et al., 2022; Tran et al., 2011; Zhang et al., 2012a). In recent years, genome editing technologies have provided efficient ways for high-precision molecular breeding of crops, and many target traits can be designed and improved accurately (Huang et al., 2020; Oliva et al., 2019; Zeng et al., 2020). The clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9) system has been used to knock out or fine-tune *Wx* expression (Ma et al., 2015). Existed literatures have demonstrated that editing different regions of *Wx* gene can produce materials with varying AC, which inevitably affects rice ECQ. By targeting *Wx* coding sequences or transcription start site (TSS), several glutinous lines were created with no significantly altered agronomic traits (Fu et al., 2023; Fei et al., 2019; Zhang et al., 2018, 2022). However, this kind of function-knockout strategy has much possibility to produce only null alleles and thus generate glutinous lines (Zhang et al., 2018). Soft rice with moderate AC is expected in rice quality improvement. The *cis*-regulatory elements in upstream region of *Wx* have been confirmed the effects on its gene expression. Editing the intron splicing site in *Wx* 5′untranslation region (5′UTR) downregulated its expression and thus fine-tuned grain AC (Yang et al., 2022; Zeng et al., 2020). And it is also effective to create moderate lines through targeting the *cis*-regulatory elements (CREs) of *Wx* promoter region (Huang et al., 2020; Zeng et al., 2020). However, more novel variations with the ability to influence *Wx* expression and thus moderate AC are desired to fine-tune rice quality for different breeding goals.

In this study, we used CRISPR/Cas9 system to finely edit *Wx* promoter and 5′UTR. We created 14 distinct variations in the upstream region, of which MT7 and MT13 significantly downregulated expression of *Wx* at mRNA and protein. MT7 was a very low expression allele, but differing from the null allele *wx*. MT13 expressed mRNA and protein of *Wx* at low levels. Thus, MT7 rice was more similar to glutinous rice with white and fully opaque appearance, and MT13 rice was semi-translucent soft rice, but they were significantly different from known glutinous rice and soft rice in ECQ, respectively. Our study provides two novel *Wx* allelic variations and germplasms for breeding.

2. Materials and methods

2.1. Plant materials and growth conditions

In this study, we obtained 14 homozygous editing lines, named MT1-MT14, showing different *Wx* allelic variations through gene editing technology. Near-isogenic lines (NILs) with different alleles of *Wx* (NIL-Wx^a, NIL-Wx^b, and NIL-*wx*), which were developed in the genetic background of *indica* variety Yangfunuo 4 (YFN 4) (Zhang et al., 2012b), were used as controls. All materials were planted in paddy fields in Beijing during summer, and in Lingshui, Hainan Province, during winter.

2.2. Vector construction and transformation

Mutants were produced in the background of NIL-Wx^a. Wx^a in NIL-Wx^a was used to create novel *Wx* alleles by CRISPR/Cas9-mediated promoter and 5′UTR editing. After the CREs in Wx^a promoter sequence predicted by Plant-CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot, 2002), we designed fifteen target sites (T1-T15, Table S1) by CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/news.php) and divided them into two groups: T1-T7 and T10 were the first group, and T8-T15 were the second group. Each group was integrated into a CRISPR/Cas9 targeted genome editing tool, as described by Ma et al. (2015), and the two plasmids were transformed into *Agrobacterium* separately. Transgenic plants were generated from Wx^a-NIL calli via *Agrobacterium*-mediated transformation and selected by culture medium supplemented with hygromycin (50 mg/ml). Transgene-free plants were selected through polymerase chain reaction (PCR)-mediated examination of hygromycin-resistant gene and *Cas9* gene. Mutations of positive plants were identified by comparing the target site sequences with those of Wx^a-NIL by sequencing the PCR products. The primer sequences used in these experiments were listed in Table S2. The finally obtained 14 homozygous editing lines showing different *Wx* allelic variations were named MT1 to MT14.

2.3. Examination of mRNAs and proteins

Total RNAs were isolated from mature grains by using RNAprep Pure Plant kit (TIANGEN, Beijing, China), treated with RNase-free DNase I, and used to synthesize cDNA with SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on an ABI StepOnePlus™ instrument by using Power SYBR Green PCR Mastermix (ABI, USA). All assays were followed the manufacturer's instructions.

GBSSI proteins were extracted from endosperms of mature grains. The endosperms were supplemented with buffer A (0.055 M Tris-HCl, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, pH 6.8) (1 mL/10 endosperms), grind evenly, and pass through a 400-mesh sieve. 1 ml of buffer A was added to the filtrate and shook well. After centrifuging at 1500×*g* to remove the supernatant, 1 ml of acetone was added to the sediment and shook well. After centrifugation and vacuum drying, 5 mg extract was mixed with 50 μL buffer A and performed a boiling water bath. After the mixture cooling down, 100 μL buffer A was added into the mixture and gently mixed. After centrifugation at 12,000×*g* for 5 min, the supernatant was subjected to 10% SDS-PAGE. The proteins in gels were visualized by Coomassie Brilliant Blue staining. The grey value of the protein bands was measured by using the ImageJ software.

2.4. Quantification of amylose

Iodine colorimetry was used to determine the amylose content through measuring the absorbance of the purple complex formed between iodide ion and amylose (Juliano et al., 1981; William et al., 1958). The milled rice was sieved through a 100-mesh sieve. Exactly 10 mg milled rice flour was gelatinized 16 h in 100 μL of absolute alcohol and 900 μL of 1.0 M NaOH at 30 °C. Then, 5 μL of the gelatinized sample solution, 5 μL of distilled water and 990 μL of I_2/KI solution (2%) were mixed together and the mixture stood at room temperature for 20 min. Absorbance values of the reaction solutions at a wavelength of 680 nm were measured to calculate the ACs through standard curve.

2.5. Quantification of brown rice proteins and lipids

The grain protein content (PC) and lipid content (LC) were assayed according to GB 5009.5–2016. The brown rice flour passing 100-mesh sieve was used for determination and each sample was measured three biological replicates. PC was determined by Kjeldahl method by using an automatic Kjeldahl apparatus K-370 (BUCHI, Switzerland) and a nitrogen conversion factor of 6.25 was used to calculate the PC. Using Soxhlet extractor, the LC in each brown rice flour sample was extracted with petroleum ether and calculated based on the weight difference before and after sample extraction.

2.6. Scanning electron microscopy of starch granules

The rice grains were broken by artificial percussion and cross-section was stuck on the copper nails. The morphology of endosperm and starch granules was observed by the scanning electron microscope (SEM).

2.7. Quantification of gel consistency

Gel consistency (GC) of grains was evaluated according to the procedure described in GB/T 22294-2008. 100 mg of the polished rice powder which passed through a sieve with a pore size of 0.15 mm (100-mesh) was placed into a 15 × 150 mm tube and wetted with 0.2 mL of 95% ethanol containing 0.025% thymol blue and 2.0 mL of 0.2 M KOH. These tubes were incubated in boiling water for 8 min, cooled at room temperature for 5 min, and stand in ice water for 20 min. GC was measured in mL as the length (mm) of the gel spreading in the tubes when laid flat on the graph for 1 h.

2.8. Evaluation of starch pasting properties

Pasting properties of rice starch were measured by Rapid Viscosity Analyzer (RVA, Newport Scientific, Australia). We added 3.0g of refined rice flour and 25 mL of distilled water in the RVA-measurement aluminum tank. The program settings were as follows: quickly mix for 10 s; hold at 50 °C for 1 min; rise to 95 °C at a rate of 12 °C/min and hold at 95 °C for 5min; drop at a constant speed to 50 °C and hold at 50 °C for 1.5min. Except for the first step, the remaining parts were maintained at 160 rpm. Data analysis was carried out by TWC3.0 software (Thermal Cycle Win-Dows).

3. Results

3.1. Two novel Wx allelic variations

In order to create novel *Wx* allelic variations, we analyzed the upstream region of about 2.0-kb from Wx^a, which contains a 0.7-kb promoter region and a 1.3-kb intron-containing 5′UTR, and five potential *cis*-regulatory elements (CREs): one A-box, two TATA-boxes and two CAAT-boxes, were identified. Fifteen target sites covering these regions were designed for multiplex editing. These target sites were integrated into two plasmids: T1-T7 and T10 were the first group and T8-T15 were the second group, and transformed into *Agrobacterium* separately (Fig. 1A). Transgenic plants were generated from NIL-Wx^a via *Agrobacterium*-mediated transformation. Cas9-free plants from the transgenic (T_1) segregating families were PCR-selected and self-crossed to generate genotype homozygous lines. Finally, we got 14 homozygous editing lines showing different *Wx* allelic variations (MT1-MT14), which had similar phenotypes of plants, panicles and tillers with NIL-Wx^a (Wild-type, WT) (Fig. 1B; Fig. S1). Examination of AC demonstrated that the AC of MT7 and MT13 reduced significantly compared with that of NIL-Wx^a (Fig. 2A). MT7 showed a 91 bp-deletion around the first CAAT-box localized from 626 bp to 630 bp, 1bp-insertion in the second CAAT-box localized from 807 bp to 810 bp and three 1 bp insertions around the second CAAT-box. MT13 had a 4 bp-deletion in the A-box, a 186 bp-deletion around the first TATA-box, a 31 bp-deletion following the second TATA-box, and two fragment-deletions in the regions outside of these CREs (Fig. 1B). The two *Wx* allelic variations, which had not reported in previous studies about *Wx* non-coding region editing (Huang et al., 2020; Zeng et al., 2020; Zhang et al., 2022), represented two novel *Wx* alleles.

Then, we evaluated GBSSI mRNA and protein levels in the mature endosperm of MT7 and MT13. In MT7, the expression levels of GBSSI mRNA and protein were almost undetectable. MT13 expressed significantly lower levels of the mRNA and protein than those of Wx^a (Fig. 2B and C; Fig. S2). Thus, the two novel *Wx* alleles significantly down-regulated *Wx* expression both at mRNA and protein levels.

3.2. The two novel allelic variations great change contents of amylose, proteins and lipids in grains

We further evaluated grain phenotypes and starch features of MT7 and MT13 using NIL-Wx^a (WT), NIL-Wx^b and NIL-*wx* as control. MT7 and MT13 had similar plant and grain type to NIL-Wx^a, NIL-Wx^b and NIL-*wx*, although had some difference in 1 or 2 dimensions of grain type (grain length, grain width and grain thickness) among them (Fig. 3A–E; Table S3). The thousand-grain weight of MT7 and MT13 was similar to that of NIL-*wx*, and slightly lower than NIL-Wx^a and NIL-Wx^b (Fig. 3F).

Iodine colorimetry was used to estimate the AC of grains. This assay demonstrated that MT7 and MT13 had 5.87 ± 0.46% and 9.61 ± 0.25% amylose in respective grains (Fig. 4A; Table S3), which was significantly higher than that of NIL-*wx* (increasing by 166.12% in MT7 and 335.87% in MT13), and much lower than those of NIL-Wx^b (decreasing by 64.39% in MT7 and 41.68% in MT13) and NIL-Wx^a (decreasing by 78.83% in MT7 and 65.33% in MT13). Thus, MT7 and MT13 lines were in very low AC categories (5–12%). MT13 resembled the famous 'soft rice' *Wx* allele Wx^{mp} (7 ± 3%), which showed amino acid residue substitution at exons 4 and 10 as compared with Wx^a (Yang et al., 2013; Zhou et al., 2021). Following significantly decreased AC in kernels, PCs of MT7 and MT13 kernels were much lower than that of the donor NIL-Wx^a, but close to that of NIL-Wx^b; LCs in the two novel allelic lines were much higher than those of NIL-Wx^a and NIL-Wx^b, but lower than that of NIL-*wx* (Fig. 4B and C).

It is known that AC in rice endosperm determines the transparency degree of brown and milled rice, which is a major measurement of rice appearance and commercial quality (Zhang et al., 2017; L. Zhang et al., 2019b; Zhu et al., 2012). Unlike the transparent brown rice of the donor NIL-Wx^a, MT7 brown rice appeared white and fully opaque, and look like NIL-*wx* brown rice; MT13 brown rice was semi-translucent (Fig. 5A), which was similar with rice varieties carrying 'soft rice' allele Wx^{mp} reported previously (Xu et al., 2021; Zhou et al., 2021). SEM demonstrated that starch granules from NIL-Wx^a and NIL-Wx^b were morphologically similar to each other, both of which were polygonal with sharp edges and smooth surfaces with little cavities among the granules. Like NIL-*wx* starch granules, MT7 starch granules were irregular and had a great number of cavities in the granules. MT13 mainly contained regular starch granules with a few MT7-like starch granules. As compared to the glutinous rice, MT13 had an improved transparency because fewer air cavities within the starch granules (Fig. 5B).

3.3. Assessment of grain ECQ by RVA and gel consistency

Rice ECQ can be comprehensively evaluated by the pasting properties (RVA profile) and gel consistency (GC). RVA is a heating and cooling viscometer that provides information on the pasting properties (Maw and Kenji, 2018), which comprises mainly the RVA profile, especially breakdown value (BDV), setback value (SBV) and consistence value (CSV), can effectively reflect ECQ: high BDV, and low SBV and CSV are all representative characteristics of high ECQ rice (Bao et al., 2000; Manshan et al., 2005; He et al., 2015). The GC of rice flour is an important index of the colloidal properties of rice which reflects the extension length of rice gel after gelatinization and cooling: the higher the GC, the softer and better ECQ of rice (Gani et al., 2013).

We determined the RVA profiles of the two novel allelic lines and the donor NIL-Wx^a along with NIL-Wx^b and NIL-*wx* (Fig. 6, Table S4). Compared with NIL-Wx^a, both MT7 and MT13 showed significant changes in RVA profiles, with MT7 being closer to that of NIL-*wx* and MT13 being closer to that of NIL-Wx^b (Fig. 6A). MT13 line had the highest BDV values, and the lowest SBV value among the five lines. MT7 showed a BDV value between NIL-Wx^b and NIL-*wx* with much closed to NIL-Wx^b, and the second lowest SBV value the among the five lines (Fig. 6A and B). The CSV of the five lines showed the same trend as AC (Figs. 4A and 6C), which was in agreement with other studies suggesting a significant positive correlation between CSV and AC (Manshan et al., 2005). Furthermore, MT13 line had a GC value similar to NIL-Wx^b, while GC value of MT7 line was comparable with that of NIL-*wx*, which were both superior to that of NIL-Wx^a (increasing by 63.93% in MT7 and 49.01% in MT13 as compared with that of NIL-Wx^a) (Fig. 6E). These results indicated that the ECQ of two novel allelic lines had greatly

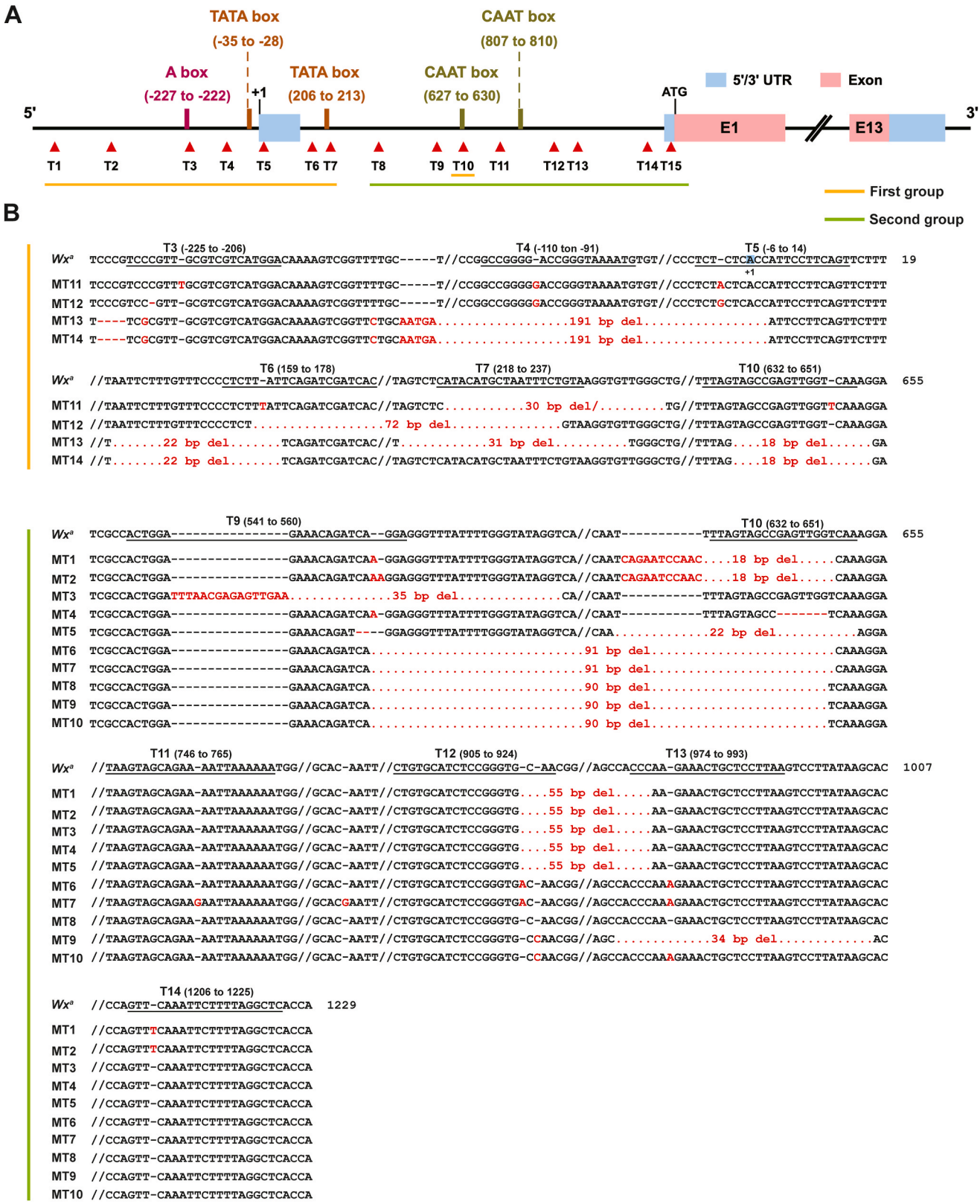

Fig. 1. Generating novel *Wx* alleles via editing promoter and 5′UTR by using CRISPR/Cas9. (A) Schematic diagram showing these predicted *cis*-acting elements and target sites (T1-T15) for editing promoter and 5′UTR of Wx^a. The red triangles indicate the positions of the targets. (B) Nucleotide variations of 14 homozygous mutant lines. The parts marked in red indicate areas which were different Wx^a. '-' and 'del', base deletion; '//' and non-displayed areas mean the same sequence with Wx^a. The sequences of T1, T2 and T15 were not displayed, for these areas were not edited. The letter 'A' in blue was transcription start site (TSS) and its location was defined as +1. The yellow and green lines were used to distinguish the targets connected to different vectors and the corresponding mutants. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

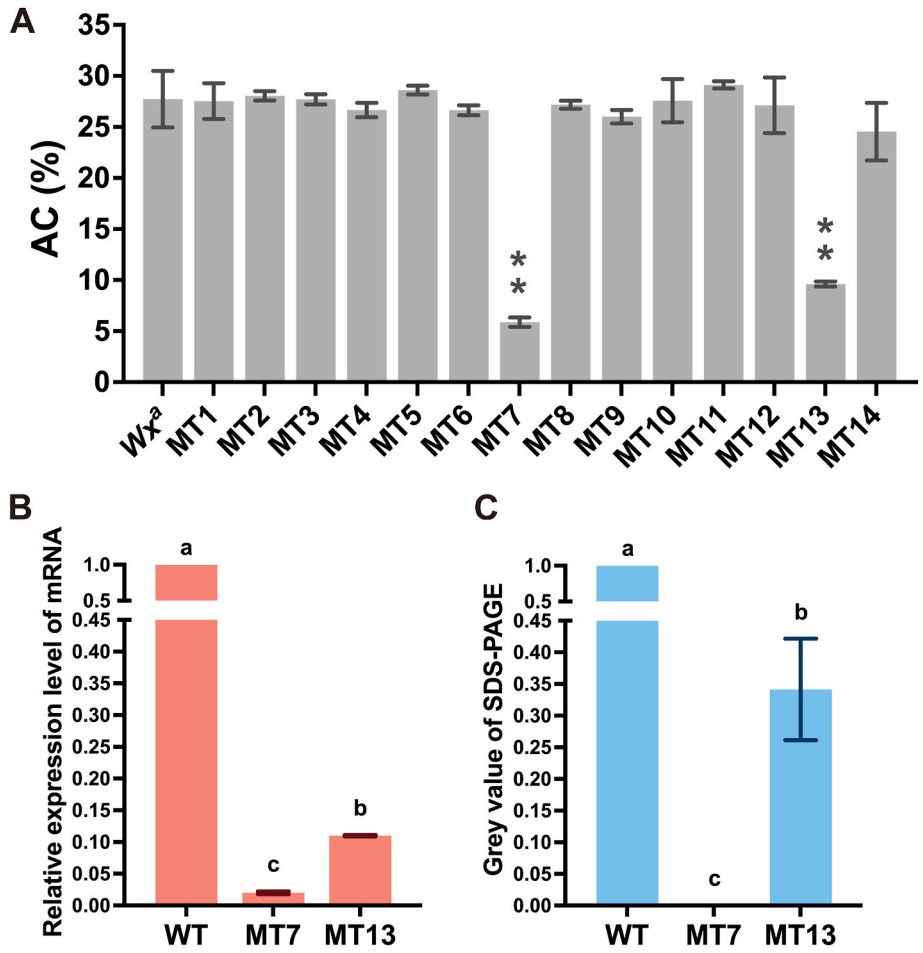

Fig. 2. Amylose content of the mutants and *Wx* expression levels of MT7 and MT13. (A) Amylose content (AC) in mature grains of edited lines and wild-type (WT) planted in Beijing. Error bars represent means ± SD ($n = 3$). ** indicates significant differences at $P < 0.01$ level (one-way ANOVA). (B) Relative expression level of *Wx* mRNA in grains of WT, MT7 and MT13. The value of WT was regarded as 1 unit. (C) GBSSI level in grains of WT, MT7 and MT13. The protein level was showed by grey value of protein bands resolved by SDS-PAGE and grey values were measured by using the ImageJ software. The value of WT was regarded as 1 unit. The SDS-PAGE images of proteins in Fig. S2. The error bars in (B) and (C) are means ± SD ($n = 3$) and the different letters indicate significant differences at $P < 0.05$ (one-way ANOVA).

improved than that of NIL-Wx^a.

4. Discussion

Wx has been considered as a master module for rice quality improvement programs (Deng et al., 2022; Tran et al., 2011; Zhang et al., 2012a), and its different allelic variations are used to create multifarious rice varieties with distinct ECQ for consume needs. The allelic variations fine-tuning *Wx* expression is urgently needed both to creating new ECQ germplasms and further understanding expression regulation of *Wx*. We created 14 distinct mutations in upstream region of *Wx*, of which MT7 and MT13 significantly downregulated expression of *Wx* at mRNA and protein levels. MT7 expressed *Wx* transcripts and proteins at hardly detectable levels, but generated 5.87 % amylose in grains; MT13 expressed about 1/10 mRNA and 1/3 protein of Wx^a, respectively, and generated 9.61 % amylose in grains. Both of them were in very low (5–12%) category.

ECQ is widely considered as a major criterion for the rice quality and the most important trait affecting consumer acceptability of rice (Cho et al., 2013; Maw and Kenji, 2018). AC, GC and RVA characteristic values, especially BDV, SBV, CSV of RVA, are used for comprehensive evaluation of rice ECQ due to their significant correlation with the texture of rice (Tan et al., 1999). It has been well known that low AC, high GC, high BDV, and low SBV and CSV are all representative characteristics of high ECQ rice with soft texture and great palatability, which is favored by Chinese rice consumers (Wang et al., 2003). In recent years, rice with lower amylose content (5%–12%) has been very popular. After steaming, this type of rice, with the transparency between conventional rice and glutinous rice, has a glossy and translucent appearance, a soft texture and good elasticity. So, it becomes known as 'soft rice' or 'semi-glutinous rice' (Shi et al., 2002; Wang et al., 2021). The two novel allelic lines, MT7 and MT13 with improved ECQ, had the characteristics of glutinous rice and soft rice, respectively. MT13 had a semi-translucent transparency with the GC value similar to NIL-Wx^b, while MT7 line was comparable with NIL-*wx* in not only the fully opaque endosperm but also the GC value (Figs. 5A and 6E). According to the RVA profiles (Fig. 6; Table S4), The BDV of MT7 was between NIL-Wx^b and NIL-*wx*, and that of MT13 was the highest among the five lines. The SBVs of the two novel allelic lines were lower than that of NIL-Wx^b and NIL-*wx*, while that of MT13 was the lowest among the five lines. The CSVs of MT13 was higher than that of MT7 and both of them were higher than that of NIL-*wx* and lower than that of NIL-Wx^b. It has been suggested that the hardness of rice is significantly positively correlated with the SBV, and negatively correlated with the BDV, while the stickiness of rice is significantly negative with CSV (Manshan et al., 2005). So, it can be inferred that the texture of MT7 exhibited softer yet less sticky characteristics compared with NIL-*wx*, while the texture of MT13 was stickier and softer than NIL-Wx^b. Therefore, we have created two novel

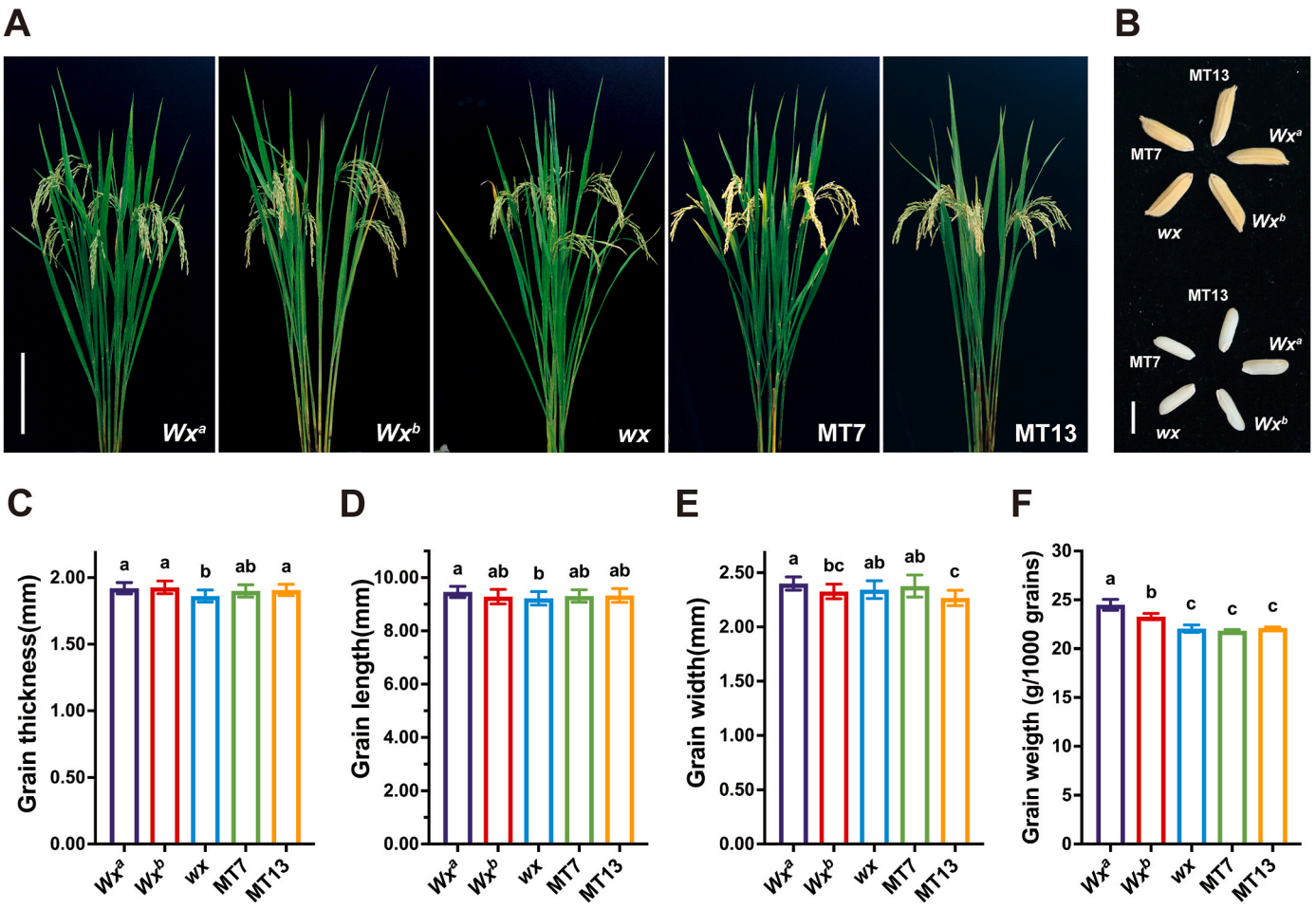

Fig. 3. Agronomic characteristic of the two novel *Wx* allelic lines MT7 and MT13, and the three references NIL-Wx^a, NIL-Wx^b and NIL-*wx*. (A) Plant phenotype at grain maturity stage. Bar = 20 cm. (B) Grain size and shape. Bar = 5 mm. (C–E) Grain thickness (C), length (D) and width (E) ($n = 20$). (F) 1000-grain weight. Error bars are means ± SD. The different letters indicate significant differences at $P < 0.05$ by one-way ANOVA.

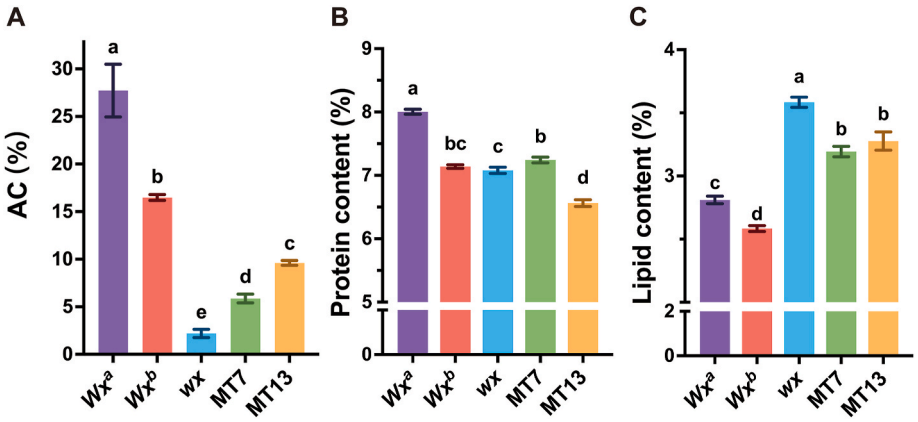

Fig. 4. Amylose content, protein content, lipid content of mature grains of the two novel *Wx* allelic lines MT7 and MT13 and the three references NIL-Wx^a, NIL-Wx^b and NIL-*wx*. (A) Amylose content (AC). (B) Protein content. (C) Lipid content. Error bars are means ± SD ($n = 3$). The different letters indicate significant differences at $P < 0.05$ by one-way ANOVA.

alleles, which can lead to different rice texture and ECQ, for diversified rice breeding needs.

Except for amylose, lipids and proteins are also the vital factors for ECQ. Lipids can affect the gelatinization properties of starch (Zhang et al., 2019c) and it is widely believed that within a certain range, the higher the fat content is, the better the palatability of cooked rice (Zhou et al., 2002). Although proteins contribute to the nutritional value of rice, high-protein rice may be unacceptable to some consumers because high-protein increases hardness, reduces the stickiness, and affects milled rice color (Juliano and Institute, 2003). Except for the differences in AC, MT7 and MT13 showed significantly changes in PC and LC. Compared with NIL-Wx^a, MT7 and MT13 lines had reduced PC and increased LC (Fig. 4, Table S3). The PC of MT13 was the lowest among the five lines, while that of MT7 was slightly higher than that of NIL-*wx*

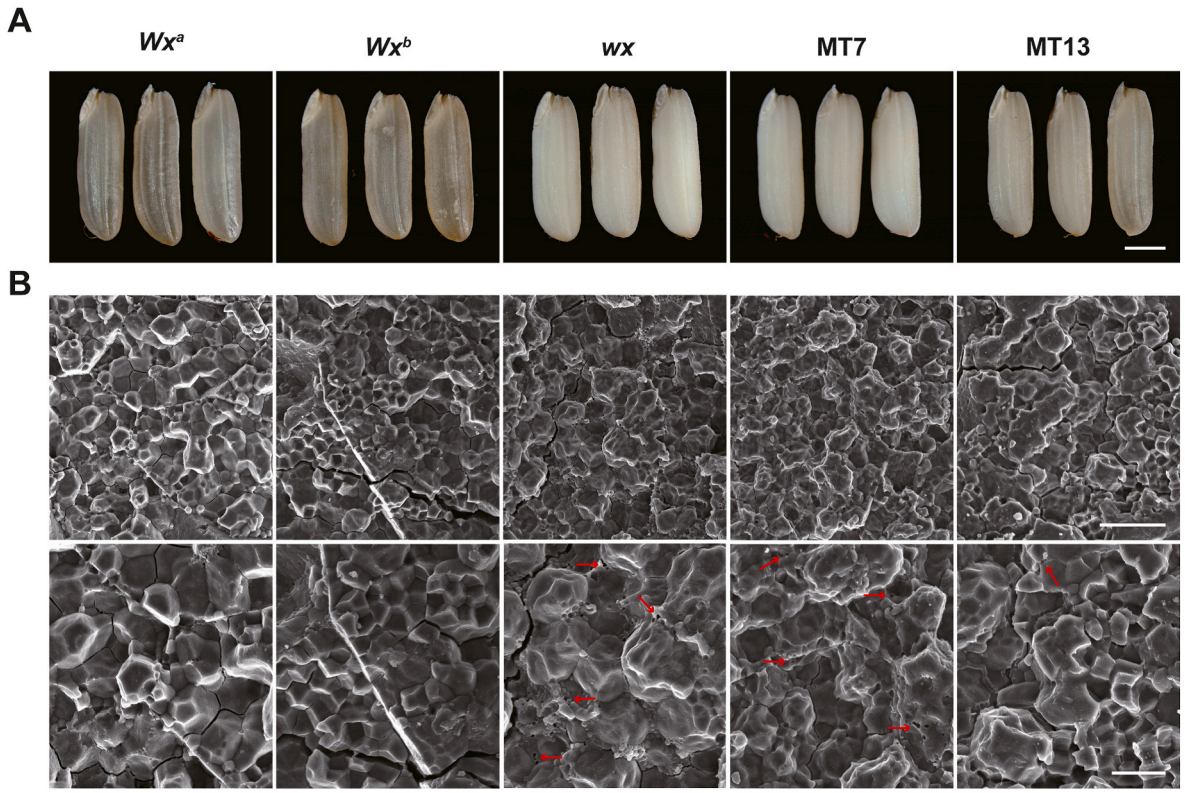

Fig. 5. The appearance and morphology of starch granules of the two novel *Wx* allelic lines MT7 and MT13 and the three references NIL-Wx^a, NIL-Wx^b and NIL-*wx*. (A) Appearance of brown rice. bar = 1 mm. (B) Micrographs of grain transverse sections obtained by scanning electron microscopy. The magnification of top row is 1000 × , bar = 25 μm; the magnification of bottom row is 2000 × , bar = 10 μm. Arrows indicate air spaces in a starch granule. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and comparable with that of NIL-Wx^b. The LC between MT7 and MT13 showed no obvious difference, second only to that of NIL-*wx*. Assuming only from the perspective of the contents of these two components, both MT7 and MT13 would exhibit softer and smoother characteristics, and due to the greater decrease in PC of MT13, its texture would become softer. Therefore, we speculated that some variations of RVA profiles and the improvement of ECQ of the two novel allelic lines were not only due to changes in AC, but also the contribution from PC and LC.

Among these 14 mutants, some lines had some similar sequence variations but significant differences in AC and *Wx* expression. For instance, MT6 and MT7 had similar bases variations, except that MT7 had two additional G-insertions around the second CAAT-box (Fig. 1B), but MT6 appeared not alter the AC and GBSSI level obviously (Fig. S3). The binding of CAAT-box by Nuclear Factor Ys (NF-Ys) is proposed to be a significant mechanism for its transcription activation function (Romier et al., 2003). In rice, NF-YB1 has been reported directly attaching to the CAAT-box of several sucrose transporter gene promoters and activates their transcription *in vivo* (Bai et al., 2016). NF-YB1-YC12-bHLH144 complexes and OsMADS14/NF-YB1 complexes were also reported activates *Wx* expression via NF-YB1 binding to a G-box in *Wx* promoter (Bello et al., 2019; Feng et al., 2022). Furthermore, MT13 and MT14 shared a similar fragment deletion pattern except MT13 having an additional 31 bp-deletion following the second TATA-box (Fig. 1A and B), but MT14 had little effect on grain AC and GBSSI level (Fig. S3). Several reports showed that mutations caused abnormal splicing of the intron 1 of *Wx* or amino acid residue substitutions of this gene reduced *Wx* function, thus decreasing AC in rice grains (Wang et al., 1995; Yang et al., 2013; Zhou et al., 2021). In order to exclude unexpected effects of *Wx* promoter and 5′UTR editing on intron 1 splicing and the coding region, we examined the intron 1 splicing and found the intron 1 was cut properly in MT6, MT7, MT13, MT14 and NIL-Wx^a as compared with NIL-Wx^b (Fig. S4). In addition, our sequence analysis of the coding regions did not detect sequence variations among MT7, MT13 and NIL-Wx^a (Fig. S5). Together, these results suggest that these variations in upstream region of the *Wx* alleles MT7 and 13 should be important targets to dissect transcriptional regulation of *Wx,* although further work needs to clarify it.

5. Conclusion

Through finely editing promoter and 5′UTR of Wx^a under a multi-sgRNA-in-one strategy by CRISPR/Cas9 system, we generated 14 *Wx* alleles, of which MT7 and MT13 significantly down-regulated *Wx* expression and decreased AC as compared with their donor NIL-Wx^a. Besides of the AC, MT7 and MT13 significantly reduced protein content and increased lipids content of grains compared with Wx^a. MT7 and MT13 are in very low AC categories (5–12%) which are suitable for soft rice breeding (Fig. 7). MT7 rice has white and fully opaque appearance similar to glutinous rice, and MT13 rice resembles semi-translucent soft rice, but they are significantly different from known glutinous rice and soft rice in ECQ, respectively.

CRediT authorship contribution statement

Pei Zhao: Writing – original draft, Investigation, Formal analysis, Data curation. **Yuxia Liu:** Investigation, Data curation. **Zhuyun Deng:** Resources, Data curation. **Lingtong Liu:** Formal analysis, Data curation. **Tengwei Yu:** Investigation. **Gege Ge:** Investigation. **Bingtang Chen:** Investigation. **Tai Wang:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Ethics approval and consent to participate

Not applicable.

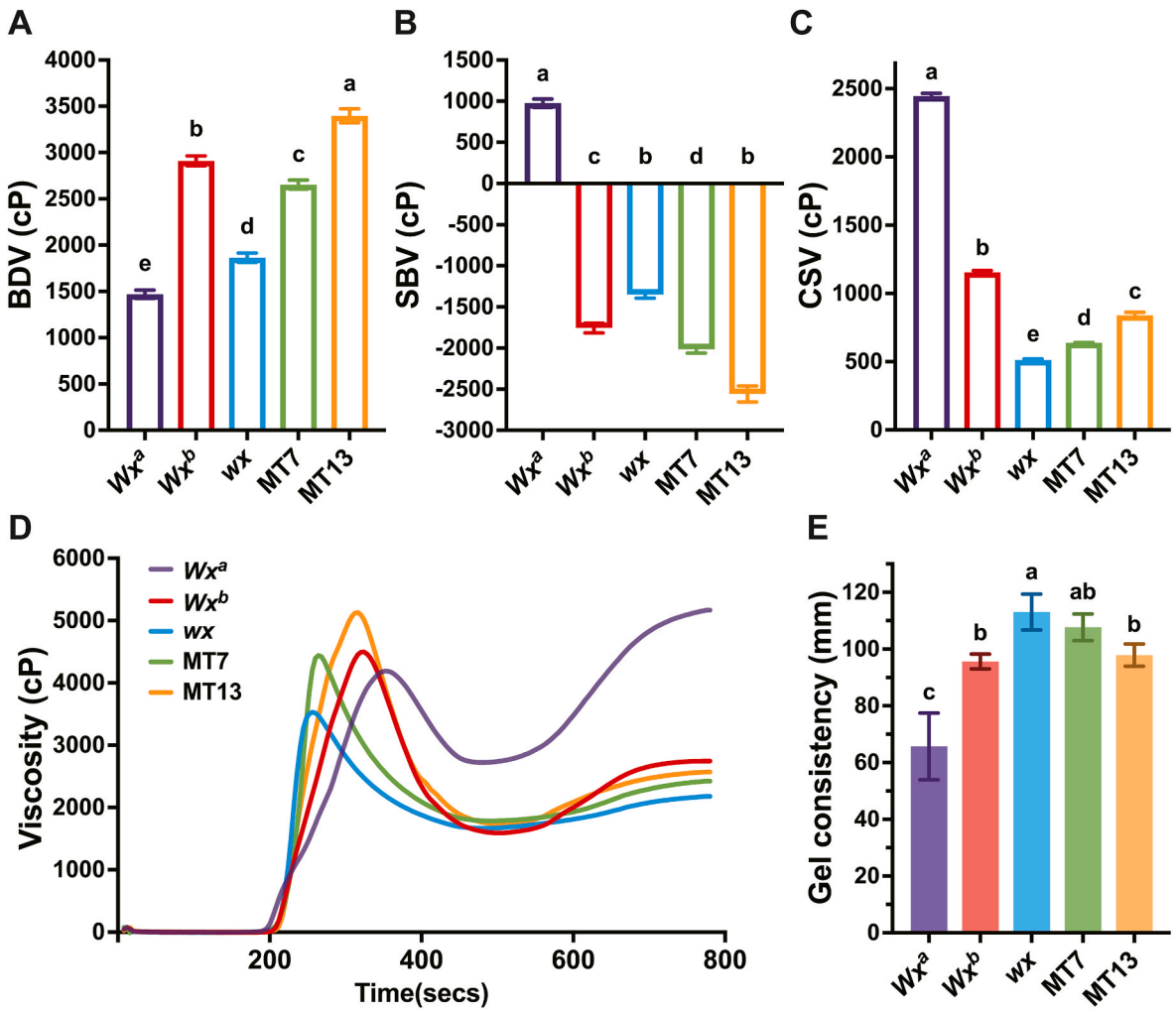

Fig. 6. Assessment of rice eating and cooking qualities by rapid viscosity analyzer (RVA) and gel consistency of the two novel *Wx* allelic lines MT7 and MT13 and the three references NIL-Wx^a, NIL-Wx^b and NIL-*wx*. (A–C) RVA characteristics. BDV, breakdown value; SBV, setback value; CSV, consistence value. cP, centi Poise. (D) RVA profiles of rice flours, viscosity unit. (E) Gel consistency. Error bars are means ± SD ($n = 3$). The different letters indicate significant differences at $P < 0.05$ by one-way ANOVA.

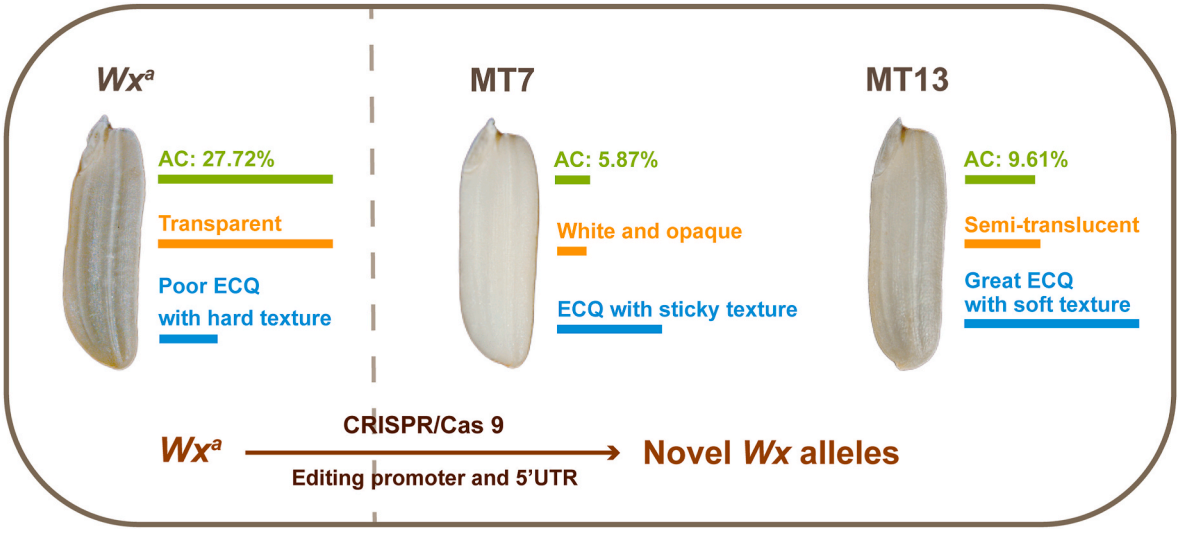

Fig. 7. Graphical abstract of the main difference between the two new alleles and NIL-Wx^a control. The green, yellow, and blue bars represent the amylose content (AC), transparent, and eating and cooking quality (ECQ) of grains, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tai Wang reports financial support was provided by Chinese Academy of Sciences. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2024.154384.

Data availability

All relevant data are provided in this article and its supporting information.

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