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Precise Editing of the *Wx* Gene Promoter via Prime Editing to Develop Novel Rice Germplasm with Low Amylose Content

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Abstract

Rice (*Oryza sativa* L.) is a staple food, and its eating quality is largely determined by amylose content (AC), which is controlled by the *Wx* gene. Current low-AC alleles often lead to excessive reductions, limiting their utility. The promoter region, particularly the pyrimidine-rich region (PRR) from -45 to -7, is hypothesized to fine-tune *Wx* expression, yet its functional motifs remain unexplored. In this study, we applied prime editing (PE) to precisely edit the *Wx* promoter in the japonica cultivar Huaidao 5. We generated two novel alleles, $Wx^{1T/32d}$ (deleting PRR1 [-45 to -19] and PRR2 [-11 to -7]) and Wx^{5d} (deleting only PRR2), which are absent in natural germplasm. These edits resulted in significant, fine-tuned reductions in AC (3.95% and 2.77%, respectively) without affecting grain transparency. Our results identify PRR1 and PRR2 as important functional motifs whose deletion synergistically downregulates AC. Furthermore, the edited lines exhibited improved taste without compromising agronomic performance. This study not only provides novel insights into the regulatory motifs of the *Wx* promoter but also demonstrates the precision and potential of PE for improving rice grain quality.

Keywords: rice; amylose content; prime editing; promoter editing; *Wx*

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1. Introduction

Rice (*Oryza sativa* L.) is one of the world's most important food crops, and quality improvement is a central goal in breeding programs [1–3]. Amylose content (AC) is a critical factor influencing eating and cooking quality [4,5], primarily regulated by the *Wx* gene, which encodes granule-bound starch synthase I (OsGBSSI). A strong positive correlation exists between *Wx* gene expression levels and AC [6].

To date, several low AC alleles (AC 8–12%) with coding region variations have been identified, such as Wx^{mp}, $Wx^{op/hp}$, Wx^{mq}, Wx^{mw} and Wx^{la} [7–11]. These alleles endow rice with superior palatability and serve as invaluable genetic resources for rice breeding [4,10–17]. However, these coding region variants exhibit significant limitations: First, coding region mutations often cause excessive amylose reduction, rendering fine-grained regulation difficult. Second, the exploration of coding region alleles has approached a bottleneck, failing to meet the demands of precise regulation. Although *Wx* promoter-targeted research has opened new avenues, currently identified promoter mutations still lack elite alleles capable of stably and precisely regulating AC, leading to a critical shortage of effective strategies for improving rice eating and cooking quality [18]. Evidently, the scarcity of elite allelic variations has become a core bottleneck hindering the precise regulation of rice AC and quality improvement. Studies have shown that the *Wx* promoter might be a TATA-less promoter, featuring a potential pyrimidine-rich region (PRR). The PRR could play a crucial role in regulating gene expression, as it may be associated with various environmental stimuli (e.g., dehydration, light, temperature, and hormones) and participate in modulating gene transcription [19]. Therefore, studying the PPR may facilitate the discovery of novel alleles.

In crop genetic improvement, traditional breeding is constrained by lengthy cycles and genetic linkage drag. While next-generation gene-editing technologies like CRISPR/Cas9 have overcome recombination efficiency limitations of traditional breeding, editing the *Wx* coding region often leads to *waxy* mutations with AC plummeting to 0–2%. Additionally, off-target effects induced by double-strand breaks (DSBs) hinder its application [20,21]. In contrast, promoter editing for targeted gene expression regulation offers an ideal approach to avoid drastic phenotypic shifts [22]. The Prime Editing (PE) system, developed in 2019, offers three key advantages for crop improvement: (1) Reverse transcriptase-mediated single-base substitutions and precise small insertions/deletions avoid frameshift mutations in coding regions; (2) Direct targeting of core promoter elements enables quantitative transcriptional regulation; (3) Complete elimination of DSB-induced chromosomal aberrations, reducing off-target effects to less than 10% of traditional CRISPR technologies [23]. This "non-destructive editing" approach provides a transformative tool for precise quality trait improvement.

In this study, we applied PE to precisely modify the *Wx* promoter in japonica rice Huaidao 5, creating two novel low-AC alleles ($Wx^{1T/32d}$ and Wx^{5d}) not found in nature. These alleles significantly reduced AC (3.95% and 2.77%, respectively) while maintaining grain transparency. We identified PRR1 and PRR2 as key functional motifs whose synergistic deletion fine-tunes AC. The edited lines showed improved taste without yield penalty, demonstrating PE's precision for quality breeding. This study provides new regulatory motifs and showcases PE's potential for rice improvement.

2. Materials and Methods

2.1. Plant Materials

The recipient parent used in this study was Huaidao 5 (*Oryza sativa* L. subsp. *japonica*), an elite rice variety widely cultivated for direct-seeding rice production in the Huaibei region of Jiangsu Province, China. This variety exhibits moderate plant height, compact plant architecture, and strong lodging resistance. Huaidao 5 carries the Wx^{b} allele with an AC of approximately 15%–16%. This study employed Huaidao 5 as the receptor parent and utilized PE technology for precise editing of the Wx gene promoter.

2.2. Prime Editing Workflow

2.2.1. Target Design

The pyrimidine-rich region (PRR) from −45 to −7 bp upstream of the transcription start site (TSS) in the *Wx* gene promoter may play an important role in gene expression regulation. Therefore, site-specific knockout of this region may generate new *Wx* alleles capable of fine-tuning AC. Based on alignment between the target editing type and wild-type genotype, the target site was set within the PRR to achieve deletion of bases at this locus. By analyzing the sequence characteristics of this site, the PAM sequence was determined to be NGG for recognition by Cas9 nickase. Subsequently, the editing window was defined, and potential off-target effects were evaluated. Prime editing guide RNAs (pegRNAs) were designed containing: (i) a spacer sequence fully complementary to the target DNA; (ii) a primer binding site (PBS) complementary to the 3′ end of the target DNA to prime reverse transcription; and (iii) a reverse transcription template (RTT) containing a 5-bp deletion sequence. The PE-Designer online tool (https://pe-designer.genome.sg/) was used to predict the editing efficiency and specificity of pegRNAs, which were subsequently optimized based on the prediction results.

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2.2.2. Construction of Gene Editing Vectors

Synthesis of Target Gene Amplification Primers

Primers for PCR amplification were designed as follows:

QC5bp-L:
5′-cagtGGTCTCatgcatgaaggaatggtgagagagggtttcag-3′
QC5bp-R:
5′-cagtGGTCTCatcaagcaccgactcggtgccac-3′

PCR amplification, sequencing, and enzymatic digestion-ligation

PCR amplification was performed in a 50 μL reaction volume using the following program. Amplification products were separated by electrophoresis on a 1.5% agarose gel. Bands of expected size were excised and purified using a gel extraction kit. The recovered DNA (designated rDNAT1) was sequenced to confirm correctness, then subjected to enzymatic digestion and ligation with the vector to obtain the final construct (Tables 1–4).

Table 1. PCR reaction components.

Component	Volume
Forward primer (10 μM)	2 μL
Reverse primer (10 μM)	2 μL
2× Taq PCR Mix	25 μL
ddH$_2$O	20 μL
Template	1 μL
Total	50 μL

Table 2. PCR program.

Step	Cycles
94 °C for 5 min	1
94 °C for 30 sec	30
50 °C for 45 sec	30
72 °C for 22 sec	30
72 °C for 10 min	1
16 °C for 30 min	1

Table 3. Digestion-ligation reaction components.

Component	Volume
Nuclease-free Water	8 μL
10× Buffer	2 μL
BsaI/Eco31I	1 μL
T4 DNA ligase	10 μL
pHU-PE-Lncas9(K34) (4 U/μL)	4 μL
rDNAT1	4 μL
Total	20 μL

Table 4. Digestion-ligation conditions.

Step	Cycles
37 °C for 20 min	1
37 °C for 10 min	5
20 °C for 10 min	5
37 °C for 20 min	1
80 °C for 5 min	1

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Transformation of competent cells with ligation products

Escherichia coli competent cells were removed from −80 °C storage and thawed rapidly on ice. A 50 μL aliquot of competent cells was mixed with 5 μL of ligation product, gently flicked to mix, and incubated on ice for 20–30 min. Heat shock was performed at 42 °C for 90 sec to facilitate DNA uptake, followed by immediate chilling on ice for 2 min. Then, 1 mL of pre-warmed LB medium was added, and cells were incubated at 37 °C with shaking for 1 h to allow recovery and expression of the antibiotic resistance gene. The transformation mixture was plated on LB agar containing kanamycin and incubated at 37 °C overnight (12 h) with inverted plates.

Colony PCR identification

Ten single colonies were randomly picked and subjected to colony PCR identification. Amplification products were sequenced to screen for positive clones and obtain the target plasmid. Identification primers were (Table 5):

PE-max-F: 5′-atcttcaaaagtcccacatcg-3′

PE-max-R: 5′-cattcgccatgccgaagcat-3′

Table 5. PCR reaction components.

Component	Volume
Forward primer (10 μM)	1 μL
Reverse primer (10 μM)	1 μL
2× Taq PCR Mix	12.5 μL
ddH_2O	9.5 μL
Template	1 μL
Total	25 μL

The PCR procedure is consistent with the aforementioned description.

2.2.3. Genetic Transformation

Plasmid transformation and agrobacterium detection

One microliter of plasmid was added to 50 μL of Agrobacterium tumefaciens strain EHA105 competent cells, mixed thoroughly, and electroporated. One milliliter of LB liquid medium was added, and cells were incubated at 30 °C with shaking at 180 rpm for 30 min. Fifty microliters of the bacterial suspension was plated on LB agar and incubated at 30 °C in the dark for 48 h. Single colonies were picked for PCR and electrophoresis verification to confirm correct amplification bands (Table 6).

Table 6. PCR reaction components.

Component	Volume
Forward primer (10 μM)	1 μL
Reverse primer (10 μM)	1 μL
2× Taq PCR Mix	10 μL
ddH_2O	7 μL
Template	1 μL
Total	20 μL

The PCR procedure is consistent with the aforementioned description.

Rice genetic transformation

Rice seeds without mold spots and with normal germ pores were selected and surface-sterilized sequentially with 75% ethanol for 1 min and 15% sodium hypochlorite for 20 min, followed by three rinses with sterile water. Sterilized seeds were inoculated on callus induction medium and cultured at 26 °C under light for 20 days. Agrobacterium was resuspended to an OD600 of 0.2 and used to infect calli for 10–15 min, followed by co-cultivation for 48–72 h. Subsequently, selection culture, secondary selection, differentiation culture, and rooting culture were performed sequentially to obtain transgenic plants. Leaf DNA was extracted, and positive plants were identified using the PCR method described above to determine the editing type.

2.3. Analysis of Wx Gene Expression and AC in Transgenic Lines

T0 generation positive seedlings were recovered by hydroponic culture and then transplanted to the field for cultivation following standard management practices. Seeds were harvested at maturity and planted in Hainan Province for T1 generation, with 30 plants per line. Homozygous positive plants were identified by sequencing. Samples were collected at 15 days after grain filling (DAF) for quantitative real-time PCR (qRT-PCR) analysis of *Wx* gene expression. After maturation, AC and GBSSI protein content were measured, and the eating quality value was determined.

2.3.1. AC Determination

Polished rice was ground and passed through a 100-mesh sieve. A 0.1000 g sample was weighed and subjected to alkaline treatment and iodine staining according to standard methods. Absorbance at 620 nm was measured, and AC was calculated using a standard curve.

2.3.2. qRT-PCR ANALYSIS

Total RNA was extracted from grains at 15 DAF using a plant total RNA extraction kit and reverse-transcribed into cDNA. qRT-PCR was performed using gene-specific primers to analyze Wx gene expression levels.

2.3.3. GBSSI Protein Quantification

Total protein was extracted from mature brown rice using a plant seed total protein extraction kit. GBSSI protein expression was detected by Western blot analysis with three biological replicates. Average values were calculated to determine differences in GBSSI protein between mutants and the control.

2.4. Investigation of Agronomic Traits in Transgenic Lines

Agronomic traits were investigated and compared between homozygous edited lines and the control, including heading date, plant height, panicle length, seed setting rate, 1000-grain weight, grain length, grain width, and grain thickness.

3. Results

3.1. Screening of Homozygous Gene Editing Lines

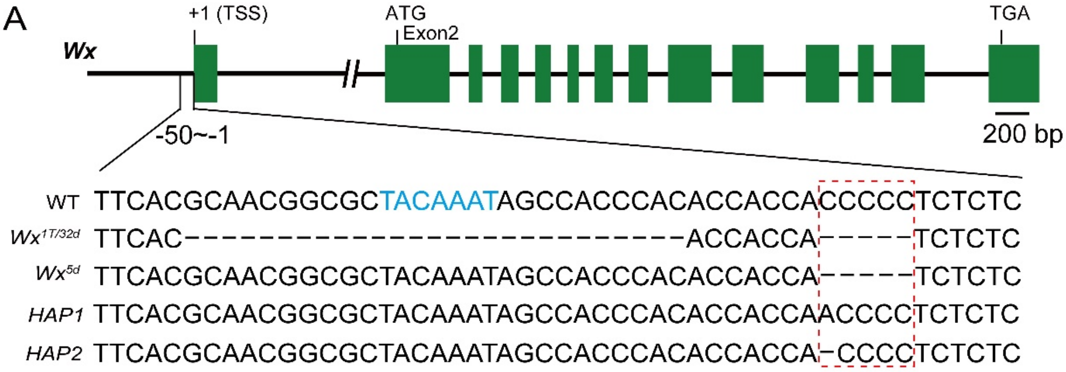
Through precise editing of the *Wx* gene promoter using PE, a total of 15 T0 generation single plants were obtained, of which 10 were mutant plants, yielding a mutation rate of 66.67%. Through generational advancement and genotype detection, two different homozygous editing types were successfully screened in the T2 generation. The specific editing types were as follows:

$Wx^{1T/32d}$: A total of 32 bp sequence was deleted, including 27 bp of PRR1 (−45 to −19) and the PRR2 sequence (−11 to −7);

Wx^{5d}: Only the 5-bp PRR2 motif was deleted.

3.2. *Wx* novel Alleles Created by Prime Editing Are Extremely Rare in Natural Rice Populations

To verify the frequency of these editing types in natural populations, the promoter sequences of 3,032 rice accessions from the SNP-Seek database (https://snp-seek.irri.org/) were analyzed. The target editing region was found to be highly conserved in natural populations: only 0.63% (19/3,032) carried the HAP1 variant (C-to-A mutation at −11), and 0.20% (6/3,032) carried the HAP2 variant (C deletion at −11). The remaining accessions had promoter sequences identical to Huaidao 5 (Figure 1), indicating that the structural variations *Wx*$^{1T/32d}$and *Wx*^{5d} created by the PE system in this study represent novel alleles not previously reported in nature.



B

Subpopulation	admix	aro	aus	ind1A	ind1B	ind2	ind3	indx	japx	subtrop	temp	trop
WT	99	75	201	209	203	285	475	610	83	112	291	364
HAP1	4	2	0	0	3	0	0	4	0	0	2	4
HAP2	0	0	0	0	0	0	0	0	0	0	0	6
Wx$^{1T/32d}$	0	0	0	0	0	0	0	0	0	0	0	0
Wx^{5d}	0	0	0	0	0	0	0	0	0	0	0	0

Figure 1. Two mutants obtained by precisely editing the core region of the *Wx* promoter using the PE system. (A) Structure of the *Wx* gene and editing types of mutants; (B) Variation types of natural germplasm in this region.

3.3. Deletion of PRR1 and PRR2 Motifs in the *Wx* Promoter Reduces AC and Enhances Eating Quality

AC determination of the mutants showed that the AC of both mutants decreased significantly compared to the control. The AC of *Wx*$^{1T/32d}$ decreased from 16.35% in the wild type to 12.40% (a reduction of 3.95%), while *Wx*^{5d} decreased to 13.58% (a reduction of 2.77%). Grain transparency showed no significant changes (Figure 2A,B). These results directly demonstrate that the PRR1 and PRR2 motifs in the promoter region play important roles in *Wx* gene expression and AC regulation, and that the combined deletion of PRR1 and PRR2 exhibits an additive regulatory effect, achieving gradient fine-tuning of AC.

Molecular detection showed that the mRNA transcription level of the *Wx* gene and OsGBSSI protein level in the mutants were consistent with the trend of AC changes (Figure 2C–E), validating the effectiveness of PE editing at the transcription-translation level and revealing that PRR2 may be a core cis-acting element for *Wx* gene transcription initiation. Taste value determination showed that the wild type, *Wx*$^{1T/32d}$, and *Wx*^{5d} had taste values of 72.6, 76.6, and 74.2, respectively. The *Wx*$^{1T/32d}$ mutant with the lowest AC exhibited the highest taste value (Figure 2F), consistent with transcription-translation levels.

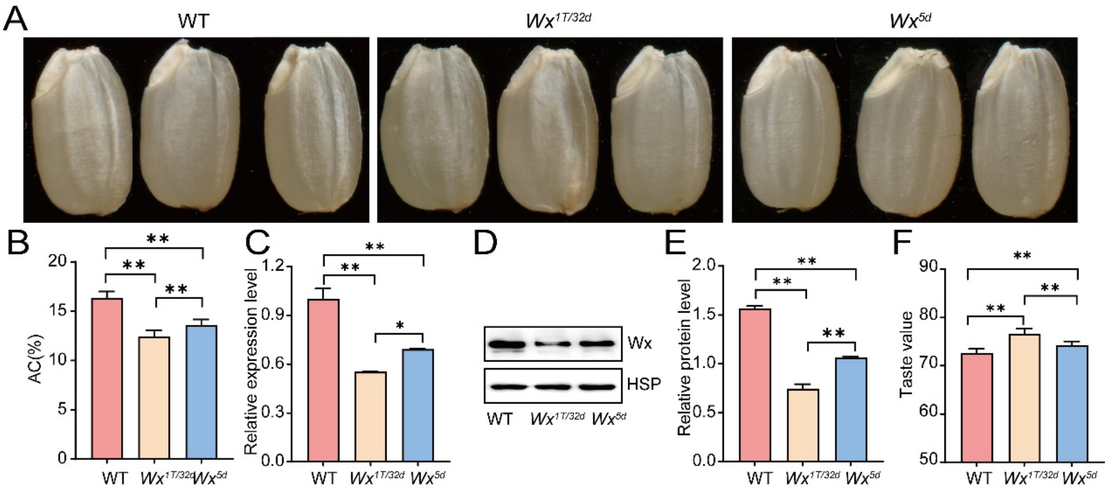


Figure 2. Analysis of grain appearance quality, as well as transcriptional and translational levels of the *Wx* gene in mutants. (A) Comparison of brown rice appearance between mutants and WT; (B) Amylose content of mutants and WT. **: $P < 0.01$; (C) Relative expression level of *Wx* in mutants and WT; (D) Western blot results of mutants and WT; (E) Relative protein expression in mutants and WT; (F) Comparison of taste values between mutants and WT.

3.4. Prime Editing of the Wx Promoter Improves Grain Quality Without Compromising Agronomic Traits

Analysis of variance (ANOVA) of eight core agronomic traits, including plant height, growth period, seed setting rate, panicle length, 1000-grain weight, grain length, grain width, and grain thickness, showed no significant differences between the two mutants and the wild type (Figure 3A,B), fully demonstrating that PE-mediated targeted editing of the *Wx* promoter can achieve directed improvement of rice quality while maintaining stable agronomic traits.

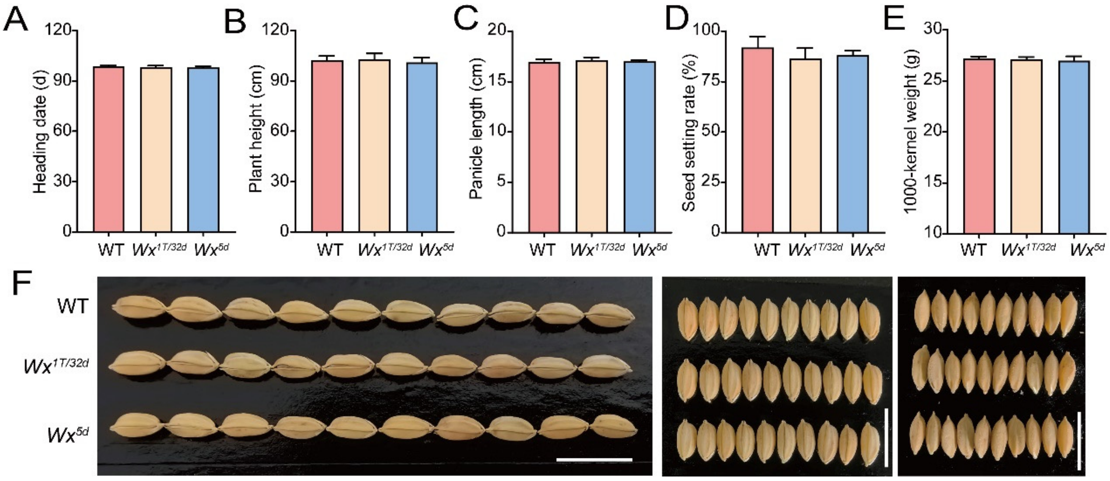


Figure 3. (A) Statistical comparison of five traits between mutants and WT. (B) Comparison of grain length, grain width, and grain thickness between mutants and WT.

4. Discussion

With increasing consumer demand for high-quality rice, grain quality has become a major breeding objective in rice improvement. As the major gene controlling AC, the *Wx* gene has been extensively studied, with different gene loci and polymorphic sites functionally validated. To date, at least 10 different natural *Wx* alleles have been cloned: Wx^{lv}、Wx^{a}、Wx^{b}、Wx^{in}、Wx^{mp}、$Wx^{op/hp}$、Wx^{mq}、Wx^{mw}、Wx^{la}, and *wx* [7–9]. Timely reduction of AC plays an important role in improving rice quality. The utilization of the *Wx*^{b} allele (AC ~16%) has greatly improved rice eating quality, while the soft rice allele *Wx*^{mp} (AC ~10%) has further enhanced rice quality, with the Nanjing series soft rice varieties developed using this allele being widely promoted in Jiangsu Province. However, mining of *Wx* alleles is approaching saturation, and gene editing, with its capability for site-specific knock-in and knock-out, has become a powerful tool for creating novel *Wx* alleles.

In this study, we employed the PE system to precisely target the core region of the rice *Wx* gene promoter (PRR1 and PRR2) [24,25], successfully generating two novel low-AC mutants (*Wx*$^{1T/32d}$ and *Wx*^{5d}). Compared to traditional CRISPR/Cas9 technology, the PE system demonstrated three major advantages:

(1) Site-specific modification capability: PE achieves precise insertion/deletion through pegRNA-guided reverse transcriptase-mediated editing, avoiding the risk of chromosomal translocation caused by Cas9-induced DSBs.

(2) Gradient expression regulation: *Wx*$^{1T/32d}$ (deletion of 32 bp including PRR1 and PRR2) and *Wx*^{5d} (deletion of only the 5-bp PRR2 motif) showed AC reductions of 3.95% and 2.77%, respectively, exhibiting a dose-dependent effect (Figure 2B). This fine-tuning capability is difficult to achieve with traditional knockout technologies—for example, CRISPR-edited *Wx* alleles often result in AC dropping sharply to 0–2% (waxy phenotype) [20,21], which cannot meet market demand for "moderate AC".

(3) Extremely low off-target risk: The nickase mechanism of PE reduces off-target rates to less than 1/10th of that of CRISPR [23]. In the future, the newly developed PE3b system could be introduced with additional sgRNAs to expand the editing window [26], thereby applying this strategy to larger regulatory regions of the *Wx* promoter to improve AC regulation precision.

This study is the first to discover that the combined deletion of PRR1 and PRR2 in the *Wx* promoter can produce an additive effect (Figure 2B–D), but the specific molecular mechanism remains unclear. We speculate that: (1) the PRR2 may serve as a binding site for certain transcription factors, and its deletion may weaken the recruitment efficiency of these factors to PRR1, leading to decreased stability of the transcription initiation complex and reduced AC; (2) given the spatial proximity of PRR1 and PRR2 (−45 to −19 bp and −11 to −7 bp), they may be located within the same chromatin loop, so combined deletion may disrupt local chromatin open states, inhibiting RNA polymerase II binding and reducing AC; (3) PRR2 may be a target for certain histone acetyltransferases, and its deletion may interfere with epigenetic modifications in this region, thereby reducing AC. Future studies could employ ChIP-qPCR to detect changes in protein binding in mutants or utilize DNase I hypersensitive site sequencing (DNase-seq) to resolve alterations in chromatin accessibility.

The mutants obtained in this study achieved precise quality improvement. *Wx*$^{1T/32d}$ (AC = 12.40%) and *Wx*^{5d} (AC = 13.58%) exhibited taste values of 76.6 and 74.2 (Figure 2F), respectively, significantly superior to the taste value of 72.6 for traditional *Wx*^{b}. Combined with their stable agronomic traits (Figure 3) and transparent grain appearance (Figure 2A), these mutants provide valuable genetic resources and germplasm for future quality breeding.

5. Conclusions

This study represents the first successful application of PE system to create low-AC alleles (*Wx*$^{1T/32d}$ and *Wx*^{5d}) that are scarce in nature, revealing the synergistic regulatory effect of PRR1 and PRR2 and providing novel targets for molecular mechanism studies of amylose synthesis. Simultaneously, this work demonstrates the unique advantages of PE technology: through precise editing of core regulatory elements in promoters, quantitative regulation of target traits can be achieved without affecting other agronomic traits. The new germplasm with improved taste, transparent appearance, low AC, and unaffected agronomic traits obtained in this study provides important genetic materials and novel genes for rice quality improvement. Future research should further explore the regulatory mechanisms of related motifs, mine additional 5′ UTR regulatory regions, and apply this strategy to elite varieties with different genetic backgrounds to promote the precision and efficiency of crop quality breeding.

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Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AC	amylose content
PRR	pyrimidine-rich region
OsGBSSI	granule-bound starch synthase I
PE	prime editing
DSBs	double-strand breaks
pegRNAs	Prime editing guide RNAs
PBS	primer binding site
RTT	reverse transcription template
DAF	days after grain filling
qRT-PCR	quantitative real-time PCR
ANOVA	Analysis of variance

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