Targeted Deletion of the \textit{Wx} Gene First Intron \textit{via} CRISPR/Cas9 Significantly Increases Grain Amylose Content in Rice With a \textit{Wx}^b Allele, but Not in Rice With a \textit{Wx}^a Allele

\textbf{Xingdan Liu}  
Hunan Agricultural University  [https://orcid.org/0000-0001-5371-7492]

\textbf{Qi Ding}  
Hunan Agricultural University

\textbf{Wenshu Wang}  
Hunan Agricultural University

\textbf{Yanling Pan}  
Hunan Agricultural University

\textbf{Chao Tan}  
Hunan Agricultural University

\textbf{Yingbo Qiu}  
Hunan Agricultural University

\textbf{Ya Chen}  
Hunan Agricultural University

\textbf{Hongjing Li}  
Hunan Agricultural University

\textbf{Yinlong Li}  
Hunan Agricultural University

\textbf{Naizhong Ye}  
Hunan Agricultural University

\textbf{Nan Xu}  
Hunan Agricultural University

\textbf{Xiao Wu}  
Hunan Agricultural University

\textbf{Rongjian Ye}  
Hunan Agricultural University

\textbf{Jianfeng Liu}  
Hunan Agricultural University

\textbf{Chonglie Ma}  [machonglie@sinochem.com]  
Hunan Agricultural University

\textbf{Original article}

\textbf{Keywords:} Wx gene, GBSSI, CRISPR/Cas9, intron, amylose conten, rice

\textbf{Posted Date:} August 18th, 2021

\textbf{DOI:} https://doi.org/10.21203/rs.3.rs-816375/v1

\textbf{License:} This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background: Rice Waxy (Wx) gene plays a major role in seed amylose synthesis, and consequently controls grain amylose content. The expression of Wx gene is highly regulated at both transcriptional and post-transcriptional levels. Particularly, the GT/TT polymorphism at the S' splicing site of its 1st intron greatly affects this intron's splicing efficiency and defines two predominant Wx alleles, Wx<sup>a</sup> and Wx<sup>b</sup>. Wx<sup>a</sup> rice often has intermediate to high amylose content, whereas Wx<sup>b</sup> rice has low to intermediate amylose content. A previous study indicates that rice Wx<sub>1</sub> intron significantly enhances gene expression when it is inserted into the S' UTR (untranslated region) of a foreign gene. By deleting Wx<sub>1</sub> intron with the CRISPR/Cas9 technology, we intended to create a totally novel Wx allele, and further to investigate how the intron removal affects Wx gene expression and rice grain amylose content.

Results: CRISPR/Cas9-mediated targeted deletion of Wx<sub>1</sub> intron was performed on 4 rice inbreds, KY131(Wx<sup>a</sup>), X32(Wx<sup>b</sup>), X35(Wx<sup>a</sup>) and X55(Wx<sup>b</sup>). Complete deletion of the 1st intron occurred in 8.6%-11.8% of the primary transformants of these 4 inbreds. Transgene-free, homozygous mutants were obtained. Their grain amylose content and Wx gene expression were analyzed. Compared to the amylose content of wild type plants, mutants' amylose content was significantly increased from 13.0% to about 24% in KY131 and X32 which both carried the Wx<sup>b</sup> allele. However, no significant difference in amylose content was observed between wild type plants and mutants of X35 and X55 which carried the Wx<sup>a</sup> and Wx<sup>b</sup> allele, respectively. Results of Wx gene expression analysis on wild type plants and mutants showed a high consistence with their amylose content results. Mutants of KY131 and X32 accumulated much more steady mRNA transcripts than their wild type plants, while steady mRNA level remained somehow unchanged between wild type plants and mutants of X35 and X55. Grain quality including appearance quality and ECQ(eating and cooking quality) that are tightly linked to amylose content was also evaluated on wild type plants and mutants, and data were presented and analyzed.

Conclusions: This study presents a novel and fast strategy to increase amylose content for rice inbreds carrying a Wx<sup>b</sup> allele. Our data strongly suggest that rice Wx<sub>1</sub> intron regulates Wx gene expression mainly at the post-transcriptinal level, not as previously thought that it influences Wx gene transcription as well. In addition, removal of the first intron creates a completely novel Wx allele. Further studies on this new Wx allele would provide invaluable insights into the regulation of Wx gene expression, which will help researchers to engineer more new alleles that leads to the breeding of rice cultivars with better eating and cooking quality.

Background

Improving grain quality is one of the most important goals in rice (Oryza sativa L) breeding programs. Rice quality refers to the basic characteristics of rice in commodity circulation. It includes four main aspects, milling quality, appearance quality, cooking and eating quality and nutritional quality. Among them, appearance quality, cooking and taste quality (ECQ) are particularly important(Lau et al., 2015). ECQ is determined from amylose content (AC), gel consistency (GC), gelatinization temperature (GT), and viscosity (Phing Lau et al., 2016; Tian et al., 2009). Starch accounts up to 90 % of the dry weight in the rice grain(Zhou et al., 2002). Amylose, a constituent of starch, is a major indicator of ECQ in rice (Fitzgerald et al., 2009; Li et al., 2016; Tian et al., 2009). According to the content of amylose, rice was divided into four types, glutinous (AC < 2%), soft, intermediate to low and high (AC > 25%) (Pandey et al., 2002). The higher amylose content in rice grain is, the less sticky and harder the cooked rice is, resulting in poor taste (Jobling, 2004; Juliano, 1992). However, rice with too low amylose content is too sticky and soft. Thus, it is more popular with intermediate to low amylose content in rice.

The Waxy (Wx) gene encodes granule-bound starch synthase I (GBSSI), which was cloned by Wang et al in 1990(Sano, 1984; Sano et al., 1985; Wang et al., 1990). The Wx gene is a major gene controlling amylose content in rice endosperm, and plays a decisive role on rice ECQ(Tian et al., 2009). At present, there are great differences in amylose content among cultivated rice varieties. Such vast differences mainly come from Wx gene allele variation. At least 9 Wx natural alleles have been discovered and identified in rice. Those alleles include Wx<sup>1</sup>, Wx<sup>2</sup>, Wx<sup>3</sup>, Wx<sup>op</sup> / Wx<sup>mp</sup>, Wx<sup>mp</sup>, Wx<sup>dp</sup> / Wx<sup>dp</sup>, Wx<sup>mp</sup> / Wx<sup>mp</sup>, Wx<sup>dp</sup> / Wx<sup>dp</sup>, and wx (Chen et al., 2008; Dobo et al., 2010; Mikami et al., 2008; Patrick D. Larkin 2003; Teng et al., 2012; Zhang et al., 2021; Zhang et al., 2019; Zhou et al., 2021). DNA sequence difference between Wx alleles, cause variation in Wx gene expression and enzymatic activity, which leads to the difference of amylose content and quality of rice. Researchers have designed different strategies to improved rice quality, including both transgenic methods and marker-assisted selection (MAS) breeding (Jin et al., 2010; Kimiko Itoh et al., 2003; Liu et al., 2005; Phing Lau et al., 2016; Rie Terada et al., 2000; Yu et al., 2009). Especially, introducing Wx<sup>b</sup> (AC<sub>1</sub>16%) and Wx<sup>a</sup> (AC<sub>1</sub>20%) alleles into rice germplasm with high amylose content via MAS or traditional breeding greatly expedites rice quality improvement.

With the development of rice consumption specialization, the demand for amylose content in rice is increasingly diversified, which requires new ways to modulate amylose content or create new Wx alleles. The third generation genome editing technology – CRISPR/Cas9 was developed in 2012(Jinek et al., 2012; Richter et al., 2012), which has been rapidly developed and widely used in the improvement of agronomic traits(Chen et al., 2019; Fiaz et al., 2019; Gao, 2021). Up to now, CRISPR/Cas9 system can realize gene regulation efficiently and conveniently, such as knock out, knockin, substitution, single base editing and epigenetic modification, and it also can manipulate gene translation and expression by editing gene uORF and promoter(Chen et al., 2019; Rodriguez-Leal et al., 2017; Zhang et al., 2018a; Zong et al., 2017). Most importantly, the T-DNA insertion site and gene editing target sites are in different locations, and homozygous edited mutants free of transgenic components could be selected through progeny separation(Zhu et al., 2020). Compared with the traditional transgenic methods, the CRISPR/Cas9 technology is more prone to the changes of natural mutations and has more potential to be applied in production practice. Recently, CRISPR/Cas9 mediated rice Wx gene editing have been intensively reported. These reporters can be grouped into 4 categories. First, researchers have created new glutinous rice by completely knocking out the Wx gene (Ma et al., 2015; Zhang et al., 2018b). Second, by editing the key cis-acting elements on the Wx gene promoter, researchers have achieved fine-tune of AC in the Wx<sup>b</sup> background (Huang et al., 2020a; Zeng et al., 2020). Third, researchers have tried to regulate rice AC by manipulating the splicing efficiency of Wx gene at the post-transcriptional level (Zeng et al., 2020). Fourth, researchers have used CRISPR/Cas9-mediated base editor to is modulate rice AC by modifying GBSSI enzyme activity(Xu et al., 2020). These studies have not only generated a plenty of new Wx alleles and added precious new knowledge about the regulation of
rice Wx gene expression and the modulation of GBSSI enzyme activity. In addition, a previous study shows that the first intron of the rice Wx gene greatly enhances foreign gene expression in rice protoplasts, but not in tobacco protoplasts. When it is inserted into the 5' UTR region between the CaMV 35S promoter and GUS (β-glucoronidase) coding sequence, GUS expression is increased 15-fold in rice protoplasts (Li et al., 1995), strongly suggesting that the first intron of the Wx gene might play an important role in regulating the Wx gene expression, presumably at the transcriptional level.

In the study, we exploited the CRISPR/Cas9 gene editing technology to remove the entire first intron of the Wx gene. The intron removal created a totally novel Wx allele, and significantly increased the amylose content in rice inbreds with the Wxb allele, while the amylose content was not changed much in rice inbreds with the Wxa allele.

**Results**

**CRISPR/Cas9-mediated deletion of Wx 1st intron**

To investigate how Wx1st intron regulates amylose content of rice grains, we decided to remove the entire 1st intron using CRISPR/Cas9 technology. We chose four rice inbreds with different genetic background as our testing materials. Kongyu131 (KY131) was an elite japonica inbred and carried a typical Wxb allele. The other three X32, X35 and X55 were indica inbreds from our breeders, carrying Wxa, Wxa or Wxl allele, respectively. The typical Wxl allele of X55 carried the same GT polymorphism as Wxa allele at the well-defined GT/TT polymorphism site that differentiates Wxa and Wxb alleles (Zhang et al., 2019). We designed two target sites, Target1 and Target2 which was located at the 5' and 3' of Wx 1st intron, respectively (Fig. 1A). An edit vector (Fig. 1B) expressing CRISPR/Cas9, two gRNAs (guide RNA, gRNA1 for target1 and gRNA2 for target2), and CP4/EPSPS (as selection marker) was delivered into rice cells through Agrobacterium-mediated transformation. A total of 314 glyphosate-resistant transgenic plants were generated for these 4 rice inbreds (Table 1). Our PCR assays on all the 314 primary transformants detected 33 mutants with one or two copies of the first intron removed, deletion efficiency ranging from 8.6-11.85% (Fig. 1C, Table 1). These 33 intron deleted mutants included 12 for KY131, 8 for X32, 5 for X35 and 8 for X55 (Fig. 1C, Table 1).

| CRISPR/Cas9-mediated target deletion of Wx 1st intron |
|------------|----------------|----------------|----------------|
| Inbred Name | No. of T0 plant tested | No. of 1st intron deleted lines (deletion efficiency, %) | No. of T1 lines tested |
| KY131      | 104             | 12 (11.5)      | 4              |
| X32        | 68              | 8 (11.8)       | 4              |
| X35        | 58              | 5 (8.6)        | 2              |
| X55        | 84              | 8 (9.5)        | 4              |

We further investigated if off-target occurred in our experiments. Using the online software CRISPR-P2.0 (http://crispr.hzau.edu.cn/CRISPR2/), we identified two potential off-target sites for both gRNAs used in this study. PCR amplification and DNA sequencing of predicted off-target sites were performed on both T0 mutants and partial of their T1 offsprings. As shown in Supplementary Table S1, no off-target was detected at the putative off-target loci in both tested T0 plants and their T1 offsprings.

**Identification of transgene-free, homozygous mutants**

T1 seeds of the primary transformants with normal seed setting rate and other agronomic traits were selected for further screening of transgene-free, homozygous mutants. As shown in Tables 1, 4, 4, 4 and 4 target mutant lines were botained for KY131, X32, X55 and X35, respectively. Sequencing these 14 lines confirmed that the first intron of their Wx genes was completely deleted. Further analyses indicated that these 14 mutants could be categorized into 3 mutant types according to their difference in the number of nucleotides deleted. These three mutant types were named as M1(-1041bp), M2(-1042bp), M3(-1043bp), respectively, with M2 as the predominant one (9/14) (Fig. 1D, Table 2). Alignment of the DNA sequence of these 3 mutant types with that of WT also revealed that, besides precise deletion of the entire first intron (1021bp), extra 20-, 21- or 22bp were deleted in M1, M2 or M3 mutant, respectively (Fig. 1D). We predicted that the 5'UTR of M1, M2 and M3s mRNA would respectively be 20-, 21- and 22bp shorter than that of wild type.
physicochemical properties of rice grains derived from mutant lines KY131-CR-1, KY131-CR-3, X32-CR-1, X32-CR-2 and their corresponding WT KY131 and X32 mutants. Results showed that the increased level of amylose content was consistent in both generations (Fig. 3A). Thus, we concluded that the extra 20-22bp deletion in the 5` UTR of intron deleted mutants had not much influence on Wx gene expression in a WT background. However, the relative expression levels of Wx gene between X35 and X55 WT plants (both have the Wxa allele) and their intron-deleted mutants did not show much difference (Fig. 2A), demonstrating that removal of the first intron does not affect the Wx gene expression in a Wxa background.

As aforementioned that the 5`UTR of Wxa allele, the grain amylose contents of transgene-free, homozygous mutants (first generation) and together with their corresponding WT plants was measured, and the results were shown in Table 2. The amylose content of KY131 and X32 mutants significantly increased from 13% to about 24%, while the amylose content of X35 and X55 mutants had no significant difference (Table 2, Fig. 2B). These amylose content results were well correlated to our Wx gene expression data. Significantly increased relative expression of Wx gene was only observed in KY131 and X32 mutants that showed significant AC increase, but not in X35 and X55 mutants that showed similar AC as their corresponding WT plants (Fig. 2A). We further analyzed the grain amylose content of second generation KY131 and X32 mutants. Results showed that the increased level of amylose content was consistent in both generations (Fig. 3A,3B), suggesting that the amylose content change in mutants were genetically stable.

### Table 2

| Background | Mutant line | No. of transgene-free plants tested | Mutation type | AC (%) |
|------------|-------------|-------------------------------------|---------------|--------|
| KY131      | 12          | WT                                  | 13.8 ± 0.22 a |
| KY131-CR-1 | 8           | M2                                  | 22.5 ± 0.07 b |
| KY131-CR-2 | 6           | M2                                  | 22.7 ± 0.36 b |
| KY131-CR-3 | 12          | M2                                  | 24.3 ± 0.12 b |
| KY131-CR-4 | 11          | M2                                  | 21.5 ± 0.40 b |
| X32        | 12          | WT                                  | 11.2 ± 0.06 a |
| X32-CR-1   | 8           | M1                                  | 23.3 ± 0.37 b |
| X32-CR-2   | 8           | M2                                  | 22.0 ± 0.65 b |
| X32-CR-3   | 12          | M2                                  | 21.3 ± 0.34 b |
| X32-CR-4   | 9           | M1                                  | 23.2 ± 0.25 b |
| X55        | 12          | WT                                  | 28.1 ± 0.68 a |
| X55-CR-1   | 8           | M2                                  | 28.1 ± 0.10 a |
| X55-CR-2   | 6           | M2                                  | 28.9 ± 0.69 a |
| X55-CR-3   | 7           | M2                                  | 28.3 ± 0.64 a |
| X55-CR-4   | 10          | M2                                  | 27.6 ± 0.39 a |
| X35        | 12          | WT                                  | 25.5 ± 0.68 a |
| X35-CR-1   | 9           | M3                                  | 25.5 ± 0.43 a |
| X35-CR-2   | 12          | M2                                  | 25.3 ± 0.34 a |

Notes: M1, M2, M3 represent mutants with 1041bp, 1042bp, 1043bp deletion, respectively (Fig. 1D). AC values reported are mean ± SD. Letters following the AC mean values stands for the levels of difference, a, not significantly different, b, significantly different (P < 0.01, t-test).

**Wx gene expression was significantly increased in KY131 and X32 mutants**

To investigate the expression of Wx gene in intron-deleted mutants, the relative amount of Wx gene mRNA in 10 DAP (days after pollination) seeds of different mutant plants and WT plants was detected by qRT-PCR. Results were shown in Fig. 2. Compared to KY131 and X32 WT plants that both carried Wxa allele, the intron-deleted mutants of KY131 and X32 plants accumulated about 1-2 times more stable mRNA (Fig. 2A), suggesting that removal of the first intron significantly affects Wx gene expression in a WT background. As aforementioned that the 5`UTR of Wxa allele, the grain amylose content of KY131 and X32 mutants significantly increased from 13% to about 24%, while the amylose content of X35 and X55 mutants had no significant difference (Table 2, Fig. 2B). These amylose content results were well correlated to our Wx gene expression data. Significantly increased relative expression of Wx gene was only observed in KY131 and X32 mutants that showed significant AC increase, but not in X35 and X55 mutants that showed similar AC as their corresponding WT plants (Fig. 2A). We further analyzed the grain amylose content of second generation KY131 and X32 mutants. Results showed that the increased level of amylose content was consistent in both generations (Fig. 3A,3B), suggesting that the amylose content change in mutants were genetically stable.

**Deletion of Wx 1st intron substantially increased grain amylose content of KY131 and X32 mutants**

The grain amylose content of transgene-free, homozygous mutants (first generation) and together with their corresponding WT plants was measured, and the results were shown in Table 2. The amylose content of KY131 and X32 mutants significantly increased from 13% to about 24%, while the amylose content of X35 and X55 mutants had no significant difference (Table 2, Fig. 2B). These amylose content results were well correlated to our Wx gene expression data. Significantly increased relative expression of Wx gene was only observed in KY131 and X32 mutants that showed significant AC increase, but not in X35 and X55 mutants that showed similar AC as their corresponding WT plants (Fig. 2A). We further analyzed the grain amylose content of second generation KY131 and X32 mutants. Results showed that the increased level of amylose content was consistent in both generations (Fig. 3A,3B), suggesting that the amylose content change in mutants were genetically stable.

**Grain quality evaluation and physicochemical property analysis of mutant grains**

It is well established that grain amylose content is closely linked to grain quality and affects grain physicochemical properties such as GC and GT(Tian et al., 2009). To examine whether the deletion of the first intron of Wx gene would affect other physicochemical properties of rice grains, we measured the physicochemical properties of rice grains derived from mutant lines KY131-CR-1, KY131-CR-3, X32-CR-1, X32-CR-2 and their corresponding WT KY131 and X32.
X32. Compared with grains of the WT plants, grains of 4 mutant plants showed no significant difference in gelatinization temperature/ASV (alkali spreading value) (Table 3). However, their amylose content increase did slightly decrease the Gel consistency of rice grains (Table 3) as expected. The RVA (Rapid Visco Analyzer) pasting property values also reflect similar results (Table 3, Fig. 3B). In addition, milling quality was also not much different between the grains of KY131 and X32 WT plants and their mutants (Table 3). Grain transparency evaluation demonstrated that polished rice grains of X32 intron-deleted mutants had better transparency than that of X32 WT plants, indicating appearance quality was improved in X32 intron-deleted mutants (Table 3, Fig. 4A, 4B). However, the improvement of appearance quality observed in X32 mutants was not found in KY131 intron-deleted mutants (Table 3, Fig. 4C, 4D). We further inspected whether amylose content change influences the structure of starch granules by scanning electron microscopy (SEM) of transverse mature endosperm sections. Our results revealed no obvious difference in the morphology of starch granules from the endosperm between mutants and wild types (Fig. 4E-P).

Table 3
Appearance quality, milling quality and physiochemical properties of K131 and X32 mutant lines (2nd generation of transgene-free homologous mutant plants)

| Properties                | K131          | X32           |
|---------------------------|---------------|---------------|
|                           | K131(WT)      | K131-CR-1     | K131-CR-3   | X32(WT) | X32-CR-1 | X32-CR-2 |
| Brown rice rate (%)       | 82.2 ± 0.14a  | 81.9 ± 0.08a  | 82.7 ± 0.02a | 77.8 ± 1.2a | 77.5 ± 0.77a | 77.6 ± 0.52a |
| Polished rice rate (%)    | 70.6 ± 0.34a  | 70.2 ± 0.58a  | 70.7 ± 0.44a | 65.2 ± 1.16a | 66.6 ± 0.45a | 65.1 ± 0.86a |
| Head rice rate (%)        | 66.8 ± 2.08a  | 62.4 ± 0.57a  | 64.5 ± 1.55a | 59.7 ± 0.40a | 54.0 ± 0.94a | 54.8 ± 1.34a |
| Grain length(mm)          | 4.2 ± 0.09a   | 4.7 ± 0.01a   | 4.5 ± 0.06a  | 5.6 ± 0.11a  | 5.6 ± 0.02a  | 5.6 ± 0.01a  |
| Grain width(mm)           | 2.7 ± 0.04a   | 2.6 ± 0.01a   | 2.7 ± 0.01a  | 2.0 ± 0.01a  | 1.9 ± 0.00a  | 1.9 ± 0.00a  |
| Length/ Width ratio       | 1.6 ± 0.03a   | 1.8 ± 0.01a   | 1.7 ± 0.02a  | 2.8 ± 0.04a  | 3.0 ± 0.01a  | 3.0 ± 0.00a  |
| Chalkiness rate (%)       | 10.0 ± 1.33a  | 14.1 ± 2.86a  | 13.9 ± 1.58a | 14.7 ± 1.43a | 8.8 ± 0.50a  | 9.7 ± 0.95a  |
| Chalkiness degree (%)     | 2.9 ± 0.20a   | 3.7 ± 0.66a   | 3.6 ± 1.15a  | 4.9 ± 0.77a  | 2.8 ± 0.27a  | 3.4 ± 0.34a  |
| Transparency grade        | 2 ± 0.00a     | 2 ± 0.00a     | 2 ± 0.00a    | 1 ± 0.00a    | 1 ± 0.00a    | 1 ± 0.00a    |
| AC (%)                    | 13.7 ± 0.22a  | 21.9 ± 0.07b  | 23.9 ± 0.12b | 13.1 ± 0.12a | 23.3 ± 0.17b | 20.7 ± 0.57b |
| GC (mm)                   | 84.6 ± 1.26a  | 61.2 ± 3.24b  | 54.8 ± 1.35b | 97.4 ± 1.92a | 82.4 ± 2.46b | 82.7 ± 1.67b |
| ASV                       | 6.0 ± 0.00a   | 6.9 ± 0.06a   | 6.5 ± 0.31a  | 1.3 ± 0.10a  | 1.2 ± 0.00a  | 1.1 ± 0.13a  |
| PKV(RVU)                  | 317.9 ± 8.38  | 198.1 ± 8.79  | 232.7 ± 2.63 | 322.3 ± 1.93 | 237.9 ± 3.35 | 251.8 ± 5.82 |
| HPV(RVU)                  | 176.4 ± 17.69 | 154.4 ± 10.41 | 172.6 ± 4.62 | 131.9 ± 5.85 | 1437 ± 5.92  | 151.6 ± 2.53 |
| BDV(RVU)                  | 141.5 ± 10.40 | 43.7 ± 6.13   | 60.0 ± 3.83  | 190.4 ± 7.45 | 94.2 ± 2.74  | 100.2 ± 5.38 |
| CPV(RVU)                  | 286.3 ± 20.19 | 260.0 ± 7.63  | 298.4 ± 3.05 | 202.4 ± 3.59 | 270.3 ± 4.05 | 274.3 ± 4.13 |
| SBV(RVU)                  | -31.6 ± 12.76 | 61.9 ± 1.34   | 65.7 ± 1.56  | -119.9 ± 5.5 | 32.4 ± 0.70  | 22.4 ± 1.80  |
| CSV(RVU)                  | 109.9 ± 2.50  | 105.6 ± 6.79  | 125.7 ± 5.4  | 70.5 ± 3.22  | 126.6 ± 2.2  | 122.7 ± 4.22 |
| PetT(Min)                 | 6.1 ± 0.17    | 6.6 ± 0.16    | 6.4 ± 0.08   | 5.6 ± 0.08   | 6.0 ± 0.07   | 5.9 ± 0.12   |
| PeT(°C)                   | 75.5 ± 0.44   | 74.6 ± 0.89   | 75.5 ± 0.43  | 84.8 ± 0.03  | 84.2 ± 0.98  | 82.6 ± 0.46  |

Notes: Values reported are mean ± SEM. Letters following the mean values stands for the levels of difference, a, not significantly different, b, significantly different (P < 0.01, t-test). AC, amylose content; GC, gel consistency; ASV, alkali spreading value; PV, peak viscosity; HPV, through viscosity or hot paste viscosity; CPV, final viscosity or cool paste viscosity; BDV, breakdown viscosity (BDV = PV-HPV); SBV, setback viscosity (SBV = CPV-PV); CSV, consistency viscosity (CSV = CPV-HPV); PeT, peak time; PaT, pasting temperature. All the viscosity parameters were expressed in rapid visco units (RVU).

Conclusions

The amylose content (AC) in rice endosperm is an important factor affecting the rice eating and cooking quality (ECO) (Tian et al., 2009). However, vast differences in regional consumer preference, market demand and functionality makes ECO hard to define and standardize. In general, South Asians favor long, slender rice with a high AC and hard gel consistency (GC), while Southeast Asians have a preference for long grains with intermediate AC and soft GC. Such differences often hinder the spread of elite varieties to other countries whose rice markets demand rice with a different AC. Developing efficient, low-cost technology to modulate grain AC will provide a reliable solution to overcome this barrier. In addition, the development of specialized rice consumption creates more diversified demands for rice AC. For example, changing market demands more and better quality rice specialized for rice wine industry and for patients with diabetes or high blood pressure in recent years (Sun et al., 2017). Therefore, it is of great commercial value to develop biotechnological methods to accurately adjust rice grain AC to satisfy diverse consumers.

With the rapid advance of CRISPR/Cas-mediated gene editing technologies (Gao, 2021), researchers have already rushed to exploit these novel powerful technologies for accurate modulation of rice grain AC. Studies including knocking out the Wx gene to produce glutinous rice (Ma et al., 2015; Xu et al., 2015; Zhang et al., 2018b), fine-tuning Wx gene expression via regulatory promoter editing, and modulating the enzyme activity of GBSSI via base editing have been carried out (Huang, et al., 2021; Xu et al., 2020; Zeng et al., 2020). These studies have paved new avenues...
for rice AC improvement, shed new lights on the understanding of the molecular regulation of Wx gene expression and the modulation of GBSSI enzyme activity, and importantly generated an array of novel Wx alleles that have valuable application potential. In this study, we developed a new strategy to modify rice AC. We deleted the entire first intron of the rice Wx gene in both Wx<sup>a</sup> and Wx<sup>b</sup> backgrounds using the CRISPR/Cas9 technology. Removal of the first intron from inbreds KY131 and X32 with a Wx<sup>b</sup> allele significantly increased grain AC, by more than 10%, from 13% to about 24.0% (Table 2 Fig. 2). However, such a phenomena was not observed in inbreds X35 and X55 carrying a Wx<sup>a</sup> allele. Grain AC of WT X35, X55 and their intron-deleted mutants remained about the same ( Table 2, Fig. 2). Our method will provide an efficient and rapid way to convert intermediate AC rice cultivars (mostly with a Wx<sup>b</sup> allele) to high AC cultivars, which facilitates elite rice cultivars of intermediate AC adapted to new plantation regions and consumer markets that favor high AC cultivars.

Deletion of the entire first intron generated a complete novel Wx allele which has other implications. First, removal of the first intron allowed us to gain insights into its regulation on Wx gene expression. Previous studies have showed that the GT/TT SNP at the 5’ splicing junction of the first intron is a major post-transcriptional regulation factor to affect rice grain AC(Cai et al., 1984; Isshiki; et al., 1998, Samadder et al., 2008). By eliminating the splicing process of the first intron, we demonstrated that rice seeds accumulated about 1–2 times more amount of stable mRNA and their amylose content increased by about 10% (from 13% to about 24%) after the first intron removal under Wx<sup>b</sup> background (Table 2, Fig. 2), while stable mRNA accumulation and seed AC remained about the same after the first intron deleted under the Wx<sup>a</sup> background (Table 2, Fig. 2). This gives us a quantitative view on how much the G to T SNP post-transcriptionally affects the expression of the Wx gene. A previous study also demonstrates that the first intron of the rice Wx gene stimulates the expression of a foreign gene in rice protoplasts when it is placed in the 5’ UTR region between CaMV 35S promoter and GUS coding sequence, indicating that the first intron could act as a transcriptional enhancer(Li et al., 1995). This notion might have solid ground given the fact that quite a few first introns with big size (~1.0kb or more) and located within the 5’ UTR of highly expressed genes such as rice Actin1, maize Ubi1, indeed function as transcriptional stimulators(Rose, 2008, 2019). However, our data demonstrated that rice seeds carrying the Wx<sup>a</sup> gene with or without the first intron accumulated about the same amount of stable mRNA and produced about the same amount of amylose (Table 2, Fig. 2), strongly suggesting that the first intron of the rice Wx gene does not have transcriptional enhancement. Second, the new Wx allele without first intron will provide us an excellent starting material to further engineer new Wx alleles that could overcome the high temperature imposed adverse effects on rice grain quality. It has been well established that majority of japonica rice carry the Wx<sup>b</sup> allele and produce rice grain with intermediate AC, while majority of indica rice have the Wx<sup>a</sup> allele and produce rice grain with intermediate to high AC. In recent years, consumer preference is increasingly changing to favor intermediate AC rice in China market, which promotes the Wx<sup>b</sup> allele rapidly spreading into indica rice cultivars. However, previous studies have pointed out that under elevated temperature, the expression of Wx gene is down-regulated in some japonica cultivars, resulting in producing less GBSSI protein and leading to lower grain AC and lower grain quality. Presumably, the selection of the first intron 5’ splicing site of the Wx<sup>b</sup> gene is compromised under high temperature (Larkin and Park, 1999; Zhang et al., 2014a). The novel Wx allele created in this study may provide a promising solution to attenuate the adverse effect of high temperature on grain quality. Although removal of the first intron causes AC increase, further adjustment of the AC of the AC of the first intron deleted mutants to a suitable AC could be achievable using previously reported gene editing strategies(Huang et al., 2020a; Huang et al., 2021; Xu et al., 2020; Zeng et al., 2020). Engineering new Wx alleles that perform better under high temperature will be invaluable to rice production, particularly as global warming increasingly poses threats on the world food security.

In conclusion, our study presented a novel and efficient strategy to modify rice grain amylose content, particularly, to generate high amylose content rice from a rice carrying a Wx<sup>b</sup> allele. By deleting the entire first intron, we created a completely novel Wx allele. Further analyses on the first intron deleted mutants provided new insights into the regulation of Wx gene expression. And the new Wx allele could serve as an excellent material for further engineering new Wx alleles that perform better under high temperature and improve rice eating and cooking quality.

**Abbreviations**

- **AC**: amylose content
- **bp**: base pairs
- **CRISPR**: clustered regularly interspaced palindromic repeats
- **Cas**: CRISPR – associated protein
- **ECQ**: eating and cooking quality
- **EPSPS**: 5-enolpyruvylshikimate-3-phosphate synthase
- **GBSSI**: granule-bound starch synthase I
- **GC**: gel consistency
- **GT**: gelatinization temperature
- **gRNA**: guide RNA
- **GUS**: β-glucoronidase
- **PAM**: Protospeaker adjacent motif
- **qRT-PCR**: quantitative reverse transcriptase -PCR polymerase chain reaction

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js
Materials And Methods

Plant materials and growth conditions

The rice variety KY131 (KY131, Wx<sup>b</sup> allele, *Oryza sativa* L. ssp. *japonica*) was an elite inbred broadly cultivated in the Northeast China region. X32 (Wx<sup>a</sup> allele, *Oryza sativa* L. ssp. *indica*), X35 (Wx<sup>lv</sup> allele, *Oryza sativa* L. ssp. *indica*) and X55 (Wx<sup>a</sup> allele, *Oryza sativa* L. ssp. *indica*) were inbred lines provided by our breeders. The transgenic rice lines and transgene-free edited rice plants were all grown in the standard greenhouse (16-h light at 30°C/8-h night at 22°C) in the Life Science and Technology Center, China National Seed Group Co., LTD, Wuhan, China.

Construction of the CRISPR/Cas9 vector and plant transformation

The CRISPR/Cas9 vector targeting the first intron of the *Wx* gene was constructed as previously described (Zhang et al., 2014b). The vector used in this study was based on the vector pCambia1300 backbone. The editing vector pZZT477 contained a Cas9 expression cassette driven by sugarcane Ubi4 promoter, a *CP4-EPSPS* gene cassette (as selection marker) driven by CaMV 35S promoter, Two sgRNA expression cassettes respectively driven by rice U3 or U6 snRNA promoters (Fig. 1). The editing vector pZZT477 was transferred into *Agrobacterium* tumefaciens strain EHA105 by electroporation and consequently delivered into KY131, X32, X35 and X55 cells via *Agrobacterium*-mediated transformation as previously described (Hiei et al., 1997).

Molecular Characterization of the Mutant Plants

Rice genomic DNA was extracted by using a DNA Quick Plant System (TransGen Biotech, Beijing, China). 50 ng of genomic DNA were used as a template to perform PCR amplification using Taq polymerase (Tiangen, Beijing, China). Genomic DNA of all transgenic herbicide resistant T0 plants was examined by PCR using the specific primers *CP4F/CP4R*. The primer sets T1-F/T1-R and T2-F/T2-R were designed to flank the designated target sites to make sure the sequence of rice cultivars, the primer sets JCF/JC-R were digested to obtain homozygous mutants, respectively. The PCR primer sets used for the PCR/RE assay were as listed in Supplementary Table S2. To examine the deletion details, PCR fragments from transgene-free homozygous plants were sequenced on ABI3730XL Capillary Sequencer.

Selection of transgene-free homozygous mutants

According to the Chinese National Standards Ministry of Agriculture Announcement No. 953-6-2007, Announcement GB/T 19495.5—2004 Genetically Modified Product Testing, the rice leaves of T1 transgenic plants were sampled for GMO testing. An Applied Biosystems 7900HT instrument was used to conduct quantitative real-time PCR for detecting transgene residues. To ensure there were no transgene residues in selected homozygous mutants, we used 13 primer pairs to amplify T-DNA components including CaMV35S promoter, Ssuβi4 promoter, NOS terminator, OCS terminator, CaMV35S terminator, Cas9, and Epsps-CP4, and vector backbone region. Only homologous mutants with PCR product negative for all 13 primer pairs would be selected for further analysis.

RNA Extraction and RT-PCR analysis

Total RNA was extracted from grains after 10 days of grain-filling using the TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using the Transcripter First Strand cDNA Synthesis Kit (Roche). Quantitative RT-PCR analyses were conducted to amplify *Wx* gene on an Applied Biosystems 7900HT instrument with 2× SYBR Green PCR Master Mix (Applied Biosystems).

Scanning Electron Microscopy of Starch Granules

Rice grains were dried in an oven at 42°C for 2 days and cooled in a desiccator. Cross sections of the samples were manually snapped and sputter-coated with gold palladium on copper studs. Magnifications of 500× and 2000× were used to observe endosperm and starch granule morphology.

Evaluation of Rice Quality

The rice quality was measured following the procedure described in GB/T 15683-2008 and NY/T 83-2017, and each sample was tested for 3 times. RVA pasting properties were detected with a Rapid Visco Analyzer (RVA) according to the manufacturer’s instruction (NewPort Sci. Co., Australia).

Statistical Analysis

The data were analyzed by using Student’s unpaired t-test in the Microsoft Excel. Differences were considered to be significant at P < 0.05 or P < 0.01.
Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information files. The materials used and/or analysed during the current study are available from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by The National Key Research and Development Program of China (2016YFD0102000) and “Breeding of major new varieties of main grain crops” program (2020ABA016) from Department of science and technology of Hubei Province.

Authors’ contributions

LX. designed the experiments and conducted partial vector construction, plant transformation, \textit{Wx} gene expression analysis and other required works. DQ, WW, PY, TC, CY, XN and LH contributed in vector construct, plant transformation, molecular analysis works. WX, YR participated in grain quality analysis. LY, QY and YN were responsible for plant growth and management. LJ and MC wrote the manuscript and coordinated all the experiments.

Acknowledgement

We were very grateful of Dr. Yizhon Cai, Ms. Jing Wei and other colleagues who contributed to this work, but was unable to be listed as co-authors, for their invaluable helps in this work.

References

1. Cai, X., Wang, Z., Xing, Y., Zhang, J., and Hong, M. (1984). Aberrant splicing of intron 1 leads to the heterogeneous 5’UTR and decreased expression of \textit{waxy} gene in rice cultivars of intermediate amylose content. The Plant Journal \textit{14}, 459–465.
2. Chen, K.L., Wang, Y.P., Zhang, R., Zhang, H.W., and Gao, C.X. (2019). CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture. Annual Review of Plant Biology, Vol 70, 667-697.
3. Chen, M.-H., Bergman, C.J., Pinson, S.R.M., and Fjellstrom, R.G. (2008). \textit{Waxy} gene haplotypes: Associations with pasting properties in an international rice germplasm collection. Journal of Cereal Science \textit{48}, 781-788.
4. Dobo, M., Ayres, N., Walker, G., and Park, W.D. (2010). Polymorphism in the GBSS gene affects amylose content in US and European rice germplasm. Journal of Cereal Science \textit{52}, 450-456.
5. Fiaz, S., Ahmad, S., Noor, M.A., Xu, Peng, Wang, Younas, A., Riaz, A., Riaz, A., and Ali, a.F. (2019). Applications of the CRISPR/Cas9 System for Rice Grain Quality Improvement Perspectives and Opportunities. International Journal of Molecular Sciences \textit{20}, 888.
6. Fitzgerald, M.A., McCouch, S.R., and Hall, R.D. (2009). Not just a grain of rice: the quest for quality. Trends Plant Sci \textit{14}, 133-139.
7. Gao, C. (2021). Genome engineering for crop improvement and future agriculture. \textit{Cell} \textit{184}, 1621-1635.
8. Hiei, Y., Komari, T., and Kubo, T. (1997). Transformation of rice mediated by Agrobacterium tumefaciens. Plant Mol Biol \textit{35}, 205-218.
9. Huang, L., Li, Q., Zhang, C., Chu, R., Gu, Z., Tan, H., Zhao, D., Fan, X., and Liu, Q. (2020a). Creating novel \textit{Wx} alleles with fine-tuned amylose levels and improved grain quality in rice by promoter editing using CRISPR/Cas9 system. Plant Biotechnol J \textit{18}, 2164-2166.
10. Huang, L., Nese Sreenivasulu, and Liu, Q. (2020b). Waxy Editing Old Meets New. Trends Plant Sci \textit{25}, 963-966.
11. Huang, X., Su, F., Huang, S., Mei, F., Niu, X., Ma, C., Zhang, H., Zhu, X., Zhu, J.K., and Zhang, J. (2021). Novel \textit{Wx} alleles generated by base editing for improvement of rice grain quality. J Integr Plant Biol.
12. Isshiki, M., Morino, K., Nakajima, M., Okagaki, R.J., Wessler, S.R., Izawa, T., and Shimamoto, K. (1998). A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the S’ splice site of the first intron The Plant Journal \textit{75}, 133-138.
13. Jin, L., Lu, Y., Shao, Y.F., Zhang, G., Xiao, P., Shen, S.Q., Corke, H., and Bao, J.S. (2010). Molecular marker assisted selection for improvement of the eating, cooking and sensory quality of rice (\textit{Oryza sativa L.}). Journal of Cereal Science \textit{51}, 159-164.
14. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science \textit{337}, 816-821.
15. Jobling, S. (2004). Improving starch for food and industrial applications. Curr Opin Plant Biol \textit{7}, 210-218.
16. Juliano, B. (1992). Structure, chemistry, and function of the rice grain and its fractions. Cereal Foods World, \textit{772}-772.
17. Kimiko Itoh, Hiroko Ozaki, Kyoko Okada, Hidetaka Hori, Yasuhito Takeda, and Mitsui, T. (2003). Introduction of Wx Transgene into Rice wx Mutants Leads to Both High- and Low-Amylose Rice. Plant and Cell Physiology 44, 473-480.

18. Larkin, P.D., and Park, W.D. (1999). Transcript accumulation and utilization of alternate and non-consensus splice sites in rice granule-bound starch synthase are temperature-sensitive and controlled by a single-nucleotide polymorphism. Plant Mol Biol 40, 719-727.

19. Lau, W.C., Rafii, M.Y., Ismail, M.R., Puteh, A., Latif, M.A., and Ramli, A. (2015). Review of functional markers for improving cooking, eating, and the nutritional qualities of rice. Front Plant Sci 6, 832.

20. Li, H., Prakash, S., Nicholson, T.M., Fitzgerald, M.A., and Gilbert, R.G. (2016). The importance of amylose and amylopectin fine structure for textural properties of cooked rice grains. Food Chem 196, 702-711.

21. Li, Y., Ma, H., Zhang, J., Wang, Z., and Hong, M.-m. (1995). Effects of the first intron of rice Waxy gene on the expression of foreign genes in rice and tobacco protoplasts Plant Science 108, 181-190.

22. Liu, Q.Q., Yu, H.X., Chen, X.H., Cai, X.L., Tang, S.Z., Wang, Z.Y., and Gu, M.H. (2005). Field Performance of Transgenic indica Hybrid Rice with Improved Cooking and Eating Quality by Down-regulation of Wx Gene Expression. Molecular Breeding 76, 199-208.

23. Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., Xie, Y., Shen, R., Chen, S., Wang, Z., Chen, Y., Guo, J., Chen, L., Zhao, X., Dong, Z., and Liu, Y.G. (2015). A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. Mol Plant 8, 1274-1284.

24. Mikami, I., Uwatoko, N., Ikeda, Y., Yamaguchi, J., Hirano, H.Y., Suzuki, Y., and Sano, Y. (2008). Allelic diversification at the wx locus in landraces of Asian rice. Theor Appl Genet 117, 979-989.

25. Pandey, M.K., Rani, N.S., Madhav, M.S., Sundaram, R.M., Varaprasad, G.S., Sivarajan, A.K., Bohra, A., Kumar, G.R., and Kumar, A. (2012). Different isoforms of starch-synthesizing enzymes controlling amylose and amylopectin content in rice (Orzysa sativa L.). Biotechnol Adv 30, 1697-1706.

26. Patrick D. Larkin, a, W.D.P. (2003). Association of waxy gene single nucleotide polymorphisms with starch. Molecular Breeding 72, 335–339.

27. Phing Lau, W.C., Latif, M.A., M.Y.R., Ismail, M.R., and Puteh, A. (2016). Advances to improve the eating and cooking qualities of rice by marker-assisted breeding. Crit Rev Biotechnol 36, 87-98.

28. Richter, C., Chang, J.T., and Fineran, P.C. (2012). Function and regulation of clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated (Cas) systems. Viruses 4, 2291-2311.

29. Rie Terada, Midori Nakajima, Masayuki Isshiki, Ron J. Okagaki, Wesseler, S.R., and KoShimamoto, a, (2000). AntisenseWxGenes with Highly Active Promoters Effectively SuppressWxGene Expression in Transgenic Rice. Plant and Cell Physiology 41, 881-888.

30. Rodriguez-Leal, D., Lemmon, Z.H., Man, J., Bartlett, M.E., and Lippman, Z.B. (2017). Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing. Cell 177, 470-480

31. Rose, A.B. (2008). Intron-mediated regulation of gene expression. Curr Top Microbiol Immunol 326, 277-290.

32. Rose, A.B. (2019). Introns as Gene Regulators: A Brick on the Accelerator. Front Genet 9, 672.

33. Samadder, P., Sivamani, E., Lu, J., Li, X., and Qu, R. (2008). Transcriptional and post-transcriptional enhancement of gene expression by the 5' UTR Intron of rice rubi3 gene in transgenic rice cells. Mol Genet Genomics 279, 429-439.

34. Sano, Y. (1984). Differential regulation of waxy gene expression in rice endosperm. Theor Appl Genet 68, 467-473.

35. Sano, Y., Maekawa, M., and Kikuchi, H. (1985). Temperature effects on the Wx protein level and amylose content in the endosperm of rice. Journal of Heredity 76, 221-222

36. Sun, Y., Jiao, G., Liu, Z., Zhang, X., Li, J., Guo, X., Du, W., Du, J., Francis, F., Zhao, Y., and Xia, L. (2017). Generation of High-Amylose Rice through CRISPR/Cas9-Mediated Targeted Mutagenesis of Starch Branching Enzymes. Front Plant Sci 8, 298.

37. Teng, B., Zeng, R., Wang, Y., Liu, Z., Zhang, Z., Zhu, H., Ding, X., Li, W., and Zhang, G. (2012). Detection of allelic variation at the Wx locus with single-segment substitution lines in rice (Orzysa sativa L.). Molecular Breeding 30, 583-595.

38. Tian, Z., Qian, Q., Liu, Q., Yan, M., Liu, X., Yan, C., Liu, G., Gao, Z., Tang, S., and Zeng, D. (2009). Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities. Proc Natl Acad Sci USA 106, 21760-21765.

39. Wang, Z.Y., Wu, Z.L., Xing, Y.Y., Zheng, F.G., Guo, X.L., Zhang, W.G., and Hong, M.M. (1990). Nucleotide sequence of rice waxy gene. Nucleic Acids Res 18, 5898.

40. Xu, R.F., Li, H., Qin, R.Y., Li, J., Qiu, C.H., Yang, Y.C., Ma, H., Li, L., Wei, P.C., and Yang, J.B. (2015). Generation of inheritable and "transgene clean" targeted genome-modified rice in later generations using the CRISPR/Cas9 system. Sci Rep. 5, 11491.

41. Xu, Y., Lin, Q., Li, X., Wang, F., Chen, Z., Wang, J., Li, W., Fan, F., Tao, Y., Jiang, Y., Wei, X., Zhang, R., Zhu, Q.H., Bu, Q., Yang, J., and Gao, C. (2020). Fine-tuning the amylose content of rice by precise base editing of the Wx gene. Plant Biotechnol J 19, 11-13.

42. Yu, H.X., Liu, Q.Q., Xu, L., Lu, M.F., Yang, X.J., Gong, Z.Y., Cai, X.L., Zhang, Y.S., Zhang, C.Q., Wang, Z.Y., and Gu, M.H. (2009). Quality characteristics and field performance of selectable marker-free transgenic rice with antisense Wx gene and improved quality derived from the elite parents of hybrid indica rice. Journal of Cereal Science 50, 370-375.

43. Zeng, D., Liu, T., Ma, X., Wang, B., Zheng, Z., Zhang, Y., Xie, X., Yang, B., Zhao, Z., Zhu, Q., and Liu, Y.G. (2020). Quantitative regulation of Waxy expression by CRISPR/Cas9-based promoter and 5UTR-intron editing improves grain quality in rice. Plant Biotechnol J 18, 2385-2387.

44. Zhang, C., Yang, Y., Chen, S., Liu, X., Zhu, J., Zhou, L., Lu, Y., Li, Q., Fan, X., Tang, S., Gu, M., and Liu, Q. (2021). A rare Waxy allele coordinately improves rice eating and cooking quality and grain transparency. J Integr Plant Biol 63, 889-901.

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js
45. Zhang, C., Zhu, J., Chen, S., Fan, X., Li, Q., Lu, Y., Wang, M., Yu, H., Yi, C., Tang, S., Gu, M., and Liu, Q. (2019). Wx(lv), the Ancestral Allele of Rice Waxy Gene. Mol Plant 12, 1157-1166.

46. Zhang, H., Duan, L., Dai, J.S., Zhang, C.Q., Li, J., Gu, M.H., Liu, Q.Q., and Zhu, Y. (2014a). Major QTLs reduce the deleterious effects of high temperature on rice amylose content by increasing splicing efficiency of Wx pre-mRNA. Theor Appl Genet 127, 273-282.

47. Zhang, H., Si, X., Ji, X., Fan, R., Liu, J., Chen, K., Wang, D., and Gao, C. (2018a). Genome editing of upstream open reading frames enables translational control in plants. Nat Biotechnol 36, 894-898.

48. Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., Mao, Y., Yang, L., Zhang, H., Xu, N., and Zhu, J.K. (2014b). The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12, 797-807.

49. Zhang, J., Zhang, H., Botella, J.R., and Zhu, J.K. (2018b). Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the Waxy gene in elite rice varieties. J Integr Plant Biol 60, 369-375.

50. Zhou, H., Xia, D., Zhao, D., Li, Y., Li, P., Wu, B., Gao, G., Zhang, Q., Wang, G., Xiao, J., Li, X., Yu, S., Lian, X., and He, Y. (2021). The origin of Wx(la) provides new insights into the improvement of grain quality in rice. J Integr Plant Biol 63, 878-888.

51. Zhou, Z., Robards, K., Helliwell, S., and Blanchard, C. (2002). Composition and functional properties of rice. International Journal of Food Science and Technology 37, 849-868.

52. Zhu, H.C., Li, C., and Gao, C.X. (2020). Applications of CRISPR-Cas in agriculture and plant biotechnology. Nature Reviews Molecular Cell Biology 21, 661-677.

53. Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., Qiu, J.L., Wang, D., and Gao, C. (2017). Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. Nat Biotechnol 35, 438-440.

Figures

**Figure 1**

CRISPR/Cas9-mediated deletion of Wx 1st intron. A: Position and sequence of the two gRNA target sites on the genomic regions of Wx gene. Introns are shown as lines; exons shown as boxes; The PAM motifs (CCN) are underlined. B: Schematic diagram of the edit vector pZZT477.
s EPSPs (5-enolpyruvylshikimate-3-phosphate synthase) gene; Ubi4, sugarcane Ubi4 promoter; Cas9, CRISPR/Cas9 gene; U3, U6, rice U3 and U6 snRNA promoter. C: Detection of mutations in the first Intron of Wx gene via PCR assay in T0 generation. D: Sequencing results of the first Intron deleted mutant lines, the PAM motifs are underlined, target sequences are highlighted in red, dotted lines indicate deletions. The WT indicate wild type, the M1, M2, M3 are different homozygous lines in T1 generation.

Figure 2

Wx gene expression, amylose content of endosperms in wild type and T1 mutants. A: Relative expression level of Wx gene in wild type and mutants of 1st generation of transgene-free homologous plants. The expression of WT plants was arbitrarily set to 1, and the relative expression value of mutants was obtained by comparing the mutant expression to WT expression. B: Amylose content of endosperms in wild type and 1st generation plants of transgene-free homologous mutants. Data is presented as mean ± SD, **indicates significant difference at P <0.01 (t test).
Figure 3

Amylose content and Gel consistency of endosperms in wild type and 2nd generation of mutant plants (T2). A: Amylose content of endosperms in wild type and 2nd generation of mutant plants (T2). B: Gel consistency of endosperms in wild type and 2nd generation of mutant plants (T2). Data is presented as mean ± SD. **indicates significant difference at P < 0.01 (t test).
Figure 4

The appearance and morphology of starch granules of intron deleted mutants. A-D: Milled rice from different mutants. A: X32, B: mutant X32-CR-2, C: KY131, D: mutant K131-CR-1. E-P: SEM images showing the morphology of the starch granules. E, I, M were X32, F, J, N were mutant X32-CR-2, G, K, O were KY131 and H, L, P were mutant K131-CR-1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- **SupplementaryTables.docx**