Brief Communication

Creating novel Wx alleles with fine-tuned amylose levels and improved grain quality in rice by promoter editing using CRISPR/Cas9 system

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In rice, the Waxy (Wx) gene encoding GBSSI (granule-bound starch synthase I) controls amylose synthesis in endosperm and is the primary factor influencing grain eating and cooking quality (ECQ) (Sano, 1984; Tian et al., 2009). The natural allelic variations within the Wx locus are the main cause of the broad diversity of amylose content (AC) and ECQ in modern rice (Zhang et al., 2019). Our recent study revealed a high-to-low AC selection trend globally in the course of rice domestication (Zhang et al., 2019). In recent decades, there have been advances in modulating AC and improving ECQ in both indica and japonica rice using certain Wx alleles, particularly Wx\(^b\) and Wx\(^a\) with low-to-intermediate AC (Lau et al., 2016). Generally, it is expected that mild regulation of AC can be implemented by fine-tuning of the Wx expression to improve rice ECQ.

Recent advances in the CRISPR/Cas system make it possible to knockout, knock-in, base edit or fine-tune expression of target genes (Chen et al., 2019; Li et al., 2020). Editing conserved cis-acting elements on a promoter, particularly the core regions vital for the initiation of RNA polymerase II transcription machinery (Haberle and Stark, 2018), is considered the most practical strategy to reduce transcript abundance. Although Wx expression could be eliminated to generate glutinous rice following the editing of coding sequences (Zhang et al., 2018), to date no study has reported the successful fine-tuning of Wx expression.

We predicted the cis-acting elements on the Wx promoter using PLACE (https://www.dna.affrc.go.jp/PLACE/?action=new place). Seven target sites were selected for editing using CRISPR-GE (https://bio.tools/crispr-ge), including six sites (S1–S6) near or containing the predicted elements and one (S7) in the core promoter near a predicted TATA box (Figure 1a). The designed single-guide RNAs for every two target sites, S1 and S2, S2 and S3, S3 and S4, or S5 and S6, were integrated into one CRISPR/Cas9 vector, and that of only S7 was constructed by itself. The constructs were introduced via Agrobacterium into the japonica cultivar Nipponbare carrying the Wx\(^a\) allele. A total of 49 individuals with target mutations were identified from 115 T\(_0\) transformants. Among the 29 lines in T\(_1\) generations with homozygous mutated Wx alleles, only the S7-edited plants exhibited significant decreases in AC in the endosperm compared with the wild type (Figure 1b). It is noteworthy that AC was not significantly altered in lines in which the S3- and S4-predicted motifs were edited (Figure 1b). We speculated that it might be due to multiple copies of these motifs on the Wx promoter (Figure 1a) (Wang et al., 2013), and, through this redundancy, other elements may complement the functions of disrupted motifs in the edited lines.

We further identified six homozygous edited lines without transgene via PCR and sequencing in T\(_2\) generation from the S7-edited plants, including two mutated Wx\(^a\) alleles with single-base substitution (Wx\(^b\rightarrow\text{CIA}\) and Wx\(^b\rightarrow\text{AC}\)), one with base insertion (Wx\(^b\rightarrow\text{I1}\)) and three with nucleotide deletion (Wx\(^b\rightarrow\text{d2}\), Wx\(^b\rightarrow\text{d8}\) and Wx\(^b\rightarrow\text{d15}\)) (Figure 1c). Excluding a slight decrease in kernel weight, all the lines had no significant differences in major agronomic traits and grain phenotype (Figure 1d) compared with the wild type. First, the transcript abundance of six novel Wx alleles was compared with that in Nipponbare (Wx\(^a\)) and its near-isogenic line (NIL) (wx) carrying the null wx allele (Zhang et al., 2019). As there will be Wx precursor mRNA (pre-mRNA) present due to the G-to-T variation in the splice site of intron 1 of the Wx\(^a\) allele (Cai et al., 1998), three pairs of primers (Figure 1a,e) were designed to evaluate the abundance of pre-mRNA, mature mRNA and total mRNA of Wx, respectively. The results revealed an overall downward trend in both precursor and total Wx mRNA in edited lines compared with the wild-type Wx\(^a\), while the mature Wx mRNA exhibited irregular changes, including dramatic reductions in Wx\(^b\rightarrow\text{CIA}\), Wx\(^b\rightarrow\text{I1}\), Wx\(^b\rightarrow\text{d2}\) and Wx\(^b\rightarrow\text{d8}\), and significant increases in Wx\(^b\rightarrow\text{d8}\) (Figure 1e). A dual-luciferase reporter assay in tobacco also showed consistent transcriptional activity results for S7-edited Wx promoters (Figure 1f). This indicated that mutating the S7 site in the Wx core promoter modulated the transcription levels.

Significantly, the edited lines exhibited different AC in different growth seasons. As shown in Figure 1g, in the winter in Lingshui, Hainan, China, the AC of six S7-edited lines declined markedly compared with the wild type, consistent with the change in Wx expression (Figure 1h). However, no significant or slight changes were observed in either AC or GBSSI abundance in edited lines grown in the summer at Yangzhou, Jiangsu (Figure 1g and h). The overall temperature in the winter in Lingshui was lower than that in summer.
Novel rice Wx alleles by promoter editing

(a) Schematic representation of the rice Wx gene with promoter editing sites.

(b) Table showing various mutations and their effects on the number of lines and AC (15.50-16.84).

(c) Diagrams illustrating the TATA box, PAM, and target site S7.

(d) Photographs showing Wx(WT) and Wxmut1(V10).

(e) Bar graphs comparing the relative expression levels of Wx precursor RNA (Pref/PreR), Wx mature RNA (MatF/MatR), and total Wx RNA (TotF(Exon4)/TotR(Exon7)).

(f) Bar graphs comparing the LUC/REN ratio across different conditions.

(g) Winter (Lingshui) and Summer (Yangzhou) amylose content analysis.

(h) Gel images showing GBSSI and HSP expression.

(i) Graph showing viscosity over time for different Wx conditions.

(j) Graph showing amylose content (%) at different temperatures.

(k) Graph showing relative expression level at different temperatures.
the summer in Yangzhou, which could be the major cause of the difference in AC. The Wx^{db} line was therefore selected to further verify this in greenhouse with different maximum temperatures during grain filling (31°C, 28°C and 20°C). The AC of the Wx^{db} line decreased under all three temperature conditions, while the AC changes under 31°C and 28°C were consistent with those from Yangzhou (summer) and Lingshui (winter), respectively (Figure 1j). Moreover, the AC decrease in Wx^{db} at 20°C was even greater than that at either 28°C or 31°C, which was consistent with the change in Wx expression under different temperatures (Figure 1k). This supported the supposition that temperature during grain filling was the key factor causing the difference in AC and GBSSI accumulation in the edited lines.

In summary, we generated six novel Wx alleles by editing the region near the TATA box of the Wx promoter, in turn down-regulating Wx expression and fine-tuning grain AC. Among them, Wx^{db} shows potential for breeding, as it showed a moderate and stable decrease in AC under various growth conditions (Figure 1g) and similar pasting properties (Figure 1i). The relative expression levels of Wx in developing endosperm. The locations of primers in brackets are arrowed in panel (a). (f) In vitro transcription activity of Wx promoter (–1463 bp to +1299 bp) from Wx2 and its edited alleles in tobacco using dual-luciferase reporter assay. Vector, pGreenII 0800-LUC. (g and h) Comparison of rapid viscosity analyser spectra of rice flours from plants in Lingshui (winter). (j and k) AC in mature grains and mature developing seeds under different temperature treatments. Wx^{lcv}, Wx^{b-A/C}, Wx^{h-17}, Wx^{d2}, Wx^{db} and Wx^{d15} indicate the edited lines, and Wx^{mp} (10.57% of AC) and wx (glutinous) are near-isogenic lines carrying corresponding Wx alleles under the Nipponbare (Wx1) background. At least three plants of each line were sampled for each measurement. Error bars are means ± SD (n = 3), * and ** indicate significant differences at P < 0.05 and P < 0.01 levels, respectively, based on one-way ANOVAs.

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Conflict of interest

The authors have declared no conflict of interest.

Author contributions

Q.Q. L., L. H., Q.F. L. and C. Z. conceived the project. L. H., R. C., Z. G., H. T., D. Z. and X. F. performed the research and analysed the data. L. H., Q.Q. L. and Q.F. L. wrote the manuscript.

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