Brief Communications

Quantitative regulation of Waxy expression by CRISPR/Cas9-based promoter and 5’UTR-intron editing improves grain quality in rice

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In cereal crops, grain starches are composed of different proportions of amylose and amylpectin, which determine the cooking and eating qualities. The amylose synthesis is controlled by the Waxy (Wx) gene encoding a granule bound NDP-glucose-starch glucosyltransferase (Shure et al., 1983). In rice (Oryza sativa L.), the varied activities of natural Wx alleles regulate different amylose contents (AC), gel consistency (GC) and pasting viscosity of grain starches; these factors together influence the grain appearance, cooking/eating quality and starch physical characters (Zhang et al., 2019). Wx is a strong allele mainly distributing in indica (an O. sativa subspecies) cultivars producing high ACs (25%–30%) (Wang et al., 1995). While Wx2, presenting mainly in japonica (another subspecies) cultivars, is a weak allele producing moderate ACs (15–18%) (Ishiki et al., 1998). Generally, rice grains with higher ACs and lower GC values have poor eating quality, while those with moderate ACs (15–20%) and higher GC values (60–80 mm) give better taste for most consumers. Using the successive backcrossing methods, Wx6 can be introgressed into indica varieties to improve the grain quality. However, the traditional breeding methods are time consuming and difficult to break close linkage drags with undesirable traits.

We previously employed CRISPR/Cas9 to target the Wx coding region to generate glutinous rice (Ma et al., 2015). However, this kind of function-knockout strategy produces only null gene alleles, and when Wx is targeted generally glutinous lines are generated. Studies on generating various quantitative variations of traits by genome editing are rare. To rapidly improve rice grain quality, here, we developed CRISPR/Cas9 editing strategies to generate new Wx alleles producing various ACs by quantitative regulation of its expression, using an elite indica variety Tian Feng8 (TFB) as a test. TFB is a widely used parent in hybrid rice breeding for its high-yield performance, but its grain quality (and of the resultant hybrids) is poor due to higher AC (ca. 25%) and lower GC (56 mm; see below).

Disruption of promoter sequences by genome editing may change agronomic traits (Li et al., 2017; Rodriguez-Leal et al., 2017). Therefore, we selected a ca. 2.0-kb upstream sequence of Wxα in TFB for targeting, which contains a 0.9-kb promoter regulatory region and a 1.1-kb intron-containing 5’untranslated region (UTR) (Figure 1a). The first strategy is based on transcriptional regulation, thus we analysed the promoter sequence using Plant-CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and identified three putative cis-regulatory elements (CREs), Endosperm-box, A-box and CAAT-box. We designed four pairs of targets (T1–T8) in this region (Figure 1a) using CRISPR-GE (Xie et al., 2017) for multiplex editing. The second strategy we explored is for post-transcriptional regulation by targeting the 5’UTR intronic splicing site (5’UISS) of Wxα with a target T9 to alter the intron-splicing pattern and efficiency. In addition, a coding-exon editing (with a target T10) was done to produce glutinous rice. Using our CRISPR/Cas9 system (Ma et al., 2015), we prepared six constructs for the double-target or single-target editing (Figure 1a, b), and used them for Agrobacterium-mediated transformation of TFB.

From transgenic (T1) segregating families, we PCR-selected transgene-free plants and further identified 23 homozygous mutant T2-lines (Figure 1b). These lines had base insertions or deletions at the targets, or fragment deletions (or a 36-bp substitution) between the paired targets, which removed the putative CREs, respectively (Figure 1b). In two lines (UISS-1, UISS-6) by the 5’USS-editing, the intronic splicing site (GT) was deleted (Figure 1c). We named some of these Wx mutant alleles that showed obviously down-regulated expression largely affecting AC (see below) as follows: Wxα-dE (Endosperm-box deleted in T1T2-2 line), Wxα-dU (unknown element deleted in T3T4-2), Wxα-dS (A-box deleted in T5T6-5), Wxα-dC1, Wxα-dC2 and Wxα-dC3 (CAAT-box deleted in T7T8-4, T7T8-5 and T7T8-6, respectively), and Wxα-dS1, Wxα-dS2 and Wxα-dS3 (splicing-site deleted/impaired in UISS-1, UISS-2 and UISS-6) (Figure 1b). We selected a T10-edited mutant (TFBg) for analyses.

To investigate the splicing patterns of the 5’UISS-edited lines, we performed reverse transcription (RT)-PCR and cDNA-sequencing. UISS-1 (Wxα-dS1), UISS-2 (Wxα-dS2) and UISS-6 (Wxα-dS3) generated multiple alternatively or atypically spliced transcripts with various frequencies, similar to Wxα in a japonica variety KY131 (Figure 1c). Three transcripts (Wxα-dS1-3, Wxα-dS2-4 and Wxα-dS4) were found to retain the non-spliced intron. These major alternative splicing events with size differences were confirmed by gel electrophoresis (Figure 1d). Obviously, the splicing-site deletion in UISS-1 and UISS-6 resulted in the altered intron-splicing patterns (and suppressed splicing of some transcripts). However, the 2-bp deletion near the 5’UISS in UISS-2 also produced an alternative
Improvement of rice grain quality by quantitative regulation of Wx expression via promoter and 5'UTS editing using CRISPR/Cas9. (a) Structure of Wxa and target sites at the promoter region (T1–T8 in four pairs), the intronic splicing site within the 5'untranslated region (5'UTS; T9) or a coding exon (T10). F-RT/R-RT and F-qRT/R-qRT, primers for RT-PCR and qRT-PCR, respectively. (b) Nucleotide variations (in red) at the targets (protospacer adjacent motif in italic) of homozygous mutant lines (T2) from an indica variety TFB carrying Wxa. '-' and 'del', base deletion; 'in', base insertion; 'subs', base substitution (AGACACAAATTCCTTCAGTTCTTTGTCTATCGGGCT). Sequences between the targets are omitted. Lower-case letters, the intron. TFBg, a glutinous line. (c, d) cDNAs (RNAs from 15-day-old seeds) showing mRNA splicing by sequencing 24 clones each line (c) and agarose-gel analysis (d). Asterisks indicate spliced-out nucleotides. KY131, a japonica variety with Wxb having a G-to-T mutation at the splicing site (SS). Actin 1, a control. (e) Measurements of Wx expression, amylose content (AC) and gel consistency (GC). Bars, SD (n = 3). Samples without a same letter show significant difference by Duncan's test (P < 0.05). (f) Polished grains of TFB and representative edited lines. (g) Rapid visco analysis profiles of grain starches of the lines. HHZ, an indica variety (with Wxa and 17.1% AC) as a comparison. cP (centi Poise), viscosity unit.

Figure 1
quantitative trait alleles with fine-tuned transcriptional and post-transcriptional regulations of gene expression activity. We expect that application of these grain-improved lines having desirable AC and GC levels and their exploitation in hybrid rice breeding will provide rice products with better quality to meet consumer’s preferences. As CREs and 5’UTR introns are present in many genes, our study provides a promising breeding method for improvement of important traits in crops and other organisms.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Y.-G.L. and Q.Z. designed the studies. D.Z., T.L., X.M., B.W., Z.Z., Y.Z., X.X., B.Y. and Z. Z. performed the experiments. D.Z., T.L., X.M., B.W., Z.Z., Q.Z. and Y.-G.L. analysed data. Y.-G.L. and Q.Z. wrote the paper.

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