

CRISPR/Cas9-Mediated Siprolamin mutagenesis increase the content of functional amino acids in foxtail millet (*Setaria italica*)

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Abstract

Foxtail millet is a small diploid C4 crop that possesses superior nutritional properties. It provides a high content of essential amino acids, vitamins, and minerals. In this study, we used CRISPR/Cas9 to simultaneously edit two α -prolamin genes in foxtail millet. Analysis of the derived homozygous lines revealed a decrease in prolamin content. Surprisingly, the content of FAAs such as lysine, taurine, GABA, tryptophan, and sialic acid as well as glucose, fructose, sucrose, and maltose, was significantly increased. Additionally, peak viscosity, final viscosity, trough viscosity, and other parameters showed significant differences, suggesting that the food quality of the gene editing lines may have been altered. The editing lines grew normally, with only a slight decrease in 1000-grain weight compared to the wild type. Overall, we successfully created new germplasm with significantly increased FAAs content through gene editing, providing a valuable reference for the selection of functional foxtail millet varieties.

Main Text

In this study, through RNA-seq analysis of foxtail millet grain development, we identified a prolamin gene, *Seita.8G190200* (*Si8G18810*), which exhibited higher expression during the dough and maturity stages compared to the ovule and milk stages^[5]. We performed phylogenetic tree analysis and characterized the gene structure and conserved motifs of the prolamin gene family in foxtail millet (Fig. 1a and Supplementary Fig. S1). To further investigate the biological functions of this gene, we designed sgRNA targeting *Seita.8G190200* and constructed the CRISPR-Cas9 vector (Fig. 1b). Through genetic transformation of Ci846, we obtained 35 transgenic seedlings and identified five editing types, including two homozygous lines (*ko1* and *ko2*) with insertion mutation types in the α -prolamin genes, along with three heterozygous lines (*ko3*, *ko4*, and *ko5*) (Supplementary Fig. S2). Subsequently, potential sgRNA-dependent off-target sites for *Seita.8G190200* were predicted using the web tool CRISPR-GE (<http://skl.scau.edu.cn/>). Two potential off-target sites containing 3-nt and 5-nt mismatches relative to *Seita.8G190200* were evaluated, and no off-target events were detected across the 14 lines tested (Table S1). Phenotypic comparison between the gene editing lines (*ko1* and *ko2*) and the wild type revealed that while *ko1* showed no significant difference in grain length compared to the wild type, its grain width was significantly reduced. In contrast, *ko2* exhibited significant reductions in both grain length and width (Fig. 1c). The thousand grain weight of both *ko1* and *ko2* was significantly lower than that of wild type (Fig. 1c).

Through histological staining with iodine solution, toluidine blue, and Sudan IV, we comparatively analyzed the wild type and two gene editing lines (*ko1* and *ko2*) (Supplementary Fig. S3). The endosperm of *ko1* and *ko2* exhibited enhanced starch deposition (brown/black staining), with starch constituting the predominant component. Toluidine blue staining clearly visualized endosperm cell structures (dark blue staining). Lipids were predominantly localized in the aleurone layer and embryo (orange-red staining). Near infrared grain analysis confirmed that, compared to the wild type, the gene editing lines exhibited significantly reduced starch and moisture content but elevated total protein and lipid levels (Supplementary Fig. S4).

More importantly, amino acids serve as the fundamental building blocks of proteins and represent the cornerstone of life. Amino acid metabolism plays a vital role in various biological processes. Through quantitative analysis of three biological replicates, we revealed a significant reduction in prolamin content in the gene editing lines *ko1* and *ko2* compared to the wild type, with decreases of 29.90% and 28.47%, respectively (Fig. 1d). We performed comprehensive amino acid profiling of triplicate seed samples from the wild type and the gene editing lines (*ko1*, *ko2*) using LC-MS/MS, including essential amino acids, non-essential amino acids and several non-proteinogenic amino acids. The gene editing lines grains exhibited significant increases in multiple functional amino acids relative to the wild type (Fig. 1e). Additionally, many amino acids with unknown functions were significantly increased in the gene editing lines and may participate in important biological processes (Supplementary Fig. S5).

Functional amino acids are crucial for human health. For example, taurine provides anti-aging and lipid-lowering benefits^[6], while γ -aminobutyric acid (GABA) plays a critical role in mood regulation^[7]. Multiple amino acids act synergistically to maintain human physiological homeostasis. Compared with the wild type, the gene editing lines *ko1* and *ko2* showed significantly increased levels of all essential and non-essential amino acids, except for tryptophan in *ko1* which showed no significant difference (Fig. 1e). Notably, taurine (2-Aminoethanesulfonic-Acid) content reached 4867.19 ng/g in *ko1* and 4885.42 ng/g in *ko2*, approximately double that of the wild type (2551.01 ng/g). Significant increases in kynurenine and kynurenic acid levels were observed in the gene editing lines. Compared with the wild type, sialic acid (N-Acetylneuraminic-Acid) content increased by 27.28% and 67.84%, GABA (γ -Aminobutyric-Acid) levels rose dramatically by 227.44% and 406.88%, serotonin (5-HT) content increased by 12.01% and 74.09%, succinic acid levels elevated by 89.53% and 119.56%, (S)- β -Aminoisobutyric Acid (BAIBA) content showed an approximately 1000-fold increase in *ko1* and *ko2* respectively. These findings suggest significant implications for the nutritional quality of foxtail millet grains in the gene editing lines.

To further investigate changes in storage substances of foxtail millet grains, we analyzed soluble sugar components in the gene editing lines and the wild type through three biological replicates, identifying 18 saccharides including 12 monosaccharides, 5 disaccharides, and 1 trisaccharide. All sugar components showed significant increases in the gene editing lines compared to the wild type, except for N-acetylglucosamine (Supplementary Fig. S6). Compared with wild type, glucose content of *ko1* and *ko2* was significantly increased by 230.29% and 425.89%, fructose content was significantly increased by 261.65% and 474.72%, and mannose content was significantly increased by 15.87% and 17.35%, respectively. Sucrose content increased by 3.08% and 10.95%, and maltose content increased by 1058.80% and 3018.08%, respectively. Raffinose represents a prominent naturally occurring trisaccharide, biosynthesizes through the enzymatic linkage of galactose, glucose, and fructose, it is also designated as melitriose. Interestingly, the contents of fructose and glucose were both increased, but the raffinose content of *ko1* and *ko2* was significantly reduced by 17.86% and 10.85%, respectively, compared with the wild type (Fig. 1f).

It has been reported that rice eating quality shows a negative correlation with protein content, higher protein content generally corresponds to inferior quality. Furthermore, quality depends on both protein

quantity and composition, particularly the negative impacts of glutenin and prolamin^[8]. To evaluate changes in eating quality of the gene editing lines, we analyzed their starch pasting properties using RVA (Fig. 1g). The results showed that compared to the wild type, peak viscosity was significantly increased in *ko1* and *ko2*. Trough viscosity and final viscosity were also markedly elevated. Breakdown viscosity and setback viscosity showed no significant differences (Supplementary Fig. S7). These findings indicate that knockout of the gene not only improves the nutritional quality of foxtail millet but also affects its eating quality.

Phenotypic analysis revealed that the gene editing lines exhibited not only reduced grain size and altered storage substance composition but also significantly decreased plant height and shorter panicles compared to wild type (Fig. 1h, i). However, no significant differences were observed in flag leaf length, width and panicle diameter compared to wild type (Supplementary Fig. S8). Thus, knocking out the gene will affect plant growth. This study on foxtail millet prolamins demonstrates that the *Seita.8G190200* plays crucial roles in regulating prolamin content and functional amino acid levels, potentially serving as an important target for improving both nutritional and eating quality of foxtail millet. These findings provide scientific foundations for developing novel nutritionally fortified functional foxtail millet products, breeding new foxtail millet varieties enriched with functional amino acids for human health benefits. In maize, zein is also known as prolamin. The cloning of the *O2* gene in maize marked a major breakthrough in zein research, as it is a key transcription factor regulating the expression of zein genes^[9,10]. Research on zein has provided preliminary insights into the molecular mechanisms regulating prolamin genes. Therefore, we hypothesize that the molecular mechanisms regulating prolamin genes in foxtail millet are also associated with the *O2* gene family. In subsequent studies, we need to further investigate the molecular mechanisms that regulate the accumulation of grain storage substances.

Declarations

Conflict of interest

The authors have no conflicts of interest to declare.

Author Contribution

J.W, X.C and R.Z planned and designed the manuscript. R.Z, J.W and X.C wrote the manuscript. R.Z and other authors performed experiments or edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability

All data used in this work are publicly available.

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Figures

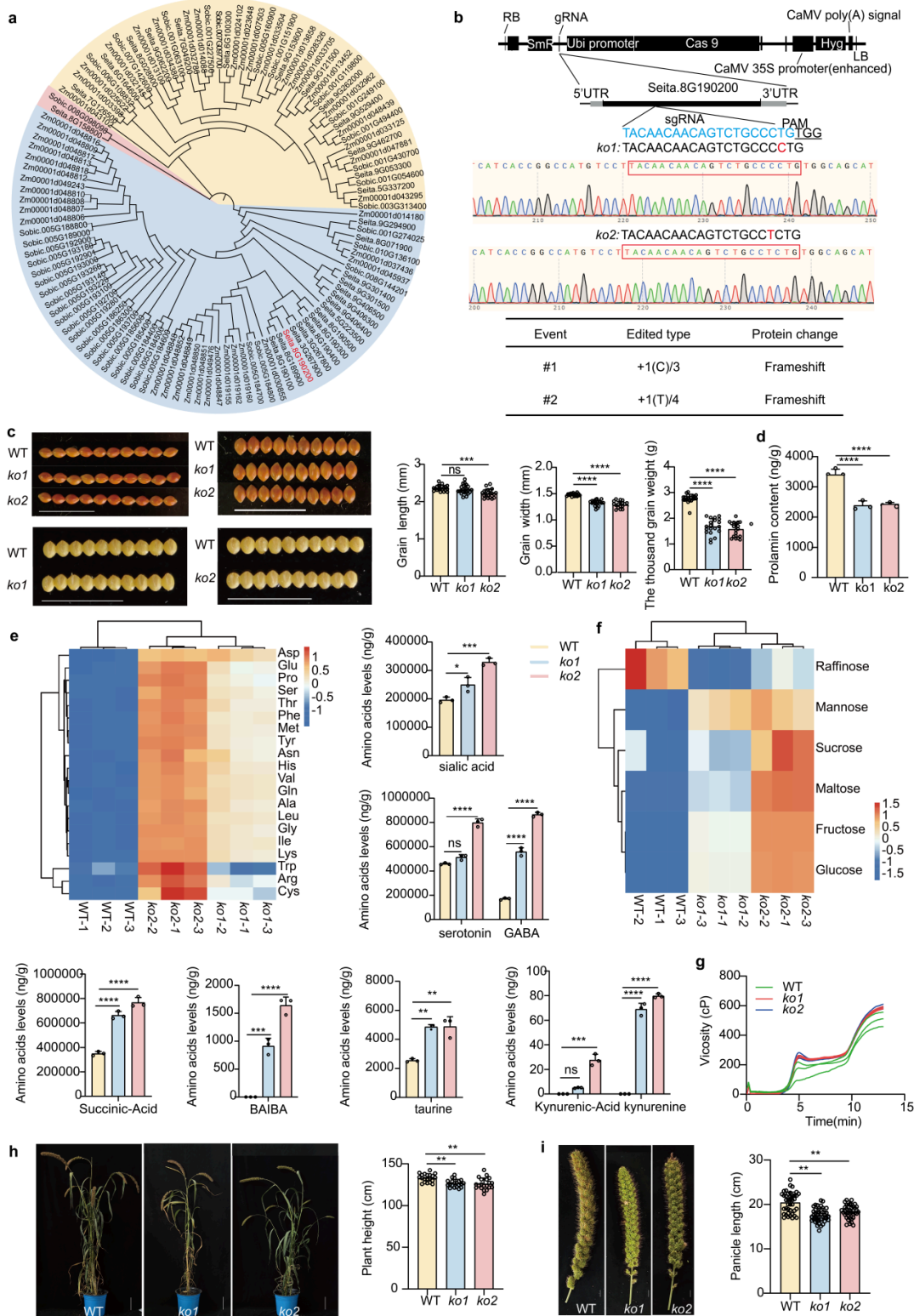


Figure 1

Knockout of *Seita.8G190200* can increase the content of functional amino acids and soluble sugars in foxtail millet grains while slightly affecting plant growth. (a) Phylogenetic tree of prolamins in foxtail millet, maize, and sorghum. (b) The target sites of the CRISPR-Cas9 vector and the editing types of the two knockout lines, *ko1* and *ko2*. (c) Phenotype of grain from wild type, *ko1*, and *ko2*, along with statistical results of grain length, grain width, and thousand grain weight. Scale bar = 1 cm. (d)

Significantly reduced prolamin protein content in *ko1* and *ko2* compared to wild type. (e) Heatmap comparison of essential and non-essential amino acid content in wild type and gene editing lines. Bar chart showing the changes in the content of functional amino acids in the grains of gene editing lines compared to the wild type. (f) Heatmap comparison of sugar content between gene editing lines and the wild type. (g) Paste viscograms of wild type, *ko1* and *ko2*. (h) Phenotypic analysis and statistical comparison of wild type, *ko1* and *ko2*. Scale bar=10 cm. (i) Panicle analysis and statistical comparison of wild type, *ko1* and *ko2*. Scale bar=1 cm. The mean $\pm$ SE of three biological replicates in (d and e) are shown. The significant differences are established by Tukey multiple comparison test. Each little circle indicates phenotype value of each sample. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$. ns, no significant difference.

Supplementary Files

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