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De novo creation of popcorn-like fragrant foxtail millet[FA]

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Aroma plays a crucial role in the sensory appeal and enjoyment of food, and it significantly impacts the grain quality of cereals, thereby influencing the value and consumption of agricultural products. For example, aromatic rice varieties like Basmati and Jasmine are renowned for their distinct aroma and command almost double the price of regular rice in the market. Despite the higher cost, they continue to be popular in both domestic and international rice markets (Shan et al., 2015). Thus, improving aromatic traits has become a key objective for crop breeding programs.

Aromatic rice is characterized by releasing more than 200 volatile compounds from its grains, with 2-acetyl-1-pyrroline (2-AP) being the key compound responsible for its distinctive aroma (Okpala et al., 2019). Fragrance in rice is known to be regulated by the *BADH2* gene, which encodes betaine dehydrogenase (Chen et al., 2008). The reduction in BADH2 activity or the presence of non-functional BADH2 enzymes results in the production of 2-AP and the release of fragrances (Chen et al., 2008). This function of BADH2 has been proven to be conserved across various crops, including rice, soybean, and sorghum (Yundaeng et al., 2013; Okpala et al., 2019; Qian et al., 2022). In recent years, gene editing technologies, such as clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), have been utilized to create novel plant materials with specific traits. In various crops, including rice, sorghum, wheat, soybean, and cotton, CRISPR/Cas9-mediated editing of the *BADH2* gene has been successful in inducing fragrance (Zhang et al., 2022; Imran et al., 2023).

Foxtail millet (*Setaria italica*) is domesticated in China and has a long history of cultivation in arid and semi-arid regions, mainly in East Asia (He et al., 2023). In Northern China, dehulled foxtail millet grain, also known as Xiaomi in Mandarin Chinese, is a traditional staple. People enjoy consuming it in various forms, such as congee and steamed Xiaomi, with a strong demand for fragrant aroma and taste. Besides its traditional use, millet-based processed foods, including millet cookies, millet noodles, and millet wine, have been developed (Birania et al., 2020). The inclusion of fragrance in millet can greatly enhance its utilization in the food industry. However, no naturally fragrant variety of foxtail millet has been discovered.

In the rice genome, there are two members of the *BADH* family. According to He et al. (2015), *BADH1* is closely correlated with salt tolerance, while *BADH2* is responsible for conferring fragrance to rice. To identify the *BADH2* gene in foxtail millet, we screened the foxtail millet genome for proteins exhibiting significant similarity to rice BADH and constructed a phylogenetic tree (Figure S1). Through this analysis, we identified two *BADH* genes in foxtail millet, namely *Seita.6G151100* and *Seita.7G127300*. *Seita.6G151100* showed higher protein homology with the *BADH2* genes of sorghum, maize, and rice, with sequence identities of 91.90%, 91.29%, and 86.51%, respectively. Conversely, *Seita.7G127300* exhibited higher homology with *BADH1*. As a result, *Seita.6G151100* was defined as *SiBADH2*, suggesting its possible role in conferring fragrance to foxtail millet.

We then analyzed the expression pattern of the *SiBADH2* gene using RNA-seq data from a non-fragrant variety, Yugu1. The analysis revealed that *SiBADH2* was expressed throughout all growth stages, with particularly higher expression levels during the seedling and flowering stages and the lowest expression level at the shooting stage (Figure S2). Moreover, *SiBADH2* was highly expressed in leaves and panicles, while its expression was relatively low in roots. These expression patterns are in line with previous reports on rice and sorghum (Okpala et al., 2019; Zhang et al., 2022).

To find aromatic millet germplasm resources, we screened 1,200 worldwide accessions of foxtail millet and its wild relatives, *Setaria viridis* (Table S1), for variations in *SiBADH2* using high-depth resequencing data. We found 84 single nucleotide polymorphisms (SNPs)/indels in the *SiBADH2* gene region, of which 81 were in the intron and untranslated regions (UTRs), and only three SNPs were in the exon. However, all these three SNPs were synonymous mutations and did not affect the gene's function (Figures 1B, S3). Based on these 84 variations, the 1,200 accessions were classified into 14 haplotypes (Table S2). Among them, Hap5, Hap8, Hap9, Hap10, Hap11, Hap12, Hap13, and Hap14 only existed in wild foxtail millet (*Setaria viridis*), while Hap1, Hap2, Hap3, Hap4, Hap6, and Hap7 were mainly present in the cultivated varieties (Figure S3). To further investigate the aromatic traits, we randomly selected 10–20 accessions from each haplotype, including cultivars and landraces from 10 countries or regions representing different alleles of *BADH2*, to conduct sensory evaluations for aroma (Table S3). We found that none of them were aromatic. These findings indicated that all the identified variations (SNPs/indels) in *BADH2* did not introduce any mutations that could affect the gene's function. Therefore, our understanding is that aromatic foxtail millet can only be

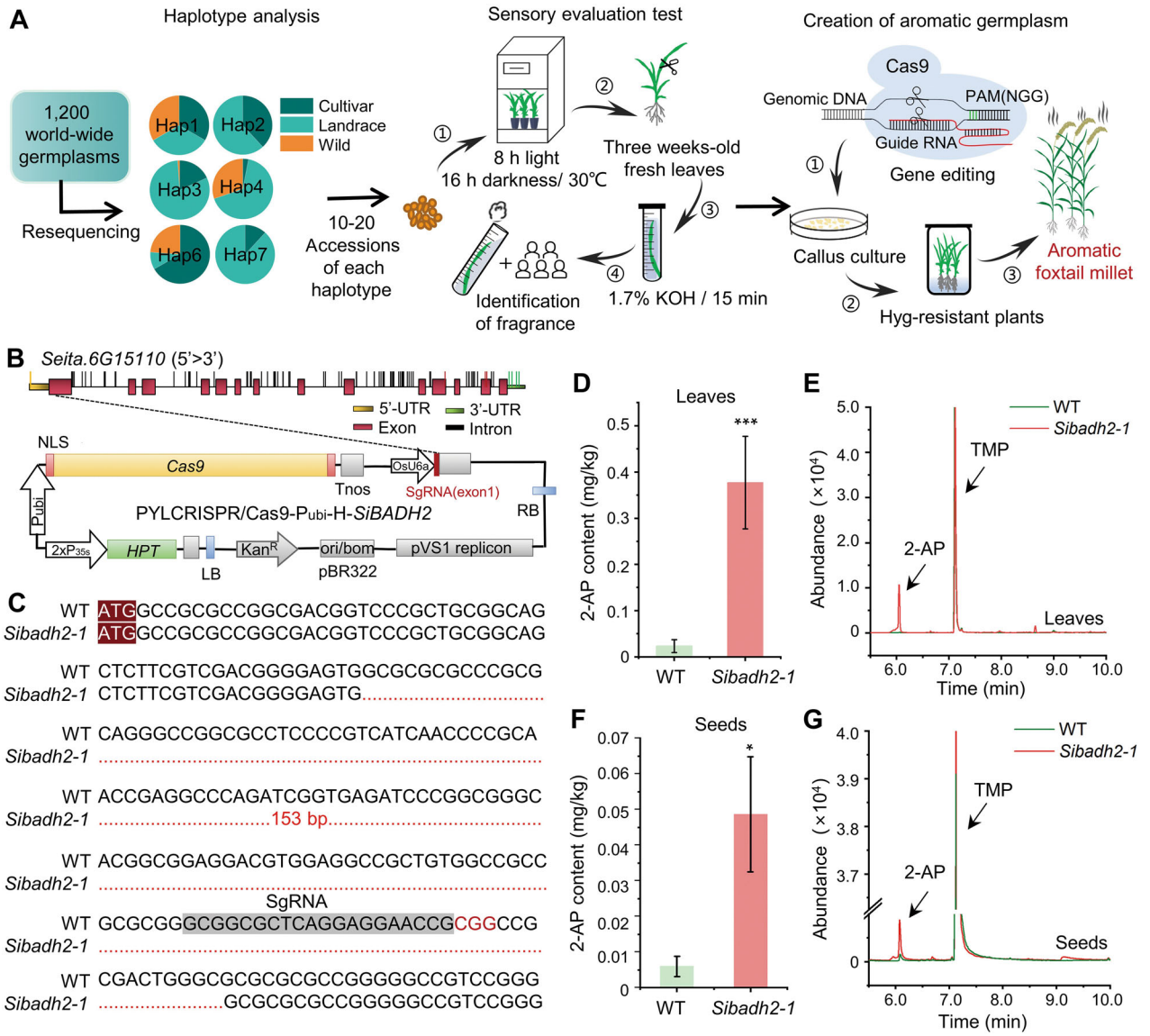

Figure 1. Creation of the aromatic foxtail millet using the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology

(A) Experimental design flow chart. Plant screening for resistance involved the use of hygromycin (Hyg). **(B)** *SiBADH2* gene structure and schematic diagram of the pYLCRISPR/Cas9-Pubi-H-*SiBADH2* vector. The lines on the gene structure represent the variation sites of the *SiBADH2* gene among 1,200 foxtail millet germplasms. The yellow, black, red, and green lines represent the variation site on the 5′-untranslated region (UTR), intron, exon, and 3′-UTR, respectively. **(C)** DNA sequence of the gene-edited *SiBADH2* mutants in the Ci846 background. Red bases show the protospacer adjacent motif (PAM) sequence, the dotted line means base deletions. **(D)** 2-acetyl-1-pyrroline (2-AP) content in mature stage leaves of the mutants and the wild type (WT). **(E)** Total ion chromatograms (TIC) of 2-AP and 2,4,6-trimethyl pyridine (TMP) (as internal standard) in leaves of *Sibadh2-1* and wild type. **(F)** 2-AP content in dried mature seeds for the mutants and wild type. **(G)** TIC of 2-AP and TMP in seeds of *Sibadh2-1* and WT. Data in **(D)** and **(F)** are shown as the means ± *SD* ($n = 3$). *P* values were calculated using the two-tailed Student's *t* test. * and *** represent $P < 0.05$ and $P < 0.001$, respectively.

achieved through mutation breeding or gene editing, as naturally aromatic germplasms seem to be absent.

To develop aromatic foxtail millet, we utilized the CRISPR/Cas9-mediated genome editing technology to target and edit the *SiBADH2* gene in the non-fragrant variety, Ci846 (Figure 1A). The target site was designed within the first exon. We cloned the single guide RNA (sgRNA) with the target sequence into the CRISPR/Cas9 vector pYLCRISPR/Cas9-Pubi-H, placing it under the control of the *OsU6a* promoter (Figure 1B). Subsequently, we introduced the vector into foxtail millet via *Agrobacterium*-mediated gene transformation into embryonic calluses (Figure 1A). We obtained in total 28 T_0-generation plants, 21 of which were identified as positive transgenic plants through polymerase chain reaction amplification (Table S4). Further sequencing analysis revealed that only two plants exhibited genomic editing of the target gene with a heterozygous genotype. From the progeny of these two edited plants, we selected nine homozygous individuals for further

investigation. By comparing DNA sequences, we found that all nine homozygous plants possessed a 153-bp deletion at the target site, which we refer to as *Sibadh2-1* (Figure 1C). This deletion resulted in the elimination of amino acid residues 20–70 in the aldehyde dehydrogenase domain, causing a lack of one alpha-helix and two beta-sheets in the three-dimensional structure of the BADH2 protein (Figure S4). Finally, we identified five Cas9-free lines from the progeny, making them suitable breeding materials (Figure S5). We then cultivated these materials in the greenhouse for subsequent experiments.

To verify the fragrance of the *BADH2* gene-edited line, we conducted a fragrance test on fresh leaves of 3-week-old seedlings. We exposed the leaves of the *Sibadh2-1* line to 1.7% KOH solution for 15 min. As expected, the leaves emitted a strong popcorn-like fragrance compared to the wild type. To quantify the fragrance, we measured 2-AP content using gas chromatography-mass spectrometry (GC-MS), with 2,4,6-trimethyl pyridine (TMP) as an internal control. The 2-AP content was measured in the leaves at the later filling stages. The results showed that the mutants had a 2-AP content of 0.38 mg/kg, while the wild type had almost undetectable levels of 2-AP (Figure 1D, E). Moreover, in mature seeds, a 2-AP peak was detected in the homozygous line *Sibadh2-1*, with a content of 0.05 mg/kg, while the wild type exhibited negligible levels of 2-AP (Figure 1F, G). These findings indicate the successful development of a popcorn-like fragrant foxtail millet germplasm through genome editing of *SiBADH2*.

Simultaneously, we assessed 12 agronomic traits of both the *BADH2* gene-edited line and the wild type in the greenhouse. We found that 11 of these traits, including plant height, leaf length, leaf width, heading date, panicle length, panicle diameter, panicle weight, grain weight per panicle, grain length, grain width, and 1,000-grain weight, showed no significant difference between the wild-type and the edited lines, while the stem diameter was slightly higher in the edited lines compared to the wild type, possibly due to the greenhouse growth environment (Figure S6).

We also designed a target in the 5′-UTR region of *SiBADH2.* In the T_0 generation, we obtained 27 plants, among which we identified 19 individual edited plants, including 10 heterozygous mutations and nine biallelic (two distinct variants) mutations (Table S5). To isolate edited homozygous lines, we focused on the biallelic mutations and obtained three homozygous mutation types in the T_1 generation: a 1-bp insertion, a 32-bp deletion, and a 46-bp deletion. In these edited lines, the *SiBADH2* expression levels were 1.38–3.05 times lower than the wild type (Figure S7). Surprisingly, despite the significant reduction in *SiBADH2* expression, no fragrances were detected in either fresh leaves at the seedling stage or dry leaves at maturity in the subsequent phenotypic determination through organoleptic testing.

In conclusion, to date, despite screening a wide range of foxtail millet accessions and conducting sensory evaluations, no naturally occurring 'fragrance-alleles' were discovered in foxtail millet. However, we successfully created an aromatic foxtail millet variety with popcorn-like fragrance in both seeds and leaves using the CRISPR/Cas9 technology to edit the first exon of the *SiBADH2* gene. This ground-breaking study not only reasserts the conserved function of the *BADH2* gene across diverse crops but also marks the generation of the first aromatic foxtail millet variety. The development of this aromatic foxtail millet serves as a valuable genetic resource for future efforts to enhance aroma traits in foxtail millet through breeding and biotechnology approaches.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

X.D., Y.Z., and Q.H. designed the experiments; Y.Z. and S.Z. performed the experiments; X.M., Y.S., G.J., S.T., H.Z., and C.W. provided technical support; Y.Z., X.D. and Q.H. wrote the manuscript. All authors read and approved this manuscript.

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SUPPORTING INFORMATION

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Figure S1. Phylogenetic tree depicting the evolutionary relationships among the BADH protein family in plants

Figure S2. Expression pattern of *SiBADH2* in different stages and tissues of non-fragrant variety Yugu1

Figure S3. Haplotype analysis of the *SiBADH2* gene based on the examination of 1,200 foxtail millet germplasms

Figure S4. Protein alignment of SiBADH2 in the wild type and the edited line

Figure S5. The gel electrophoretics of Cas9-free T_1 generation

Figure S6. The agronomic traits of *Sibadh2-1* and wild type

Figure S7. Targeted editing of the 5′-untranslated region of *SiBADH2* using clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) in foxtail millet

Table S1. Twelve hundred foxtail millet germplasm resources from the Chinese Gene Bank

Table S2. Haplotypes of *SiBADH2* gene in 1,200 foxtail millet germplasm resources

Table S3. List of varieties for fragrance identification

Table S4. Primers used in this study

Table S5. The editing type of the 5′-untranslated region target in T_0 generation