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Production of aromatic three-line hybrid rice using novel alleles of *BADH2*

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Abstract

Aroma is a key grain quality trait that directly influences the market price of rice globally. Loss of function of betaine aldehyde dehydrogenase 2 (*OsBADH2*) affects the biosynthesis of 2-acetyl-1-pyrroline (2-AP), which is responsible for aroma in fragrant rice. The current study was aimed at creating new alleles of *BADH2* using CRISPR/Cas9 gene editing technology under the genetic background of the japonica Ningjing 1 (NJ1) and indica Huang Huazhan (HHZ) varieties. Sensory evaluation and analysis using headspace solid-phase microextraction gas chromatography–mass spectrometry (HS-SPME-GC-MS) showed that the grains of the four homozygous T_1 lines with new alleles of *BADH2* (*nj1-cr*^{BADH2}*-1*, *nj1-cr*^{BADH2}*-2*, *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2*) produced moderate fragrance and had significantly increased 2-AP content compared with wild-types. Moreover, there were no significant differences in the amylose content and gelatinization temperature among the four lines with new alleles of *BADH2* to the wild-types. Thereafter, we crossed the HHZ background new alleles of *BADH2* with CMS line Taonong 1A (TN1A) to produce a three-line hybrid variety B-Tao-You-Xiangzhan (BTYXZ) with increased grain aroma. The 2-AP content in grains of the improved BTYXZ-1 and BTYXZ-2 reached at 26.16 and 18.74 μg/kg, and the gel consistency of BTYXZ-1 and BTYXZ-2 increased significantly by 9.1% and 6.5%, respectively, compared with the wild-type Tao-You-Xiangzhan (TYXZ). However, the γ-aminobutyric acid (GABA) content in the improved three-line hybrid rice BTYXZ-1 (5.6 mg/100 g) and BTYXZ-2 (10.7 mg/100 g) was significantly lower than that of the TYXZ. These results demonstrated that CRISPR/Cas9 gene editing technology could be successfully utilized in improving aroma in non-fragrant japonica and indica varieties. In addition, the newly developed *BADH2* alleles provided important genetic resources for grain aroma improvement in three-line hybrid rice.

Introduction

Globally, there is an increase in demand in the consumption of aromatic and superior grain quality rice (Calingacion *et al*., 2014). Aroma, or scent or fragrance is one of the unique and key indicators that determine the cooking and eating traits of rice grain (Sakthivel *et al*., 2009). The desirability and popularity of aromatic rice cultivars among rice consumers has resulted in a high market price compared with non-aromatic cultivars (Okpala *et al*., 2019; Shan *et al*., 2015). Based on aroma, rice grain samples are classified as non-aromatic, slightly aromatic, moderately aromatic and strongly aromatic (Jana *et al*., 2011).

Previous studies have revealed that 2-acetyl-1-pyrroline (2-AP) is the major compound contributing to popcorn-like fragrance in rice, whose accumulation is controlled by genetic characteristics of the rice plant (Bergman *et al*., 2000; Fitzgerald *et al*., 2009; Hashemi *et al*., 2015; Hinge *et al*., 2016). Griglione *et al*. (2015) analysed and quantified volatile organic compounds in two aromatic and four non-aromatic rice cultivars and found that 2-AP is the main compound associated with aroma in rice. However, four other amine heterocycles: pyrrole, 1-pyrroline, 2-acetylpyrrole and 5-oxo-2, 3, 4, 5-tetrahydropyridine have been reported to strongly correlate with the production of 2-AP (Daygon *et al*., 2017). The biosynthesis of 2-AP occurs in all organs of aromatic rice plant except the roots (Okpala *et al*., 2019). 2-AP biosynthesis and accumulation is also influenced by various environmental stress conditions and crop management practices (Bhattacharjee *et al*., 2002; Yang *et al*., 2012). Shading during the grain filling stage, irrigation regimes, application of nitrogenous fertilizers, temperature, drought and salinity have been widely reported to influence biosynthesis and accumulation of 2-AP (Mo *et al*., 2015; Poonlaphdecha *et al*., 2012; Wang *et al*., 2013; Wijerathna *et al*., 2014; Yoshihashi *et al*., 2005). In addition, plant growth regulators, time of harvest and post-harvest handling have also been reported to contribute to variation in 2-AP content in rice plant (Duan *et al*., 2009; Goufo *et al*., 2010, 2011).

Genetic studies have revealed that aroma trait is controlled by a single recessive gene *BADH2*, which is located in the long arm of chromosome 8 between the SSR markers RM223 and RM515

(2.34 cM) (Bourgis *et al*., 2008; Bradbury *et al*., 2005; Hashemi *et al*., 2015). However, other researchers have reported that aroma in rice grains is a polygenic trait (Aluko *et al*., 2004; Amarawathi *et al*., 2008; Wan *et al*., 2006). Sequence alignment of this gene between aromatic and non-aromatic rice cultivars identified multiple types of fragrant alleles that were responsible for the aroma in rice, such as *badh2-E2*, *badh2-E7* and *badh2-E4/5* (Kovach *et al*., 2009; Shao *et al*., 2011; Shi *et al*., 2008, 2014). Despite the multiple alleles in the *BADH2* gene, Kovach *et al*. (2009) demonstrated that *badh2.1*, a single recessive allele, is the predominant allele in almost all aromatic rice including Jasmine and Basmati varieties. The corresponding *OsBADH2* dominant gene encodes Betaine aldehyde dehydrogenase (BADH2) that catalyses the conversion of γ-aminobutyraldehyde (GAB-ald) into γ-aminobutyric acid (GABA), which eventually inhibits 2-AP biosynthesis in non-fragrant rice. However, a mutation in this gene and subsequent loss of function of BADH2 enzyme in fragrant rice results in low levels of GABA accumulation, consequently leading to an increase in 2-AP accumulation and therefore aroma (Bradbury *et al*., 2005; Chen *et al*., 2008). Through map-based cloning and complementary experiments, Chen *et al*. (2008) confirmed that mutation in *BADH2* gene is responsible for fragrant phenotype. In addition, RNA interference (RNAi)-directed down-regulation of *OsBADH2* (Niu *et al*., 2008) and artificial microRNA-induced down-regulation of *OsBADH2* (Chen *et al*., 2012) resulted in aroma production in rice.

High demand and low supply of high-quality fragrant rice has led to an increase in the price of aromatic rice in the global markets (Giraud, 2013). This has prompted rice breeders to develop novel high yielding fragrant rice cultivars to meet the ever-growing consumer demand for fragrant rice. Conventional breeding or marker-assisted breeding techniques utilized in introgression of fragrant genes and breeding for aromatic rice genotypes tends to be inefficient, labour intensive and time-consuming (Shan *et al*., 2015). With advancements in plant biotechnology, genome editing technologies have made it possible to improve the existing aromatic rice varieties and create aromatic rice from non-aromatic rice varieties. RNAi and transcription activator-like effector nuclease (TALEN) technologies have been successfully utilized in reducing the expression levels of *OsBADH2*, thereby increasing the level of 2-AP and ultimately, aroma in rice grains (Chen *et al*., 2012; Khandagale *et al*., 2020; Shan *et al*., 2015). However, incomplete inhibition of the expression of *OsBADH2* and regulatory issues of transgenic plants limited the application of these techniques for aroma improvement in rice. Recently, clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) system has been utilized successfully to bioengineer *OsBADH2* in both aromatic and non-aromatic rice varieties (Ashokkumar *et al*., 2020; Shao *et al*., 2017). This technique has become popular in rice aroma improvement due to its efficiency in creating precise mutations, ability to edit multiple genes and possibility of obtaining transgene-free plants (Fiaz *et al*., 2019a).

Therefore, the objectives of the present study were: (a) to create new alleles of *BADH2* using CRISPR/Cas9 gene editing technology in the genetic background of the japonica rice variety, Ningjing 1 (NJ1) and the indica rice variety, Huang Huazhan (HHZ), (b) to evaluate grain quality and agronomic traits among the mutant lines and (c) to apply the new alleles of *BADH2* for improving the grain aroma of three-line hybrid rice variety Tao-You-Xiangzhan (TYXZ).

Results

BADH2 genotyping among the receptor varieties

The *BADH2* gene in NJ1, HHZ and Taonong 1A (TN1A) cultivars was amplified using PCR-based primers and sequenced using Sanger method. The sequencing data were analysed using SnapGene software. The comparative analysis results of *BADH2* gene sequence in NJ1, HHZ and Nipponbare (reference cultivar) showed that the open reading frame (ORF) was highly homologous among the three cultivars and they were all typical non-aromatic rice. On the other hand, the *BADH2* gene sequencing results in cv. TN1A and TN1B portrayed a 8-bp deletion and three single-nucleotide polymorphisms (SNPs) in exon 7 along its ORF (Figure 1a).

CRISPR/Cas9-induced mutagenesis of *BADH2* gene

To create new alleles of *BADH2*, CRISPR/Cas9 vector carrying rice U6 promoter and a single-guide RNA (sgRNA) for targeting the second exon of *BADH2* gene was constructed (Figure 1b). Mutation in this exon has been shown to result in high 2-AP accumulation in grains of rice plant (Shi *et al*., 2008). The CRISPR/Cas9 construct of *BADH2* was inserted into NJ1 and HHZ by *Agrobacterium*-mediated transformation. Using PCR-based screening and Sanger sequencing, five types of homozygous new alleles of *BADH2* were identified in the T_0 generation: *nj1-cr*^{BADH2}-1 and *hhz-cr*^{BADH2}*-1* contained one nucleotide insertion (thymine**)**, *nj1-cr*^{BADH2}-2 contained one nucleotide insertion (adenine), *nj1-cr*^{BADH2}-3 contained two nucleotide deletion, *hhz-cr*^{BADH2}-2 contained seven nucleotide deletion and *hhz-cr*^{BADH2}-3 contained one nucleotide deletion (Figure 1c).

Generation of T-DNA-free new alleles of *BADH2*

To generate non-transgenic new alleles of *BADH2*, we tracked the segregation of T-DNA harbouring the desired *BADH2* gene mutations in the T_1 population. Firstly, using hygromycin medium selection method, we germinated T_1 seeds of cv. NJ1 and cv. HHZ in a petri dish containing hygromycin medium. Seeds which developed into both root and shoot were classified as transgenics while the ones which exhibited only shoot growth were classified as T-DNA-free candidate lines (Figure 1d). To further confirm the absence of T-DNA, the T-DNA-free candidate lines were transferred to hygromycin-free petri dishes containing nutrient solution and allowed to grow fully into roots and shoots. They were then screened for T-DNA based on PCR detection of *Cas9* and *HPT*. The absence of T-DNA was confirmed by negative PCR results of both *Cas9* and *HPT* (Figure 1e).

Performance of new alleles of *BADH2* on major agronomic traits

To evaluate the agronomic performance in the new alleles of *BADH2* lines, the T_1 progenies of cv. NJ1 and cv. HHZ background together with the wild-type plants were cultivated in the experimental paddy fields at the China National Rice Research Institute. Phenotypically, grain length and a thousand-grain weight of *nj1-cr*^{BADH2}*-1* and *nj1-cr*^{BADH2}*-2* lines increased significantly compared with the wild-type. The grain width of *nj1-cr*^{BADH2}*-1* line increased significantly while that of *nj1-cr*^{BADH2}*-2* line did not change when compared with the wild-type (Figure 2b,d). Other agronomic traits, such as plant height, number of effective tillers, number of grains per panicle and grain

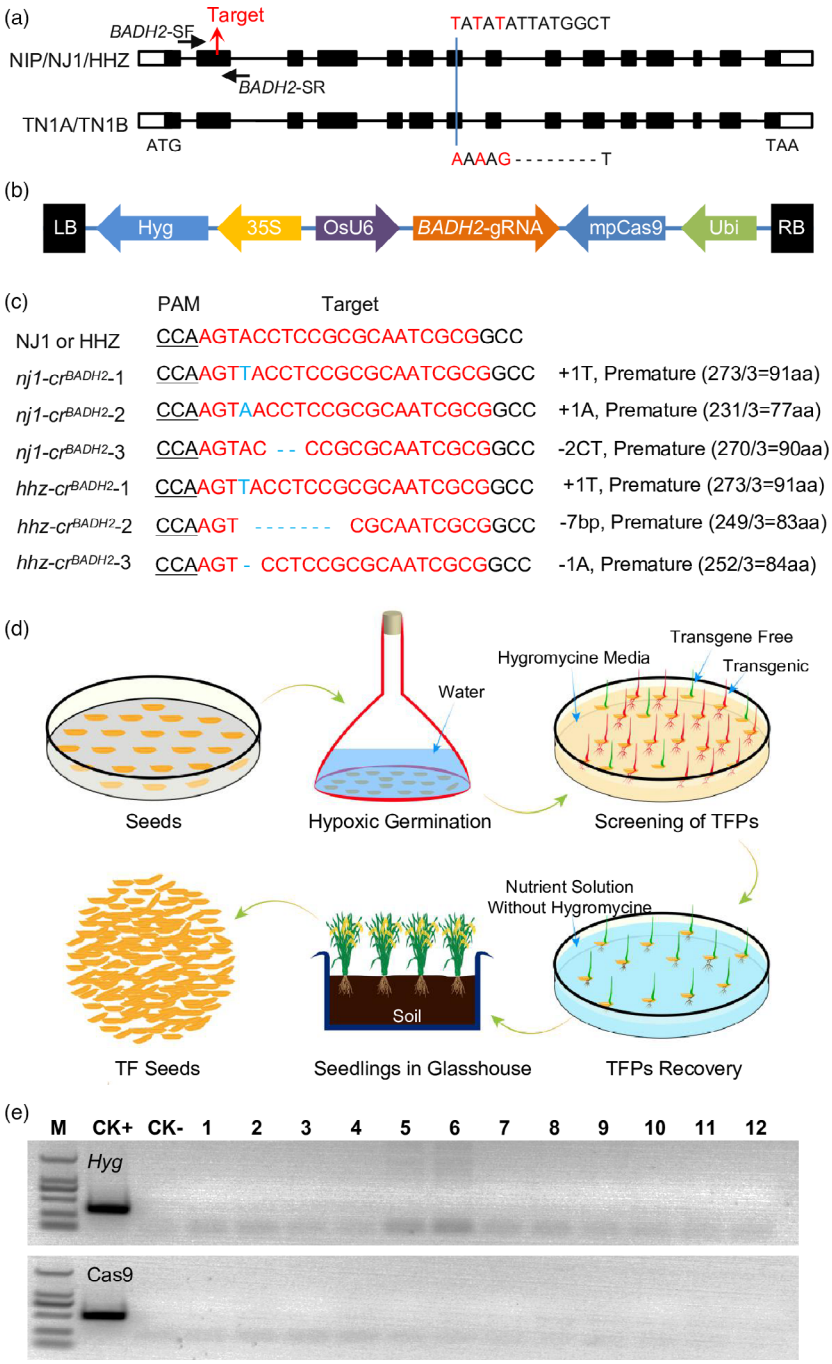
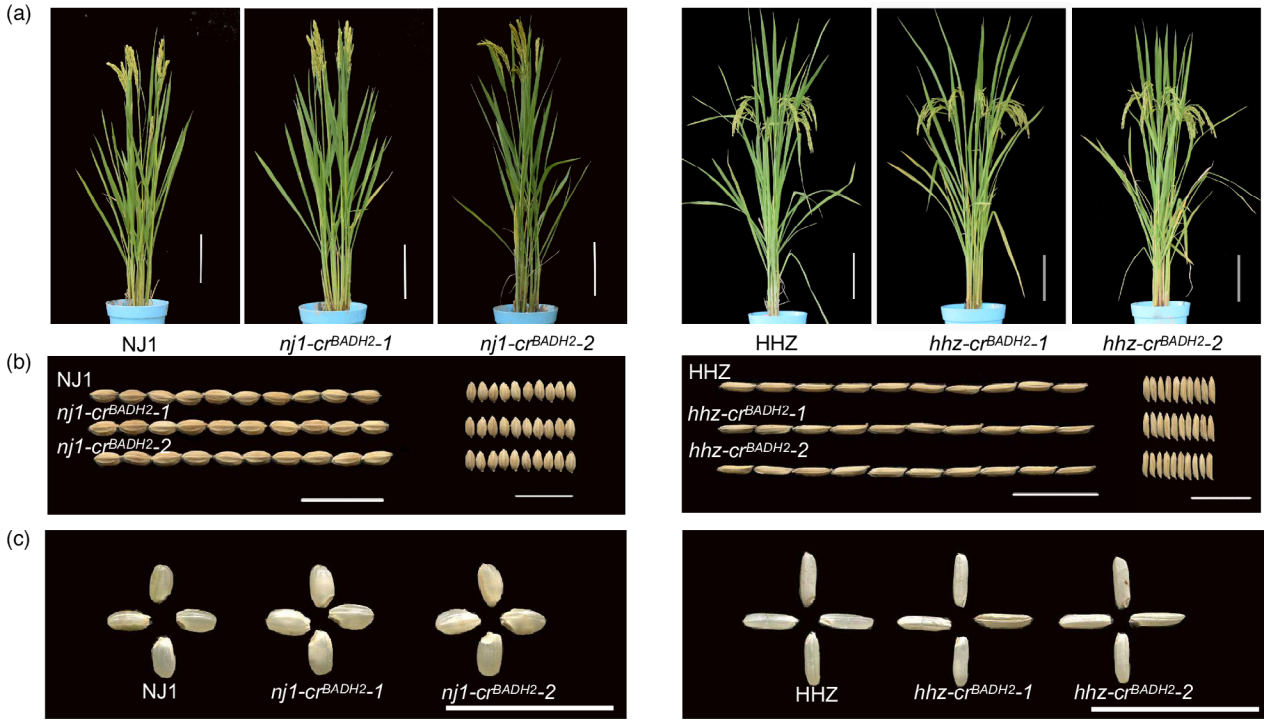


Figure 1 Identification of new alleles of *BADH2* and selection of T-DNA-free plants. (a) The structure of *BADH2* gene. The primers BADH2-SF and BADH2-SR were used for genotyping. (b) The structure of the T-DNA region of the Cas9/guide RNA (gRNA) vector. Marker gene Hygromycin (Hyg) was driven by the CaMV35S (35S) promoter whereas the gRNA was driven by the rice U6 promoter and the mpCas9 was driven by the Ubiquitin (Ubi) promoter. LB, Left border. RB, Right border. (c) Identification of mutation in BADH2 by sequencing of the target site in T_0 transgenic individuals. PAM, Protospacer adjacent motif. (d) Schematic diagram of TFPs screening process. (e) Detection of transgenic vector framework by specific hygromycin and Cas9 primers. M, DL2000 DNA marker.

thickness of the *nj1-cr*^{BADH2}*-1* and *nj1-cr*^{BADH2}*-2* lines were not significantly different compared with the wild-type (Figure 2a,d). Similarly, under the genetic background of cv. HHZ, grain thickness and a thousand-grain weight of the *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2* lines decreased significantly compared with the wild-type (Figure 2b–d). In addition, the seed setting rate and plant height of two lines were decreased significantly, while the number of effective tillers increased significantly compared with the wild-type (Figure 2a,d). Although the number of grains per panicle (yield) increased in *hhz-cr*^{BADH2}*-1* line and decreased in *hhz-cr*^{BADH2}*-2* line, these changes were not significantly different when compared with the wild-type (Figure 2d).

Fragrance and 2-AP content in *BADH2* edited lines

Rice grains harvested from the four homozygous T_1 lines (*nj1-cr*^{BADH2}*-1*, *nj1-cr*^{BADH2}*-2*, *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2*) were evaluated for 2-AP accumulation and fragrance. Firstly, we used 1.7% KOH solution for sensory evaluation of fragrance. The results showed that the grains of the four new lines of *BADH2* produced moderate fragrance while grains of the wild-type control varieties NJ1 and HHZ did not produce any fragrance. Additionally, gas chromatography-mass spectrometry (GC-MS) was used to determine the 2-AP content in the mature seeds harvested from the four homozygous T_1 lines, and used 2, 4, 6-



(d)

Varieties	Agronomic traits							
	Grain length/mm	Grain width/mm	Grain thickness /mm	1000 grain weight/g	Seed setting rate/%	Grain number per panicle	Effective tillers	Plant height/cm
NJ1	6.97±0.03	3.27±0.01	2.40±0.12	26.07±0.06	80.08±0.96	147.23±18.99	9±2	91.64±2.33
nj1-cr^{BADH2}*-1*	7.35±0.01**	3.38±0.01**	2.39±0.06	27.90±0.34**	81.53±0.92	144.71±6.64	10±3	90.26±2.61
nj1-cr^{BADH2}*-2*	7.23±0.01**	3.26±0.01	2.43±0.03	27.49±0.39**	73.21±3.27*	117.44±10.19	11±2	89.68±1.83
HHZ	8.84±0.01	2.11±0.01	1.96±0.08	19.90±0.17	90.34±0.83	181.25±27.04	9±1	106.65±2.76
hhz-cr^{BADH2}*-1*	8.92±0.06	2.10±0.01	1.88±0.06*	17.69±0.05**	83.88±1.99**	199.40±45.72	14±1**	102.12±1.97**
hhz-cr^{BADH2}*-2*	8.89±0.03	2.06±0.03*	1.89±0.07*	18.15±0.35**	79.16±5.18*	136.36±18.02	13±1**	94.64±5.41**

Figure 2 Agronomic traits of new alleles of *BADH2* germplasm. (a) Plant morphology of new alleles of *BADH2* *nj1-cr*^{BADH2}*-1*, *nj1-cr*^{BADH2}*-2*, *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2* in NJ1 and HHZ, respectively. (b) Comparison of grain length and grain width among new alleles of *BADH2* lines and wild-type. (c) Brown rice of new alleles of *BADH2* lines and wild-type. (d) Statistical analysis of agronomic traits. Values are means ± SD. *$P < 0.05$; **$P < 0.01$ ($n = 10$, two-tailed Student's *t*-test, three independent experiments). Bars = 20 cm in (a), 2 cm in (b), 1 cm in (c).

trimethyl pyridine (TMP) as internal standard due to its molecular similarity to 2-AP in GS-MS. A 2-AP peak at about 6.5 min was detected in *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2* lines, but no 2-AP peak was detected from the wild-type (HHZ), which is non-fragrant (Figure 3a). All the four new alleles of *BADH2* created by CRISPR/Cas9 technology produced 2-AP in the grains. The 2-AP content in *nj1-cr*^{BADH2}*-1* and *nj1-cr*^{BADH2}*-2* reached 46.45μg/kg and 82.73μg/kg, respectively. Similarly, the 2-AP content in *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2* reached 18.19μg/kg and 28.41μg/kg, respectively. However, none of the two wild-type varieties (NJ1 and HHZ) produced any detectable 2-AP content in the grains (Figure 3b). Moreover, we constructed a standard curve of 2-AP peak area using the 2-AP standard product and determined 2-AP content in the grains of above samples (Figure S1a,b). The results displayed that the content of 2-AP measured by the external standard method and the internal standard method showed the same trend (Figure S1c).

To further understand the relationship between *BADH2* gene mutation and aroma in rice, we determined the RNA expression levels in the four new alleles of *BADH2* and the wild-type varieties. Total RNA was extracted from seeds at 5th, 10th, 15th and 30th days after flowering, followed by quantitative analysis of *OsBADH2* using Quantitative Real-Time PCR (qRT-PCR) technology. The results revealed that *OsBADH2* expression level increased with seed maturation from 5th to 30th days after flowering. However, the expression levels of *OsBADH2* in the four new alleles of *BADH2* lines were reduced compared with the wild-type (Figure 3c). These results indicated that the editing of *BADH2* gene resulted in significant down-regulation of the *BADH2* gene expression levels in the four new allelic lines of *BADH2*, which ultimately influenced the increase of 2-AP content.

HS-SPME-GC-MS analysis of the untargeted volatiles in seeds

Metabolite analysis by dynamic Headspace Solid-phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) detected a total of 1032 volatile metabolites in mature grains

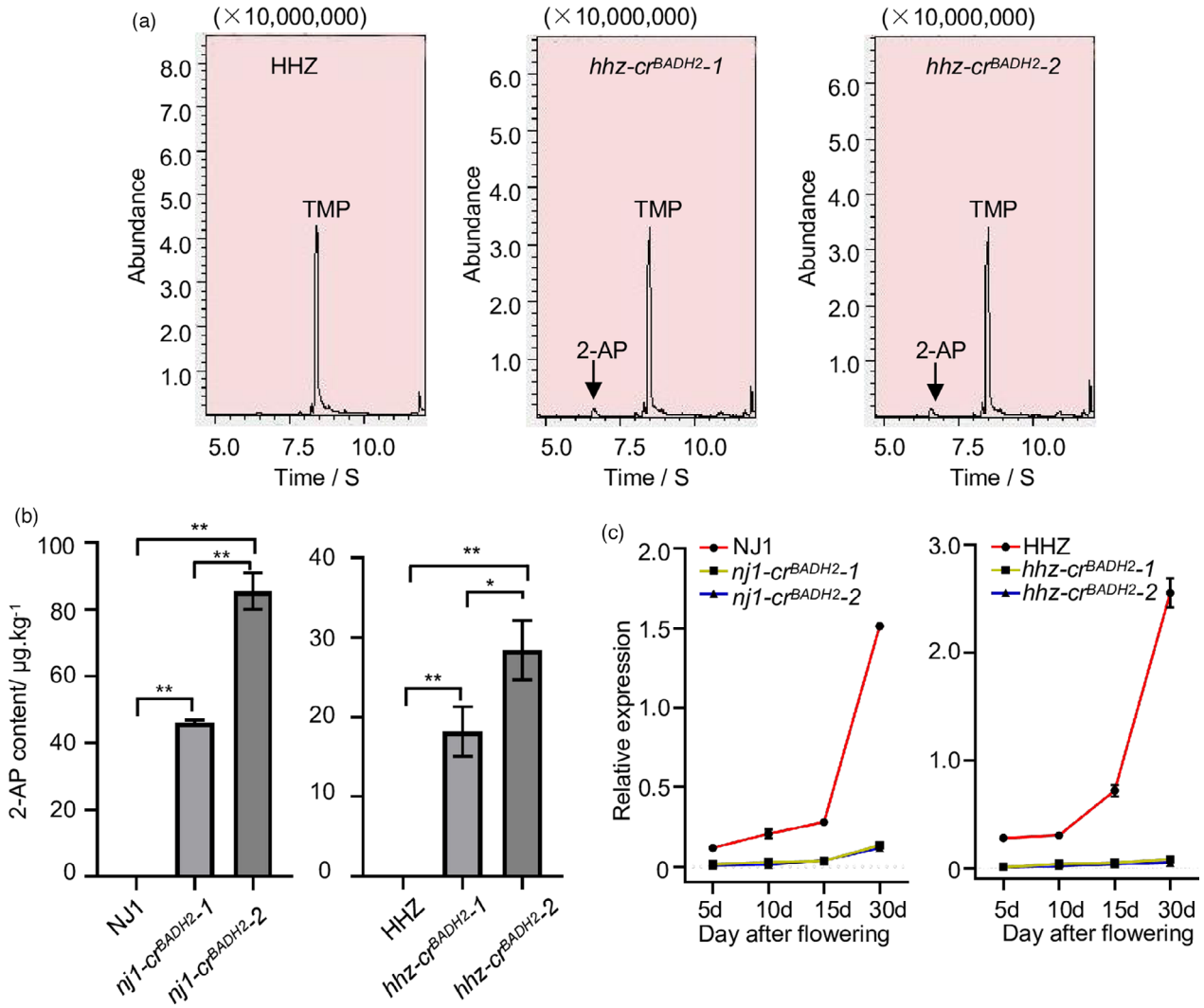


Figure 3 Fragrant identification of new alleles of *BADH2* lines. (a) Total ion chromatograms (TIC) of 2-AP and TMP (as internal standard) in the new alleles of *BADH2* lines and control. (b) 2-AP content of the new alleles of *BADH2* lines and control. (c) Relative expression level of *BADH2* in the caryopsis of NJ1, *nj1-cr*^{BADH2}*-1*, *nj1-cr*^{BADH2}*-2*, HHZ, *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2* lines. Values are means ± SD. **P* < 0.05; ***P* < 0.01 (*n* = 3 in (b, c), two-tailed Student's *t*-test, three independent experiments).

of the new alleles of *BADH2* lines and the corresponding wild-type varieties (Figure 4a, Figure S3a). Further, differential metabolite screening was performed by the variable important in projection (VIP) value of the first principal component of the orthogonal partial least squares-discriminant analysis (OPLS-DA) model>1 and a *T*-test ($P < 0.05$), and by also considering that the levels of the selected metabolites portrayed a synchronous increasing or decreasing trend in new allelic lines of *BADH2* compared with the corresponding wild-type. The results showed that there were 218 different metabolites in the HHZ (HHZ, *hhz-cr*^{BADH2}*-1*, *hhz-cr*^{BADH2}*-2*) group (Figure 4b, Figure S2), which mainly included aromatics (27.1%), ester (20.6%), alkanes (15.1%) and heterocycle compounds (12.4%). Other chemical compounds, such as alkenes, alcohols, aldehydes, amines and halogenated compounds were also identified. Likewise, a total of 229 different metabolites were detected in NJ1 (NJ1, *nj1-cr*^{BADH2}*-1*, *nj1-cr*^{BADH2}*-2*) group (Figure S3b, Figure S4), of which the largest proportion was accounted in aromatic compounds (29.7%). Except for halogenated and amines, the rest of the groups were consistent with HHZ group, though the content of each component was not equal. Volcano plot analysis revealed that the 2-AP compound was still the main difference between the allelic of *BADH2* lines and the wild-type non-fragrant rice (Figure 4c, Figure S3c). Meanwhile, we also identified some aromatic-related metabolites that have been annotated, such as benzaldehyde (bitter almond odour), neophytadiene (an important aroma substance in tobacco leaves), isophorone (a food flavour that smells like mint), benzeneacetaldehyde (aroma similar to hyacinth and a sweet aroma of fruit after dilution), undecanal (the aroma of rose, flower, fruit and sweet orange in the diluted state) (Larranaga *et al.*, 2016; Ghosh *et al.*, 2020; Aslankoohi *et al.*, 2016; Liu *et al.*, 2018; Grimm *et al.*, 2001). From the two sets of data, we found that some alkanes, including heptadecane, octadecane and eicosane were abundant in the wild-type, but their content in the new allelic lines of *BADH2* was significantly reduced. These substances are abundantly present in flue-cured tobacco, with moderate smoke (Figure 4d, Figure S3d).

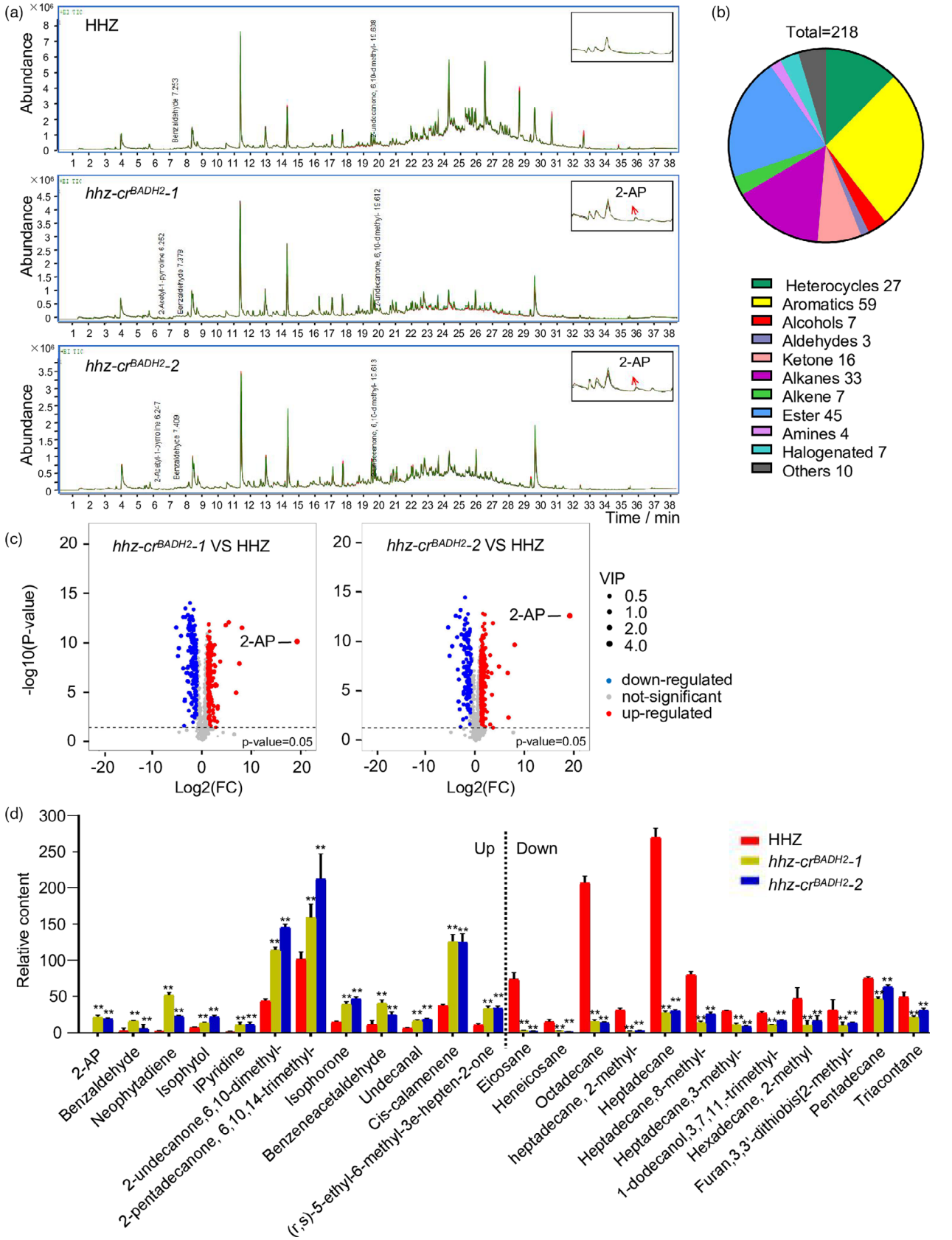


Figure 4 HS-SPME-GC-MS analysis of the untargeted volatiles in HHZ background seeds. (a) Total ion chromatography (TIC) of new allelic lines of *BADH2* and HHZ control in metabolomics. (b) Graphical representation of differential volatiles detected from new allelic lines of *BADH2* and HHZ control seeds. (c) Volcano Plot differential volatile metabolites analysis of new allelic lines of *BADH2* and HHZ control. (d) Major differential volatiles relative content in seeds. Values are means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 6$, two-tailed Student's *t*-test, three independent experiments).

Effects of mutation in *OsBADH2* on rice grain quality

We investigated the effects of *OsBADH2* mutation on different grain quality features among the four new alleles of *BADH2* lines. Compared with the wild-type varieties, no significant changes were found in the amylose content (Figure 5a), gel consistency (Figure 5b), starch viscosity (Figure 5c) and gelatinization characteristics of starch (Figure 5d) in the four new alleles of *BADH2* lines. These results indicated that the CRISPR/Cas9 editing of *OsBADH2* generated new alleles of *BADH2* with improved grain aroma, and without affecting other grain quality traits.

Development of promising hybrid rice with improved aroma in grains

After confirming that the *hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2* allele lines can significantly increase the content of 2-AP in the grains, we crossed each of these two new alleles of *BADH2* lines with a three-line fragrance sterile line, TN1A, to obtain BTYXZ (BTYXZ-1 = *hhz-cr*^{BADH2}*-1*×TN1A and BTYXZ-2 = *hhz-cr*^{BADH2}*-2*×TN1A) hybrids with improved fragrance. The results showed that in terms of aroma improvement, only one-quarter of the grains of the conventional cross-combination of TYXZ had aroma, whereas all the grains of the

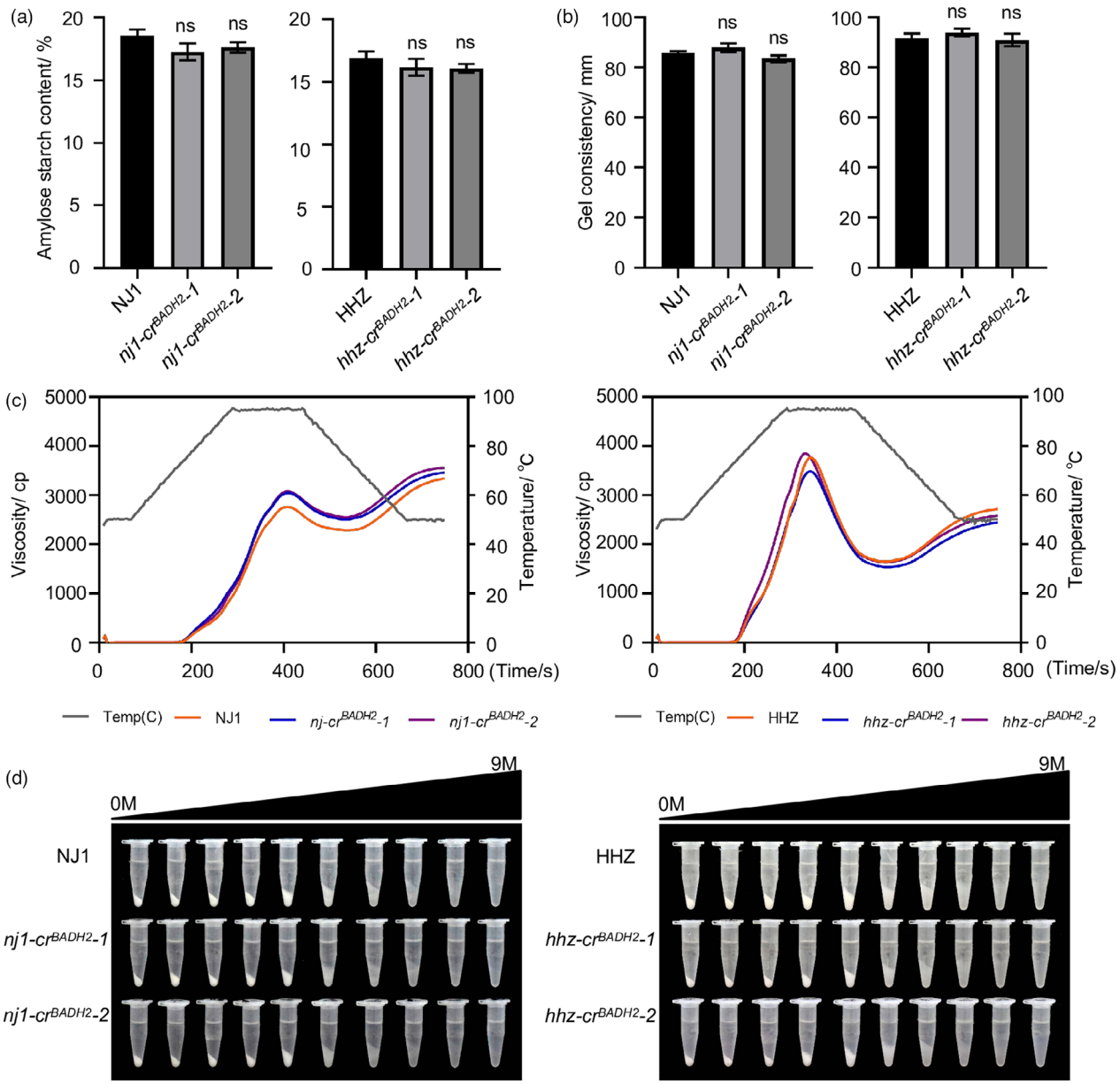
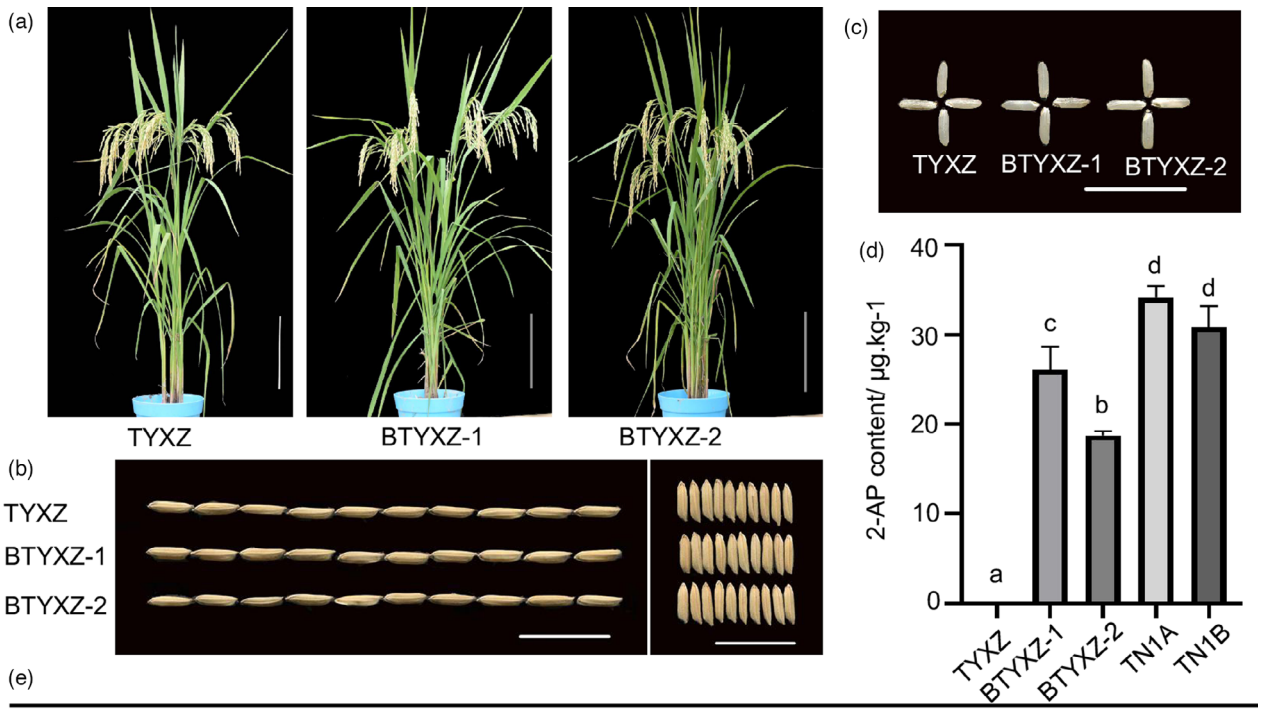


Figure 5 Determination of physicochemical properties of grain starch in new alleles of *BADH2*. (a) Amylose content. (b) Gel consistency. (c) Pasting properties of endosperm starch of NJ1, HHZ and new alleles of *BADH2* lines. The viscosity value at each temperature is the average of three replicates. The grey line indicates the temperature change during the measurements. (d) Gelatinization characteristics of starch in urea solutions, starch powder of the NJ1, HHZ and new alleles of *BADH2* lines was mixed with varying concentrations (1–9 M) of urea solutions. Values are means ± SD. ns, no significance (n = 3, two-tailed Student's t-test, three independent experiments).



(e)

Varieties	Agronomic traits						
	Grain length/mm	Grain width/mm	Grain thickness/ mm	1000-grain weight/g	Seed setting rate/%	Grain number per panicle	Plant height/cm
TYXZ	9.78±0.03	2.31±0.02	2.09±0.05	25.64±0.24	82.20±1.33	198.81±13.55	111.37±4.09
BTYXZ-1	9.86±0.05	2.37±0.04	2.12±0.04	26.69±0.25*	78.33±3.66	169.97±37.50	110.98±4.92
BTYXZ-2	9.73±0.06	2.39±0.01	2.10±0.06	25.95±0.01	79.72±1.63	157.75±24.71	106.99±2.62*

Figure 6 The new alleles of *BADH2* increased the content of 2-AP in BTYXZ hybrid lines. (a) The Plant morphology of TYXZ, BTYXZ-1 and BTYXZ-2. (b) Comparison of grain length and grain width between TYXZ and BTYXZ. (c) Brown rice of TYXZ and BTYXZ. (d) 2-AP content of the TYXZ, BTYXZ improvement cross, TN1A and TN1B. Values are means ± SD. Different letters indicate significant difference $P < 0.05$ ($n = 3$, Two-way ANOVA multiple comparison, Tukey test, three independent experiments). (e) Statistical analysis of agronomic traits. Values are means ± SD. *$P < 0.05$ ($n = 10$, two-tailed Student's *t*-test, three independent experiments). Bars = 20 cm in (a), 2 cm in (b), 1 cm in (c).

improved BTYXZ-1 and BTYXZ-2 contained fragrance. The content of 2-AP in TYXZ grains was found below detection limit in GC-MS analysis, while 2-AP content in the seeds of improved BTYXZ-1 and BTYXZ-2 was 26.16 and 18.74 μg/kg, respectively (Figure 6d). When compared with the TYXZ variety, the two newly bred BTYXZ hybrid lines (BTYXZ-1 and BTXZ-2) did not show any significant difference in plant morphology, grain physical appearance and other yield-related traits (Figure 6a–c,e). Therefore, these results demonstrated that the new alleles of *BADH2* lines created under the genetic background of HHZ can be widely utilized in improving the aroma of three-line hybrid rice TYXZ without affecting its agronomic features and yield performance.

Starch physicochemical properties in the improved BTYXZ lines

We evaluated the physicochemical properties of rice endosperm starch in the grains of the newly bred three-line hybrids. The results showed that the gelatinization temperature and amylose content of BTYXZ-1 and BTYXZ-2 did not change significantly than TYXZ (Figure 7a,b). The gel consistency of BTYXZ-1 and BTYXZ-2 increased significantly by 9.1% and 6.5%, respectively, compared with TYXZ (Figure 7c). RVA spectrum analysis showed that the maximum viscosity (PV) and final viscosity (CPV) of BTYXZ-1 were lower than TYXZ, but the trend of the viscosity curves of the two lines was consistent (Figure 7d). Similarly, starch pasting properties in BTYXZ-2 and TYXZ were not significantly different. Gelatinization characteristics of starch in BTYXZ-1 and BTYXZ-2 lines did not change significantly when compared with the wild-type (Figure 7e).

Evaluation of rice texture and taste in the improved BTYXZ lines

Since the improved TYXZ lines (BTYXZ-1 and BTYXZ-2) had extremely significant changes in gel consistency compared with the wild-type control (TYXZ), we conducted grain texture analysis on fresh cooked rice and retrograded cooked rice. As shown in Figure 8a, the hardness of fresh and retrograded cooked rice of BTYXZ-1 and BTYXZ-2 lines was significantly lower than that of TYXZ. In addition, the adhesion of fresh cooked rice increased significantly in the two improved lines compared with TYXZ.

(a)

Sample	Gelatinization onset temperature To/°C	Gelatinization peak temperature Tp/°C	Gelatinization endset temperature Tc/°C	Enthalpy of gelatinization ΔH/.g^{-1}
TYXZ	66.37±0.06	71.46±0.10	78.41±0.41	8.15±0.17
BTYXZ-1	66.35±0.03	71.34±0.01	77.82±0.18	8.14±0.23
BTYXZ-2	66.16±0.04**	71.35±0.01	78.06±0.22	8.04±0.05

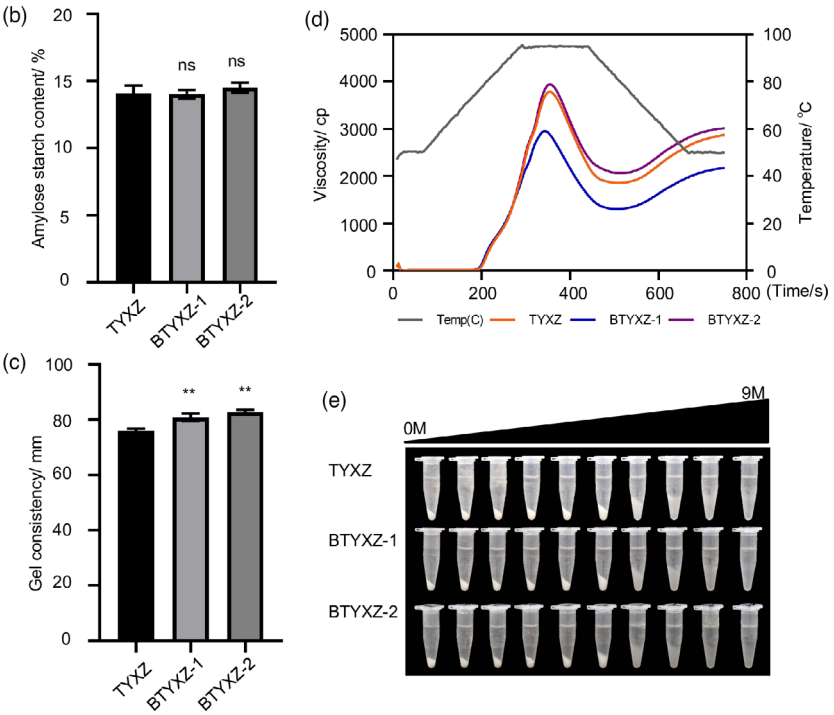


Figure 7 Determination of starch physicochemical properties in fragrance improved BTYXZ hybrid lines. (a) Determination of gelatinization temperature of TYXZ and BTYXZ. (b) Amylose content. (c) Gel consistency. (d) Pasting properties of endosperm starch of TYXZ and BTYXZ. The viscosity value at each temperature is the average of three replicates. The grey line indicates the temperature change during the measurements. (e) Gelatinization characteristics of starch in urea solutions, starch powder of the TYXZ and BTYXZ was mixed with varying concentrations (1–9 M) of urea solutions. Values are means ± SD. $^{**}P < 0.01$; ns, no significance ($n = 3$ in (a–c), two-tailed Student's *t*-test, three independent experiments).

We further evaluated whether the taste of BTYXZ-1 and BTYXZ-2 rice grains changed with the changes in grain texture. We measured the rice taste by using a rice taste analyser machine (Satake Company Limited-Japan) and recorded the test scores in the Chinese Indica Rice Testing Database based on appearance, taste and comprehensive scores (Figure 8b–d). The results showed that compared with TYXZ, the appearance score and taste score in BTYXZ-1 line decreased significantly (Figure 8b,c). Moreover, the appearance score in BTYXZ-2 line did not change significantly when compared with TYXZ. Further, the taste score in BTYXZ-2 line decreased significantly when compared with TYXZ (Figure 8b,c). Furthermore, the comprehensive scores in BTYXZ-1 and BTYXZ-2 lines did not change significantly when compared with TYXZ. The comprehensive score in TYXZ was 76.2 while the comprehensive scores in BTYXZ-1 and BTYXZ-2 lines were 76.63 and 76.66, respectively (Figure 8d). Based on these results, it is found that the use of new alleles of *BADH2* (*hhz-cr*^{BADH2}*-1* and *hhz-cr*^{BADH2}*-2*) in the background of HHZ can significantly accelerate the improvement of aroma in TYXZ. Although the hardness, adhesion, appearance and taste changed in the improved BTYXZ lines, the overall taste quality did not change significantly.

GABA content decreased in the BTYXZ hybrid lines

GABA is a type of inhibitory neurotransmitter and the development of rice varieties having its high concentration is one of the key research objectives of functional rice development (Shimajiri *et al*., 2013; Zhao *et al*., 2017). Previous reports have indicated that the GABA content in fragrant rice is significantly lower than that in non-fragrant rice (Karladee and Suriyong, 2012; Khandagale *et al*., 2020). Therefore, in this study, we used HPLC to quantitatively analyse the GABA content in brown rice of the modified BTYXZ lines and wild-type cv. TYXZ. At a detection peak of about 4.63 min, the GABA content in the brown rice of the wild-type reached 70.8 mg/100 g while that of the modified BTYXZ-1 and BTYXZ-2 fragrant hybrid lines reached 5.6 mg/100 g and 10.7 mg/100 g, respectively (Figure 9a–d). These results showed that the GABA content in the improved BTYXZ-1 and BTYXZ-2 hybrid lines was significantly lower than that of the wild-type. At the same time, when the GABA content in both the wild-type and the two improved hybrid lines was compared with the 2-AP content, the results demonstrated that the 2-AP content in the brown rice was negatively correlated with the GABA content (Figures 6d and 9d).

Discussion

Aroma is one of the major grain quality traits that directly influences the market price of rice. The *BADH2* gene is the main gene that controls the aroma in rice, and its loss of function leads to the accumulation of an aromatic compound 2-AP which in turn results in increased fragrance in rice. Different types of *BADH2* mutations cause variations in 2-AP accumulation (Shan *et al*., 2015; Shao *et al*., 2017). The discovery and identification of new *badh2* mutants from different genetic backgrounds and different allele provide an important genetic resource for further research on fragrance improvement in rice. Breeding of new rice varieties

through conventional breeding methods is laborious and time-consuming (Ashokkumar *et al*., 2020). The application of CRISPR/Cas9-based gene editing technology in plant breeding is an auspicious alternative to the conventional and marker-based breeding due to the ability to precisely and efficiently edit genomes of diverse living organism (Fiaz *et al*., 2019a; Manghwar *et al*., 2019). To date, CRISPR/Cas9-based gene editing technology has been used to create novel rice genotypes exhibiting desirable agronomic, nutritional and grain-related traits, such as male sterility (Barman *et al*., 2019; Zhou *et al*., 2016), reduced plant height (Hu *et al*., 2019), reduced cadmium content in grain (Tang *et al*., 2017), reduced grain amylose content (Zhang *et al*., 2018) and improved grain weight (Xu *et al*., 2016).

In this study, we sequenced the *BADH2* gene in the three receptor cultivars (NJ1, HHZ and TN1A) and successfully edited it using CRISPR/Cas9 system. The absence of detectable mutation confirmed the lack of aroma in NJ1 and HHZ. However, the presence of mutation in exon 7 of *BADH2* gene in cv. TNIA confirmed that this variety is capable of producing aromatic compound 2-AP and therefore fragrance (Figure 1a). Similar results showing allelic variation in *BADH2* gene between fragrant and non-fragrant rice varieties were reported by Chan-in *et al*. (2020). Our gene editing results demonstrated that the use of CRISPR/Cas9-based targeted mutagenesis and PCR-based screening for visible mutations was efficient and convenient to create new alleles of *BADH2* lines (Figure 1b,c). The mutational screening T_2 progenies revealed the stable inheritance of *OsBADH2* mutations in the T_0 and T_1 generations.

The major *BADH2* alleles regulating fragrance in rice lie in the 7-bp deletion in exon 2 (Shi *et al*., 2008) and in the 8-bp deletion along with 3 SNPs (Bradbury *et al*., 2005). In this study, we created a total of five new alleles of *BADH2* which portrayed a significant increase in 2-AP accumulation and no obvious changes in agronomic traits (Figures 1c and 3b), and all of the five new alleles of *BADH2* were carrying new/unreported mutations in exon 2. Therefore, the five transgenic individuals contain novel *BADH2* alleles of rice aroma gene (*OsBADH2*) and provide new germplasm resources that can be applied in genetic improvement of aroma in rice breeding. Based on literature search, no previous work involving breeding of three-line hybrid rice using *BADH2* novel alleles generated through CRISPR/Cas9 technology has been reported. Therefore, in our study, the crossing of CMS TN1A variety with the new alleles of *BADH2* lines developed in the genetic background of HHZ significantly increased 2-AP accumulation, which ultimately improved the fragrance in the grains of the newly bred hybrid lines (BTYXZ) (Figure 6d). Compared with the wild-type (TYXZ), the BTYXZ lines had no significant changes in amylose content and gelatinization temperature (Figure 7a,b). The gel consistency increased greatly, the hardness of both fresh cooked rice and retrograded cooked rice reduced significantly, and the adhesiveness of fresh cooked rice increased significantly (Figures 7c and 8a). The appearance score and taste score decreased significantly, but the comprehensive score did not change significantly (Figure 8b–d). The above results indicate that the creation of new alleles of *BADH2* and crossing the edited lines with CMS TN1A can potentially improve the aroma in the three-line hybrid rice variety, and without adversely affecting the eating and cooking qualities of rice grain. In addition, the results of this study offer new ideas, new methods and new genetic resources for the improvement of aroma in hybrid rice cultivars.

CRISPR/Cas9 gene editing technology provides an opportunity to develop and select transgene-free plants (Boettcher and McManus, 2015). The creation of CRISPR-edited transgene-free plant has somehow increased the social and ecological acceptability of CRISPR-edited crops (Ahmad *et al*., 2021; Bartkowski *et al*., 2018). Consequently, some countries, such as US have accepted transgene-free CRISPR-edited crops for commercialization after stringent regulatory measures (Waltz, 2016). The absence of T-DNA vector following the negative PCR results of both Cas9 and *HPT* (Figure 1e) indicated that transgene-free homozygous mutants could be easily obtained in the T_1 generation, as the inheritance of T-DNA and the targeted gene was relatively independent. In addition, these results suggest that the T-DNA-free mutants obtained are genetically stable as they cannot undergo further induced mutation within their genome. Moreover, the T-DNA-free edited lines address one of the important biosafety concerns surrounding transgenic crops. The potential of generating CRISPR-edited transgene-free plants has also been previously reported by Nekrasov *et al*. (2017) and Barman *et al*. (2019). Therefore, we hope that these new alleles of *BADH2* lines will be globally adopted by rice farmers to meet the soaring demand of aromatic rice.

The results of this study demonstrate that mutation of *OsBADH2* greatly increased the 2-AP accumulation in the grains even though the increase was significantly different between the two edited cultivars (NJ1 and HHZ). The 2-AP's accumulation in the *OsBADH2* edited lines of cv. NJ1 was significantly higher than those of cv. HHZ (Figure 3b). It is speculated that different mutations of *BADH2* influence the biosynthesis of 2-AP differently. The concentration of aroma in fragrant rice largely depends on the genetic and environmental factors that influence the accumulation of 2-AP (Hu *et al*., 2020). Therefore, the differences in genetic background, growth periods and environmental adaptability could also be the contributing factors to the significant differences in the 2-AP's accumulation of two edited cultivars. Moreover, different *BADH2* alleles in the same genetic background showed significant differences in the 2-AP accumulation in rice grain (Figure 3b). These results further confirmed that different *BADH2* allele mutations have different effects on the accumulation of aroma in rice. However, the grain aroma in the BTYXZ-1 hybrid lines was higher than that in BTYXZ-2 hybrid lines (Figure 6d). This could be caused by the segregation of the progenies bred through the hybridization of the fragrant male sterile line TN1A with each of the HHZ mutant line, which caused allelic variation in the *BADH2* alleles thus resulting in differences in the aroma content between the two hybrid lines.

Similarly, the successful generation of novel alleles, possessing improved grain aroma, using various genome editing tools has been reported in previous studies (Khandagale *et al*., 2020; Shan *et al*., 2015; Tang *et al*., 2021; Usman *et al*., 2020). These authors induced the mutations of *BADH2* in different genetic backgrounds, which all led to the increase of 2-AP content in grains, but the accumulation levels of 2-AP in the grains of different allele lines are different. The 2-AP content in one of NJ1 novel allele ($nj1\text{-}cr^{BADH2}\text{-}2$) was almost the same with what was reported by Tang *et al*. (2021). However, the concentration of 2-AP in the grains of hybrid lines, NJ1 and HHZ mutant lines was low as compared with those reported by Shan *et al*. (2015) and Usman *et al*. (2020). This could be attributed to differences in the genetic background among the different varieties used to manipulate the *BADH2* gene. Secondly, the editing of different regions within *OsBADH2* sequence by these authors could also cause the differences in the contents of 2-AP in grain.

(a)

	Sample	Hardness /N	Maximum adhesion/N	Adhesion /mj	Cohesion/ Ratio	Elasticity /mm	Adhesiveness /N	Chewability /mj
Fresh cooked rice	TYXZ	11.49±0.99	-0.46±0.12	0.22±0.07	0.31±0.03	0.60±0.05	3.59±0.54	2.17±0.48
	BTYXZ-1	9.37±1.01**	-0.45±0.05	0.25±0.04*	0.30±0.03	0.62±0.08	2.78±0.53	1.76±0.56
	BTYXZ-2	9.76±0.57**	-0.51±0.11	0.26±0.07**	0.29±0.03	0.57±0.02	2.81±0.31	1.60±0.19*
Retrograded cooked rice	TYXZ	21.52±1.08	-0.11±0.07	0.02±0.01	0.32±0.03	0.80±0.08	6.93±0.96	5.55±1.12
	BTYXZ-1	19.01±1.38**	-0.20±0.13	0.05±0.02	0.33±0.03	0.74±0.03	6.27±0.89	4.68±0.75
	BTYXZ-2	18.26±0.76**	-0.19±0.03	0.04±0.03	0.35±0.02	0.74±0.07	6.32±0.42	4.71±0.63

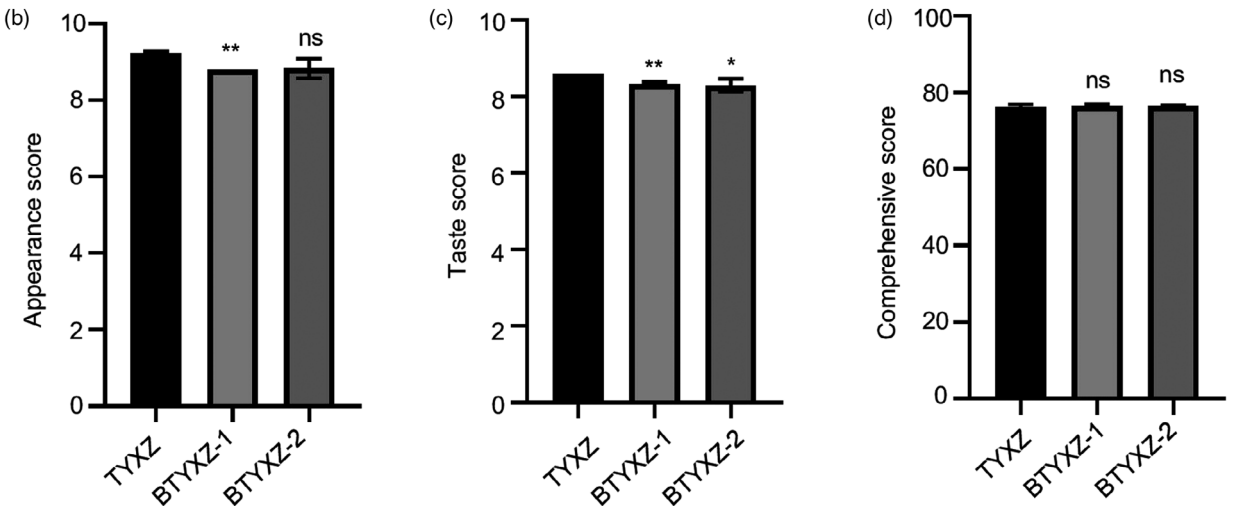


Figure 8 The texture and taste evaluation of fragrance improvement BTYXZ hybrid lines. (a) Texture analysis of fresh rice and retrograded rice. (b) TYXZ and BTYXZ appearance evaluation. (c) TYXZ and BTYXZ taste evaluation. (d) TYXZ and BTYXZ comprehensive evaluation. Values are means ± SD. $^*P < 0.05$; $^{**}P < 0.01$; ns, no significance ($n = 6$ in (a), $n = 3$ in (b–d), two-tailed Student's *t*-test, three independent experiments).

Genetic studies have demonstrated that a single recessive *BADH2* gene is associated with rice aroma (Bradbury *et al*., 2005). The expression levels of *BADH2* gene in aromatic rice are lower compared with non-aromatic rice cultivars (Hinge *et al*., 2016; Prodhan *et al*., 2017). According to previous reports, down-regulation of *OsBADH2* in non-aromatic rice using transcription activator-like effector nucleases (TALENs) and RNA-mediated interference (RNAi) techniques resulted in reduced transcript levels of this gene (Khandagale *et al*., 2020; Shan *et al*., 2015). Likewise, in the present study, similar expression patterns of *OsBADH2* were observed among the four new alleles of *BADH2* (Figure 3c). Subsequently, the reduced expression of *OsBADH2* in NJI and HHZ cultivars resulted in the elevation of 2-AP content in the four *osbadh2* mutant alleles (Figure 3b).

Several studies have been carried out to establish key aromatic volatile compounds related to the biosynthesis and accumulation of 2-AP in rice. For instance, Hinge *et al*. (2016) identified a total of 88 volatile compounds in two aromatic and a non-aromatic rice cultivars. Based on the comparative analysis of the volatile compounds contained in the mature seeds of the new alleles of *BADH2* lines and the wild-types, the gene-edited lines had some fragrance compounds, such as benzaldehyde, neophytadiene, isophytol and pyridine. These substances showed remarkably greater abundance in the mutant lines than the wild-types, and these compounds could have influenced 2-AP's biosynthesis in the mutant lines and aroma uniqueness in each of the mutant lines. Among the novel fragrance compounds profiled, pyridine shares structural homology with 2-AP. Similar findings were also recorded by Ashokkumar *et al*. (2020).

The breeding of GABA-rich rice is a popular direction in the current and future functional rice research. Previous studies have reported that GABA has a number of health benefits in the human body, such as relieving fatigue (Kanehira *et al*., 2011), lowering blood pressure (Vaz *et al*., 2015), inhibiting the proliferation of cancer cells (Song *et al*., 2016) and delaying ageing (Prud'homme *et al*., 2017). The GABA is mainly obtained from food for the human body (Nikmaram *et al*., 2017). Non-aromatic rice cultivars have been reported to contain approximately 30–40% higher levels of GABA than aromatic rice cultivars (Karladee and Suriyong, 2012; Khandagale *et al*., 2020). The BADH2 enzyme, coded by *OsBADH2*, catalyses the conversion of γ-aminobutyraldehyde (GAB-ald) to GABA. Conversely, the loss of function of BADH2 enzyme inhibits the conversion of GAB-ald to GABA (Bradbury *et al*., 2008). The two BTYXZ hybrid lines were found to contain significantly lower amount of GABA compared with the wild-type cultivars in the grains, and different allelic mutations of *BADH2* showed different effects on the accumulation of GABA in rice (Figure 9d). Therefore, the results of this study demonstrated that the editing of *OsBADH2* negatively affected the biosynthesis and accumulation of GABA in the grains. Although, we successfully improved the aroma production in the three-line hybrid rice cultivar (TYXZ), there was

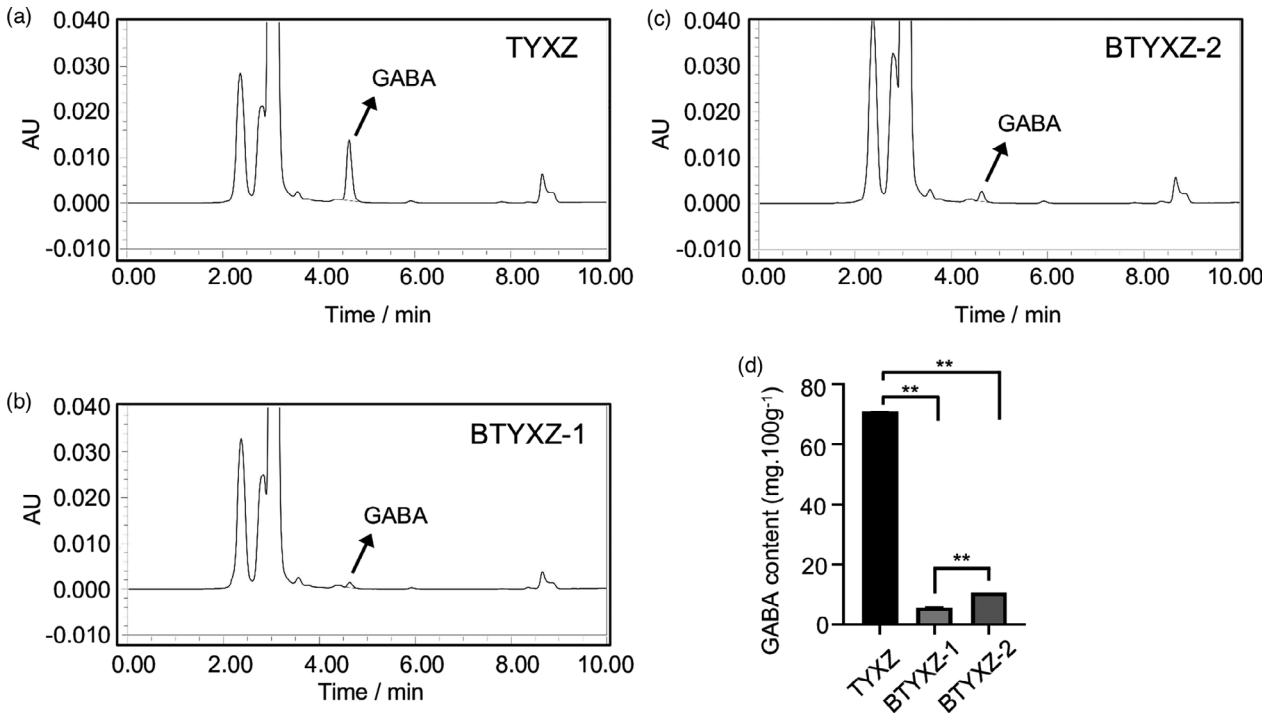


Figure 9 Determination of GABA content in BTYXZ hybrid lines. (a–c) Determination peak of GABA in TYXZ (a), BTYXZ-1 (b) and BTYXZ-2 (c) brown rice. (d) GABA content of the brown rice of TYXZ, BTYXZ-1 and BTYXZ-2. Values are means ± SD. $^{**}P < 0.01$ ($n = 3$, two-tailed Student's *t*-test, three independent experiments).

a significant decrease in GABA, which is a key nutritional compound in rice. There might also be other nutritional elements that were lost due to the induced mutation of *OsBADH2*.

In conclusion, the results of this study have demonstrated that site-directed mutagenesis of *OsBADH2* by CRISPR/Cas9 system can successfully create new alleles of *BADH2* with improved aroma in the rice grain. In addition, the crossing of new allelic lines of *BADH2* with TN1A showed good compatibility in three-line hybrid rice breeding for aroma enhancement. Furthermore, several different volatile compounds were profiled in the mature seeds of both new allelic lines of *BADH2* and the wild-types. Aroma consists of diverse and complex volatile compounds, and understanding their metabolic pathways is an interesting area for future research in enhancing the fragrance production in rice. Although the mutation of *OsBADH2* negatively affected the GABA synthesis and subsequent accumulation, further research should be carried out to create and screen excellent allele combinations with both enhanced aroma and GABA accumulation in rice grain.

Methods

Experimental materials and growth conditions

The rice varieties used in this study included *Oryza sativa* japonica cultivar Ningjing 1 (NJ1), *Oryza sativa* indica cultivar Huang Huazhan (HHZ), a three-line sterile line Taonong 1A (TN1A) and a maintainer line Taonong 1B (TN1B) (*Oryza sativa* indica cultivar). The gene-edited materials together with the wild-types were cultivated in the field during the normal rice growing season at the China National Rice Research Institute (Fuyang, Hangzhou: 30°03′N, 119°57′E). Water and fertilizers were applied according to the standard field cultivation methods.

Vector construction and rice transformation

The *BADH2* gene sequence of the four cultivars, NJ1, HHZ, TN1A and TN1B, was first analysed. Based on the sequencing results, a target site (5′-CGCGATTGCGCGGAGGTACT-3′) at the second exon of *BADH2* gene was selected. The final CRISPR/Cas9 vector was transformed into the NJ1 and HHZ rice varieties via the *Agrobacterium*-mediated transformation method (Hiei *et al.*, 1994). (See Appendix S1 for detailed steps).

Mutation detection and screening of T-DNA-free plants

For the mutation detection, all the transformed T_0 plants were genotyped using a specific PCR-based primer pair (Table S1), which amplified the target site. Mutations were identified by comparing the amplicon sequences of the putative transgenic plants with those derived from the two wild-type cultivars (NJ1 and HHZ). Homozygous T_1 progenies were screened for the presence or absence of T-DNA using both PCR assays and hygromycin plate resistance screening methods (See Appendix S1 for detailed steps).

Evaluation of agronomic performance

Agronomic and yield-related performance of T_1 progenies and the corresponding wild-type (WT) varieties was evaluated at the rice maturity stage. Ten plants with similar growth were selected to investigate plant height, number of effective tillers, grain number per panicle, seed setting rate, grain length, grain width, grain thickness and 1000-grain weight. Ten seeds in three

replicates were randomly selected for measuring grain length, grain width and grain thickness. The 1000-grain weight was measured randomly by selecting 1000 seeds in three replicates.

RNA isolation and real-time PCR analysis

Total RNA was isolated from developing seeds on the 5th, 10th, 15th and 30th day after flowering using the Plant RNA Extraction Kit (Bio Teke, China). Each of the RNA sample was then synthesized to cDNA using ReverTra Ace qPCR RT Kit (TOYOBO, Japan). Gene expression was determined by qRT-PCR using the Light-Cycler 480 system (Roche, Basel, Switzerland) with three technical replicates. *OsActin* (*Os03g0718150*) was used as an internal control (Peng *et al*., 2014). The primer sequences used in this analysis are listed in Table S1. (See Appendix S1 for detailed steps).

Sensory test for aroma and quantification of 2-AP content

The KOH immersion method was used for the sensory evaluation test for aroma among the new alleles of *BADH2* and the WT plants according to Sood (1978) with slight modifications. The quantitative analysis of the aromatic compound 2-AP was determined in seeds of both the mutants and WT plants by headspace solid-phase microextraction (HS-SPME) coupled with GC-MS method (Hinge *et al*., 2016). The samples were prepared according to Chen *et al*. (2012) with slight modifications. Each sample had three replicates. For the internal standard quantification, the 2-AP content was calculated as follows:

$$2-\text{AP content} = (2-\text{AP area} \times \text{TMP content})/\text{TMP peak area}$$

Where TMP = 2, 4, 6-trimethyl pyridine; 2-AP = 2-acetyl-1-pyrroline.

Likewise, for the external standard quantification, the steps were as follows: First, 3 mg of 2-AP standard solution, dissolved in approximately 10% of toluene (CAS NO: 85213-22-5, Toronto Research Chemicals, Canada), followed by dilution with dichloromethane to prepare concentrations of 0.75, 1.5, 3 and 6 μg/mL standard solution. 10 μL of the each of the standard solution was added to the sample bottle (containing 100 mg of rice flour) in turn then sealed tightly. Each sample was repeated three times. The chromatographic column and chromatographic conditions were consistent with the internal standard method, and a standard equation was constructed based on the 2-AP area and the injected 2-AP content (see Appendix S1 for detailed steps).

Extraction and quantification of GABA content

High-performance liquid chromatography (HPLC) method was used for the quantification of GABA in both mutants and WT plants. The extraction of the sample was carried out according to Komatsuzaki *et al*. (2007), with slight modifications. 2.5 g of powdered sample of brown rice was placed in a 50 mL centrifuge tube followed by the addition of 20 mL of 80% ethanol solution and mixed well. After 30 min of ultrasonic vibration, the samples were vortexed for 2 min, then allowed to stand for 5 min. This was followed by centrifugation at 5000 rpm at room temperature for 5 min. The supernatant was then aspirated into a 50 mL volumetric flask. This extraction step was repeated once. 1 mL of aliquots of rice extract was added into a test tube with a stopper and mixed with 0.2 mL of $NaHCO_3$ solution (0.4 g $NaHCO_3$ dissolved in 10 mL ddH_2O, ready to use) and 0.2 mL of DABS-CI solution (20.0 mg 4-dimethylaminoazobenzene-4-sulfonyl chloride dissolved in 10 mL of acetonitrile, ready for use). After mixing, the test tube was placed in a water bath at 70 °C for 20 min and then passed through a 0.45 μm water phase filter membrane. The samples and standards were diluted with acetonitrile into 0.2, 0.5, 2, 4, 20, 100 mg/L standard solutions. One milliliter of each sample and the standard was loaded into tubes in three replicates. The tubes were then placed in the HPLC machine for GABA analysis. The peaks of the standard solutions were then detected at a wavelength of 436 nm. Each sample had three biological replicates and their averages were computed using Microsoft Excel software. A standard curve of the relationship between area and concentration was calculated. The GABA content was quantified according to the standard curve equation. The HPLC conditions were as follows: Column: C18 column, Detection wavelength: 436 nm, Column temperature: 30 °C, Injection volume: 10 μL, Mobile phase: Acetonitrile: Sodium acetate trihydrate ratio = 35:65, Flow rate: 1.0 mL/min.

Untargeted metabolomics analysis

Mature seeds of new allelic lines of *BADH2* and wild-types were dehusked and milled into rice flour using Udy cyclone mill (Cyclotec 1093 sample mill, Tecator, Sweden). Two grams of each sample was added into a bottle and then transferred to a 20 mL headspace sample bottle for HS-SPME-GC-MS analysis. The samples were then shaken at a speed of 450 rpm for 15 min and at a constant temperature of 60 °C. A 50/30 μm DVB/CAR/PDMS extraction head was inserted into the headspace of the sample, and headspace extraction for 40 min at an extraction temperature of 90 °C. The samples were analysed at 250 °C for 5 min, followed by GC-MS separation and identification. (see Appendix S1 for detailed steps).

Evaluation of starch physicochemical properties

The seeds of both the mutants and WT plants were dehusked and milled into rice flour using Udy cyclone mill. To obtain uniform granule size, the milled flour samples were sieved through a 100-mesh sieve.

Amylose content

Three sample replicates and standard solutions were prepared according to the method of Fiaz *et al*. (2019b) and analysed through rapid flow auto analyser (AA3, SEAL, Germany).

Gel consistency

Three sample replicates were prepared and their GC was measured according to the method of Fiaz *et al*. (2019b).

Pasting properties

The pasting properties of the milled rice flours were evaluated using a Rapid Visco-Analyzer (Techmaster, Newport Scientific, Warriewood, Australia) as previously reported by Zhang *et al*. (2013). Each sample was prepared and measured in triplicates.

Taste value of rice

The taste value of cooked rice was determined using SATAKE STA1B Taste analyser (Satake, Japan) as previously reported by (Li *et al*., 2018). Each sample was prepared and measured in triplicates.

Evaluation of grain texture

Determination of cooked rice texture properties

Rice texture was measured as described previously by Li *et al*. (2016) with minor modifications. The Texture Profile Analysis (TPA) settings were as follows: Test speed, 60 mm/s; Compression degree, 60%; Test height, 17 cm; Trigger force 0.1 N. Texture measurements were conducted 10 times for each cooking sample. Extreme values were discarded and an average of six values was calculated. Parameters recorded from the test curves were hardness (HN), adhesion (ADN), cohesion (CON), elasticity (SN) and stickiness (see Appendix S1 for detailed steps).

Thermodynamic characteristics analysis of rice flour

The thermodynamic properties of rice flour were analysed by differential scanning calorimetry (DSC) using a DSC 200 F3 thermal analyser (Netzsch Instruments NA LLC, Burlington, MA) as previously reported by Zhang *et al*. (2013) with slight modifications. The DSC properties were obtained including parameters, such as initial gelatinization temperature (T_0), peak temperature (T_p), end temperature (T_c) and enthalpy of gelatinization (ΔH). The DSC curves of samples were obtained using Stare Default DBV9.10 software. (see Appendix S1 for detailed steps).

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Conflict of Interest

The authors declare no competing interests.

Author contributions

S.H. (Shikai Hu) and S.H. (Suozhen Hui) designed experiments and analysed data; S.H. (Suozhen Hui), H. L., A.M.M, L.Z., J.C., S.A., C.L., J.W., G.J., L.X., G.S., Z.S., S.T., and J.W. performed the experiments; P.H., S.H. (Shikai Hu), S.H. (Suozhen Hui) and A.M.M wrote the manuscript and prepared the illustrations; P.H., S.H. (Shikai Hu) and X.W. conceived the idea and supervised the project.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 The detailed steps of the methods involved in this study.

Figure S1 Determination of 2-AP by external standard method

Figure S2 Heatmap analysis of seeds volatile metabolites in HHZ background

Figure S3 HS-SPME-GC-MS analysis of the untargeted volatiles in NJ1 background seeds

Figure S4 Heatmap analysis of seeds volatile metabonomics in NJ1 background

Table S1 Primers used in this study.