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Short communication

Development and application of two novel functional molecular markers of *BADH2* in rice

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ABSTRACT

Background: Fragrance is one of the most important quality traits in rice, and the phenotype is attributed to the loss-of-function betaine aldehyde dehydrogenase (*BADH2*) gene. At least 12 allelic variations of *BADH2* have been identified, and some of these have been applied to rice fragrance breeding using traditional molecular markers and Sanger sequencing techniques. However, these traditional methods have several limitations, such as being very expensive, imprecise, inefficient, and having security issues. Thus, a new molecular marker technology must be developed to improve rice fragrance breeding.

Results: In this study, more than 95% of the cultivated fragrant rice varieties belonged to a 7-bp deletion in exon 2 (*badh2-E2*) or an 8-bp deletion and 3-bp variation in exon 7 (*badh2-E7*). Both allelic variations resulted in the loss of function of the *badh2* gene. We developed two novel SNP molecular markers, SNP_badh2-E2 and SNP_badh2-E7, related to the alleles. Their genotype and phenotype were highly cosegregated in the natural variation of rice accessions, with 160 of the 164 fragrant rice varieties detected with the two markers. These markers cosegregated with the fragrance phenotype in the F_2 population.

Conclusions: Two functional SNP molecular markers of *badh2-E2* and *badh2-E7* allelic variations were developed. These functional SNP molecular markers can be used for genotype and genetic improvement of rice fragrance through marker-assisted selection and will significantly improve the efficiency of fragrant rice breeding and promote commercial molecular breeding of rice in the future.

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1. Introduction

Rice is one of the world's most important crops, feeding up to approximately half of the world's population and widely planted worldwide. Fragrance is considered a crucial grain quality trait, and a series of fragrant rice varieties have been successfully bred, including Basmati rice (Pakistan and India), Jasmine rice (Thailand), Wuxiangjing 9, and Suyunuo (China) [1,2,3]. Currently, fragrant rice is highly favored by consumers because of a special fragrance with a premium market price, revealing broad prospects and great commercial value for this product.

At present, 2-acetyl-1-pyrrolidine (2-AP) is considered to be the main determinant of the rice fragrance trait among over 200 volatile compounds that have been detected in rice [4]. A dominant BADH2 allele encoding betaine aldehyde dehydrogenase (BADH2) inhibits the synthesis of 2-AP, and the recessive allele induces 2-AP formation [5]. *BADH2* has been confirmed to be the main determinant of rice fragrance. It is located in chromosome 8, comprises 15 exons, and encodes a protein consisting of 503 amino acids [5,6]. This protein was confirmed to possess aldehyde dehydrogenase activity, which may catalyze the oxidation of betaine aldehyde, 4-aminobutyraldehyde, and 3-aminopropionaldehyde [5,7]. For nonfragrant rice, *BADH2* catalyzes the oxidation of 4-aminobutyraldehyde, which is the precursor of 2-AP. This process leads to the inhibition of 2-AP synthesis, and rice loses its fragrance [8]. In contrast, for fragrant rice, the loss of function of the BADH2 protein cannot catalyze the oxidation of 4-aminobutyraldehyde, resulting in the accumulation of 2-AP [8,9].

During the breeding of crops, germplasm resource innovation and method improvement are crucial to success. In recent years, a large number of yield-related, disease resistance-related and quality-related functional genes in rice have been cloned through scientists' efforts from countries worldwide [10,11]. Molecular marker-assisted selection is available in crop breeding, and molecular markers

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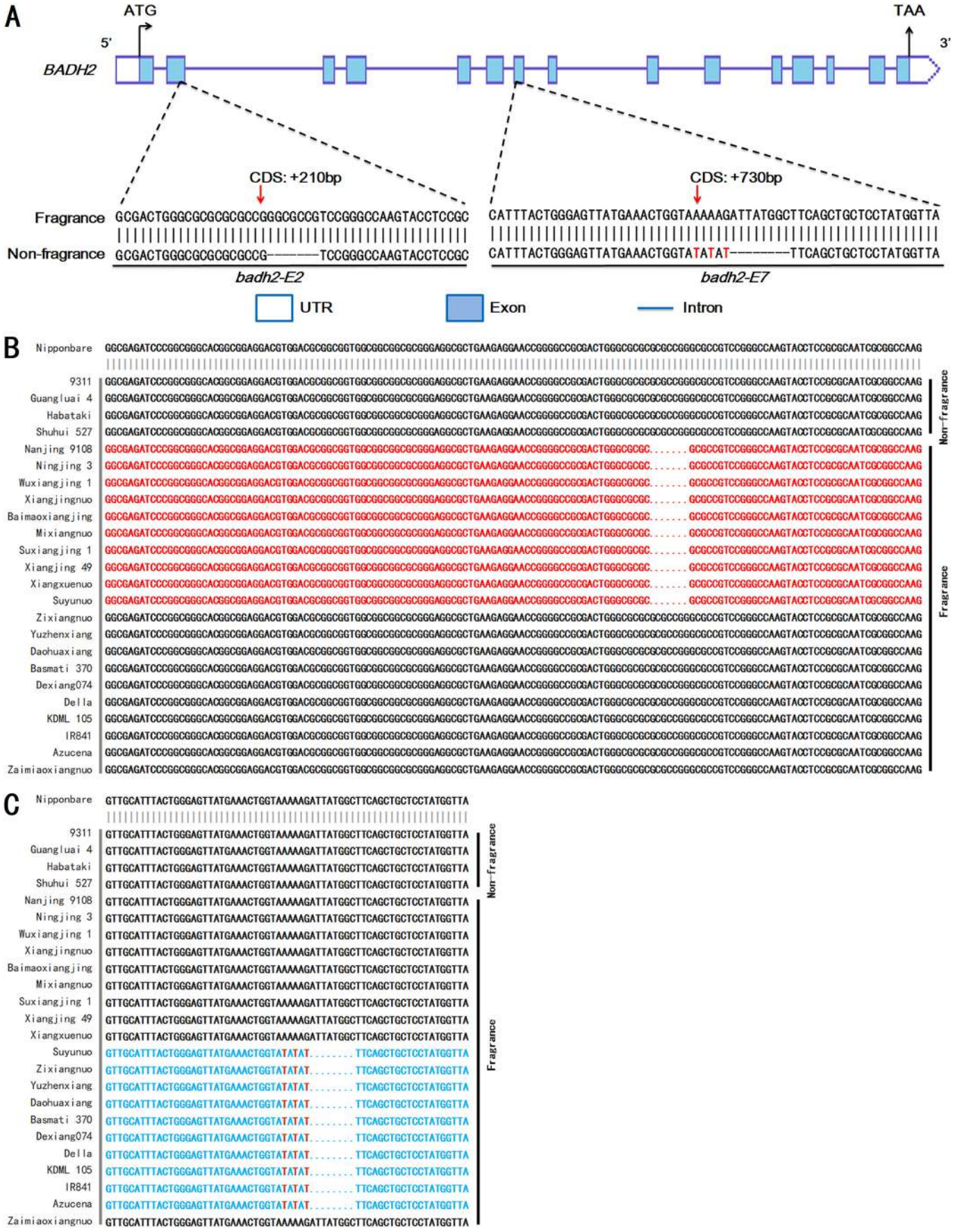

Fig. 1. Allelic variation of *BADH2* in popularly cultivated fragrant rice. (A) Allelic variation of *BADH2* in exon 2 and exon 7. (B) *badh2-E2* allelic variation in popularly cultivated fragrant rice is shown in red. (C) *badh2-E7* allelic variation in popularly cultivated fragrant rice is shown in blue. A total of 23 rice accessions were selected, and 2 targeted regions of *BADH2* were resequenced. The sequences of Nipponbare and 9311 were obtained by a BLAST search from TIGR.

associated with different functional genes are constantly developed and used by breeders. Currently, more than 12 allelic variations of *BADH2* have been identified in the gene codon region [8,12]. The resequenced segments of *BADH2* covered 5.8 kb of the *japonica* sequence in many fragrant rice accessions, functional SNP sites have been detected, and genotype analysis has shown that there are 27 haplotypes of *BADH2* [12]. Moreover, rice domestication has shown that *BADH2* was domesticated during the evolution of rice and that the fragrance gene originated from *japonica* rice, not *indica* rice [8,12,13]. The genetic basis and regulatory mechanism of rice aroma have been analyzed previously; however, there is a new issue regarding how to apply this functional gene to the breeding of fragrant rice. Breeders have undertaken much exploration. Among 12 allelic variations, molecular markers correlated with *badh2-E2*, *badh2-E4/5* (803-bp deletion between exon 4 and exon 5), *badh2-E7*, *badh2-E12* (3-bp insertion in exon 12), *badh2-E13* (3-bp insertion in exon 13), and *badh2-E14* (1-bp insertion in exon 14) were developed by different researchers [2,3,14]. The genetic markers FMbadh2-E4/5 [2], FME14I [3], FMbadh2-E2, and FMbadh2-E7 [14] were validated as functional markers using 14–22 fragrance rice varieties and two F_2 rice populations, and these markers were cosegregated with the fragrance phenotype. All previously developed functional markers were based on polymerase chain reaction (PCR) and gel electrophoresis technology [2,3,14,15].

The most critical step for breeding is in the accurate identification of agronomic traits or phenotypes of the plant. For fragrant rice breeding, breeders identify rice using their sensory organs with chewing or KOH incubation methods [16]. However, these methods may not be reliable owing to several shortcomings, including long-term identification, which may lead to taste and olfactory fatigue for breeders and involve high labor, money, and time costs. In addition, qualitative and quantitative analysis methods have been used for rice fragrance analysis, such as GC–MS, which is difficult to operate and is extremely expensive [8,17].

Because of the complexity regarding the determination of the fragrance phenotype and the efficiency of rice fragrance breeding, marker-assisted selection (MAS) is a preponderant method for fragrant gene screening. A new generation of SNP molecular marker technology has emerged owing to single nucleotide polymorphism being widespread in the genome of all species, and the SNP detection platform has been improved, *e.g.*, LGC SNP Line and Array Tape SNP Line. In the present study, we developed two functional SNP molecular markers covering *badh2-E2* and *badh2-E7* allelic variation sites that are suitable for most popularly cultivated fragrant rice varieties. The results from this study may develop a faster, safer, and more accurate process for fragrant rice breeding with lower costs, which will be helpful for commercial molecular breeding in the future.

2. Materials and methods

2.1. Plant materials

A total of 168 fragrant rice and 20 nonfragrant rice varieties were included in the present study (**Table S1**). All plants were grown in the field in Chongqing at Yangtze Normal University in April 2017. Leaves were obtained for DNA extraction at the seedling stage and rice seeds were harvested for rice fragrance phenotype analysis at the mature stage. "Zixiangnuo" is a local *japonica*-type rice with a high 2-AP value from Hu'nan Province in China, and it was used as a positive control for the fragrance phenotype analysis. "Guangluai 4" is a local *indica*-type rice without fragrance from Guangdong Province in China and was used as a native control for the fragrance phenotype analysis. In addition, four F_2 (**Table S1**) populations generated from cross breeding between fragrant and nonfragrant rice ("Nanjing 9108" × "Habataki," "Ningjing 3" × "Shuhui 527," "Yuzhenxiang" × "Habataki," and "Daohuaxiang" × "Shuhui 527") were selected for further verification of functional molecular markers.

2.2. Fragrance determination

Fragrance determination was performed using the KOH incubation method [16]. Rice seeds were harvested in the field and dried, and then the seed coat was removed with a rice husker. Approximately 2.0 g of husked rice was placed into a 50 mL centrifuge tube and mixed with 10 mL of 1.7% KOH solution. The tube was covered tightly and incubated for 10 min at room temperature (25–30°C). The sample was then smelled by at least three individuals to evaluate whether the sample was fragrant or nonfragrant.

2.3. DNA extraction

Genomic DNA was extracted from rice leaves by the simplified CTAB method using an automatic liquid workstation (Freedom Evo 150, TECAN, Switzerland), which increased the efficiency and decreased the genotyping costs of high-throughput DNA extraction. Rice leaves were lyophilized, placed into a 96-well deep-hole plate (approximately 100 mg of sample per well) with two steel beads (diameter = 4 mm), and ground with an automated tissue homogenizer (Geno/Grinder®, Thermo Scientific, USA). A total of 500 μL of CTAB buffer was added to each well and incubated at 65°C for 30 min. Then, 400 μL of chloroform/isoamyl alcohol solution (24:1) was added, and the plate was vortexed and centrifuged for 10 min at 12000 × g. The supernatant was transferred to a new 96-well deep hole plate, 500 μL of isopropanol was added, and it was incubated at 4°C for 30 min. Finally, the sample was centrifuged for 5 min at 12,000 rpm, after which the supernatant was discharged, and the DNA dissolved with 500 μL of ddH_2O. The DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA).

2.4. Cloning and sequencing

To confirm the detailed sequence variation in different fragrant rice varieties, we designed primers using Primer Premier v5.0 (**Table S2**) that covered exon 2 and exon 7 of *BADH2* to clone the target fragments. PCR was carried out in a total volume of 50 μL using Phanta Max Super-Fidelity DNA Polymerase (Vazyme, China) according to the manufacturer's instructions. PCR cycling was performed in a Thermal Cycler T100 (Thermo, USA) according to the manufacturer's instructions. A total of 23 popularly cultivated rice varieties were selected and sequenced using the Sanger sequence method. The sequences of Nipponbare and 9311 were obtained from TIGR (http://rice.plantbiology.msu.edu/), and then used as reference sequences.

2.5. Development of allele-specific markers

The DNA sequences of *BADH2* were obtained from NCBI (www.ncbi.nlm.nih.gov/) using a BLAST search. The sequence alignments of the donors and recipients were performed using DNAMAN 6.0. A total of 50-bp sequences were extracted upstream and downstream of the polymorphism sites for marker design. A tri-primer containing two allele-specific primers and one allele-flanking common primer was performed using BatchPrimer 3 (http://probes.pw.usda.gov/batchprimer3/) [18] for each SNP site (parameters: Tm-optimum: 57°C; product size-optimum: 50-bp). A Fam or Hex fluorescent tag sequence (Fam: 5′-GAAGGTGACCAAGTTCATGCT-3′; Hex: 5′-GAAGGTCGGAGTCAACGGATT-3′) was linked at the 5′-terminus of the two allele-specific primers. All primer oligos were ordered from Life Technologies (Shanghai, China).

2.6. Kompetitive allele-specific PCR for SNP genotyping

Genotyping assays were conducted using the LGC SNP Line (LGC, Britain), a 384-well format, and were established as 3.0 μL reactions (20 ng template DNA, 1.5 μL 2 × Kompetitive Allele-Specific PCR

Table 1
The functional SNP molecular markers developed for the genotyping of the popularly cultivated fragrant rice varieties.

Marker name	Primer type	Primer sequence (5′ → 3′)
SNP_badh2-E2	Unfavorable allele-specific	TACTTGGCCCGGACGGCGCC
	Favorable allele-specific	TACTTGGCCCGGACGGCGCG
	Allele flanking common	GAGGCGCTGAAGAGGAACCG
SNP_badh2-E7	Unfavorable allele-specific	AACCATAGGAGCAGCTGAAG
	Favorable allele-specific	AACCATAGGAGCAGCTGAAA
	Allele flanking common	GGTTGCATTTACTGGGAGTT
Fam-tail		GAAGGTGACCAAGTTCATGCT
Hex-tail		GAAGGTCGGAGTCAACGGATT

(KASP) Mastermix, 0.17 μM KASP primer, and ddH_2O added to 3.0 μL). PCR was performed using Waterbath thermal cycling in accordance with the following protocol: 94°C for 15 min; 10 touchdown cycles (94°C for 20 s, touchdown at 65°C, and − 0.9°C per cycle for 1 min); and 29 amplification cycles (94°C for 20 s and 57°C for 1 min) [19]. Finally, we used PHERAstar for plate reading. All data were uploaded to Kraken™ for data analysis and reporting.

3. Results

3.1. Identification of loss-of-function badh2 allelic variation in popularly cultivated fragrant rice

The most recognized allelic variation types of *BADH2* were *badh2-E2* and *badh2-E7* (Fig. 1A). The results showed that 10 of the 20 fragrant rice varieties belonged to *badh2-E2,* which has a 7-bp deletion in exon 2 (Fig. 1B), and the other 10 fragrant rice varieties belonged to *badh2-E7,* which contain an 8-bp deletion and 3-bp variation in exon 7 (Fig. 1C). Both *badh2-E2* and *badh2-E7* allelic variations were found in Suyunuo (Fig. 1B and C). However, there was no variation in the two target regions for the Zaimiaoxiangnuo variety (Fig. 1B and C). Thus, most of the popularly cultivated fragrant rice varieties shared a similar hereditary basis and *BADH2* allelic variation, and Zaimiaoxiangnuo may be attributed to another allelic variation of *BADH2,* which rarely occurs in the natural variation.

3.2. Development of functional SNP molecular markers

We designed two functional markers for the exon 2 and exon 7 variation sites. Detailed information is shown in Table 1. The resequenced rice varieties were used for functional marker verification, and the DNA of four F_1 hybrid individual plants was used as the control (Table 2 and Fig. 1B). We applied the allele-specific primer and allele-flanking primer using BatchPrimer 3. The favorable allele primer was linked with a Hex fluorescent tag sequence, and the unfavorable allele primer was linked with a Fam fluorescent tag sequence (Table 1). The presence of functional markers was examined using the LGC SNP Line. The genotype analysis results showed that the Hex fluorescent signal was detected in 19 of the 20 fragrant rice varieties, and that the F_1 hybrid rice DNA fluorescence signal was heterozygous. However, nonfragrant rice varieties were detected with the Fam fluorescence signal of the two functional SNP molecular markers (Fig. 2 and Table 2). The genotype data generated from the functional SNP molecular markers were consistent with the resequencing results (Fig. 1B, C, Fig. 2, and Table 2). Thus, we successfully developed functional SNP molecular markers and forecasted that they could meet the requirements for popularly cultivated fragrant rice identification.

Table 2
Elementary validation of the developed functional SNP molecular markers using the resequenced rice varieties.

No.	Cultivar name	Phenotype	Genotype	SNP_badh2-E2	SNP_badh2-E7
				SNP call	SNP call
1	Nipponbare	Nonfragrance	*BADH2*, wild type	C:C	G:G
2	9311	Nonfragrance	*BADH2*, wild type	C:C	G:G
3	Guangluai 4	Nonfragrance	*BADH2*, wild type	C:C	G:G
4	Habataki	Nonfragrance	*BADH2*, wild type	C:C	G:G
5	Shuhui 527	Nonfragrance	*BADH2*, wild type	C:C	G:G
6	Nanjing 9108	Fragrance	*badh2-E2* allele	G:G	G:G
7	Ningjing 3	Fragrance	*badh2-E2* allele	G:G	G:G
8	Wuxiangjing 1	Fragrance	*badh2-E2* allele	G:G	G:G
9	Xiangjingnuo	Fragrance	*badh2-E2* allele	G:G	G:G
10	Baimaoxiangjing	Fragrance	*badh2-E2* allele	G:G	G:G
11	Mixiangnuo	Fragrance	*badh2-E2* allele	G:G	G:G
12	Suxiangjing 1	Fragrance	*badh2-E2* allele	G:G	G:G
13	Xiangjing 49	Fragrance	*badh2-E2* allele	G:G	G:G
14	Xiangxuenuo	Fragrance	*badh2-E2* allele	G:G	G:G
15	Suyunuo	Fragrance	*badh2-E2* allele *badh2-E7* allele	G:G	A:A
16	Zixiangnuo	Fragrance	*badh2-E7* allele	C:C	A:A
17	Yuzhenxiang	Fragrance	*badh2-E7* allele	C:C	A:A
18	Daohuaxiang	Fragrance	*badh2-E7* allele	C:C	A:A
19	Basmati 370	Fragrance	*badh2-E7* allele	C:C	A:A
20	Dexiang 074	Fragrance	*badh2-E7* allele	C:C	A:A
21	Della	Fragrance	*badh2-E7* allele	C:C	A:A
22	KDML 105	Fragrance	*badh2-E7* allele	C:C	A:A
23	IR841	Fragrance	*badh2-E7* allele	C:C	A:A
24	Azucena	Fragrance	*badh2-E7* allele	C:C	A:A
25	Zaimiaoxiangnuo	Fragrance	*badh2-other* allele	C:C	G:G
26	F1 hybrid individual	Nonfragrance	Heterozygosis	G:C	A:G
27	F1 hybrid individual	Nonfragrance	Heterozygosis	G:C	A:G
28	F1 hybrid individual	Nonfragrance	Heterozygosis	G:C	A:G
29	F1 hybrid individual	Nonfragrance	Heterozygosis	G:C	A:G
30	NTC	N.A.	ddH2O	N.A.	N.A.
31	NTC	N.A.	ddH2O	N.A.	N.A.

N.A. means these data are not available.

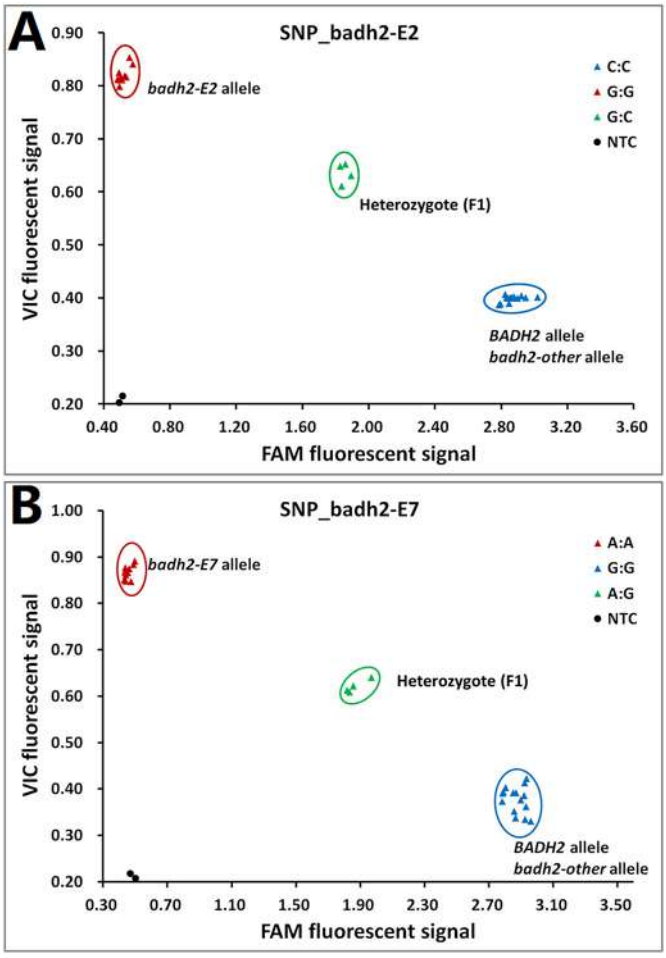

Fig. 2. Elementary validation of the developed functional SNP molecular markers using the resequenced rice varieties. (A) Genotype of the resequenced rice varieties using SNP_badh2-E2. (B) Genotype of the resequenced rice varieties using SNP_badh2-E7. F1 hybrid individual plants and ddH2O were used as the controls.

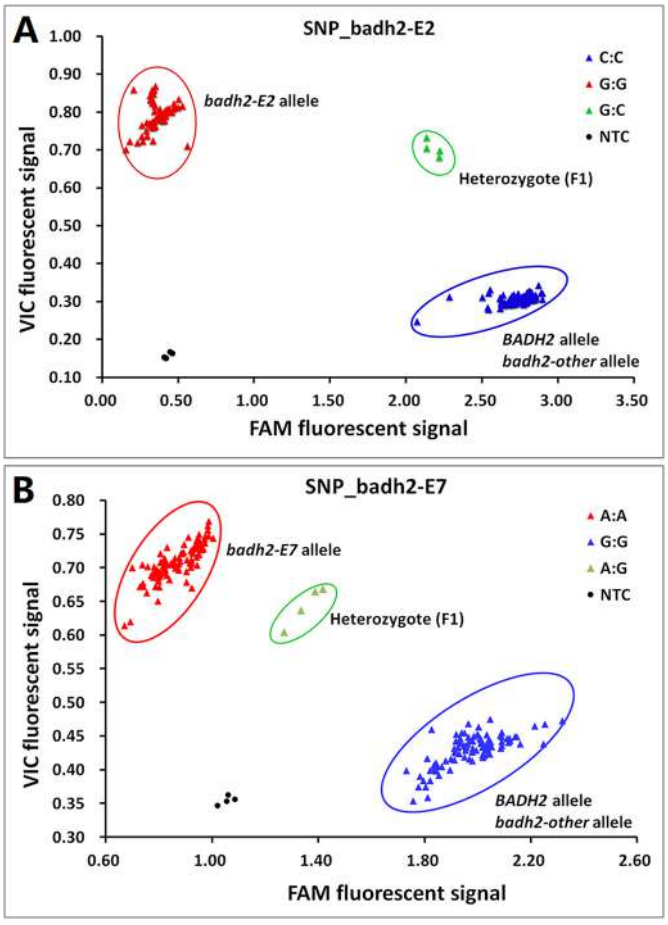

Fig. 3. Genotyping of *BADH2* with functional SNP molecular markers in 168 popularly cultivated fragrant rice varieties and 20 nonfragrant rice varieties. A. Fragrant rice varieties of *badh2-E2* allelic variation detected by SNP_badh2-E2. B. Fragrant rice varieties of *badh2-E7* allelic variation detected by SNP_badh2-E7. F_1 hybrid individual plants and ddH_2O were used as the controls.

3.3. Application of functional molecular markers

To further verify the accuracy of the functional markers for the identification of fragrant rice, the two functional molecular markers were used to genotype 188 germplasms (168 fragrant rice and 20 nonfragrant rice), which were collected from CNRRI and SICAU-RRI. All phenotypes were analyzed using the KOH incubation method (**Table S3**). The results showed that 69 accessions belonged to the *badh2-E2* allelic variation (Fig. 3A) and 92 accessions belonged to the *badh2-E7* allelic variation (Fig. 3B), with more than 97% of the popularly cultivated fragrant rice varieties identified. Four fragrant rice accessions could not be identified using the developed functional markers (Fig. 3 and Table 3). The results showed that the phenotype and genotype were highly correlated, with most of the popularly cultivated fragrant rice varieties belonging to the *badh2-E2* and *badh2-E7* allelic variations, including Basmati and Jasmine, which are popular worldwide. The genetic basis of cultivated rice has been narrowed in combination with domestication and artificial selection, and a component of rare alleles has a low gene frequency (Table S3).

The four F_2 populations derived from the cross between fragrant and nonfragrant rice varieties were genotyped with SNP_badh2-E2 and SNP_badh2-E7 (Fig. 4). SNP_badh2-E2 was used to genotype the F_2 population derived from Nanjing 9108 and Ningjing 3 (*badh2-E2* allelic variation donor) crossed with Habataki and Shuhui 527 (recipient), respectively (Fig. 4A, B, and Table S4). SNP_badh2-E7 was used to genotype the F_2 population derived from Yuzhenxiang and Daohuaxiang (badh2-E7 allele variation donor) crossed with Habataki and Shuhui 527, respectively (Fig. 4C, D, and Table S4). For each F_2 population, more than 90 individuals were randomly selected, and Mendel's inheritance was exhibited by phenotype analysis with 3:1 segregation ($\chi_c^2 < \chi^2_{(1,\,0.05)}$). Genotyping analysis revealed that all F_2 populations exhibited 1:2:1 segregation of the Fam fluorescent signal:

Table 3
Segregations of the phenotype and genotype in 4 F_2 populations.

SNP_badh2-E2	Nanjing 9108 × Habataki		Ningjing 3 × Shuhui 527	
	Phenotype ratio	Genotype ratio	Phenotype ratio	Genotype ratio
Expected ratio	3:1	1:2:1	3:1	1:2:1
Actual value	140:48	50:90:48	68:26	23:45:26
χ^2 value	0.007	0.383	0.227	0.362
SNP_badh2-E7	Yuzhenxiang × Habataki		Daohuaxiang × Shuhui 527	
	Phenotype ratio	Genotype ratio	Phenotype ratio	Genotype ratio
Expected ratio	3:1	1:2:1	3:1	1:2:1
Actual value	136:52	48:88:52	72:22	21:51:22
χ^2 value	0.574	0.936	0.057	0.702

badh2-E2 allele and *badh2-E7* allele were crossed to two wild type (*BADH2*) rice accessions, respectively. $\chi^2_{(1,\,0.05)} = 3.84$ and $\chi^2_{(2,\,0.05)} = 5.99$.

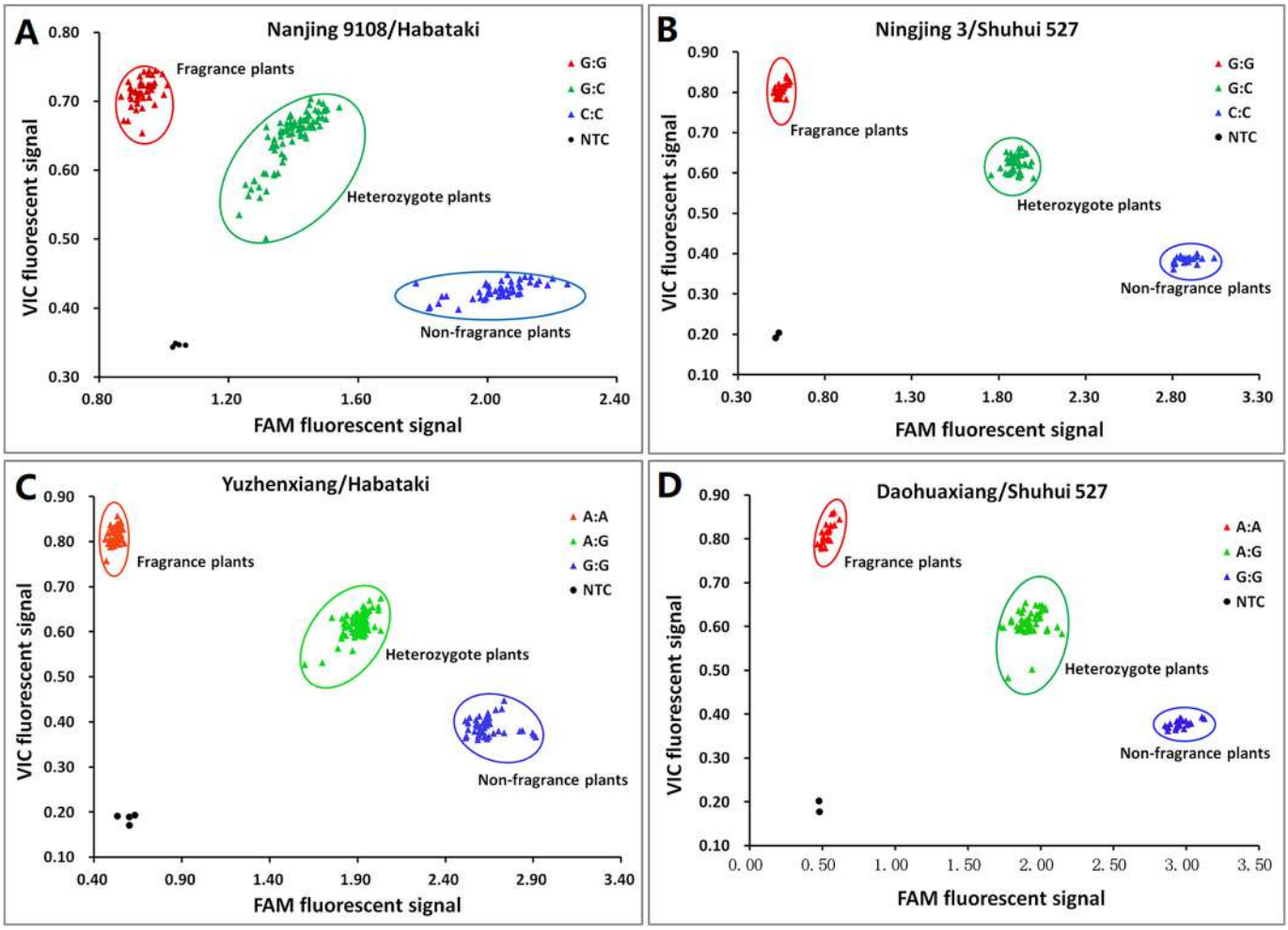

Fig. 4. Verification and application of the functional SNP molecular markers among separate populations. A. The F_2 population derived from the cross between Nanjing 9108 and Habataki genotyped by SNP_badh2-E2. B. The F_2 population derived from the cross between Ningjing 3 and Shuhui 527 genotyped by SNP_badh2-E2. C. The F_2 population derived from the cross between Yuzhenxiang and Habataki genotyped by SNP_badh2-E7. D. The F_2 population derived from the cross between Daohuaxiang and Shuhui 527 genotyped by SNP_badh2-E7. The parental plants and ddH_2O were used as the controls.

Heterozygosis: Hex fluorescent signal ($\chi_c^2 < \chi^2_{(2,\ 0.05)}$) (Table 3). Furthermore, the phenotypic identification results were closely matched with genotyping analysis data for all individual plants in the four F_2 populations (Table 3). Thus, *BADH2* was found to belong to a recessive inheritance of fragrance control in rice, and functional markers SNP_badh2-E2 and SNP_badh2-E7 are suitable for fragrance gene screening in rice breeding and germplasm identification.

4. Discussion

Phenotype identification has been universally used to screen prepotent individuals during traditional breeding processes of plants. The rice fragrance phenotype is usually identified using chewing, KOH incubation, and hot water extraction methods [16]. However, these methods are labor intensive and may not be reliable owing to the sensory insensitivity of the individuals, as well as being harmful to the breeder's tongue and nose [2,3,8]. The phenotype of grain-related traits has been typically determined after harvest, which has led to wasted time and inefficiency. In addition, quantitative and qualitative analysis methods such as high performance liquid chromatography and GC–MS have been used to measure the concentration of 2-AP, with 2-AP considered as a volatile flavor component of fragrance [8,17]. However, these methods are not suitable for the large amount of segregation of population screening required in the field.

Over the past decades, many functional genes that significantly regulate crop agronomic traits have been identified, and breeders have made great efforts to apply them in practice [10,11]. MAS is an economical and efficient breeding strategy for target trait selection, and molecular markers of functional genes have been reported one by one. Molecular markers closely linked to fragrance genes for assisted selection have shown an efficient and accurate route in fragrant rice breeding [2,3,14,15]. *BADH2* has been proven to exclusively regulate rice aroma [5], with multiple allelic variations found in natural rice accessions [8,12]. Some associated molecular markers of loss-of-function alleles have been developed by researchers and detected using gel electrophoresis methods, such as SSRs and CAPS [2,3].

In fragrance breeding, at least eight loss-of-function allelic variations have been found. InDel molecular markers were developed by breeders for the partial identification of variations and have been detected using gel electrophoresis [2,3,8,12]. In the present study, we developed two functional SNP molecular markers that covered the *badh2-E2* and *badh2-E7* allelic variations and genotypes and phenotypes were cosegregated. Compared to molecular markers, whose detection depends on gel electrophoresis, newly generated markers operated easily without any toxic substances, such as ethyl bromide, $AgNO_3$, and formaldehyde. The SNP molecular markers developed based on the KASP method produced a high-throughput route for target gene screening with lower costs and reduced labor, resulting in a sharp

increase in efficiency. In addition, determining the genotype using the LGC SNP Line, which converted the data into fluorescence signals, made the data more visual and readable. In brief, accurate genotype data were obtained with fewer costs and greater labor safety.

In China, rice has a long cultivation history, and fragrant rice germplasm is widespread. For the nonfunctional *badh2* response to rice fragrance, any allelic variation might lead to a new nonfunctional *badh2*, and *BADH2* has been knocked out successfully using TALEN and CRISPR/Cas [20,21]. The results showed that the *badh2-E2* and *badh2-E7* alleles were distributed widely in cultivated fragrant rice. Thus, SNP_badh2-E2 and SNP_badh2-E7 can be widely applied to fragrant rice breeding with strong advantages (Fig. 3, Fig. 4, Table S3, and Table S4). However, 4 of the 164 fragrant rice varieties could not be identified using SNP_badh2-E2 and SNP_badh2-E7 molecular markers (*i.e.*, a total of 160 rice varieties were identified). In addition, no variation was found in two target regions of the 4 varieties by sequencing. Therefore, we speculated that *BADH2* was domesticated in natural variations and that some rare variation of the loss-of-function *badh2* allele existed in cultivated rice. These results were consistent with previous studies [10,12]. Hence, new functional SNP molecular markers should be developed in future studies to adapt new allelic variations. Combined with functional SNP molecular markers for the identification and screening of fragrant rice, germplasm will be helpful for rice breeding. We believe that more new rice varieties that are of high quality and produce a high yield will be cultivated successfully in the near future.

5. Conclusions

In the present study, two novel functional SNP molecular markers associated with *badh2-E2* and *badh2-E7* allelic variations were developed using the KASP method. The functional SNP molecular markers were applied to fragrant rice germplasm identification and F_2 population screening. The genotype and phenotype were highly cosegregated in natural variation rice accessions, and 160 of the 164 fragrant rice varieties were detected using the two markers. Thus, most of the popularly cultivated fragrant rice varieties belong to the *badh2-E2* and *badh2-E7* allelic variations. We confirmed that the functional SNP molecular markers, SNP_badh2-E2 and SNP_badh2-E7, were perfectly cosegregated with the phenotype in each F_2 population. SNP_badh2-E2 and SNP_badh2-E7 can be used for genotype and genetic improvement in fragrant rice through MAS and will significantly improve the efficiency of fragrant rice breeding and promote commercial molecular breeding of rice in the future.

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Conflict of interest

The authors declare that they have no competing interests.

Supplementary material

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