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Isolation and Molecular Marker Detection of *Badh2* Gene from Aromatic Rice Germplasm Resources in Southern Henan

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Abstract

The production of aroma in aromatic rice is due to the increase of 2-acetyl-1-pyrroline (2-AP) precursor substances caused by the functional deletion of *Badh2* gene on chromosome 8, and the accumulation of 2AP makes rice produce aroma. In this study, *Badh2* gene was isolated and cloned from 18 representative aromatic rice cultivars in Southern Henan, and the bioinformatics analysis of *Badh2* gene was carried out. Meanwhile, seven functional molecular markers developed by *Badh2* gene were used to detect and analyze *Badh2* gene in 18 aromatic rice varieties from Southern Henan. The results showed that the coding region of *Badh2* gene was 1509 bp in length. It contained 15 exons and 14 introns, and encoded 503 amino acids. There are many types of variation of the *Badh2* gene in the 18 aromatic rice varieties. According to the variation of *Badh2* gene, the tested aromatic rice varieties could be divided into three groups, among which Xinxianggeng 1, Xiangnuo 25, Heixiangdao 193 and Xiangbao 2 were concentrated in group Ⅰ, while the other 14 kinds of aromatic rice were concentrated in group II. Seven functional molecular markers of *Badh2* gene were used to detect different varieties mutation types in exon 2, exon 4~5, exon 7 and exon 13 of *Badh2* gene. No aromatic rice varieties with different mutation types were found in promoter region, exon 12 and exon 14 of *Badh2* gene. Therefore, our results provide important information for understanding the genetic basis of fragrant genes in aromatic rice germplasm resources in Southern Henan and breeding new varieties of high-quality aromatic rice using molecular marker-assisted selection.

Keywords: Aromatic rice; *Badh2* gene; Gene cloning; Bioinformatics analysis; Molecular marker detection.

1. Introduction

Rice (*Oryza sativa* L.) is the staple food for more than 3 billion people in the world and provides about 25% of their energy [1, 2] which is one of the most important food crops. For most of the population in Southeast Asia, rice provides more than 35% of its energy [3, 4]. The world population is expected to grow at a rate of 25% over the next 30 years and reach 10 billion [5]. With the improvement of people's living standard, the demand for high-quality rice is increasing [6]. Rice quality traits are complex, including nutritional quality, appearance quality, cooking and eating quality, and consumers tend to pay more attention to cooking and eating quality of rice [7-9]. As one of the cultivated rice types, aromatic rice is favored by consumers at home and abroad because of its unique aroma [10]. Over the past decade, the market share of aromatic rice has gradually increased, and the price of aromatic rice has been higher [11, 12]. Therefore, it has important economic value and broad application prospects to conduct in-depth research on aromatic rice germplasm resources, cultivate new varieties with high quality and yield, and apply them in production practice.

More than 200 volatile substances have been isolated and identified in rice [10, 13]. 2-Acetyl-1-pyrroline (2-AP) is considered as one of the main volatile substances in aromatic rice [13, 14], and 2-AP can be detected at low concentrations. Previous studies have found that the aroma of gene is located on chromosome 8 of rice, and the aroma of rice is caused by the mutation of exons 2 and 7 of *Badh2* gene. In exon 7 of betaine aldehyde dehydrogenase 2 gene, there are eight deletions and three polymorphisms of deoxynucleotide mutations, which result in the inability of the protein enzymes transcribed and translated from betaine aldehyde dehydrogenase 2 gene to perform their normal functions and make common rice emit aroma that it should not have [11, 15-18]. There are different scholars who argue that the loss of 7 bases at the second exon of *Badh2* gene may be the main cause of rice fragrance [19-22]. In different aromatic rice varieties, in addition to one 7 bp deletion in exon 2, there may also be an 803 bp deletion in exons 4 and 5 of *Badh2* gene [18, 23, 24], and a mutation site exists in exons 1, 10, 13 and 14 [23-26]. Further studies revealed that there were insertion, deletion or single nucleotide mutation sites in exon 1 and intron 1 of *Badh2* gene, promoter region and its 5'-UTR region [18, 27, 28]. Aroma differences in rice are largely determined by allelic variations in the *Badh2* gene. Aroma-related rice varieties often include 8 bp deletion in exon 7 and 3 single nucleotide polymorphisms (SNPs), or 7 bp deletion in exon 2 [18, 23]. Therefore, when the *Badh2* gene mutates in the coding region or regulatory region, it can produce non-biologically active betaine dehydrogenase, resulting in the aroma of rice, which provides an important theoretical basis for the cultivation of new aromatic rice varieties.

The genetic basis of rice fragrant genes is complex. There is less than one pair of genes controlling rice aroma, or more than 2~4 pairs or more. This may be due to: (1) The genetic basis of different aroma types (such as popcorn, jasmine, violet and pecan) is different; (2) There are interactions between fragrant genes and various environmental factors (illumination, temperature, soil fertility, etc.); (3) The diversity of aroma components in rice, and the methods and techniques for identifying different types of aroma are not perfect at present [29, 30]. There are abundant aromatic rice germplasm resources in the South of Henan, among which the aroma is different. It is speculated that there may be some variation in the fragrant *Badh2* gene in aromatic rice. The *Badh2* gene was isolated and cloned from 18 representative fragrant rice varieties in Southern Henan. Sequence analysis of the *Badh2* gene was carried out. Seven functional molecular markers of the *Badh2* gene were used for detection and analysis. Therefore, the

results of this study will further reveal the genetic basis of fragrant genes in Southern Henan aromatic rice and improve important information for the cultivation of new varieties of high-quality aromatic rice.

2. Materials and Methods

2.1. Materials for Testing

All the materials in this study are *japonica* rice varieties. Among them, 18 aromatic rice resources from Southern Henan are widely representative, 14 conventional aromatic rice and 4 *waxy* aromatic rice (Table 1). All aromatic rice varieties were sown in the same experimental field of Xinyang Academy of Agricultural Sciences in 2016. Each variety was planted in 2 rows, 12 plants per row, and the row spacing was 16.5 × 26.4 cm. Routine cultivation and management of common field was carried out from sowing to seed maturity.

Table-1. Rice varieties and their sources in this experiment

Number	Variety	Source	Type
1	Xianggeng 805	Xinyang Academy of Agricultural Sciences	*Japonica* conventional aromatic rice
2	Changxianggeng 101	Xinyang Academy of Agricultural Sciences	*Japonica* conventional aromatic rice
3	Xiangnuo 1862	Henan Academy of Agricultural Sciences	*Waxy* aromatic rice
4	Zhengxianggeng 11	Henan Academy of Agricultural Sciences	*Japonica* conventional aromatic rice
5	Nongxianggeng 4	Agricultural University of Heundefinednan	*Japonica* conventional aromatic rice
6	Xiangnuo 25	Institute of Agricultural Sciences, Xixian County, Henan Province	*Waxy* aromatic rice
7	Xiangbao 2	Xinyang Institute of Agriculture and Forestry	*Japonica* conventional aromatic rice
8	Exiang 1	Xinyang Institute of Agriculture and Forestry	*Japonica* conventional rice
9	Xiangfeng 916	Xinyang Academy of Agricultural Sciences	*Japonica* conventional aromatic rice
10	Huaibinxiangxiangdao	Institute of Agricultural Sciences, Huaibin County, Henan Province	*Japonica* conventional aromatic rice
11	Nongxianggeng	Agricultural University of Heundefinednan	*Japonica* conventional aromatic rice
12	Xinxianggeng 1	Xinyang Academy of Agricultural Sciences	*Japonica* conventional aromatic rice
13	Xiangbao 1	Xinyang Institute of Agriculture and Forestry	*Japonica* conventional aromatic rice
14	Xinxiangruo 933	Xinyang Academy of Agricultural Sciences	*Waxy* aromatic rice
15	Heixiangruo 1926	Xinyang Institute of Agriculture and Forestry	*Waxy* aromatic rice
16	Xiangxiang 1	Institute of Agricultural Sciences, Huaibin County, Henan Province	*Japonica* conventional aromatic rice
17	Heixiangdao 193	Xinyang Institute of Agriculture and Forestry	*Japonica* conventional aromatic rice
18	Exiang 2	Xinyang Institute of Agriculture and Forestry	*Japonica* conventional rice

2.2. Isolation and Cloning of *Badh2* Gene

Genome-wide DNA extraction from all aromatic rice leaves by CTAB method [4, 31]. PCR primers such as Table 2. The PCR reaction system includes DNA template 2 μl, 10 × Buffer 2.5 μl, $MgCl_2$ 1.5 μl of 25 mmol·L^{-1}, dNTP 2.0 μl of 2.5 mmol·L^{-1}, forward and reverse primers (12.5 mmol·L^{-1}) of 1.0μl, Taq enzyme of 5 U·μl^{-1} of 0.4 μl, supplemented with sterile water to 20 μl. The reagents used were purchased from Shanghai Biotechnology Co., Ltd. The conditions of PCR reaction were as follows: pre-denaturation at 94℃ for 5 min, denaturation at 94℃ for 30 s, annealing at 55~61℃ for 30~90 s, extension at 72℃ for 10 min after 32 cycles, and preservation at 4℃. After the target fragment was recovered by Axygen gel recovery kit, the target fragment was connected to the carrier of pMD19-T (purchased from Takara Company). Systems: Solution I 5 μL, pMD19-T 1 μL, gel recovery product X μL, DDW 4-X pMD19-T 1 μL, gel recovery product 4 μL. Conditions: 16 ℃, overnight. The above products were

transformed into *DH5α* (purchased from Solarbio Company) and grown overnight at 37 ℃. A part of the monoclones grown on the plate was selected to grow in liquid LB medium containing ampicillin. After cultured in shaking bed at 37 ℃ and 180 rpm for 5~6 h, the positive bacteria were detected by PCR, and then sent to Anhui General Company for sequencing. Finally, the sequence was spliced completely.

Table-2. PCR amplification and sequencing primers of *Badh2* gene in rice

Number	Primer name	Primer sequence
1	Badh2 P1-F	GTCTCTCCTCCAACATGCTC
2	Badh2 P3-R	CAAGCGTCTCTAGTCTAGCC
3	Badh2 P3-F	GTGCGTATCCTCTGTTCTGG
4	Badh2 P4-R	CCATCAGGAGAGGATAGTTC
5	Badh2 P5-F	CCTCCGTGTTAATGCAGCTC
6	Badh2 P10-R	CCGGTCATCAGCTAACTTCC

2.3. Bioinformatics Analysis Software and Related Web Sites

The nucleotides and structures of *Badh2* gene were analyzed, the amino acid sequences encoded by *Badh2* were speculated, and the properties and functional domains of the proteins were analyzed and predicted by bioinformatics. The relevant tool software and web site for *Badh2* gene sequence analysis are shown in Table 3.

Table-3. The main tools used in bioinformatics analysis of the structure and function of *Badh2* gene in rice

Commonly used resources	Web sites and software
Analysis of Physicochemical Properties of Prot	http://www.expasy.org/tools/protparam.Html
Hydrophilic and hydrophobic analysis of proteins	http://web.expasy.org/protscale
Protein signal peptide prediction	http://www.cbs.dtu.dk/services/SignalP
Prediction of transmembrane domain	http://www.cbs.dtu.dk/services/TMHMM
Prediction of protein secondary structure	http：//pbil.ibcp.fr/htm/index.php
Spatial conformation modeling of proteins	http://swissmodel.expasy.org
Analysis of Protein Functional Domains	SMART website
Construction of phylogenetic tree	MEGA6.0 Software
Basic BLAST retrieval	http:/ /www.ncbi.nlm.nih.gov/BLAST

2.4. Detection of Gene Functional Markers

Badh2 gene has many allele forms in natural population, and different alleles have different aroma. Seven functional markers of *Badh2* gene have been developed for insertion, deletion or single nucleotide mutation sites in exons 2, 4, 5, 7, 12, 13 and 14 of 5'-UTR region of *Badh2* gene [11, 28, 32]. Among them, functional marker FME14 is a sequence marker of digestion amplification polymorphism (The cleaved amplified polymorphic sequence marker, CAPS), and the rest are common PCR molecular markers. Detailed information about the amplified products, primer sequence and annealing temperature of each *Badh2* gene functional marker is shown in Table 4. All the primers were synthesized by Nanjing Kingsley Biotechnology Co., Ltd. and the preliminary tests showed that the seven functional markers of *Badh2* gene were stable in the reaction system and the results were clear and reliable.

Table-4. Functional markers information of *Badh2* gene in this experiment

Alleles	Functional markers	Primer sequence (5'-3')	Annealing temperature	Enzyme	PCR products size (bp)
Badh2-UTR	FMU1-2	F:TCCCACCACCACTCCACA	61	–	Frgrant:160
		R:ACGAAGAGCTGCCGCTGC			Non-frgrant:163
Badh2-E2	FME2	F:GGGAGGCGCTGAAGAGGA	60	–	Frgrant:100
		R:GGGTAGTCACCACCCTACCTTG			Non-frgrant:1107
Badh2-E4-5	FME4-5	F:TGCTGGATGCTTTGAGTA	55	–	Frgrant:321
		R:GTTTAGCACACCTGAAGGAAGACCA			Non-frgrant:1123
Badh2-E7	FME7	F1:CCGGTGCTCCTTTGTCATC	55	–	Frgrant:583,169
		R1:TGAAACTGGTAAAAAGATTATGGC			Non-frgrant:591,449
		F2:GAGCAGCTGAAATATATACC	55	–	
		R2:TTGCATCCTGCTCGTCTGG			

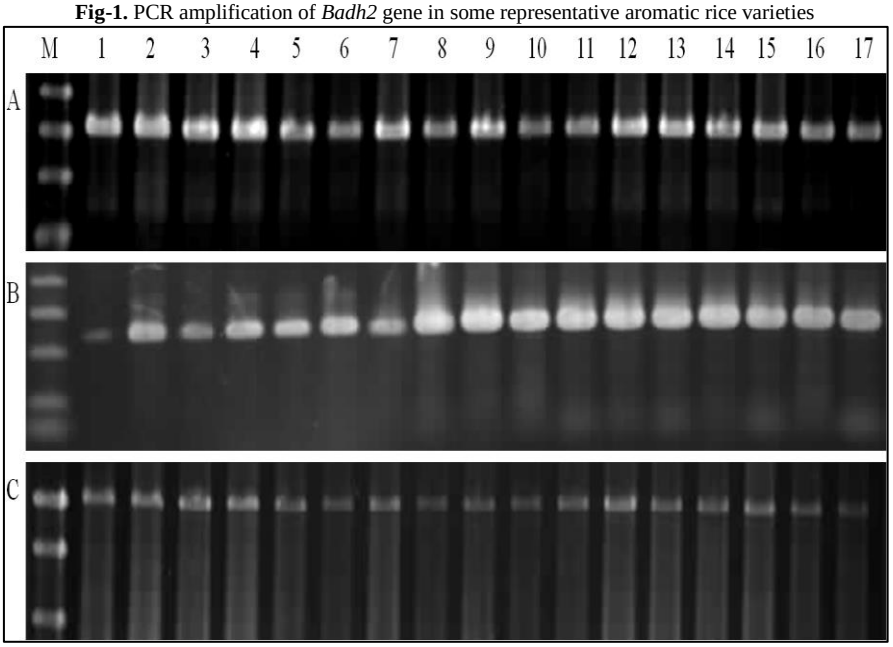
Badh2-E12	FME12	F:TTGGTCCAGTGCTCTGTGTG	58	–	Frgrant:189
		R:GCACCAGCCAGACCATAAC			Non-frgrant:192
Badh2-E13	FME13	F:TTGGTCCAGTGCTCTGTGTG	58	–	Frgrant:195
		R:GCACCAGCCAGACCATAAC			Non-frgrant:192
Badh2-E14	FME14	F:TCGATGCCGGAATTATCTGGGTGA	61	*Bsl* I	Frgrant:60,205
		R:TCCCCACGGCTCATCGGAGG			Non-frgrant:266

3. Results and Analysis

3.1. Isolation and Cloning of *Badh2* Gene from Aromatic Rice in South Henan

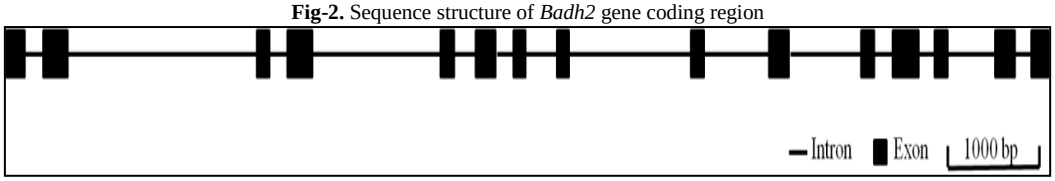
Using the leaf DNA of aromatic rice in Southern Henan as template, the expected DNA fragment could be amplified by using the primers in Table 2 to amplify the expected DNA fragment (Fig. 1). The target fragments were recovered by gel recovery kit and linked to cloning vector pMD19-T. The fragments were transformed into *DH5α* and grew overnight at 37℃. After monoclonal detection, the positive bacterial liquid was sent to Anhui General Company for sequencing. Finally, the sequence of *Badh2* gene of all 18 varieties of aromatic rice germplasm resources in Southern Henan was obtained. *Badh2* gene contains 15 exons and 14 introns (Fig. 2), which encodes about 503 amino acids. There are many types of variation in *Badh2* gene among 18 aromatic rice varieties in Southern Henan, which may be the reason for the difference of aroma among different aromatic rice varieties in Southern Henan.

Fig-1. PCR amplification of *Badh2* gene in some representative aromatic rice varieties



A, using *Badh2* P5-F and *Badh2* P10-R primers amplification; B, using *Badh2* P1-F and *Badh2* P3-R primers amplification; C, using *Badh2* P3-F and *Badh2* P4-R primers amplification; M, Marker; 1~17 respectively represent aromatic rice varieties are shown in Table 1.

Fig-2. Sequence structure of *Badh2* gene coding region



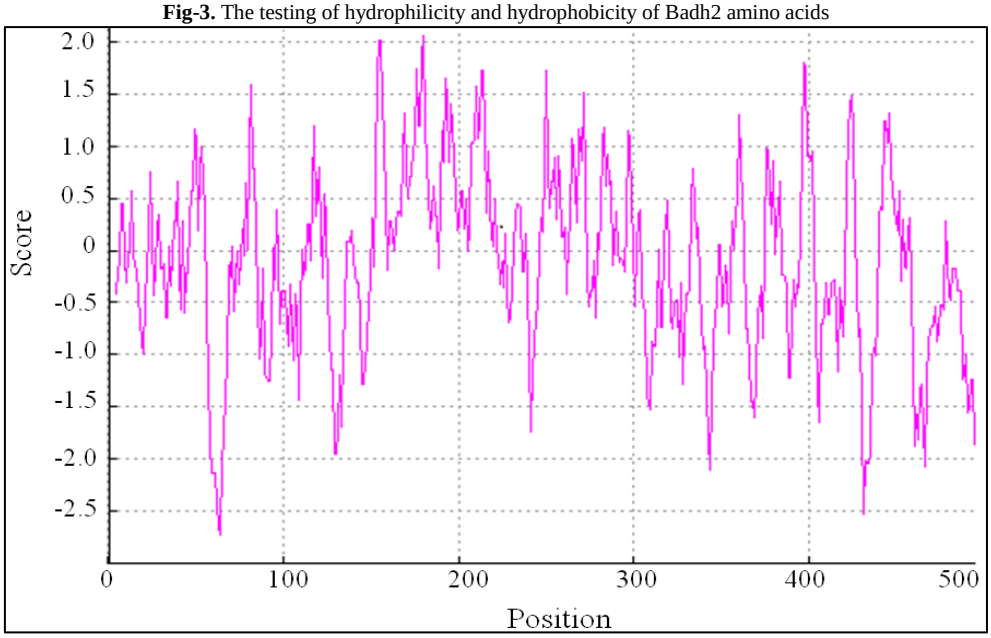
3.2. Amino Acid Sequence Coded by *Badh2* Gene and Analysis of its Physicochemical Properties

The *Badh2* allele in Xiangfeng 916 aromatic rice widely used in production was analyzed by bioinformatics. The full length of *Badh2* allele in Xiangfeng 916 aromatic rice was 7111 bp, encoding 503 amino acids. The isoelectric point of the protein was 5.36, the size of the protein was 54.68 KDa, and its instability coefficient was 72.32. The amino acid sequence analysis of *Badh2* gene showed that the content of alanine in Badh2 protein was the

highest, 11.1%. Secondly, the contents of glycine and glutamate were 8.5%. The content of methionine and histidine was 1.6% and 0.8% respectively. Among the Badh2 protein sequences, 66 negative-charged amino acids (such as aspartic acid, Asp) and glutamic acid accounted for 13.1% of the total amino acid residues, while 57 positive-charged amino acids (such as arginine, Arg) and lysine (Lys) accounted for 11.3% of the total amino acid residues. The fat index and instability index of Badh2 protein were 86.92 and 35.87, which indicated that Badh2 protein was relatively stable.

The hydrophobicity of rice Badh2 protein was predicted by Prot Scale online bioinformatics analysis tool. The results showed that the lower the score, the stronger the hydrophilicity, and the higher the score, the stronger the hydrophobicity. At the same time, the results showed that the most hydrophilic amino acid of Badh2 protein was arginine (Arg), with a score of -2.722, and the most hydrophobic amino acid was glycine (Gly) with a score of 2.067. Overall, the hydrophilic amino acids of Badh2 protein were significantly more than those of hydrophobic amino acids (Fig. 3). Therefore, Badh2 protein of aromatic rice is a relatively hydrophilic protein.

Fig-3. The testing of hydrophilicity and hydrophobicity of Badh2 amino acids



3.3. Prediction of Transmembrane Structure and Signal Peptide Analysis of *Badh2* Gene Encoding Protein

The existence of transmembrane domain of Badh2 protein in aromatic rice (www.cbs.dtu.dk/services/TMHMM) was analyzed by TMHMM. The results showed that there was no transmembrane domain of Badh2 protein (Fig. 4).

SignalP 4.0 online analysis software was used to predict the protein encoded by *Badh2* gene (http://www.cbs.dtu.dk/services/SignalP). As shown in Fig. 5, the 35th alanine residue of Badh2 protein may be the original splicing site of signal peptide, but the highest predictive score and the highest signal peptide score are 0.110 and 0.153, respectively. Both predictive values are low. Comprehensive analysis results show that Badh2 protein is likely to have no signal peptide sequence.

Fig-4. Prediction results of the transmembrane domans of Badh2 protein

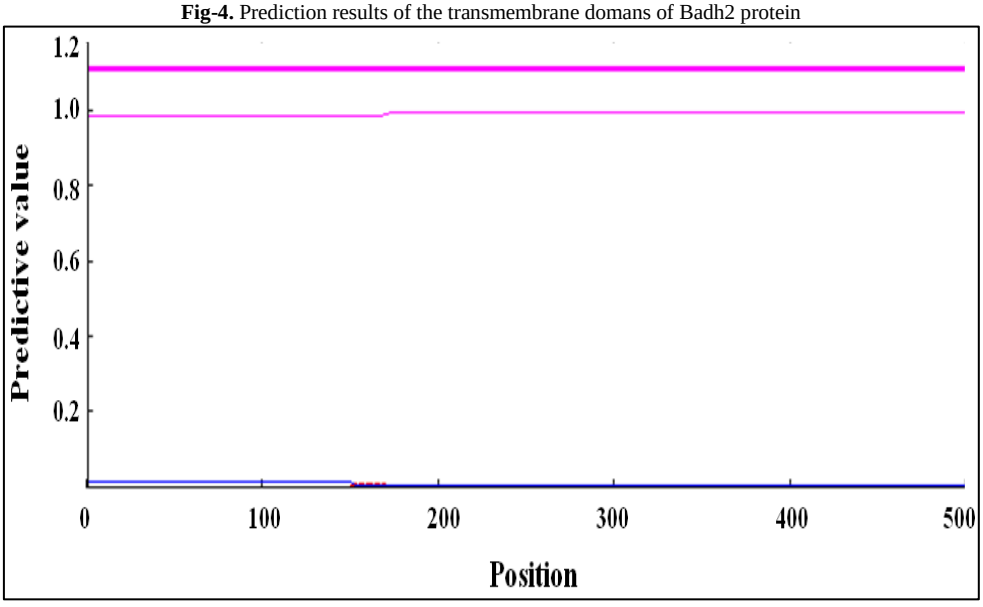
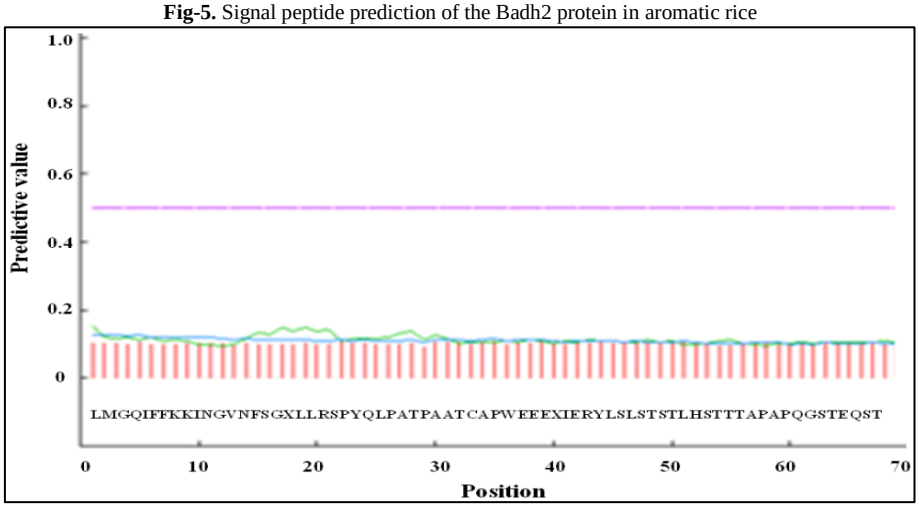


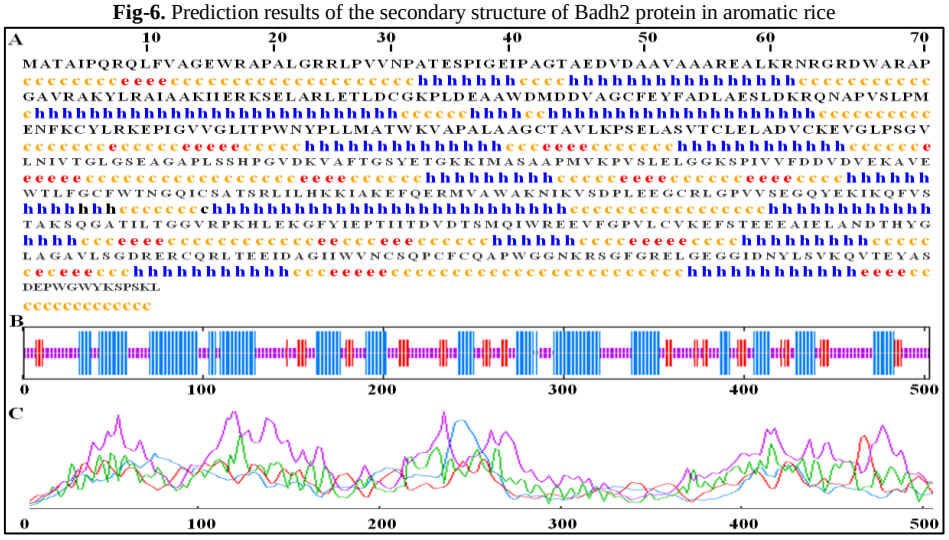
Fig-5. Signal peptide prediction of the Badh2 protein in aromatic rice



3.4. Structural Analysis of Badh2 Protein in Aromatic Rice

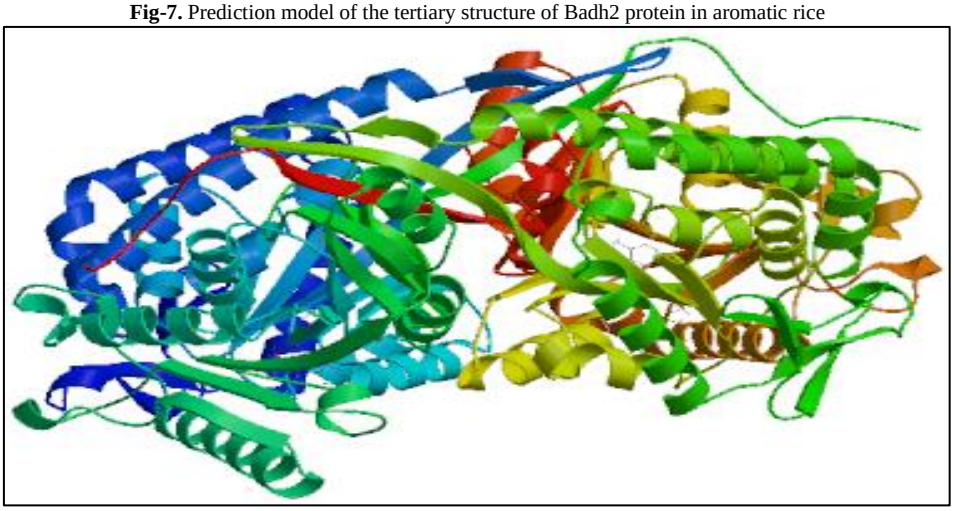
The secondary structure of Badh2 protein in rice was analyzed by online protein analysis website (https://npsa-prabi.ibcp.fr/cgi-bin). The results showed that there were 190 amino acids in Badh2 protein, accounting for 37.77% of the total sequence; 74 amino acid stretching fragments, accounting for 14.71% of the total sequence; 239 amino acid compositions were irregularly curled, accounting for 47.51% of the total sequence (Fig. 6). Using SWISS-MODEL software on-line (http:// swissmodel.expasy.org/repository), the tertiary structure of Badh2 protein was predicted and homologous modeling was carried out. The results showed that the random curl and α-helix structure occupied most of the (Fig 7), which was consistent with the secondary structure of Badh2 protein.

Fig-6. Prediction results of the secondary structure of Badh2 protein in aromatic rice



A. Sequence in secondary structure of protein; B. Histogram; C. Line are standard curve; h means alpha helix; e means extended strand; t means beta sheet; c means random coil in figure A. Blue means alpha helix; Green means beta sheet; Purple means random coil in figure B and C.

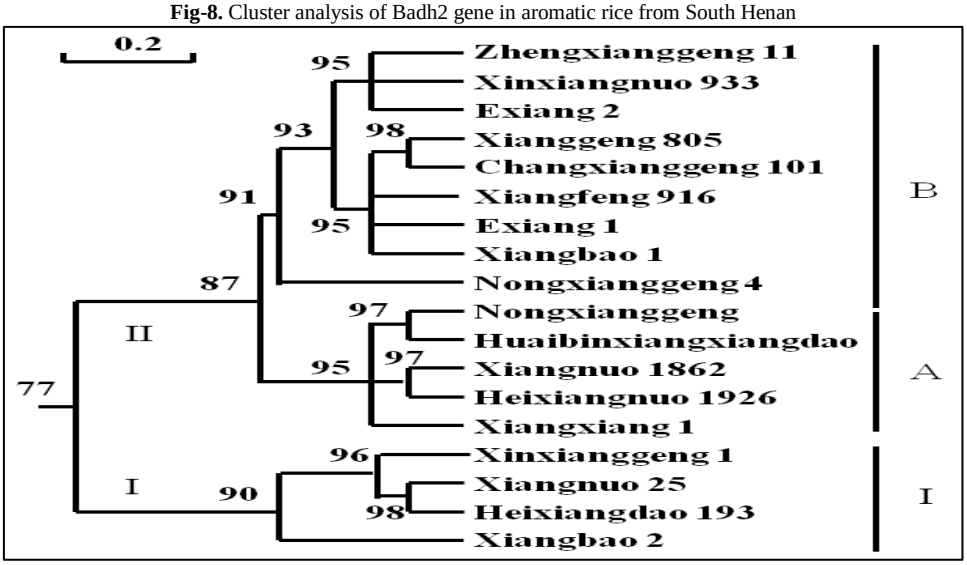
Fig-7. Prediction model of the tertiary structure of Badh2 protein in aromatic rice



3.5. Cluster Analysis of *Badh2* Gene in Southern Henan Aromatic Rice

According to the DNA sequence of *Badh2* gene of all the 18 aromatic rice germplasm resources in Southern Henan, the DNA sequence of *Badh2* gene of aromatic rice in Southern Henan was aligned by MEGA7 software, and the cluster map of *Badh2* gene of 18 aromatic rice germplasm resources in Southern Henan was drawn by MEGA7 software. The results showed as shown Fig 8: Xinxianggeng 1, Xiangnuo 25, Heixiangdao 193 and Xiangbao 2 were clustered in group I, while the remaining 14 kinds of aromatic rice were clustered in group II. In group II, Nongxianggeng, Huaibin Xiangxiang rice, Xiangnuo 1862, Hei Xiangnuo 1926 and Xiangxiang 1 gathered in group A, ZhengXianggeng, Xinxiangnuo 933, Exiang 2, Xianggeng 805, Changxianggeng 101, Xiangfeng 916, Exiang 1 and Xiangbao 1 gathered in group B, and Nongxianggeng 4 was different from group A or group B in *Badh2* gene sequence (Fig. 8).

Fig-8. Cluster analysis of Badh2 gene in aromatic rice from South Henan



3.6. Molecular Marker Detection and Analysis of Badh2 Gene in Aromatic Rice in South Henan

The genomic DNA of 18 aromatic rice varieties from South Henan was used as template. According to some of the variation loci, *Badh2* gene functional markers (Table 4) were used to amplify PCR and detected by non denaturing polyacrylamide gel electrophoresis. Functional molecular marker FME2 was used to detect the fragments. The results showed that seven aromatic rice varieties, including Xianggeng 805, Changxianggeng 101, Nongxianggeng, Xiangbao 1, Exiang 1, Nongxianggeng 4 and Xinxianggeng 1, could amplify 100 bp fragments, indicating that there was a 7 bp deficiency in exon 2 of *Badh2* gene in all seven aromatic rice varieties. Lost fragments belong to the same variation type of aroma gene. A 107 bp fragment was amplified from the other 11 fragrant rice varieties in Southern Henan, indicating that the 11 aromatic rice varieties in Southern Henan did not belong to the mutation type of exon 2 of *Badh2* gene (Table 5). *Badh2* gene functional marker FME4-5 was used for detection and analysis respectively. The results showed that Xianggeng 805 and Nongxianggeng 4 could amplify a fragment with the size of 321 bp. The remaining 16 aromatic rice varieties in Southern Henan province all amplified a fragment with the size of 1123 bp. It indicates that only Xianggeng 805 and Nongxianggeng 4 of the two aromatic rice materials in Southern Henan belong to exon 4~ 5 mutation types of *Badh2* gene. Using the *Badh2* gene functional marker FME7, it was found that Zhengxianggeng 11, Xinxiangnuo 933, Xiang feng 916, Exiang2, Huaibinxiangxiangdao, Xiangnuo 1862, Heixiangnuo 1926 and Xiangxiang 1 could amplify two fragments with sizes of 583 and 169 bp. The results indicated that there was an 8 bp deletion fragment and 3 bp mutation in the 7th exon of *Badh2* gene in these 5 aromatic rice materials, which belonged to the same mutation type of fragrant gene. *Badh2* gene functional marker FME13 was used to detect and analyze the fragments. The results showed that Xiangbao 2, Xiangnuo 25 and Heixiangdao 193 could amplify 195 bp fragments, while the other 15 varieties could only amplify 192 bp fragments. *Badh2* gene was detected by functional markers FMU1-2, FME4-5, FME1 2 and FME14 of 18 fragrant rice varieties in Southern Henan, respectively. No polymorphic loci were found (Table 5). Therefore, the *Badh2* gene of 18 aromatic rice varieties in Southern Henan may not be mutated at 5'-UTR region, exon 12 and exon 14.

Table-5. Detection results of functional markers of *Badh2* gene in 18 in aromatic rice materials

Alleles	Mutation type	Fragrant rice varieties
Badh2-UTR	3 bp deletion in 5'UTR region	–
Badh2-E2	7 bp deletion in exon 2	Xianggeng 805, Changxianggeng 101, Nongxianggeng, Xiangbao 1, Exiang 1, Nongxianggeng 4, Xinxianggeng 1
Badh2-E4-5	803 bp deletion of exon 4 and 5	Xianggeng 805, Nongxianggeng 4
Badh2-E7	8 bp deletion and 3 bp	Zhengxianggeng 11, Xinxiangnuo 933, Xiangfeng

	mutation in exon 7	916, Exiang 2, Huaibinxiangxiangdao, Xiangnuo 1862, Heixiangdao 1926, Xiangxiang 1
Badh2-E12	3 bp deletion of exon 12	–
Badh2-E13	3 bp insertion of exon 13	Xiangbao 2, Xiangnuo 25, Heixiangdao 193
Badh2-E14	1 bp insertion of exon 14	–

4. Discussion

4.1. Biological Function of *Badh2* Gene

Previous studies have shown that a mutation in the *Badh2* gene on chromosome 8 causes the rice to produce fragrance, while the Badh2 protein encoded by *Badh2* gene in non-aromatic rice has the activity of betaine dehydrogenase. The loss of betaine dehydrogenase activity in the mutant type and the accumulation of 2-AP lead to the production of rice flavor [11, 22, 24]. Through sequencing and analysis of rice fragrant genes, most researchers believe that the deletion of exon 2 or 7 of *Badh2* gene results in the accumulation of 2-AP [24] and aroma production. But the fact that some rice varieties have high levels of 2-AP but no *Badh2* alleles suggests that there may be other *Non-badh2* alleles that contribute to fragrance [26]. Most researchers only focus on the differences between exons of aromatic rice and non-aromatic rice, but for eukaryotes, introns play an important role in gene expression and regulation besides exons of genes [33, 34]. The results showed that the *Badh2* gene of 18 representative aromatic rice varieties in Southern Henan contained 15 exons and 14 introns (Fig. 2), encoding about 503 amino acids, and there were many types of mutations in the *Badh2* gene of 18 aromatic rice varieties in Southern Henan. This may be the main reason for the difference of aroma among different aromatic rice varieties in the aromatic rice germplasm resources of Southern Henan. Further analysis of *Badh2* gene sequence showed that aromatic rice varieties in Southern Henan could be divided into two groups. Xinxianggeng 1, Xiangnuo 25, Heixiangdao 193 and Xiangbao 2 were clustered in group I, while the other 14 aromatic rice varieties were clustered in group II. These results provide important information for the future development and utilization of *Badh2* gene in Southern Henan.

4.2. Regulation Mechanism of *Badh2* Gene

So far, although it has been confirmed that the loss of *Badh2* gene function can lead to the production of rice aroma substance 2-AP, how Badh2 protein regulates the biosynthesis of 2-AP remains unclear. Betaine aldehyde dehydrogenase plays an important role in the biosynthesis of 2-acetyl-1-pyrrooline. However, how betaine aldehyde dehydrogenase plays a role in the synthesis of 2-acetyl-1-pyrrooline and whether other substances are involved in the regulation are still unclear and need to be further studied. Meanwhile, gamma-aminobutyraldehyde, as the precursor of 2-acetyl-1-pyrrolidine synthesis, may also play a key regulatory role in the biosynthesis of 2-acetyl-1-pyrrolidine. The loss of Badh2 protein function in aromatic rice may lead to the accumulation of gamma-aminobutyraldehyde in the body, and then convert gamma- aminobutyraldehyde to 1-pyrrolidine, and eventually synthesize a large amount of 2-acetyl-1-pyrrolidine（2-AP [22]. In non-aromatic rice, Badh2 protein has the catalytic activity of betainal aldehyde dehydrogenase, which may convert gamma-aminobutyric acid from gamma-aminobutyric acid in rice, but inhibit the synthesis of 2-AP precursor, 1-pyrrolidine, and ultimately make it impossible to synthesize 2-AP. Therefore, the biosynthetic pathway of 2-AP and related regulatory networks and mechanisms are still unclear. In this study, the *Badh2* allele of xiangfeng 916, which is widely used in production, was taken as an example to conduct bioinformatics analysis of its *Badh2* allele. The results showed that Badh2 protein was a relatively hydrophilic protein with good stability, and signal peptide sequence and transmembrane structure were not found in Badh2 (Fig. 3~5). Further analyzing the senior Badh2 protein structure prediction, the result shows that in the Badh2 protein structure, random coil and alpha helix occupy a large (Fig 7). The structure of protein plays a decisive role in its function. This study conducted structural prediction analysis of Badh2 protein, providing important clues for in-depth analysis of 2-AP biosynthesis pathway and related regulatory networks and mechanisms.

4.3. Application of *Badh2* Gene in Molecular Breeding of Aromatic Rice

Since *Badh2* gene was isolated and cloned in *japonica* rice, multiple variation sites have been found in the gene [11, 18, 24, 35], and a series of molecular markers have been designed for the identification of aroma genes, the screening of different aromatic rice varieties and the cultivation of new varieties of aromatic rice. At present, there are at least 17 mutation loci in *Badh2* gene, which are distributed in the 5'-UTR region of *Badh2* gene, the 1st exon, the junction between the 1st exon and the 1st intron, the 2nd exon, between the 4th exon and the 5th exon, and the 7th, 8th, 10th, 12th, 13th and 14th exons [11]. For these mutation sites, multiple pairs of molecular markers have been designed at the junction of the 1st exon and the 1st intron of *Badh2* gene, the 2nd exon, the 4th exon and the 5th exon, and the 7th, 12th, 13th and 14th exons to identify the mutation type of *Badh2* gene [11, 12, 15, 24, 27, 28, 36]. On the basis of these molecular markers, a total of 7 functional markers of rice flavor genes were further developed (Table 4) and verified by using isolated populations [11, 24, 27, 36]. Among them, functional marker FMU1-2 can not only detect the 8 bp deletion mutation of *Badh2* gene in the 7th exon, but also detect whether there is an 8 bp deletion in the 5'-UTR region of *Badh2* gene. Functional markers FME2-7、FME7、FME12-3、FME13 and FME14 were used to identify the variation types of aroma genes in exons 2, 7, 12, 13 and 14 of *Badh2*, respectively. Functional markers FME14 belongs to CAPS (the cleaved amplified polymorphic sequence) tag, so the PCR product detection, need the help of restriction enzymes *Bsl*.

In this study, seven functional molecular markers were used to detect the mutation types of *Badh2* gene in southern Henan aromatic rice. The results showed that there were mutations in exon 2, exon 4-5, exon 7 and exon 13 of the *Badh2* gene in 18 Southern Henan aromatic rice varieties. Interestingly, there may be no variation in the 5'-UTR region, the 12th exon, and the 14th exon of the *Badh2* gene (Table 5). The results of these molecular markers were in good agreement with the results of *Badh2* gene cluster analysis in Southern Henan (Fig. 8). Therefore, the detection and analysis of functional molecular markers of these aroma genes provide an accurate, rapid and effective means for molecular breeding of aromatic rice.

5. Conclusion

According to the variation of *Badh2* gene in 18 aromatic rice varieties in Southern Henan, the tested varieties can be divided into three groups. Xinxianggeng 1, Xiangnuo 25, Heixiangdao 193 and Xiangbao 2 are clustered in group I, while the remaining 14 fragrant rice varieties are clustered in group II. Seven functional molecular markers of *Badh2* gene were used to detect different mutation types in exon 2, exon 4-5, exon 7 and exon 13 of *Badh2* gene. No aromatic rice varieties with different mutation types were found in other locations of *Badh2* gene. Therefore, the results of this study provide an important basis for using molecular marker-assisted selection to breed new high-quality aromatic rice varieties.

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References

[1] Peng, B., Kong, H. L., Li, Y. B., Wang, L. Q., Zhong, M., Sun, L., Gao, G. J., Zhang, Q. L., Luo, L. J., *et al.*, 2014a. "*OsAAP6* functions as an important regulator of grain protein content and nutritional quality in rice." *Nat Commun*, vol. 5, p. 4847.

[2] Tian, Z. X., Qian, Q., Liu, Q. Q., Yan, M. X., Liu, X. F., Yan, C. J., Liu, G. F., Gao, Z. Y., Tang, S. Z., *et al.*, 2009. "Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities." *Proc. Natl. Acad. Sci. USA.*, vol. 106, pp. 21760–21765.

[3] Kusano, M., Yang, Z. G., Okazaki, Y., Nakabayashi, R., Fukushima, A., and Saito, K., 2015. "Using metabolomic approaches to explore chemical diversity in rice." *Molecular Plant*, vol. 8, pp. 58–67.

[4] Peng, B., Wang, L. Q., Fan, C. C., Jiang, G. H., Luo, L. J., Li, Y. B., and He, Y. Q., 2014b. "Comparative mapping of chalkiness components in rice using five populations across two environments." *BMC Genet*, vol. 15, p. 49.

[5] Hickey, L. T., Hafeez, A. N., Robinson, H., Jackson, S. A., Leal-Bertioli, S. C. M., Tester, M., Gao, C. X., Godwin, I. D., Hayes, B. J., *et al.*, 2019. "Breeding crops to feed 10 billion." Available: https://doi.org/10.1038/s41587-019-0152-9

[6] Fitzgerald, M. A., Mccouch, S. R., and Hall, R. D., 2009. "Not just a grain of rice: the quest for quality." *Trends in Plant Science*, vol. 14, pp. 133–139.

[7] Guo, T., Liu, X. L., and Wan, X. Y., 2011. "Identification of a stable quantitative trait locus for percentage grains with white chalkiness in rice." *Journal of Integrative Plant Biology*, vol. 53, pp. 598–607.

[8] Takayuki, K. and Jun, M., 2018. "Identification and characteristics of quantitative trait locus for grain protein content, TGP12, in rice Oryza sativa L." *Euphytica*, vol. 214, p. 165.

[9] Daygon, V. D., Prakash, S., Calingacion, M., Riedel, A., Ovenden, B., Snell, P., Mitchell, J., and Fitzgerald, M., 2016. "Understanding the jasmine phenotype of rice through metabolite profiling and sensory evaluation." *Metabolomics*, vol. 12, p. 3.

[10] Myint, K. M., Arikit, S., and Wanchana, S., 2012. "A PCR-based marker for a locus conferring the aroma in Myanmar rice Oryza sativa L." *Theoretical and Applied Genetics*, vol. 125, pp. 887–896.

[11] He, Q. and Park, Y. J., 2015a. "Discovery of a novel fragrant allele and development of functional markers for fragrance in rice." *Molecular Breeding*, vol. 35, p. 217.

[12] Bradbury, L. M., Fitzgerald, T. L., and Henry, R. J., 2005a. "The gene for fragrance in rice." *Plant Biotechnology Journal*, vol. 3, pp. 363–370.

[13] Daygon, V. D., Prakash, S., and Calingacion, M., 2016. "Understanding the jasmine phenotype of rice through metabolite profiling and sensory evaluation." *Metabolomics*, vol. 12, pp. 1–15.

[14] Plows, J. F., Yu, X. Y., and Broadhurst, R., 2017. "Absence of a gestational diabetes phenotype in the LepRdb/+ mouse is independent of control strain, diet, misty allele, or parity." *Scientific Reports*, vol. 7, p. 45130.

[15] Bradbury, L. M., Henry, R. J., and Jin, Q., 2005b. "A perfect marker for fragrance genotyping in rice." *Molecular Breeding*, vol. 16, pp. 279–283.

[16] Mahattanatawee, K. and Rouseff, R. L., 2014. "Comparison of aroma active and sulfur volatiles in three fragrant rice cultivars using GC–Olfactometry and GC–PFPD." *Food Chemistry*, vol. 154, pp. 1–6.

[17] Mathure, S. V., Jawali, N., and Thengane, R. J., 2014. "Comparative quantitative analysis of headspace volatiles and their association with *BADH2* marker in non-basmati scented, basmati and non-scented rice Oryza sativa L. cultivars of India " *Food Chemistry*, vol. 142, pp. 383–391.

[18] Shao, G. N., Tang, S. Q., and Chen, M. L., 2013. "Haplotype variation at Badh2, the gene determining fragrance in rice." *Genomics*, vol. 101, pp. 157–162.

[19] Chen, S., Wu, J., and Yang, Y., 2006. "The fgr gene responsible for rice fragrance was restricted within 69 kb." *Plant Science*, vol. 171, pp. 505–514.

[20] Chen, S., Yang, Y., and Shi, W., 2008. "Badh2, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance." *Plant Cell*, vol. 20, pp. 1850–1861.

[21] Niu, X., Tang, W., and Huang, W., 2008. "RNAi-directed downregulation of *OsBADH2* results in aroma, 2-acetyl-1-pyrroline, production in rice Oryza sativa L." *BMC Plant Biology*, vol. 8, pp. 300–311.

[22] Shan, Q. W., Zhang, Y., and Chen, K. L., 2015. "Creation of fragrant rice by targeted knockout of the *OsBADH2* gene using TALEN technology." *Plant Biotechnology Journal*, vol. 13, pp. 791–800.

[23] Shi, Zhao, G. C., Xu, X. L., and Li, J. Y., 2014. "Discovery of a new fragrance allele and development of functional markers for identifying diverse fragrant genotypes in rice." *Molecular Breeding*, vol. 33, pp. 701–708.

[24] Michael, J. K., Mariafe, N. C., Melissa, A. F., and Susan, R. M., 2009. "The origin and evolution of fragrance in rice Oryza sativa L." *Proc. Natl. Acad. Sci . USA*, vol. 106, pp. 14444–14449.

[25] 2015. "Discovery and mapping of genomic regions governing economically important traits of Basmati rice." *BMC Plant Biology*, vol. 15, p. 207.

[26] Amarawathi, Y., Singh, R., Singh, A. K., Singh, V. P., Mohapatra, T., Sharma, T. R., and Singh, N. K., 2008. "Mapping of quantitative trait loci for basmati quality traits in rice Oryza sativa L." *Molecular Breeding*, vol. 21, pp. 49–65.

[27] Ootsuka, K., Takahashi, I., Tanaka, K., Itani, T., Tabuchi, H., Yoshihashi, T., Tonouchi, A., and Ishikawa, R., 2014. "Genetic polymorphisms in Japanese fragrant landraces and novel fragrant allele domesticated in northern Japan." *Breeding Sci*, vol. 64, pp. 115–124.

[28] Shao, G. N., Tang, A., and Tang, S. Q., 2011. "A new deletion mutation of fragrant gene and the development of three molecular markers for fragrance in rice." *Plant Breeding*, vol. 130, pp. 172–176.

[29] Luo, W., Guo, T., and Yang, Q., 2014. "Stacking of five favorable alleles for amylase content, fragrance and disease resistance into elite lines in rice Oryza sativa L. By using four HRM-based markers and a linked gel-based marker." *Molecular Breeding*, vol. 34, pp. 805–815.

[30] Peng, B., Sun, Y. F., Chen, B. Y., Sun, R. M., Kong, D. Y., Pang, R. H., Li, X. W., Song, X. H., Li, H. L., *et al.*, 2017. "Research progress of fragrance gene and its application in rice breeding." *Chin Bull Bot*, vol. 52, pp. 797–807.

[31] Jiang, G. H., He, Y. Q., and Xu, C. G., 2004. "The genetic basis of stay-green in rice analyzed in a population of doubled haploid lines from an indica by japonica cross." *Theoretical and Applied Genetics*, vol. 108, pp. 688–698.

[32] Xu, X. L., Zhao, G. C., and Li, J. Y., 2011. "Development of molecular markers used to identify two types of fragrant rice and analysis of mutation sites of BADH2 gene in 24 varieties of fragrant rice." *Plant Diversity and Resources*, vol. 33, pp. 667–673.

[33] Fu, H., Kim, S. Y., and Park, W. D., 1995. "High-level tubule expressionand sucrose inducibility of a potato Sus4 sucrose synthasegene require 5' and 3' flanking sequences and the leader intron." *Plant Cell*, vol. 7, pp. 1387–1394.

[34] Lee, B. H., Won, S. H., Lee, H. S., Miyao, M., Chung, W. I., Kim, I. J., and Jo, J., 2000. "Expression of the chloroplast-located small heat shock protein by oxidative stress in rice." *Gene*, vol. 245, pp. 283-290.

[35] He, Q., Yu, J., Kim, T. S., Cho, Y. H., Lee, Y. S., and Park, Y. J., 2015b. "Resequencing reveals different domestication rate for *BADH1* and *BADH2* in rice Oryza sativa L." *PLoS One*, vol. 10, p. e0134801.

[36] Shi, Yang, Y., Chen, S. H., and Xu, M. L., 2008. "Discovery of a new fragrance allele and the development of functional markers for the breeding of fragrant rice varieties." *Molecular Breeding*, vol. 22, pp. 185–192.