Tibet Identification of the Fragrance Allele and Development of the Functional Markers for Fragrance in Taiguoxiaoxiangzhan

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Abstract The fragrant rice breeding has become an important direction for hybrid rice breeding program. The selection and utilization of parent materials with ideal plant type, fine grain quality, strong resistance and other excellent agronomic characteristics with strong fragrance, especially the utilization and cultivation of the fragrant rice restorer lines, is of great significance to further promote the fragrant hybrid rice breeding. Taiguoxiaoxiangzhan is a restorer line with excellent grain quality, strong resistance and fragrance, which has been widely used in fragrant rice breeding in China. In order to trace the fragrance in Taiguoxiaoxiangzhan, we identified the type of the fragrance gene in Taiguoxiaoxiangzhan was badh2/E7 which has an 8 bp deletion and three single nucleotide polymorphisms (SNPs) in exon 7, and this gene is a non-functional badh allele and that the functional Badh allele encoding betaine aldehyde dehydrogenase (BADH2) could render rice non-fragrant. Furthermore, we found that the Taiguoxiaoxiangzhan increased 2-acetyl-1-pyrroline (2-AP) content (0.108 mg/kg) compared with the non-fragrant rice varieties of Nipponbare and 9311 (<0.01 mg/kg) by the GC-MS method. We also proved that FMbadh2-E7 and InDel-E7, two molecular markers reported earlier, can be applied in different genotypes, including Badh2/Badh2 (non-fragrance homozygote), Badh2/badh2 (non-fragrance heterozygote) and badh2/badh2 (fragrance homozygote). In addition, based on sequence divergence amongst the functional Badh2 and null badh2/E7 allele, we developed two functional markers, FMbadh2-E7A and FMbadh2-E7B, which can be easily used to distinguish non-fragrant from fragrant rice. Genetic analysis shows that the segregation of Badh2/Badh2, Badh2/badh2 and badh2/badh2 in the F2 population derived from crosses between Taiguoxiaoxiangzhan and different restorer lines fitted a theoretical ratio of 1:2:1, indicating that the fragrance of Taiguoxiaoxiangzhan is controlled by a single nuclear recessive gene (badh2). In this study, the identification and genetic analysis of the fragrance gene in Taiguoxiaoxiangzhan and the development of functional markers for fragrance have been studied, which provides a theoretical foundation for the further breeding of fragrant hybrid rice with excellent quality, high yield and strong resistance by molecular marker-assisted selection technology.

Keywords Rice; Fragrance gene; Badh2; 2-AP; Molecular marker

Rice is one of the most important food crops in the world. More than 50% of the world’s population take rice as their main food source to provide the energy needed by the human body. And China is a big country in rice cultivation, production and consumption. In recent years, with the steady improvement of people’s living standards, the eating quality of rice has become an important indicator for consumers and breeders to consider. Among them, fragrance is an extremely important indicator to be evaluated (Yan et al., 2015; Zhang et al., 2015; Peng et al., 2017; Shao et al., 2017). As a special type of cultivated rice, fragrant rice is regarded as a treasure due to its unique fragrance, excellent nutrition, various amino acids and proteins, and rich trace elements needed by the human body, which is favored by the majority of consumers in domestic and foreign markets (Bradbury et al., 2008; Peng et al., 2017). However, traditional fragrant rice varieties have poor adaptability. They are extremely vulnerable to various environmental factors, so it is difficult to promote and plant. Besides, they also have many other shortcomings such as poor affinity and low yield, which limits their direct utilization in hybrid rice breeding (Wang et al., 2016; Peng et al., 2017). Therefore, the selection, identification and utilization of different fragrant rice resources and the research of fragrance genes have attracted more and more attention from botanists and rice genetic breeders at home and abroad, especially the identification and cloning of fragrance genes in some ideal...
plant type and the development of functional molecular markers, which can provide important theoretical foundation and technical support for the selection and utilization of new fragrant hybrid rice varieties, thus accelerating the breeding process of fragrant rice (Wang et al., 2008; Wang et al., 2016; Peng et al., 2017).

A large number of studies have shown that 2-acetyl-1-pyrroline (2-AP), which has a strong popcorn fragrance, is the characteristic compound and the main fragrance contributor to the fragrance of fragrant rice (Bradbury et al., 2008; He and Park, 2015). At present, a variety of methods have been established to detect the fragrance of fragrant rice materials, including the most common methods in traditional breeding process, such as chewing method, coloration method and KOH soaking method (Yan et al., 2015; Peng et al., 2017). However, these methods mainly rely on human senses to identify and evaluate the fragrance, so it is difficult to ensure the accuracy and reliability of the results, and it is impossible to carry out quantitative analysis. Using the gas chromatography-mass spectrometry (GC-MS) to detect the fragrance substance 2-AP content in fragrant rice can significantly improve the accuracy and reliability of the results, which consumes fewer samples and reagents and is simple to operate. GC-MS is also suitable for the detection of fragrance substances in large quantities of rice materials (Ying et al., 2010).

The biosynthetic pathway, genetic basis and the whole metabolic regulation mechanism of the fragrance substance 2-AP in rice are very complicated. The previous studies suggested that the fragrance might be controlled by a dominant or recessive gene in the nucleus, or it might be influenced by multiple dominant or recessive QTLs (Wang et al., 2016; Wang et al., 2017). However, a large number of recent studies have shown that the fragrance characteristics of rice are closely related to one recessive gene located on chromosome 8, which encodes a betaine dehydrogenase BADH2 (betaine aldehydedehydrogenase homologue 2, BADH2). The gene is 1509 bp in length, which contains 15 exons and 14 introns, and encodes 503 amino acids (Shan et al., 2015; Wang et al., 2016; Peng et al., 2017). At present, the gene has been successfully isolated and cloned (Bradbury et al., 2008; Shao et al., 2011; Shi et al., 2014), the mutation or knockout of which can lead to the loss of function of BADH2 protein, resulting in the accumulation of the fragrance characteristic compound 2-AP in fragrant rice and causing the rice leaves and grains to produce a strong fragrance in turn (Shan et al., 2013; Shao et al., 2017; Peng et al., 2017). At present, multiple mutation types of Badh2 gene have been discovered and identified in different fragrant rice materials through genome sequencing and comparative analysis. And corresponding specific functional molecular markers have also been developed and used in allele identification and molecular marker-assisted selection breeding of rice (Wang et al., 2008; Shao et al., 2013). The most common of these are the 8 bp deletion and 3 single nucleotide site mutations (badh2-E7) in exon 7 of the Badh2 gene (Bradbury et al., 2008), and the 7 bp deletion site mutation (badh2-E2) in exon 2 of the Badh2 gene (Shi et al., 2008). Further research found that there is a 803 bp deletion mutation (badh2-E4-5) between exon 4 and exon 5 of the Badh2 gene in different fragrant rice varieties (Shao et al., 2011), and Badh2 gene has 8 bp insertion and 3 bp deletion mutation (badh2-p-5'UTR) in the promoter region and 5'UTR region, respectively (Shi et al., 2014). Therefore, mutations in the coding region or regulatory region of the Badh2 gene can lead to the loss of function of the gene, which makes the rice produce strong fragrance.

Taiguxiaoxiangzhan, introduced by Sanming Academy of Agricultural Science, is an ideal restorer line with good restorability, excellent grain quality, strong resistance and fragrance, which has shown great potential in the breeding and utilization of fragrant hybrid rice in rice growing areas of China, especially in South China, and has important application value. In this study, the fragrance gene in Taiguxiaoxiangzhan was identified and the mutation sites were analyzed by genomic amplification, sequencing and comparative analysis. And the content of 2-AP was detected by GC-MS, so as to explore the mutation type of fragrance gene and the molecular mechanism of fragrance formation. At the same time, two pairs of specific functional markers were designed to detect the mutation type of fragrance gene, and the molecular markers studied and developed by previous researchers were analyzed and verified, which provides an important theoretical foundation for the further breeding of new fragrant hybrid rice varieties by molecular marker-assisted selection technology and by using Taiguxiaoxiangzhan and its corresponding mutation type fragrance gene.
1 Results and Analysis
1.1 Identification and mutation site analysis of fragrance gene in Taiguoxiaoxiangzhan
The complete functional Badh2 gene contains 15 exons and 14 introns, with a total length of 1509 bp (Figure 1A). In order to identify the fragrance gene and its mutation type in Taiguoxiaoxiangzhan, the complete genome sequence of its allele was performed PCR amplification, sequencing and splicing analysis (Table 1) by using 10 specific primer pairs of badh2/badh2 gene (Shi et al., 2008), and the sequence of the fragrance gene Badh2 among Taiguoxiaoxiangzhan, the non-fragrant rice varieties of Nipponbare and 9311 was carried out by DNAMAN and Clustal Omega. The results showed that the type of the fragrance gene in Taiguoxiaoxiangzhan was badh2-E7 which has an 8 bp deletion and three single nucleotide polymorphisms (SNPs) in exon 7 (Figure 1B). It was also found that, compared with Nipponbare and 9311, badh2 in Taiguoxiaoxiangzhan has a single base mutation at +898 in intron 2 (T-C), and two base deletion mutations at +838 in intron 2 (Figure 1C). Since this mutation occurs in the intron region, it does not affect the normal coding of the gene and the function of the corresponding protein. And PCR amplification analysis of Taiguoxiaoxiangzhan was carried out through the specific functional marker FMbadh2-Pro. It was found that there is also an 8 bp insertion mutation in the promoter region, which was consistent with the results of previous studies (Shi et al., 2014). At the same time, 8 bp insertion was found in the promoter region of 8 non-fragrant restorer lines (Figure 2a). It is speculated that the insertion mutation of this site does not directly affect the normal coding of Badh2 gene and the function of corresponding protein.

1.2 Specific functional markers of Badh2 gene and identification of genotypes in different materials
Through PCR amplification and sequencing analysis, we found that the type of the fragrance gene in Taiguoxiaoxiangzhan was badh2-E7. Therefore, this study tested and verified the efficiency and accuracy of the functional molecular markers FMbadh2-E7 (Shi et al., 2008) and InDel-E7 (Wang et al., 2008) for the genotypes of different materials, including fragrance homozygotes (badh2/badh2), non-fragrance heterozygotes (Badh2/badh2) and non-fragrance homozygotes (Badh2/Badh2). It was found that these markers could accurately and efficiently identify the genotypes of different materials. At the same time, these specific molecular markers were used to further identify the fragrance genotypes of Taiguoxiaoxiangzhan and 8 non-fragrant restorer lines R1-R8 (Minghui 2155, Minghui 86, Minghui 3009, Fuhui 673, Minhui 3301, Jianghui 151, R527, R498) and their F1 hybrids. It was found that the fragrance genotypes of these restorers were non-fragrance homozygotes (Badh2/Badh2), and the F1 hybrids were non-fragrance heterozygotes (Badh2/badh2) (Figure 2b; Figure 2c). In this study, based on the mutation of exon 7 site of fragrance gene Badh2, two pairs of specific functional molecular markers FMbadh2-E7A and FMbadh2-E7B were independently developed and designed to identify the mutation genotypes of badh2-E7 allele. These two pairs of markers can be used to identify the homozygous and heterozygous fragrance genotypes quickly, simply and accurately (Figure 2d; Figure 2e), which can be well applied to molecular marker-assisted selection in fragrant hybrid rice breeding and screening and identification of fragrance genes.

1.3 Determination of 2-acetyl-1-pyrroline (2-AP) content
In order to further analyze whether the mutation of the fragrance gene Badh2 is the cause of the strong fragrance of Taiguoxiaoxiangzhan, GC-MS was used in this study to analyze Nipponbare, 9311 and Taiguoxiaoxiangzhan, and the content of the fragrance characteristic compound 2-AP in the grains was detected and analyzed. The minimum detection limit was 0.01 mg/kg, and 2,4,6-trimethylpyridine (TMP) was used as the internal standard. During the detection process, 2-AP and TMP could be effectively separated in total ion chromatography of Taiguoxiaoxiangzhan (Figure 3). In the figure, peak 1 is 2-AP, and peak 2 is TMP. The results showed that the content of 2-AP in Taiguoxiaoxiangzhan was 0.108 mg/kg, while 2-AP was not detected in Nipponbare and 9311, indicating that the content was less than 0.01 mg/kg, which was lower than the detection limit. Therefore, the content of 2-AP in Taiguoxiaoxiangzhan was significantly higher than that in Nipponbare and 9311. The results showed that the fragrance of Taiguoxiaoxiangzhan mainly came from the loss of function mutation of badh2 gene and the increase and accumulation of 2-AP content.
Figure 1 Sequence alignment of the fragrance gene Badh2/badh2 among the Taiguoxiaoxiangzhan, Nipponbare and 9311

Note: A: Structure of the Badh2/badh2 locus. The functional Badh2 gene consists of 15 exons (black boxes) and 14 introns (grey boxes) with its start codon (ATG) in exon 1 and stop codon (TAA) in exon 15; B: The badh2 allele of Taiguoxiaoxiangzhan has an 8bp deletion and 3 single nucleotide polymorphisms (SNPs) in exon 7 compared with the no-fragrance rice varieties of Nipponbare and 9311; C: The badh2 allele of Taiguoxiaoxiangzhan has a 2bp deletion and one single nucleotide polymorphism (SNP) in intron 2 compared with the no-fragrance rice varieties of Nipponbare and 9311

Table 1 A list of ten primer pairs for sequencing the complete Badh2/badh2 alleles and five functional markers for genotyping

| Primer   | PCR product size (bp) | Forward primer (5’-3’)           | Reverse primer (5’-3’)           |
|----------|-----------------------|----------------------------------|----------------------------------|
| Badh2P1  | 995                   | GTCTCTCTCCAAATGCTC               | GTGGTGTCAGGTGAGGTGAGTG           |
| Badh2P2  | 972                   | CCGAAGTCGTCACCATCAGTC            | CAATCAAGCCATCGCTCACA             |
| Badh2P3  | 855                   | GTTGCTACCTCTGTGTCGG              | CAAAGCTCTACATGCTCAGCC            |
| Badh2P4  | 1025                  | CTGAGCTGGCTAAGCTAGATG             | CAATCAAGGAGAGATAGTCTC            |
| Badh2P5  | 1026                  | CCTCGGTATTGTCATGCAGTC             | CATAGCAAGTGGCAATGTC              |
| Badh2P6  | 926                   | GGGGCTGCTCTCACAGGTG               | GTCTCTCCATCAGCTCCTA              |
| Badh2P7  | 984                   | CCAAGAGGAGGAGGTCAAG              | CATGTCAGGAGAGAGAGGAAG           |
| Badh2P8  | 912                   | GGTTGTGTGCTCTCTCCAGGG             | GCAATAAGGCTGTGGAAGAGG           |
| Badh2P9  | 1023                  | TTTGAGCTCAGCTTGAAGAT             | CAGTTAATCAGCAGCAAGG             |
| Badh2P10 | 1083                  | GGCAAGCTAGATCAGTGAAG             | CCGGTCACTAAGCTTCACTCC            |
| InDel-E7 | 196 (badh2-E7)        | ATACCCCATCAATCGAAT               | GAAAAGCAACATGGAGAA              |
| 204 (Badh2) |                   |                                  |                                  |
| FMbadh2-E7 | 260 (badh2-E7)      | GGTGCGATTTACTGGAGTT              | CAGTTGAACAGGCTGTCAAG            |
| 268 (Badh2) |                   |                                  |                                  |
| FMbadh2-Pro | 206 (badh2-E7)     | ATATATCAGGCAACATTTTTATT         | GAGAACCTTTTACACTTTATGCACATC     |
| 198 (Badh2) |                   |                                  |                                  |
| FMbadh2-E7A | 145 (badh2-E7)     | ACAAGGTACAGCTATCTCCTCT          | TAACCATAAGGACAGCTGAA            |
| 153 (Badh2) |                   |                                  |                                  |
| FMbadh2-E7B | 130 (badh2-E7)     | GGTTTATGTTTCTGGAGGTTTCA         | AGAGAGTTATGAGAAAAAGAACAAAA      |
| 138 (Badh2) |                   |                                  |                                  |

Note: Badh2P1-P10, FMbadh2-E7 taken from Shi et al. (2008); InDel-E7 from Wang et al. (2008); FMbadh2-Pro from Shi et al. (2014); FMbadh2-E7A and FMbadh2-E7B are newly designed markers in this study
Figure 2 Identification of the genotypes of different rice varieties and the F1 progenies with the functional markers FMbadh2-Pro (a), InDel-E7 (b), FMbadh2-E7 (c), FMbadh2-E7A (d) and FMbadh2-E7B (e)

Note: M: 100bp ladder Marker; a: 1~2: Taiguoxiaoxiangzhan; 3~4: Nipponbare; 5~6: Minghui2155; 7~8: Minghui86; 9~10: Minghui3009; 11~12: Fuhui673; 13~14: Minhu3301; 15~16: Jianghu151; 17~18: R527; 19~20: R498; b~e: 1~2: Taiguoxiaoxiangzhan; 3~12: Nipponbare, 9311, Minghui2155, Minghui86, Minghui3009, Fuhui673, Minhu3301, Jianghu151, R527, R498; 13~20: Taiguoxiaoxiangzhan/Minghui2155, Taiguoxiaoxiangzhan/Minghui86, Taiguoxiaoxiangzhan/Minghui3009, Taiguoxiaoxiangzhan/Fuhui673, Taiguoxiaoxiangzhan/Minhu3301, Taiguoxiaoxiangzhan/Jianghu151, Taiguoxiaoxiangzhan/R527, Taiguoxiaoxiangzhan/R498

Figure 3 Total ion chromatograms (TIC) of 2-AP and TMP (as internal standard) in Taiguoxiaoxiangzhan

Note: peak 1: 2-acetyl-1-pyrroline (2-AP); peak 2: 2,4,6-trimethyl-pyridine (TMP)
1.4 Genetic analysis of the fragrance gene badh2 in Taiguoxiaoxiangzhan

Taiguoxiaoxiangzhan has the advantages of ideal plant type, fine grain quality and strong fragrance, and it has important application value in rice hybrid breeding, especially for the selection of fragrant hybrid rice. Therefore, it is of great significance to study the genetic characteristics of its fragrance gene. In this study, the parents of Taiguoxiaoxiangzhan were hybridized with 8 different excellent restorer lines R1-R8 (Minghui 2155, Minghui 86, Minghui 3009, Fuhui 673, Minhui 3301, Jianghui 151, R527, R498) to obtain F1 generation, and then obtained eight F2 generations from F1 selfing for genetic analysis of the fragrance gene. At the same time, the genotypes identification and genetic analysis of F1 and F2 were carried out by using the specific molecular markers InDel-E7, FMbadh2-E7 and independently developed and designed FMbadh2-E7A, FMbadh2-E7B. It was found that the genotypes of F1 generation are all non-fragrance heterozygotes (Badh2/badh2), while the segregation ratios of three genotypes of F2 population, non-fragrance homozygotes (Badh2/Badh2), non-fragrance heterozygotes (Badh2/badh2) and fragrance homozygotes (badh2/badh2), is 1:2:1 (Table 2), which were coincidence with the law of independent assortment, indicating that the fragrance of Taiguoxiaoxiangzhan is mainly controlled by the badh2 gene. In this study, the inheritance of fragrance gene in Taiguoxiaoxiangzhan was analyzed, which provided an important theoretical foundation for the utilization of fragrance gene in further fragrant hybrid rice breeding.

| Hybrid combinations          | Non-fragrance homozygotes (Badh2/Badh2) | Non-fragrance heterozygotes (Badh2/badh2) | Fragrance homozygotes (badh2/badh2) | p value | Functional marker |
|-----------------------------|----------------------------------------|----------------------------------------|-----------------------------|---------|------------------|
| Taiguoxiaoxiangzhan/R1      | 26                                     | 55                                     | 28                          | 0.8756  | InDel-E7         |
| Taiguoxiaoxiangzhan/R2      | 32                                     | 70                                     | 38                          | 0.8879  | InDel-E7         |
| Taiguoxiaoxiangzhan/R3      | 51                                     | 90                                     | 43                          | 0.8407  | FMbadh2-E7      |
| Taiguoxiaoxiangzhan/R4      | 49                                     | 113                                    | 58                          | 0.7880  | FMbadh2-E7      |
| Taiguoxiaoxiangzhan/R5      | 38                                     | 85                                     | 42                          | 0.7888  | FMbadh2-E7A    |
| Taiguoxiaoxiangzhan/R6      | 62                                     | 112                                    | 53                          | 0.7467  | FMbadh2-E7A    |
| Taiguoxiaoxiangzhan/R7      | 41                                     | 87                                     | 49                          | 0.9178  | FMbadh2-E7B    |
| Taiguoxiaoxiangzhan/R8      | 55                                     | 101                                    | 42                          | 0.8253  | FMbadh2-E7B    |

Note: R1: Minghui2155; R2: Minghui86; R3: Minghui3009; R4: Fuhui673; R5: Minhui3301; R6: Jianghui151; R7: R527; R8: R498; p value > 0.05, No significant difference, which indicated that the segregation ratios were coincidence with the law of independent assortment.

2 Discussion

Fragrance, favored by the majority of consumers, is an important indicator affecting the quality of rice. Therefore, fragrant hybrid rice breeding is widely concerned and valued by rice genetic breeders and botanists at home and abroad. Fragrant hybrid rice breeding has become an important direction of hybrid rice genetic breeding and will have a broad market (Wang et al., 2016). There are abundant fragrant rice germplasm resources in the world, with a long history of cultivation and a wide range of planting. A large number of collection, evaluation and identification of different types of fragrant rice germplasm resources have been carried out at home and abroad. It was found that wild rice was the main source and donor of fragrance genes, which distributed in different countries and different rice types and existed obvious differences. Wild rice is now commonly found in different cultivated rice, among which Basmati series from India and Pakistan, KDML105 from Thailand, Gongxiang from Japan, Pangxiugu from Yunnan, Xianghe from Guizhou, and Jingxiangnuo from Guangxi are all well-known fragrant rice varieties that provide rich germplasm resources for fragrant rice breeding (Wang et al., 2016; Shao et al., 2017). However, in the production process, these traditional rice resources are generally vulnerable to regional influences, and have many shortcomings such as long growth period, poor affinity and low yield, which limit their direct utilization in hybrid rice breeding and actual production promotion. Therefore, it is of great significance to screen ideal and fragrant rice parents, especially the selection and utilization of fragrant rice restorer lines, and to identify their fragrance genes and develop functional markers for improving rice quality and accelerating the breeding process of fragrant hybrid rice (Wang et al., 2016). Taiguoxiaoxiangzhan, introduced by Sanming Academy of Agricultural Science, is an ideal restorer line with good restorability, excellent grain quality, strong resistance and fragrance. We have commissioned the Rice and Products Quality Supervision and Inspection...
Center of the Ministry of Agriculture and Rural Affairs in China to test its rice quality indicators. The results showed that its chalkiness, transparency, gel consistency, amylose content and alkali digestion value have reached the first-class rice quality standard. At the same time, we used the cross combination of Taiguoxiaoxiangzhan and several excellent restorer lines, through multi generation selfing selection, resistance screening and trial production with a large number of three-line sterile lines and two-line sterile lines, finally, we have selected a number of high generation fragrant lines with good comprehensive agronomic traits and stable resistance. At present, Taiguoxiaoxiangzhan has shown great potential in the breeding and utilization of fragrant hybrid rice in rice growing areas of China, especially in South China, and has important application value.

Previous studies have shown that the fragrance of rice is mainly controlled by a single recessive gene located on chromosome 8, which encodes a betaine aldehydedehydrogenase homologue 2 (BADH2), and the mutation or knockout of this gene can lead to the loss of function of BADH2 protein, resulting in the accumulation of the fragrance characteristic compound 2-AP in fragrant rice and causing the rice leaves and grains to produce a strong fragrance in turn (Shan et al., 2015; Shao et al., 2017; Peng et al., 2017). At present, a number of allelic mutation types have been identified at this site, of which the most common are badh2-E7, badh2-E2, badh2-E4-5 and badh2-p-5'UTR (Bradbury et al., 2008; Shi et al., 2008; Shao et al., 2011; Shi et al., 2014). It has also been reported that in different local fragrant rice varieties, there are single nucleotide polymorphism (SNP) mutations at exon 13 or at the junction of exon 1 and intron 1, and there are three base deletion mutations at exon 12 of Badh2 gene (Ootsuka et al., 2014; He and Park, 2015). In this study, through the amplification and sequencing analysis, it was found that the type of the fragrance gene in Taiguoxiaoxiangzhan was badh2-E7 which has an 8 bp deletion and three single nucleotide mutations in exon 7. And through further sequence alignment and specific functional marker identification analysis, we found that there are multiple base deletions or base substitution mutations in the introns. In addition, a single nucleotide mutation (T-C) and an 8-bp insertion mutation occurred at the +898 position of intron 2 and at the -1314 position of the upstream promoter of the gene, respectively. The former positional mutation is a single base substitution mutation that exists in all three alleles (badh2-E7, badh2-E2, badh2-p-5'UTR), and the latter mutation just exists in badh2-E7 and badh2-p-5'UTR. The research results were consistent with previous reports (Shi et al. al., 2014). At the same time, 8 bp insertion was also found in the promoter region of 8 non-fragrant restorer lines. Therefore, we speculated that the insertion mutation of this site does not directly affect the function of Badh2. The loss of function of badh2-p-5'UTR allele may be related to the deletion of 5'UTR base or other site mutations.

For a long time, the identification of fragrant rice is mainly carried out by traditional chewing method, coloration method and KOH soaking method. However, these traditional methods are time-consuming and laborious, and are easily affected by weather and human senses, so it is difficult to ensure the accuracy and reliability of the results, and it is impossible to carry out quantitative analysis (Ying et al., 2010; Wang et al., 2016; Shao et al., 2017). In this study, GC-MS was used to identify and analyze fragrance substances in fragrant rice grains. This method can significantly improve the efficiency and accuracy of detection with many advantages, such as high sensitivity, simple operation, low consumption of samples and reagents. GC-MS is also suitable for the detection of the content of the fragrance substance 2-AP in large quantities of rice materials, providing important technical support for the transition of identification and evaluation of the fragrance from ‘verbal description’ to ‘numerical quantitative’ (Ying et al., 2010).

In addition, because fragrance genes are recessive genes, they can only show fragrance in homozygous state, and be likely to cause the loss of fragrance genes in heterozygous state. A large number of studies have shown that the development of functional molecular markers based on the internal structure sequence of fragrance genes has become an efficient, accurate and important auxiliary means for identification of fragrance genes and fragrant rice breeding. Using these markers for selection breeding can not only identify at the early breeding stage, such as seedling stage, but also distinguish homozygous and heterozygous genotypes, so as to speed up the efficiency of fragrant rice breeding (Wang et al., 2008; Zhang et al., 2015).
In this study, two pairs of specific functional molecular markers FMbadh2-E7A and FMbadh2-E7B were independently developed and designed based on the mutation of fragrance gene badh2-E7 at exon 7 site, which can identify homozygous and heterozygous fragrance genotypes quickly, simply and accurately. Compared with the specific molecular markers YY5-YY8, InDel-E7 and FMbadh2-E7 (Wang et al., 2008; Shi et al., 2008; Yan et al., 2015), The amplified fragments of these two specific functional markers for fragrant and non-fragrant rice varieties were about 150 bp, and the bands are 145 bp / 153 bp (badh2-E7 / Badh2, FMbadh2-E7A) and 130 bp / 138 bp (badh2-E7 / Badh2, FMbadh2-E7B). During electrophoresis, the migration of bands was fast and the difference was obvious. They can more clearly identify the size and difference of amplified fragments between fragrance and non-fragrance, which can be well applied to the breeding of fragrant rice varieties and the selection of fragrant rice resources.

3 Materials and Methods

3.1 Test materials

The materials of this study included one fragrant rice variety, Taiguxiaoxiangzhan, and ten non-fragrant rice varieties, Minghui 2155, Minghui 86, Minghui 3009, Fuhui 673, Minhui 3301, Jianghui 151, R527, R498, Nipponbare and 9311. Among which, the first 8 varieties were restorer lines with excellent agronomic traits. The materials also included F1 and F2 generations, which were derived from crosses between Taiguxiaoxiangzhan and different restorer lines. Among them, Taiguxiaoxiangzhan was introduced by us and 8 excellent restorer lines were selected by us and provided by Fujian Academy of Agricultural Sciences and Sichuan Agricultural University.

3.2 Specific functional markers

The four specific functional markers InDel-E7, FMbadh2-E7, FMbadh2-E7A, and FMbadh2-E7B used in this study are respectively designed for the 8 bp deletion and three single nucleotide polymorphisms (SNPs) in exon 7 of the fragrance gene Badh2. Among which, InDel-E7 and FMbadh2-E7 were developed by Wang et al. (2008) and Shi et al. (2008), while FMbadh2-E7A and FMbadh2-E7B were independently designed and developed by us. The marker sequences and amplified fragments (Table 1) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. These markers could be used to identify the fragrance gene Badh2.

3.3 DNA extraction and PCR amplification

In this study, an improved CTAB method was used to extract whole genome DNA from rice leaves (Wei et al., 2016; Wei et al., 2019). The reaction system was 10 μL, containing 5 μL 2× Taq PCR MasterMix, 1.0 μL (10 μmol/L) equal volume mixture of forward primer and reverse primer, 0.8 μL (50 ng/μL) DNA template and 3.2 μL ddH2O. The reaction procedure was as follows: (1) pre-denaturation at 94°C for 5 min; (2) denaturation at 94°C for 1 min, annealing at 55°C~60°C for 30 s, renaturation at 72°C for 1 min, and cycle for 35 times; (3) extension at 72°C for 7 min and storing at 4°C.

3.4 Electrophoresis detection

The PCR amplification products of specific functional markers were separated and purified by 8% non-denaturing polyacrylamide gel electrophoresis for 60~120 min with a voltage of 120 v. Nucleic acid dye Ultra GelRed and gel imaging system were used to dye and photographing observe. The PCR amplified products used for sequencing of Badh2 gene were separated by 1% agarose gel electrophoresis. The gel electrophoresis was recovered and purified by DNA gel recovery kit, and then sequenced by BioSune Biotech Co., Ltd. (Cai et al., 2016). Finally, softwares such as DNAMAN and Clustal Omega were used to analyze and compare the sequence.

3.5 Detection of 2-AP content

In this study, GC-MS was used to detect the content of the fragrance characteristic compound 2-AP in the grains of Taiguxiaoxiangzhan, and 2,4,6-trimethylpyridine (TMP) was used as the internal standard. The minimum detection limit was 0.01 mg/kg. 1 g polished fragrant rice was weighed and floured into a 5 mL volumetric flask, and then add 1 mL TMP (0.5 mg/L) extraction reagent, seal and mix well, and place in a 80°C water bath for extraction for 3 hours. After taking it out and cooling to room temperature, transfer the supernatant to the sample bottle, stand still for 30 min and wait for testing. The chromatographic column was HP-5MS (30 m×0.25
mm×0.25 μm), and the carrier gas was high-purity (purity>99.999%) helium. The constant pressure is splitless for injection, and the injection volume is 1 μL, which referred to Ying et al. (2010) with corresponding improvements.

**Authors’ contributions**

ZYH and WXY were the designers and executors of the experiment. ZYH, WXY and HJH completed data analysis and wrote the first draft of the manuscript. ZR, SW and HXP participated in the design of the experiment and analysis of the results. XXM was the creator and the person in charge of the project who directed experimental design, data analysis, manuscript writing and revision. All authors read and approved the final manuscript.

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