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Rapid Detection of Rice Fragrance Allele *badh2-E7* by Recombinant Polymerase Amplification (RPA)

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Abstract Fragrant rice is favored deeply by consumers due to the strong fragrance. Fragrant rice is mainly caused by the loss-of-function mutation of the *Betaine aldehyde dehydrogenase* 2 (*Badh2*) gene in rice. *Badh2-E7*, with 8 bp deletion and 3 bp substitution in exon 7 of *Badh2*, is the main mutation allele used in fragrant rice breeding. In this study, a genotyping method named RPA-badh2-E7 for *Badh2-E7* allele was designed. This method has the characteristics of rapid (completed amplification in 5 min), sensitivity (100-fold than conventional PCR), no strict amplification conditions required (25 °C~45 °C), and independent of PCR amplifier (only one thermostatic incubator is enough for amplification). This method greatly improved the efficiency of molecular marker-assisted selection of rice fragrant genes and breeding of fragrant rice varieties.

Keywords Rice; Fragrant gene; *badh2-E7*; RPA

Rice (*Oryza sativa* L.) is the most important grain crop in China. With the development of the times and social progress, people's requirements for the quality of rice are also increasing (Zhang et al., 2020). Aroma is an important characteristic of rice quality. Rice with aroma not only has a fragrant aroma, but also is rich in nutrients such as vitamins and aromatic amino acids, which are deeply loved by consumers (Peng et al., 2018).

The molecular regulation mechanism of aroma traits and the breeding of new varieties of fragrant rice are receiving increasing attention from scientists. As early as the 1980s, scientists conducted research on the components, formation mechanisms, and genetic characteristics of rice aroma, and found that mutations in the *Badh2* (*Betaine aldehyde dehydrogenase2*) gene encoding betaine aldehyde dehydrogenase were related to the formation of fragrant rice. The functional deficiency of *Badh2* leads to the accumulation of 2-acetyl-1-pyrrolin (2-AP) (Yang et al., 2008; Fukuda et al., 2014), a substance directly related to the aroma of rice, resulting in the rich aroma of rice (Chen et al., 2008; Kovach et al., 2009). The *Badh2* gene is located on chromosome 8 and consists of 15 exons and 14 introns. Up to now, multiple different allele variations of *Badh2* have been reported, including mutation types occurring in different regions such as exons 1, 2, 4, 5, 7, 10, 12, 13, and 14 of *Badh2*, the cleavage site of intron 1, and the 5'UTR region (Amarawathi et al., 2008; Shi et al., 2008; Shao et al., 2011; Shao et al., 2013; Ootsuka et al., 2014; Shi et al., 2014; He and Park, 2015; Cheng et al., 2018). The deletion of 8 bp and substitution of 3 bp in exon 7 of *Badh2* are the main types of variation (Figure 1), which have been applied in the breeding of many fragrant rice varieties (Sun et al., 2021).

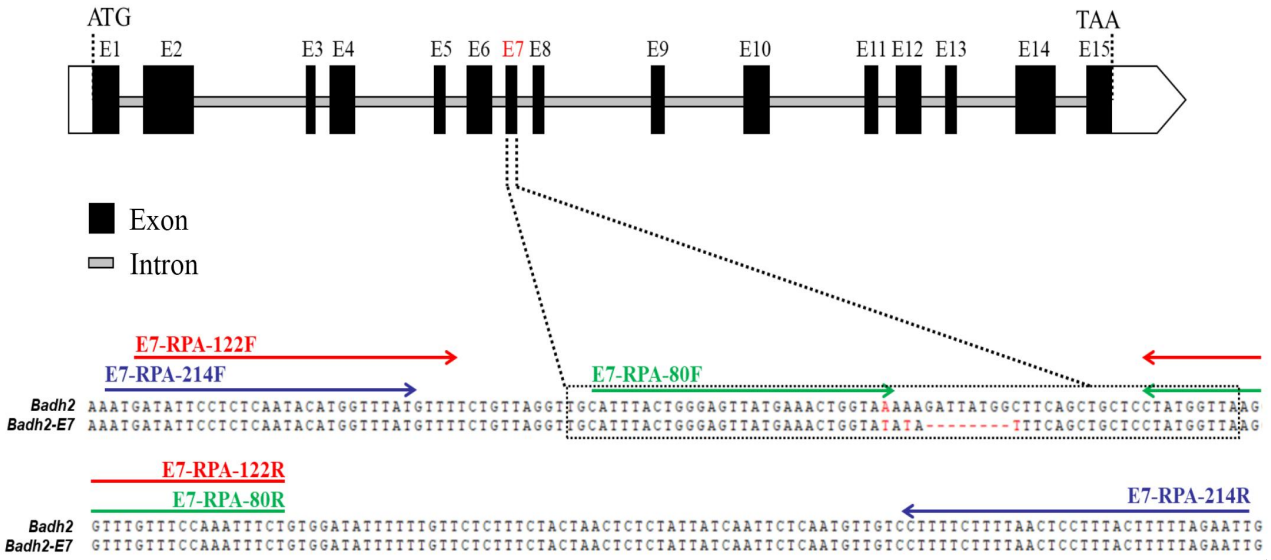

Figure 1 Design of RPA amplification primer for rice fragrance gene *badh2-E7*

In the traditional breeding process of new fragrant rice varieties, researchers often use methods such as hot water method (Hu et al., 2006; Yang et al., 2010), KOH method (Sood and Siddiq, 1978), chewing method (Berner and Hoff, 1986), and instrument measurement method (Li J.H., 2008, China Rice, (2): 8-12.) for aroma identification. These methods are either subjectively influenced, or have low accuracy, poor repeatability, and low efficiency. How to simply, accurately, and quickly identify the aroma in fragrant rice is the mainstream of contemporary research (Yan et al., 2015).

With the in-depth study of aroma genes, molecular markers have gradually become the main means of aroma detection. At present, the main method for detecting aroma genes is PCR. Recombinase polymerase amplification (RPA) technology is an emerging and rapidly developing isothermal nucleic acid amplification technology. Compared with traditional PCR technology, RPA technology has the advantages of simple operation, short amplification reaction time, and no need for specific instruments (Piepenburg et al., 2006; Zhang et al., 2022; Banerjee et al., 2023). Recently, Banerjee et al. (2023) designed a detection method for the *Badh2-E7* allele using RPA technology. However, the directional primers they designed contained an 8 bp missing part, which could only identify whether rice contained the *Badh2-E7* gene, but could not distinguish between homozygous and heterozygous lines of the *Badh2-E7* gene. This study designed a co dominant marker RPA-badh2-E7 for the *Badh2-E7* allele based on RPA technology. This method has four major advantages: (1) time-saving, amplification can be completed in 5 minutes, while conventional PCR requires 1.5 hours; (2) High sensitivity, 100 times higher than conventional PCR; (3) No strict amplification conditions are required, amplification can be performed at 25 ℃~45 ℃; (4) No PCR instrument required, only one constant temperature incubator is needed. Using RPA-badh2-E7, 36 core restoring lines of hybrid japonica rice and 1 new hybrid japonica rice variety Shenyou R1 from the Rice Center Resource Library of Shanghai Academy of Agricultural Sciences were identified. It was found that this method can effectively distinguish genotypes as homozygous Badh2, homozygous Badh2-E7, and heterozygous lines. RPA-badh2-E7 can limited identify badh2-E7, which greatly improves the efficiency of molecular marker assisted selection of rice aroma genes and the breeding of fragrant rice varieties in the future.

1 Results and Analysis

1.1 Design and quality validation of RPA amplification primers

In order to improve the molecular marker assisted selection efficiency of the aroma gene *Badh2* and accelerate the cultivation process of new aroma rice varieties, this study compared the functional variation site of the 7th exon of *Badh2* (Figure 1) and designed three pairs of primers based on the principle of RPA amplification primer design (Figure 1; Table 1).

Table 1 Primers sequences of RPA amplification

Name	Sequence (5'-3')
E7-RPA-80F	CATTTACTGGGAGTTATGAAACTGGTAA
E7-RPA-80R	AGAAATTTGGAAACAAACCTTAACCATAG
E7-RPA-122F	GATATTCCTCTCAATACATGGTTTATGTTT
E7-RPA-122R	AGAAATTTGGAAACAAACCTTAACCATAG
E7-RPA-214F	AATGATATTCCTCTCAATACATGGTTTAT
E7-RPA-214R	AATTCTAAAAAGTAAAGGAGTTAAAAGAAAAG

In order to verify the accuracy of the designed RPA primers for detecting badh2-E7, the japonica rice restoration line Shen CR1 (variety right number: CNA20191001690) selected by our research group containing the badh2-E7 aroma allele gene was used as the positive control, while the japonica rice restoration line Shen Hui 26 without the *badh2-E7* aroma allele gene was used as the negative control. The leaf DNA of Shen CR1 and Shen Hui 26 were extracted using the CTAB method, and amplified using three pairs of designed RPA amplification primers. The agarose gel electrophoresis results showed that E7-RPA-80 only had specific DNA fragments in Shen Hui 26 without *Badh2-E7*, while there were no amplification products in the variety Shen CR1 containing *Badh2-E7* (Figure 2). E7-RPA-122 and E7-RPA-214 have specific DNA amplification fragments in both fragrant and non fragrant rice. Compared to E7-RPA-214, E7-RPA-122 has better polymorphism (Figure 2), therefore E7-RPA-122 was selected as the marker for testing the *Badh2-E7* allele.

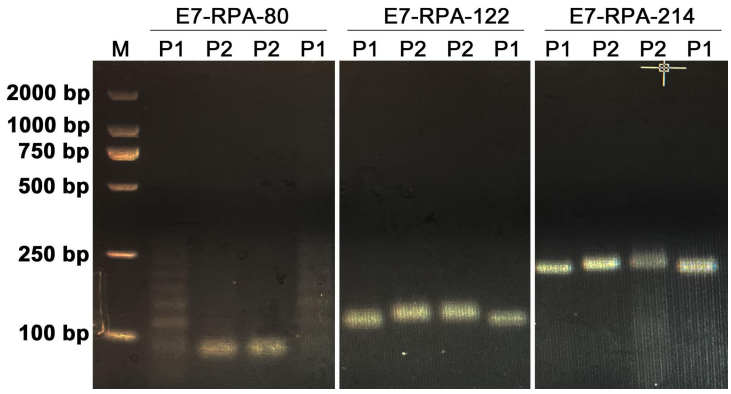

Figure 2 Quality verification of RPA amplification primers
Note: M: DL2000 Marker; P1: Shen CR1; P2: Shenhui 26

1.2 Sensitivity analysis of RPA amplification technology for detecting *badh2-E7*

Dilute the initial concentration of leaf DNA extracted from the japonica rice restoration line Shen CR1 containing the *Badh2-E7* aroma allele and the japonica rice restoration line Shen Hui 26 without the *Badh2-E7* aroma allele to 10 ng/μL. Then dilute the DNA with a 10 fold gradient. Perform amplification using conventional PCR and RPA methods respectively, and compare the sensitivity of the two methods. The results showed that the lowest detectable DNA template concentration for PCR technology was 10^{-2} ng/μL. The lowest detectable DNA template concentration for RPA amplification technology is 10^{-4} ng/μL. The sensitivity of RPA amplification technology is about 100 times that of PCR technology (Figure 3).

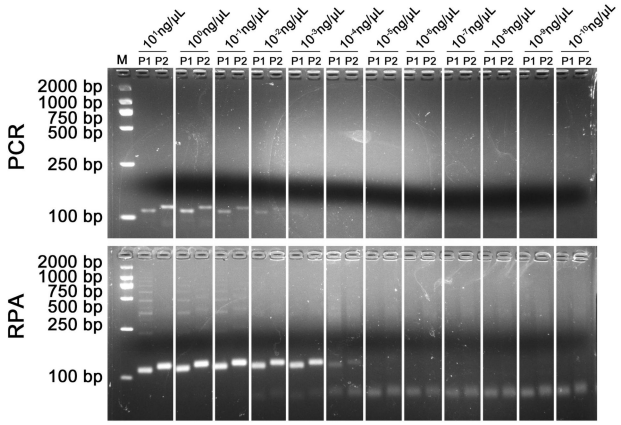

Figure 3 Evaluation of the sensitivity of the RPA-Badh2-E7

Note: M: DL2000 Marker; P1: Shen CR1 (Fragrant Rice); P2: Shen Hui 26 (Non-fragrant Rice); The template concentration of the first P1 and P2 from left to right was 10 ng/μL, and the template concentration was diluted 10 times from the second.

1.3 Optimization of RPA amplification reaction temperature and time

In order to further improve the efficiency of *Badh2-E7* genotype detection, we optimized the RPA amplification reaction conditions. Firstly, the amplification effect of RPA amplification reaction was compared under different temperature conditions, and it was found that specific DNA bands could be amplified within the temperature range of 25 °C~45 °C (Figure 4A). Therefore, we used the recommended amplification temperature of 39 °C in the product manual of the RPA isothermal amplification kit (TABAS03KIT, TwistDx) as the amplification temperature for subsequent experiments. Then, we compared the amplification effects of different RPA reaction times and found that clear DNA bands could be obtained after 5 minutes of amplification at 39 °C (Figure 4B). When using conventional PCR amplification, it often takes about 2 hours to complete the amplification reaction (Cheng et al., 2018). This result indicates that compared to conventional PCR techniques, RPA amplification can significantly shorten the amplification reaction time and does not require the use of expensive instruments such as PCR machines, greatly improving the efficiency and convenience of *badh2-E7* detection.

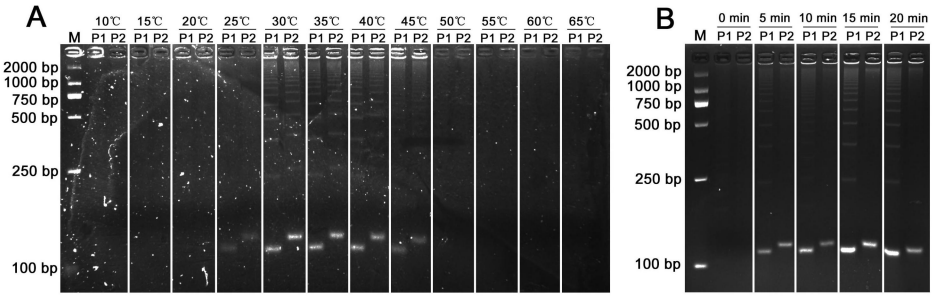

Figure 4 Optimize the temperature and time of RPA amplification reaction (A: Comparison of RPA amplification effect under different temperature conditions; B: Comparison of RPA amplification effect under different reaction times.)

Note: M: DL2000 Marker; P1: Shen CR1; P2: Shenhui 26

1.4 Application of RPA-badh2-E7 method for detecting *badh2-E7*

Using the RPA-badh2-E7 method created, 36 core restoring lines of hybrid japonica rice and 1 hybrid japonica rice Shenyou R1 (Shanghai Shendao 2022002) from the Rice Center Resource Library of Shanghai Academy of Agricultural Sciences were identified. Five restoring lines containing the aroma gene *Badh2-E7* were screened, including 'Shenfan 24', 'Shenfan 30', 'Shenfan 33', 'Shenfan 43', and 'ShenCR1' (Figure 5). Banerjee et al. (2023) designed the *Badh2-E7* allele detection marker using RPA technology, which cannot distinguish between homozygous and heterozygous Badh2-E7 genotypes. In this study, we tested the hybrid japonica rice 'Shenyou R1' (‘Shen23A’×‘Shen CR1’), it can be found that two bands can be amplified in heterozygous plants, indicating that the RPA-badh2-E7 designed in this study is a co dominant marker (Figure 5).

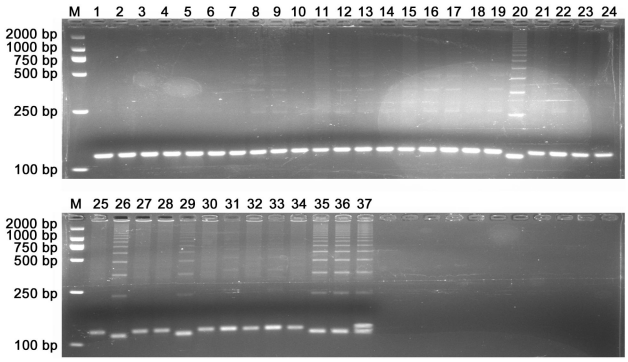

Figure 5 Identification of rice fragrant gene *Badh2-E7* by RPA amplification

Note: M: DL2000 Marker; 1:Shenfan1; 2:Shenfan2; 3:Shenfan3; 4:Shenfan4; 5:Shenfan6; 6:Shenfan7; 7:Shenfan9; 8:Shenfan10; 9:Shenfan11; 10:Shenfan12; 11:Shenfan13; 12:Shenfan14; 13:Shenfan16; 14:Shenfan17; 15:Shenfan18; 16:Shenfan19; 17:Shenfan21; 18:Shenfan22; 19:Shenfan23; 20:Shenfan24; 21:Shenfan25; 22:Shenhui26; 23:Shenfan27; 24:Shenfan28; 25:Shenfan29; 26:Shenfan30; 27:Shenfan31; 28:Shenfan32; 29:Shenfan33; 30: Shenfan34; 31:Shenfan35; 32:Shenfan36; 33: Shenfan37; 34:Shenfan38; 35:Shenfan43; 36:ShenCR1; 37:Shenyou R1

2 Discussion

The cultivation and breeding of fragrant rice has a long history. From ancient times to the present, many high-quality rice varieties with unique aromas have been cultivated both domestically and internationally, such as India's "Basmati type fragrant rice", Thailand's "jasmine fragrance type", Japan's "Gongxiang", the United States' "Jasmine85" and "Della", China's "Northeast rice flower fragrance", "Yunnan crab valley", and "Guangxi Jingxi fragrant glutinous rice" (Jain et al., 2004; Pachauri et al., 2010; Zheng et al., 2012). However, these varieties have geographical characteristics, or low yields and weak resistance (Qi et al., 2020). In recent years, with the improvement of people's living standards, the demand for high-quality fragrant rice has also increased sharply. Therefore, the breeding of more and better fragrant rice varieties has attracted increasing attention. Accurately and quickly identifying aroma traits is an important step in the breeding process of fragrant rice. Chewing method (Berner and Hoff, 1986) and KOH soaking method (Sood and Siddiq, 1978) are commonly used methods for traditional breeding to identify aroma, but the aroma characteristics themselves are greatly influenced by external environmental conditions, and subjective differences among different appraisers lead to low accuracy in aroma identification. In recent years, with the development of molecular biology and sequencing technology, molecular marker assisted selection has been widely applied in the genetic breeding of fragrant rice.

At present, the main method for detecting aroma genes using molecular markers is conventional PCR. However, conventional PCR technology requires the use of expensive PCR instruments, and the amplification time is relatively long (about 2 hours), which requires high technical requirements from experimental personnel. These to some extent limit the application of this technology in breeding (Yang and Yu, 2019). RPA isothermal amplification technology, as an emerging isothermal nucleic acid amplification technology, has higher sensitivity and specificity compared to traditional methods. It does not require special equipment and can be performed at lower temperatures (35 °C~40 °C) or even room temperature (Zhang et al., 2022; Banerjee et al., 2023). It is a molecular detection method that is expected to replace PCR. Banerjee et al. (2023) designed a detection method for the *Badh2-E7* allele using RPA technology, which can quickly and sensitively detect the presence of the *Badh2-E7* gene, but cannot distinguish between homozygous and heterozygous strains of the *Badh2-E7* gene. Unlike the study by Banerjee et al. (2023), this study designed an RPA-badh2-E7 detection method based on the deletion of the 8 bp mutation allele in the 7th exon of the aroma gene *Badh2*. The amplification reaction can be completed in as little as 5 minutes, which is much lower than Banerjee et al.'s (2023) RPA amplification time of 30 minutes and eliminates the need for RPA enzyme inactivation at 65 °C for 10 minutes. And this study explored the temperature of RPA amplification, and the results showed that RPA amplification reaction can be carried out under temperature conditions of 25 °C~45 °C. The RPA-badh2-E7 method created in this study can effectively distinguish genotypes as homozygous *Badh2*, homozygous *Badh2-E7*, and heterozygous strains. Compared with

conventional PCR, the method significantly shortens the amplification time and has a sensitivity 100 times higher than conventional PCR. Additionally, this method does not require an expensive PCR instrument and can be amplified at temperatures ranging from 25 °C to 45 °C. Using RPA-badh2-E7, 36 core restoring lines of hybrid japonica rice and one new hybrid japonica rice variety Shenyou R1 from the Rice Center Resource Library of Shanghai Academy of Agricultural Sciences were identified. It was found that this method can effectively distinguish homozygous *Badh2*, homozygous *Badh2-E7*, and heterozygous genotypes, greatly improving the efficiency of molecular marker assisted selection of rice aroma genes and the breeding of fragrant rice varieties.

3 Materials and Methods

3.1 Research materials

All rice samples used in this study are the backbone parents or varieties of hybrid japonica rice selected by the Rice Center Heterosis Utilization Research Group of the Crop Breeding and Cultivation Research Institute of Shanghai Academy of Agricultural Sciences, including 36 restoring line resources and 1 hybrid japonica rice variety.

The RPA isothermal amplification kit (TABAS03KIT) was purchased from TwistDx Co., Ltd. The RPA amplification primers were synthesized by Biotechnology (Shanghai) Co., Ltd.

3.2 Extraction of rice DNA

The DNA extraction from rice leaves was carried out using an improved CTAB method (Murray and Thompson, 1980), and the extracted DNA was measured and quantified using a NanoDropTM 2000 micro spectrophotometer. Take a leaf with a length of about 1.5 cm and place it in a 2 mL centrifuge tube; Join 750 μL 1.5×CTAB solution (1.5% CTAB, 75 mmol/L Tris-HCl, 15 mmol/L EDTA, 1.05 mol/L NaCl, ph 8.0) and a steel ball with a diameter of 6 mm; Set the frequency parameter of the plant tissue rapid grinder to 65 Hz and oscillate for 90 seconds; After grinding, the sample is incubated in a 65 °C water bath for 45 minutes, with 500 μL chloroform added, vigorously shaken, centrifuged at 12 000 r/min for 8 minutes; Transfer 500 μL of supernatant to a new 1.5 mL centrifuge tube, add an equal volume of anhydrous ethanol, mix up and down, place the centrifuge tube in a -20 °C freezer for 1 hour, centrifuge at 12 000 r/min for 8 minutes, discard the supernatant, air dry or dry at 37 °C, then add 500 μL of ddH_2O to dissolve DNA. Store the dissolved DNA in a -20 ℃ freezer for later use.

3.3 RPA reaction system

The RPA amplification system includes 29.5 μL Primer free rehydrogenation buffer, 2.4 μL each of 10 μM forward and reverse primers, 1 L DNA template, 12.2 μL sterile ddH_2O, and a total volume of 50 μL. Gently mix with a pipette and transfer the mixture to a TwistAmp reaction tube containing freeze-dried enzyme powder. Then add 2.5 μL of magnesium acetate with a concentration of 280 mmol/L, mix well, and perform constant temperature amplification. Unless otherwise specified, the amplification conditions are 39 °C and incubate for 20 minutes.

Authors' contributions

ZJH was the experimental designer and executor of this study; ZJH and ZAP completed data analysis and wrote the first draft of the paper; XWK, CC, NFA, SB, ZJM participated in experimental design and analysis of experimental results; CLM and CHW are the project conceptualizers and leaders, guiding experimental design, data analysis, paper writing, and revision. All authors read and approved the final manuscript.

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