What is the Molecular Basis of Aroma in Biriyanicheera - A Tropical Aromatic Rice Genotype

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Research Article

Keywords: Basmati, BADH2, aroma, mutation, exon, fragrance

Posted Date: August 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-827202/v1>

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Abstract

The aromatic rice cultivars possess excellent aroma generally when grown in their favourable and specific environments. An early maturing selection from a Kerala aromatic local landrace with short grains, named '*Biriyancheera*', when grown in normal tropical conditions was sufficiently fragrant. The present study focused on the analysis of aroma in '*Biriyancheera*' rice genotype through molecular methods. The seeds of two aromatic rice varieties viz., *Biriyancheera* and Gandhakasala (from Palakkad, Thrissur and Ernakulam districts) along with one non-aromatic rice variety Triveni (control) were used for the study. The *BADH2* gene was amplified in all the three rice varieties. Upon sequencing the amplified PCR products of genomic DNA, the mutation in *BADH2* gene was detected. The sequencing results of aromatic varieties revealed the presence of 8 base pair mutation in exon 7 in *Biriyancheera* and Gandhakasala, whereas this mutation was absent in the non-aromatic variety Triveni. This indicated that aroma production in *Biriyancheera* variety is due to similar mutation in *BADH2* gene as that of the popular scented rice Basmati.

1. Introduction

Aromatic rice, is considered as a special group of rice due to the presence of excellent aroma and superior grain quality. India holds the pride for being the homeland for the highly popular famous *nature's gift*, Basmati rice. With its superior, scented, long slender grain rice, Basmati is acclaimed as the major aromatic rice genotype around the globe and fetches premium price in national and international markets. At the same time, India is the homeland for many traditional aromatic rice cultivars also, which are comparable with basmati genotypes in their aroma, making many of these unrecognized landraces as an attractive low-cost alternative to the highly priced Basmati. The short grains, low yield, and lack of prominent improvement methods have kept the non-basmati cultivars, behind in the line. However, there is an immense potential for many non-basmati genotypes to hold a major share in aromatic rice market, if these genotypes are improved with high yield and other preferred qualities (Mondal et al., 2021)

Buttery et al. (1983) was the first person to analyse the aroma of cooked rice, in which 2-acetyl-1-pyrroline (2AP) was identified as a major contributor of aroma. But some studies had revealed the presence of many other volatile compounds, other than 2-AP to be responsible for the aroma in rice grains (Jezussek et al., 2002; Sansenya et al., 2018). The aroma present in different aromatic genotypes originated from diverse places and its level of expression tends to vary with genetic as well as environmental parameters.

The genomic history of the aromatic population was unraveled from the diversity found in the unique genome-wide *sativa-rufipogon* data set (Civan et al., 2020). Several molecular markers had been developed to utilise the aroma trait in rice breeding programmes (Ahn et al., 1992; Garland et al., 2000; Jin et al., 2003). Among the 25 polymorphic SSR markers across 24 aromatic rice genotypes, RM527 might be the best marker for identification and diversity estimation of aromatic genotypes followed by RM1, RM22565 and RM207 markers (Kumar et al., 2018).

The fragrance nature in rice is controlled by a recessive trait in homozygous condition, on chromosome 8 (Bradbury et al., 2005). The mutation in *BADH2* gene leads to non-functional enzyme synthesis, which is responsible for aroma production.

Bradbury et al. (2005a) showed the presence of a gene having homology with a gene coding for betaine aldehyde dehydrogenase (BAD), consisting significant polymorphisms in the coding region of fragrant plants. Later the accumulation of 2-acetyl-1-pyrroline in fragrant rice was proposed to be due to the presence of mutations resulting in loss of function of the '*fgr*' gene by introducing a stop codon in conserved amino acid sequence of BADs.

In Kerala, Wayanad Jeerakasala' and Wayanad Gandhakasala', have already been GI tagged and gained considerable attention in the international market for their suitability in multinational cuisines. However, these aromatic rice cultivars are found to possess higher fragrance, when grown in their own specific geographical niches, for their growth and proper expression of aroma and other unique characteristics. There has been decrease in aroma when the same cultivars were grown in regions other than their normal traditional growing regions. This posed difficulty in popularizing them in other major rice growing tracts of the country. If a genotype with good aroma that can be grown in normal tropical conditions is available, it can be introduced in other parts also. The early maturing selection from a Kerala aromatic local landrace with short grains, named '*Biriyanicheera*', when grown in normal tropical conditions was observed to have sufficient aroma. It has the capacity to produce sufficient aroma wherever it is grown, irrespective of the geographical or environmental conditions. In this context, the present study focuses on the molecular analysis of aroma gene in '*Biriyanicheera*' rice genotype through gene sequencing.

2. Materials And Methods

Rice varieties were used in this study are as detailed below :

- a) Biriyanicheera – test variety (newly identified and recommended aromatic rice variety for tropical areas) for the evaluation of molecular basis of aroma.
- b) Gandhakasala – standard aromatic variety (aromatic check) for comparison
- c) Triveni – standard non-aromatic variety (Non-aromatic check) for comparison
- d) Pusa basmati 1 – Popular aromatic rice variety for Validation of *BADH2* primer

The objective of the research programme was to find out the molecular mechanism of aroma producing gene in early maturing selected line of *Biriyanicheera* rice. The study was carried out at the Centre of Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Thrissur during the period 2017–2019.

2.1 Plant materials

The aromatic rice genotypes *Biriyanicheera* and Gandhakasala (check genotype) grown in Mulangunnathukavu (10.5989° N, 76.2146° E and 26 m above MSL-) of Thrissur district, Kerala districts

of Kerala along with a Non-aromatic genotype Triveni grown in Mulangunnathukavu from Thrissur, Kerala during the period, 2017–2018 were used for the study. The average days for maturity of rice genotypes *Biriyanchiseera*, Gandhakasala and Triveni variety were 105, 120 and 105 days respectively. Pusa Basmati 1, the highly popular and well studied aromatic rice variety was used for the validation of specific primers designed for *BADH2* gene.

2.2 Raising of rice plants

Seeds of aromatic rice genotypes, *Biriyanchiseera* and Gandhakasala and the non-aromatic rice genotype Triveni were germinated in petri plates lined with moist tissue paper and allowed to germinate. After a week, the germinated seedlings were transferred to a pot. Later, 21 days old seedling were transplanted into new pots at three seedlings per pot.

2.3 Molecular characterization

2.3.1 DNA isolation

Genomic DNA was isolated from young leaves of rice plants grown in pots. Fresh and green leaves yielded good quality of DNA in required quantity. The leaves were collected early in the morning from all the three genotypes. The collected leaves were immediately covered in aluminium foil and carried to the laboratory. The extraction of genomic DNA from the leaves was performed through CTAB method (Dellaporta et al., 1983).

2.3.2 Quantity and quality determination of genomic DNA

The purity of DNA was analysed using NanoDrop ND-100 spectrophotometer. Nucleic acids and proteins show absorption maxima at 260 nm and 280 nm respectively. NanoDrop recorded the absorbance at wavelengths 260 and 280 nm and purity was determined by the ratio OD ($A_{260/280}$). The OD values obtained between 1.8 and 2.0 shows the purity of the DNA, which is free from proteins. The quantity of DNA in the sample was mentioned as ng/μl. The quality was checked by agarose electrophoresis on 0.8 per cent agarose gel. The gel profile was examined for intactness, clarity of DNA band, the presence of contaminants such as RNA and proteins.

2.3.3 Designing of primers for PCR

The *BADH2* gene sequence (size = 6.1 Kb) retrieved from the Rice Genome Annotation Project was suitably divided into seven distinct regions based on the position of exons for convenient PCR amplification of exons. The primer details are mentioned in Table 1.

Table 1
Details of primers synthesized from *BADH2* gene sequence

Primer No.	Forward primer sequence	Reverse primer sequence	PCR Product length (bp)	Exons covered
1	CACTCCACACCTGACACCAC	CTGCCAACTAACGAAGCACA	980	1 & 2
2	AGCAGCATGGGACATGGTAT	ACCGGGACAGAACAAATGAG	741	4
3	GCTTTCAGCTTCTTGCTCCT	GGTCACCGGAAAGCACAG	1002	10, 11 & 12
4	TTGGCCAACGATACTCAGTG	ATGCACAAATTGTCGCGTAA	944	13, 14 & 15
5	CAGTGACCAGTGCACACTTT	ACCCAACTACACCGATAGGC	509	3
6	CCTCTCCTCCGTGTTAATGC	GGTCTAGCATCCAGCTCAG	1093	5, 6, 7 & 8
7	GTGGCAAGGAAGGCAGTTA	GGTCAGGAGCAAGAAGCTGA	707	9
1 (modified)	ACAGAACAGAGCACTCCCTCTC	CCAGATACACAATCAGCCATGC	846	1 & 2

2.3.4 Polymerase Chain Reaction (PCR)

The total volume of the PCR reaction was 10 µl constituted of *Taq* assay buffer, dNTPs, forward and reverse primers, *Taq* DNA polymerase, template DNA and sterile distilled water. The amplification profile was as follows:

The initial denaturation was carried out at 95°C for 3 minutes (min). The denaturation (94°C for 50 sec), primer annealing (around 57°C for 30 sec) and extension (72°C for 1 min), were carried out for 35 times. Final extension was carried out at 72 °C for 10 min.

2.3.5 PCR protocol using Dimethyl Sulphoxide (DMSO):

The total PCR reaction volume was 50 µl. Three per cent of DMSO (1.5 µl for 50 µl reaction) was used for the amplification in addition to *Taq* assay buffer, dNTPs, forward and reverse primers, *Taq* DNA polymerase, template DNA and sterile distilled water. The initial denaturation was carried out at 95°C temperature for 8 min. The denaturation (94°C for 3 min), primer annealing (around 55°C for 30 sec) and extension (72°C for 1 min) steps were repeated for 35 times. The quality of the amplified PCR product was checked on 1.2 per cent agarose gel by agarose gel electrophoresis. The amplified PCR products were sequenced at AgriGenome Labs Pvt. Ltd., Kochi, Kerala.

2.3.5 Analysis of sequence data

The forward and reverse DNA sequences obtained from sequencing results were used to construct contigs using CAP3 software. The contigs were then aligned with the reference *BADH2* sequence retrieved from the Rice Genome Annotation Project using CLUSTAL OMEGA software to analyze the possible mutations in the exonic regions of genomic DNA.

2.3.6 Validation of specific primers of *BADH2* gene :

The specific primers designed for *BADH2* gene were evaluated for region 1 covering the exons 1 and 2 in the three aromatic rice genotypes *Biriyanicheera*, Gandhakasala and Pusa Basmati 1 along with the non-aromatic rice genotype Triveni. The genomic DNA was used for the amplification of PCR products.

3. Results

3.1 Molecular characterization

3.1.1 DNA isolation and PCR

Fresh and young leaves of *Biriyanicheera*, Gandhakasala, and Triveni collected during early morning hours were used for DNA isolation. The quality of DNA was assessed using agarose gel electrophoresis. Single intact band of good intensity was obtained indicated that the isolated DNA was of good quality. The DNA was free from RNA and protein contamination. The concentration of the DNA was between 300 and 800 ng/μl. The A260/A280 ranged between 1.8 and 2.0 indicating good quality of genomic DNA.

PCR reaction was performed using the seven different primers amplifying the regions in genomic DNA containing the exons. The annealing temperatures for the primers ranged between 55°C and 60°C. The PCR products were electrophoresed on a 1.2 per cent agarose gel. The required specified bands were observed (Plate 1).

There was no amplification for primer 1 designed for amplifying exons 1 and 2. The amplification of the above region was attempted in different annealing temperatures of range 55°C to 65°C. Since there was no amplification of DNA with the specified primer, a new set of primer was designed to amplify the genomic DNA (exons 1 and 2). The new set of primer is mentioned below.

Forward primer: 5' ACAGAACAGAGCACTCCCTCTC 3'

Reverse primer: 5' CCAGATACACAATCAGCCATGC 3'

The PCR reaction performed with the new set of primers showed no amplification. The product amplification was attempted with certain modifications including, altered primer concentration, change of PCR buffer *etc.* PCR reaction of Triveni genotype produced a non-specific amplification, whereas the genotypes *Biriyanicheera* and Gandhakasala showed no bands in agarose gel. The reason for non-amplification was assumed to be due to the presence of GC rich regions in the genomic DNA sequence lying between exons 1 and 2. So a modified PCR reaction was performed with 3 per cent Dimethyl

Sulphoxide (DMSO). The use of DMSO in PCR amplified the genomic DNA in Triveni (non-aromatic) (Plate 2) whereas no amplification was seen in *Biriyanchiseera* and Gandhakasala.

3.1.2 PCR reaction using Phusion enzyme (High fidelity DNA polymerase)

High fidelity DNA polymerase enzyme (Phusion), capable of amplifying GC rich genomic regions, was used to amplify the exons 1 and 2. The agarose gel separation showed the presence of desired band size in Triveni genotype, whereas non-specific amplification was observed in both *Biriyanchiseera* and Gandhakasala genotypes (Plate 3). The secondary amplification of the PCR products showed non-specific amplification (Plate 4).

3.2 Sequencing of *BADH2* gene

The DNA sequence of *BADH2* gene of genotypes *Biriyanchiseera*, Gandhakasala and Triveni obtained by sequencing were used to generate the contigs by using CAP3 software. The *BADH2* gene sequence of the rice genotypes was submitted to NCBI Gen bank.

BADH2 gene in *Biriyanchiseera* rice genotype Accession number: MN337026

BADH2 gene in Gandhakasala rice genotype Accession number: MN337027

3.3 Analysis of sequence data

The genomic DNA sequence of *BADH2* gene present in all genotypes was aligned by Multiple Sequence Alignment (MSA) by using CLUSTAL OMEGA software. The reference sequence, Locus Id: LOC_Os08g32870, obtained from Rice Genome Annotation Project, was used for the comparison of sequences. MSA identified the presence of 8 base pair mutation and 3 SNPs in the exon 7 of both the genotypes *Biriyanchiseera* and Gandhakasala. The mutation was absent in non-aromatic genotype Triveni. The sequences were further compared with the reported exon 7 sequence of Basmati genotype available in NCBI. The MSA showed that mutation in both *Biriyanchiseera* and Gandhakasala were similar to the reported mutation in Basmati cultivars as mentioned Table 2.

Table 2

Sequences of exon 7 mutation in aromatic and non aromatic rice varieties

Exon 7 mutation

Reference seq	GGTAAAAAGATTATGGCTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Basmati	GGTATAT----- ATTTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Triveni	GGTAAAAAGATTATGGCTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Biriyani cheera	GGTATAT----- ATTTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
Gandhakasala	GGTATAT----- ATTTTCAGCTGCTCCTATGGTTAAGGTTTGTTTCCAAATTTCTGTG
	**** * *****

Reference seq – Reference sequence

Basmati - aromatic rice variety

Triveni - Non aromatic rice variety

Biriyani cheera - aromatic rice variety

Gandhakasala - aromatic rice variety

3.4 Validation of specific primers of *BADH2* gene

The specific primers designed for region 1 covering the exons 1 and 2 of *BADH2* gene was used for the amplification of genomic DNA. The amplification was successful only in non-aromatic genotype Triveni, whereas aromatic genotypes *Biriyani cheera*, Gandhakasala and Pusa basmati 1 showed no amplification (Plate 5). The primer 1 produced PCR band with desired band size of 846 bp in Triveni genotype.

4. Discussion

The aromatic rice varieties, 'Basmati' (popular around the globe) and 'Gandhakasala' (popular in Kerala, India) are having GI tags, indicating that for their growth and proper expression of aroma and other unique characteristics, they need their own specific geographical niches. Whereas, the test variety is having the capacity produce sufficient aroma in wherever it is grown, irrespective of the geographical or environmental conditions. Aroma detection of rice grains was performed by Sensory evaluation test by DUS (Distinctness, Uniformity, Stability) protocol prescribed by IIRR, Hyderabad. During the analysis of grains, *Biriyani cheera* had good to strong aroma, Gandhakasala had Medium to good aroma while Triveni had no aroma.

The genetic basis of rice aroma trait (*fgr*) explored by many researchers in early 1992, revealed the recessive nature of the gene and it was located at 4.5 cM distance from the marker RG28 (Ahn et al., 1992). Later, the aroma gene *fgr* was located to be between markers RM223 and RM342 (Wanchana et al., 2005). The map-based cloning and sequencing of the *fgr* region identified significant difference in the *Badh2* gene between scented and non-scented cultivars. The mutation in 7th exon of *Badh2* gene, which led to loss of *Badh2* protein, was thought to be controlling the aroma in rice. Similarly, the amplification of *BADH2* (or *fgr*) gene was attempted to understand the actual molecular reasons for the special nature of *Biriyani*cheera genotype. The sequencing of the genomic DNA consisting of exons of *BADH2* gene helped to unravel the molecular nature in *Biriyani*cheera. The occurrence of previously reported 8bp mutation (TATATATTT) in the exon 7 of *BADH2* gene in both the *Biriyani*cheera and Gandhakasala genotypes suggests that the aroma gene present in them must have followed similar path of evolution and inheritance as that of Basmati cultivars. The occurrence of 8 bp deletion in the *BADH2* gene is also reported by many researchers in various rice genotypes of the world (Bradburry et al., 2005; Chen et al., 2008). The results showed negative for the occurrence of mutations in other exons of *BADH2* gene, whereas there have been reports of presence of mutations in exons 1, 2, 10, 13, 14 and also regions between exon 4 and 5 (Shi et al., 2008; Amarawathi et al., 2008; Kovach et al., 2009; Shao et al., 2011).

The non-amplification of genomic DNA coding for exon 1 and 2 in both the aromatic genotypes was not clear. Two primers designed to amplify the exons along with the promoter regions were lying in different regions. The amplification of same region in Triveni genotype shows the functionality of the primers designed. Hence, the problem can be attributed to the major difference in genome sequence of the region, which makes it difficult to amplify the regions by the primers used. The molecular complexity of the above region can be solved through advanced methods like, 5' - genome walking or 5' - RACE (Rapid Amplification of cDNA ends). The production of aroma by *Biriyani*cheera might not be precisely due to mutation in chromosome 8. The presence of novel gene in other chromosomes can be a factor for the differential aroma production in the genotype. Amarawathi et al. (2008) has reported similar example about *BADH1* gene present in chromosome 3 and chromosome 4 as responsible for aroma production. The *BADH1* gene is involved in salt stress response in rice crop and the mechanism is unknown (Bradburry et al., 2005). Two additional genes similar to that of *BADH* has been reported on chromosome 7 (Chen et al., 2008). The other contradicting reports have suggested the presence of several genes determining the aroma in rice. For example, a major QTL on chromosome 8 and two minor QTL on chromosome 3 and 4 (Amaravathi et al., 2008), two recessive genes (Hien et al., 2006), one major QTL on chromosome 8 and two minor QTL on chromosome 4 and 12 (Lorieux et al., 1996) are some of the reported locations aroma trait. The primers designed for amplifying the promoter region, exons 1 and 2 was used to differentiate the aromatic and non-aromatic rice cultivars. The presence of clear band from PCR reaction of Triveni (non-aromatic) rice variety shows the usefulness of the primer as a marker for aroma. Similarly Bradburry et al. (2005b) has used allele specific primers for discriminating the aromatic rice varieties from the non-aromatic.

5. Conclusion

The present study indicates that aroma production in *Biriyani cheera* variety is due to similar mutation in *BADH2* gene as that of the popular aromatic rice Basmati.

Declarations

Funding information

This work was financially supported by Department of Biotechnology (DBT), Government of India.

Conflicts of interest/Competing interests:

The authors declare that there are no conflict of interests/competing interests among them.

Availability of data and material :

Available

Code of availability :

Available

Acknowledgement

Authors are grateful to the Department of Biotechnology, Govt. of India, for providing full funding, and College of Horticulture, Vellanikkara, Kerala Agricultural University, for providing facilities, to conduct this research programme.

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Figures

2.1 - <i>Birivanicheera</i> (primer 2)	3.1 - <i>Birivanicheera</i> (primer 3)
2.2 - <i>Gandhakasaala</i> (primer 2)	3.2 - <i>Gandhakasaala</i> (primer 3)
2.3 - Non-aromatic (primer 2)	3.3 - Non-aromatic (primer 3)

Figure 1

Plate 1A. BADH2 gene specific PCR bands L - DNA ladder 2.1 - Biriyanicheera (Primer 2) 2.2 - Gandhakasaala (Primer 2) 2.3 - Non-Aromatic (Primer 2) 3.1 - Biriyanicheera (Primer 3) 3.2 - Gandhakasaala (Primer 3) 3.3 - Non Aromatic (Primer 3)

Figure 2

Plate 2 Modified PCR (DMSO) for Triveni rice variety

Figure 3

Plate 3. PCR amplification using Phusion enzyme L- Ladder BC- Biriyanicheera GS- Gandhakasala NA- Triveni (Non aromatic)

Figure 4

Plate 4. Secondary PCR amplification of PCR products produced by Phusion enzyme L- Ladder BC- Biriyanicheera GS- Gandhakasala NA-Triveni (Non aromatic)

Figure 5

Plate 5. Validation of specific primers of BADH2 gene 1 - Triveni (Non Aromatic) 2 - Biriyanicheera 3- Gandhakasala 4 - Pusa Basmati 1 L – 100 bp ladder