SEQUENCE ANALYSIS OF THE MUTATION IN THE 7TH EXON OF *BADH2* GENE IN TRADITIONAL AROMATIC RICE VARIETIES IN SRI LANKA

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ABSTRACT

It is revealed that the gene responsible for the fragrance in rice is Betaine aldehyde dehydrogenease (*badh2*) *gene. An 8-bp deletion and three single nucleotide polymorphisms in the exon 7 of badh2 gene, named as badh2.1 allele, was reported to result in accumulation of a major aromatic compound, 2-acetyl 1-pyrroline (2AP) in fragrant rice. Although badh2.1 is the predominant allele in virtually all fragrant varieties, exceptions such as involvement of another genetic loci or allele had been reported by several researchers. This study was conducted aiming at detecting presence or absence of badh2.1 allele in popular traditional Sri Lankan fragrant rice varieties. A 463-bp DNA fragment was amplified covering 7th exon region and sequenced. The results showed that "Lanka Samurdi", the fragrant high yielding variety, possesses the 8bp deletion while highly fragrant three traditional rice varieties did not show the particular mutation. Hence it confirms that the aroma in most of Sri Lankan traditional fragrant varieties is not resulted by the badh2.1 allele but by another genetic factor.*

Key words: *2-acetyl-1-pyrroline, badh2.1 allele, fragrant rice*

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for more than 50 % of the world's population. Basmati type aromatic rice varieties have gained a wide acceptance in Europe and the USA. There are many other aromatic rice varieties reported in other continents. Sri Lanka has several aromatic traditional varieties that are grown in rural areas under organic farming conditions. As their genetic status is unknown they have rarely been used in variety improving breeding programs.

Genetic investigations implicated that the fragrant trait of rice was controlled by a single recessive locus (Berner and Hoff, 1986) and the underlying gene responsible for the aroma production was located on chromosome 8 (Lorieux *et al.*, 1996). The sequencing of the *badh2* locus derived from a number of aromatic rice cultivars uncovered the presence of *badh2.1* allele (Bradbury *et al*., 2005). It was reported that *badh2.1* allele consists of 8-bp deletion and 3 single nucleotide polymorphisms (SNPs) in exon 7 that leads to the introduction of premature stop codon to produce a truncated protein. This results in abrogation of the function of the enzyme, Betaine aldehyde dehydrogenease, which consequently accumulates substrate 2AP in fragrant varieties. The functional *badh2* gene codes for a mature protein with 503 amino acids (Wanchana *et al*., 2005). Identification of the gene for fragrance and availability of large aromatic rice gene pool has created a world-wide interest to look for allelic variants at this locus (Amarawathi *et al*., 2008). A 7-bp deletion in exon 2 (Shi *et al*., 2008), absence of MITE (miniature interspersed transposable element) in promoter (Bourgis, 2008), two new SNPs in the central section of intron 8 (Sun *et al*., 2008), a TT deletion in intron 2 and a repeated $(AT)_n$ insert in intron 4 of *badh2* were

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reported in various fragrant varieties (Chen, 2008).

Sequence analysis in previous studies indicated that both the traditional and modern fragrant rice varieties with diverse origins possess the same mutant allele, suggesting the donor mutation leading to fragrance probably has a single evolutionary origin (Bradbury *et al*., 2005). During this study, we examined genetic variation in a 463-bp portion of *badh2* gene covering 7th exon in four Sri Lankan traditional fragrant rice accessions. The purpose of this study was to analyze allelic variation of the 7th exon of *badh2* gene in aroma production and pave ways for its efficient manipulation and exploitation in breeding practices.

METHOD AND MATERIALS

Experimental Site and Planting Material

This research was carried out in 2011 at the Department of Biotechnology, Faculty of Plantation Management, Wayamba University of Sri Lanka.

Seeds of *Oryza sativa* L. accessions (Table 01) of which fragrance was previously evaluated by phenotypic assessment were obtained from the gene bank of Plant Genetic Resources Institute, Gannoruwa, Sri Lanka. "Bg 250", a non-aromatic rice variety obtained from Rice Research and Development Institute, Batalagoda, Sri Lanka was used as the negative control.

Genomic DNA Extraction

DNA was extracted as described in http://rgp.dna.affrc.go.jp/rgp/protocols/QTL.pdf.

Seeds from each accession were planted on wetted filter paper in a Petri dish. Leaves of three weeks old rice seedlings were used for the extraction of genomic DNA. Three to five leaves were cut into small pieces and inserted into 1.5 ml eppendorf tubes. The leaf pieces were homogenized with 300 µl of DNA extraction buffer (1 M KCl, 1 M Tris HCl, 0.5 M EDTA). Capped eppendorf tubes were incubated at 70^{0}C for 20 min. Extracts were centrifuged at 13,000 rpm for 15 min under room temperature. 100 µl of ice cold iso-propanol was added into new eppendorf tubes, and the supernatant of the above centrifuged samples were transferred into them. Solutions were mixed gently. After mixing, tubes were kept at 4^{0}C for 15-30 min and centrifuged at 13,000 rpm for 15 min under room temperature. DNA pellets were washed with 150 µl of 70 % ice cold ethanol by centrifuging at 13,000 rpm for 10 min. Supernatants were removed and pellets were air dried and dissolved in 200 µl of 1/10 TE buffer (10 mM Tris, 1 mM EDTA).

PCR Amplification

Selected rice accessions were genetically screened for fragrant gene using *badh2.1* allele specific markers viz., ESP, IFAP, INSP and EAP (Bradbury *et al*., 2005) (Table 02). PCR was performed in the 15 µl of PCR mixture containing 5 µl of genomic DNA, 1.5 µl of 10X PCR buffer, 1.2 µl of dNTPs (2.5 mM of each dNTP), 1 µl of primer mixture containing all 4 primers (20 µM of each primer) (Integrated DNA Technologies, USA) and 0.24 µl of Taq DNA polymerase (Sigma, USA) under the amplification profile consisted of initial denaturation at 95ºC for 5 min followed by 35 cycles of 1 min at 95 ºC, 30 sec at 58ºC, 1 min at 72ºC and final cycle at 72ºC for 5 min. The 7th exon region of *badh2* gene was amplified with specific primer pair (*OsBADH2* F/R) (Table 02) (Niu *et al*., 2008) following above mentioned PCR method under 55ºC annealing temperature. As replicates PCR was conducted two times using same samples. Amplified PCR products (15 µl) were electrophoresed on 1.3 % agarose gel containing 0.5 µg/ml Ethidium Bromide and visualized under UV.

Table 01: Varieties used for determination of allelic variation of *badh2* locus.

Variety name	Acc. No.
Lanka Samurdi*	08921
KuruluWee*	04903
Kuruluthuda*	04759
Suwandal *	04366
Bg 250	

*Presence of fragrance by KOH test

Table 02: DNA sequence of the tested primers specific for *badh2* locus

Primer name	Primer sequence
External Sense Primer (ESP)	5'TTGTTTGGAGCTTGCTGATG3'
Internal Fragrant Anti-sense Primer (IFAP)	5'CATAGGAGCAGCTGAAATATATACC3'
Internal Non-fragrant Sense Primer (INSP)	5'CTGGTAAAAAGATTATGGCTTCA3'
External Anti-sense Primer (EAP)	5'AGTGCTTTACAAAGTCCCGC3'
OsBADH2(F)	5'ACATAGTGACTGGATTAGGTTCTG 3'
OsBADH2 (R)	5'CATCAACATCATCAAACACCACT 3'

Purification of Amplified Product and Sequencing

The separated gel bands were cut under the ultra violet light. They were inserted separately into four eppendorf tubes. 300 µl of binding buffer (Fermentas, USA) was added to each eppendorf tube and incubated at 65^0 C for 10 min in a dry bath. Solutions were transferred into four separate columns and centrifuged at 13,000 rpm for one min. The column top was placed into a fresh tube and 30 µl of distilled water was added. Tubes were heated at 65^0 C for 5 min and centrifuged at 13,000 rpm for 1 min. Sequencing was performed at the Asiri Hospital laboratory, Colombo with the ABI Prism BigDye terminators V2.0 cycle sequencing reaction kit. A multiple sequence alignment and a phylogenetic analysis was conducted for the resulted DNA sequences, by using CLUSTAL W software.

RESULTS AND DISCUSSION

Detection of Allele Diversity by badh2.1 Specific Markers

Among 4 types of fragrant gene (*badh2.1* allele) specific markers, ESP and EAP primers generated a fragment of approximately 580 bp as a positive control in all 4 rice accessions selected for this study (Figure 01A). EAP and INSP primer pair generated a 355 bp PCR product with the "Kuruluthuda" (Acc. No.04759), "Suwandal" (Acc.No.04366) and "Kuruluwee" (Acc.No.04903) exhibiting the presence of homozygous non-fragrant genotype. A 257 bp PCR product was resulted with At405 by the ESP and IFAP primer pair, indicating the presence of the fragrant allele, *badh2.1* (Figure 01A).

Sequence Comparison

Since none of the selected traditional rice accessions which were positive for KOH fragrance assay amplified the *badh2.1* fragrant allele, 7^{th} exon region of *badh2* gene was sequenced to detect the possible variation at the respective region. Thus, all four rice accessions were amplified using a specific primer pair, (*OsBADH2* F/R), which could yield a 463 bp DNA fragment covering 7^{th} exon region of *badh2* gene (Figure 1B). Multiple sequence alignment revealed that "At 405",

the fragrant high yielding variety, possessed the 8 bp mutation and 3 SNPs as exactly similar to "Basmati 370" fragrant rice variety (Figure 02). "At 405" was derived from "At 402"/ "Basmati 442" cross. Therefore, it is further confirmed the successful introgression of mutated *badh2* allele from the donor parent of "Basmati".

However, other four traditional rice accessions did not show the particular mutation and they were exactly similar to the 7th exon region of wild type *Badh2* gene (Figure 02). With an exception to the 8-bp deletion reported as the genetic cause for aroma, it is found that some indigenous aromatic rice genotypes did not carry this deletion (Sakthivel *et al*., 2006). Similar exceptions were also observed by others in some fragrant varieties (Kuo *et al*., 2005; Navarro *et al*., 2007; Shi *et al*., 2008; Fitzgerald *et al*., 2008). Accordingly these findings have provided evidences to prove that aroma in rice is not caused merely by the 8bp deletion in 7th exon.

Analysis of Phylogenetic Relationship

Phylogenetic analysis was performed to identify the relationships of the traditional Sri Lankan fragrant rice varieties with other exotic fragrant rice varieties and the evolutionary history was inferred using the neighbor-joining method (Figure 03). There was one prominent cluster (Figure 03) in the tree with zero branch lengths. This shows that those rice varieties ("Suwandal", "Lanka Samurdi", "Basmati 370", "Kuruluwee" and "Kuruluthuda") had evolved from a common ancestor and they are closely related.

Hence, present results confirmed that, the aroma in "Suwandal", "Kuruluwee" and "Kuruluthuda" is not resulted by the *badh2.1* allele and it might be resulted by another genetic factor or another mutated allele of the *badh2* gene. Therefore, further experiments are required to detect the causal factors for the fragrance in Sri Lankan aromatic rice varieties, in order to design markers to be used in breeding programs.

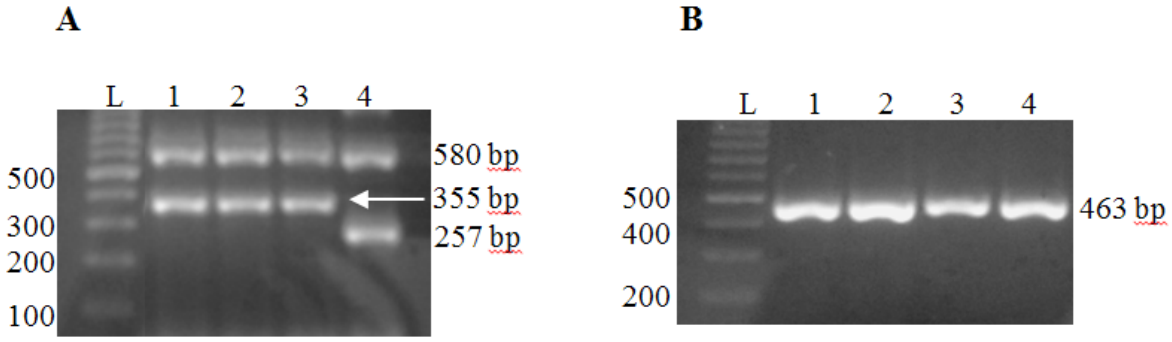

Figure 01: PCR profile generated by specific primers. A: DNA fragments generated by *badh2.1* allele specific markers B: DNA fragments generated covering 7th exon region of *badh2* gene.

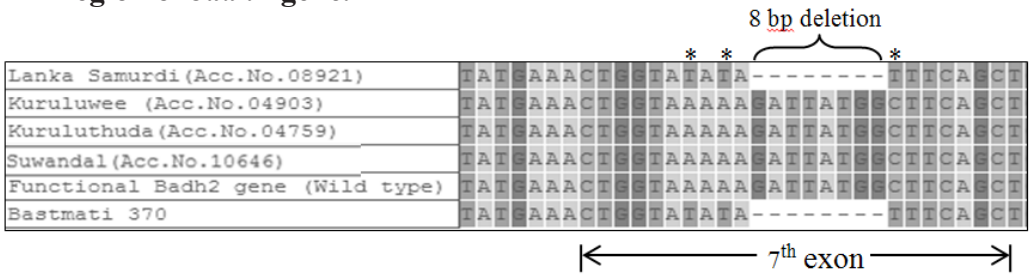

Figure 02: Partial profile of multiple sequence alignment of the 7th exon region of the *badh2* gene in "Lanka Samurdi" (At 405) (Acc.No.08921), "Kuruluwee" (Acc. No.04903), "Kuruluthuda" (Acc.No.04759), and "Bastmati 370". * indicates 3 SNPs

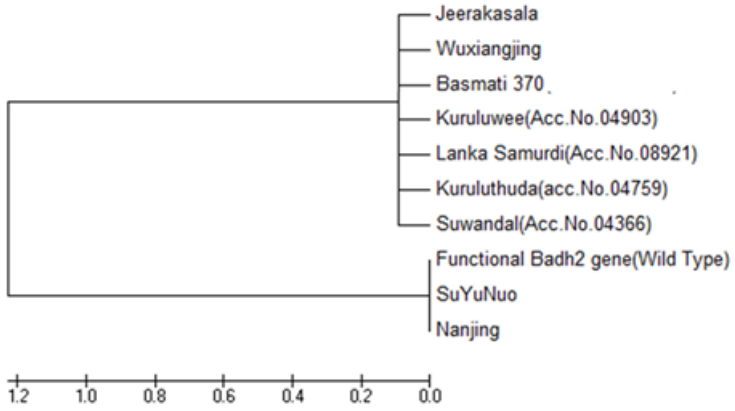

Figure 03: Dendrogram showing the phylogenetic relationship between Sri Lankan rice varieties and exotic rice varieties based on the 7th exon region of *badh2*

CONCLUSION

The sequence of 463-bp sized DNA fragment covering 7th exon region of *badh2* from Sri Lankan rice with positive control variety Basmati 370 were amplified and the fragments were sequenced. The multiple sequence alignment showed that "Lanka Samurdi" (Acc. No. 08921) possessed a mutation of an 8-bp deletion (5'-GATTATGGC-3') as that of exotic "Basmati 370", which is reported to be the mutation leading to the fragrance in rice. However, other traditional Sri Lankan fragrant rice varieties did not exhibit the above mutation in the 7th exon and hence, it can be assumed that their aroma is caused by some other genetic loci or another mutated allele of *badh2*.

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