

SHORT COMMUNICATION

Identification of fragrant gene, *fgr*, in traditional rice varieties of Sri Lanka

N.S. Kottearachchi*, E.G.D. Priyangani & D.P.S.T.G. Attanayaka

Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila.

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Abstract: A study was conducted to identify traditional rice varieties of Sri Lanka that possess the fragrant gene (*fgr*) using designed allele specific markers based on the gene sequence of mutated version of Betaine Aldehyde Dehydrogenase 2 (BAD2), which is called fragrant gene. Fifty six rice accessions including several Basmati, Suwandal, Samba and Heenati varieties, were chosen for the polymerase chain reaction (PCR) amplification with *fgr* would markers. Three fragments that would discriminate homozygous fragrant, homozygous non-fragrant and heterozygous individuals were amplified by multiplex PCR and the fragrance was evaluated phenotypically by potassium hydroxide (KOH) treatment. All positive accessions that amplified the *fgr* allele expressed the fragrance with KOH treatment. Of the traditional varieties, one accession each from Suwandal and Samba and, two accessions from Heenati were identified as the *fgr* genotypes that possessed mutated BAD2. Information from the investigation could be applied in breeding studies on aromatic rice suitable for the Sri Lankan environment, using marker assisted selection with local germplasms.

Keywords: Fragrant gene, marker assisted selection, *Oryza sativa* L., polymerase chain reaction.

INTRODUCTION

In Sri Lanka the rice breeding programmes of the past have laid much emphasis on increasing the yield potential without considering quality characteristics. Therefore, Sri Lanka imports high quality rice from other Asian countries, and in 2008, 5500 mt of Basmati type rice was imported from Pakistan under the free trade agreement¹. Importation of high valued Basmati type fragrant rice has been a requirement to support the tourist industry in the country. With the rapid improvement of people's living standards, fragrant rice is becoming more and more popular in the world rice market.

Among more than 100 volatile flavour compounds which contribute to rice aroma, 2-acetyl-1-pyrroline (2-ACP) has been identified as the primary cause for the distinctive fragrance in Basmati and Jasmine rice²⁻⁴. The linkage mapping studies²⁻⁶ have previously determined that the gene responsible for fragrance (*fgr*) or the causal factor for the elevated level of 2-ACP in rice, is located on chromosome 8. By comparison of the sequences of fragrant and non-fragrant genotypes it was found that an eight base pair deletion and three Single Nucleotide Polymorphisms (SNPs), in the betaine aldehyde dehydrogenase 2 (BAD2) enzyme, was the causal factor for the fragrance in Jasmine and Basmati rice³. Non-fragrant rice varieties appear to possess a fully functional copy of the gene encoding BAD2 enzyme. As the fragrant varieties possess the *fgr* or the mutated allele of BAD2, which is the cause for the loss of function in BAD2 enzyme, it is considered as a recessive gene. Loss of activity of BAD2 enzyme changes the direction of the biochemical pathway, which leads to the accumulation of 2-ACP. Rice along with many plant species possesses two BAD homologues, BAD1 and BAD2. The gene for BAD1 is located on chromosome 4 and it expresses a functional enzyme in both fragrant and non-fragrant rice varieties⁷.

Usually, the rice fragrance is determined by a number of sensory methods that have many limitations. Smelling of the leaves or grains after cooking is subjective and not always reliable as the ability to distinguish between fragrant and non-fragrant samples weakens with each successive analysis. Chemical methods involving smelling leaf tissue and grains after heating with water or reacting with KOH or I₂-KI⁸ solutions are mostly practiced but they might cause damage to the nasal passages and

*Corresponding author (nishagen@go.com)

senses become saturated after few analysis. Moreover all of these methods depend on personal variations. Identification of 2-ACP using gas chromatography is a reliable method, but expensive, time consuming and requires large samples of tissue which is not practical at early stages of the breeding programmes. Hence, identification of fragrance using molecular markers has advantages over traditional methods because they can be adopted to use in early seedling stages avoiding the effects of individual variations.

In Sri Lanka, only two high yielding aromatic rice varieties, namely Lanka Samurdi and At306 have been bred up to date. Unfortunately, aromatic rice cultivars, particularly Basmati rice from India and Pakistan often produce low yields because of low resistance to rice diseases and limited adaptation to a new environment⁶. Therefore, development of rice varieties suitable for the local environment with a fragrant trait is a current requirement. This can be easily achieved by introducing *fgr* gene of Basmati into local Sri Lankan rice varieties using MAS (Marker Assisted Selection). This study was conducted to discriminate fragrant and non-fragrant genotypes using *fgr* specific markers, and to investigate local traditional varieties that possess *fgr* to be used in breeding rice varieties suitable for local environments.

METHODS AND MATERIALS

Experimental site and planting material: This study was undertaken in the year 2008 at the Department of Biotechnology of the Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila. A total of 56 accessions (Table 1) was tested including Basmati, Suwandal, Samba, Heenati and other locally improved rice varieties. Seeds of all accessions were obtained from the Gene Bank of the Plant Genetic Resources Center (PGRC), Department of Agriculture, Gannoruwa, Peradeniya.

PCR Amplification: Leaves of two-week old rice seedlings planted on wetted petri dishes were used for the extraction of genomic DNA using a previously reported method⁹. A total volume of 15µL of PCR mixture consisting of 5µL of extracted DNA, 1.5µL of 10X PCR buffer, 1.2µL of 25m M deoxyribonucleotide triphosphates (dNTPs), 1µL (20 pmol/µg) of 4 types of primers (Hokkaido Systems Co Ltd., Japan) and 0.2µL of *Taq* DNA polymerase (Sigma, USA), were used for each amplification. The sequences³ of the primers used were; External Sense Primer (ESP): TTGTTTGGAGCTTGCTGATG, Internal Fragrant Anti-sense Primer (IFAP): C A T A G G A G C A G C T G A A A T A T A T A C C , Internal Non-fragrant Sense Primer (INSP):

CTGGTAAAAAGATTATGGCTTCA and External Anti-sense Primer (EAP): AGTGCTTTACAAGTCCCGC. Amplification was performed in a T-Cy thermal cycler (CreaCon Technologies, The Netherlands) under the temperature profile consisting of an initial denaturing at 95°C for 5 min followed by 35 cycles of 1 min at 95°C, 30 s at 58°C (primer annealing), 1 min at 72°C and final cycle of 5 min at 72°C. Amplified PCR products (5µL) were electrophoresed in 1.3% agarose gel containing 0.5µg/mL ethidium bromide. PCR amplification was repeated twice for the accessions that showed either the fragrant allele or fragrant phenotype, to confirm the particular data.

Fragrance evaluation: Three seeds from each accession were planted in 15 x 30 cm sized black polythene bags under standard management practices in the net house. Fragrance was evaluated according to the slightly modified method described by Sun *et al.*¹⁰. When the plants were at tillering stage, 1g of leaves was excised, cut into small pieces and incubated with 2.5 mL of 0.5% KOH in a petri dish at room temperature. After 15 min of incubation, samples were opened one by one and the odour was smelled by a three member panel. Evaluation was conducted batch wise, randomly, in order to avoid desensitization and evaluation of each sample was repeated twice. Smelled odour was categorized as fragrant if it was similar to the odour given by the positive control sample, Basmati-India (Acc.No. 11916).

RESULTS

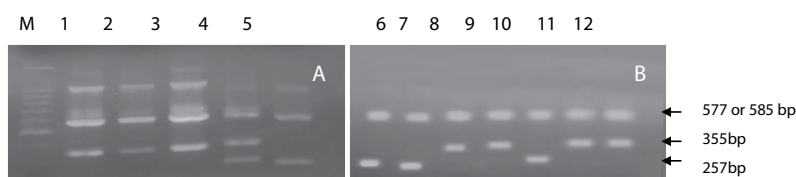
In this study, external primers, ESP and EAP generated a fragment of approximately 580bp as a positive control in all 56 samples (Figure 1 A and B). Internal primers, INSP and IFAP have given two bands when paired with external primers, ESP and EAP. IFAP primer sequence has been designed specifically for the *fgr* gene which gives fragrant genotype and INSP primer sequence has been designed specifically for the wild type sequence of *fgr* locus which gives non-fragrant genotype³. Accordingly, as shown in the Figure 1-A and B, ESP and IFAP primer pair amplified a 257bp band showing the marker for fragrant genotype. Lanka Samurdi (Acc.No.8921) in Figure 1-A and Basmati-India (Acc. No.7018), Suwandal (Acc.No.4802) and Kaluheenati (Acc.No.3989) in Figure 1-B indicated the homozygous fragrant genotypes because they amplified 257bp band. EAP and INSP primer pair amplified a 355bp band showing a marker for non-fragrant genotype. Lane 1-3 of Figure 1-A and lane 8-9 and 11-12 of Figure 1-B revealed the homozygous non fragrant genotypes because of the presence of the 355bp amplified band. Lane 4 of Figure 1-A which contained Basmati (Acc.No.11127) indicated

Table 1: Identification of the allele category based on the PCR amplification using *fgr* markers

Acc.No.	Variety	Genotype (+/-)*	Phenotype (+/-)#	Acc.No.	Variety	Genotype (+/-)*	Phenotype (+/-)#
Basmati				11041	Kalu Heenati	-	-
07018	Basmati Indian	+	+	10728	Kalu Heenati	+	+
11916	Basmati Indian	+	+	3851	Kalu Heenati	-	-
06775	Basmati Indian	-	-	4086	Kalu Heenati	-	-
07030	Basmati Nepal	+	+	4991	Kalu Heenati	-	-
06944	Basmati Pakistan	-	-	5484	Kalu Heenati	-	-
004920	Basmati Sri Lankan	-	+	3989	Kalu Heenati	+	+
006152	Basmati	+	+	12095	Kalu Heenati	-	-
11127	Basmati	+/-	-	3264	Heenati	-	-
11915	Pusa Basmathi	+	+	2087	Heenati	-	+
10984	Subramanium Basmathi	-	+	2098	Heenati	-	-
				6402	Heenati	-	-
Suwandal							
4366	Suwandal	-	-	4377	Wanni heenati	-	-
5420	Suwandal	-	-	Others			
4471	Suwandal	-	-	6316	Hatapaduru vee	-	-
4197	Suwandal	-	-	6279	Murungakayan	-	-
10729	Suwandal	-	-	6286	Suli	-	-
4802	Suwandal	+	+	11599	<i>Oryza nivara</i>	-	-
10646	Suwandal	-	+	10637	Jasmine	-	-
				4655	Rathal	-	-
Samba							
3507	Suwanda Samba	+	+	4759	Kurulu Thuda	-	+
2199	Suwanda Samba	-	-	4844	Koththamalli	-	-
9629	Suwanda Samba	-	-	6278	Mudukirial	-	-
4065	Heen Samba	-	-	4361	Suduru	-	-
3910	Heen Samba	-	-	6376	Dahanala	-	-
4239	Heen Samba	-	-	Improved			
4692	Seera Samba	-	-	11030	At306	+	+
3196	Samba	-	-	2762	Bw265	-	-
				4483	Bg400- 1	-	-
Heenati							
4124	Maha heenati	-	-	8921	Lanka Samurdi	+	+
11089	Kalu heenati	-	-	8920	Bg360	-	-

* (+) Presence of fragrant allele, (-) Absence of fragrant allele, (+/-) Heterozygous genotype

(+) Expression of fragrance by KOH treatment, (-) Absence of fragrance by KOH treatment

**Figure 1:** PCR profile generated from several rice varieties using *fgr* markers

A: Lane M:100 bp ladder DNA marker. Lane 1: Suwandal (Acc.No. 4802) Lane 2: Kalu Heenati (Acc. No.12095) Lane 3: Jasmine (Acc.No.10637), Lane 4: Basmati (Acc.No. 11127), Lane 5: Lanka Samurdi (Acc. No.8921)

B: Lane 6: Basmati-India (Acc.No.7018), Lane 7: Suwandal (Acc.No. 4802), Lane 8: Basmati-Pakistan (Acc.No.6944), Lane 9: Koththamalli (Acc.No.4844): Lane 10: Kaluheenati (Acc.No.3989), Lane 11: Rathal (Acc.No.4655), Lane 12: Kuruluthuda (Acc.No.4759)

heterozygous genotype as this lane contained both 257bp and 355bp bands.

The odour type given by the smelling of chopped leaves with 0.5%KOH was categorized as fragrant or non-fragrant as shown in Table 1. Of 10 types of Basmati tested, Subramaniam Basmati and Basmati-Sri Lanka did not amplify the 257bp fragrant band although they expressed fragrance when smelling. Five Basmati accessions demonstrated homozygous *fgr* genotype, showing only 257bp allele and they also expressed aroma phenotypically with KOH treatment. Basmati type with Acc.No.11127 revealed that it is not a pure line for the fragrance as it expressed both 257bp and 355bp bands, the heterozygous allele combination. As expected this line did not produce any fragrance with KOH treatment because of the recessive trait. Other two Basmati accessions, Acc.No.06944 and Acc.No.06775 produced neither *fgr* genotype nor fragrance phenotype even though they were labelled as Basmati.

One Suwandal accession, Acc.No.4802, one Samba accession, Acc.No.3507 and two Heenati accessions, Acc.No.10728 and Acc.No.3989 exhibited *fgr* genotype, and they did express fragrance phenotype with KOH treatment. One Suwandal accession, Acc.No.10646, one Heenati accession, Acc.No.2087 and Kurulu Thuda accession, Acc.No.4759, did not exhibit *fgr* gene although they were positive for the KOH treatment.

DISCUSSION

As multiplex PCR was conducted with four primers in a single PCR mixture, all polymorphic outcomes observable in one lane of agarose gel. This method gives an advantage by saving time and chemicals without compromising test utility for use by rice breeders in genotypic screening programmes for fragrance on a mass scale.

Of the tested improved varieties, only Lanka Samurdi and At306 exhibited 257bp *fgr* allele. Lanka Samurdi was developed in Sri Lanka, by crossing At402 variety and Basmati442 variety¹². At306, a popular aromatic commercial variety in Sri Lanka, was developed by crossing At5 with IR49517-41-1-6-2-3. Development of a fragrant line takes nearly a decade as it needs much screening and field trials (*Personal communication, B.D. Pathinayake*). Therefore, the assessment system used in this study will be useful for the development of fragrant rice within a short time period.

There is no evidence to prove that the fragrance is a quantitative trait locus (QTL) because the fragrance assessed by gas chromatography does not exhibit

normal distribution. Instead, the populations are distributed in a discrete manner^{4,7}. Five traditional rice accessions expressed 2-ACP odour when compared with the positive controls, although it was not possible to prove the presence of the *fgr* allele at BAD2 locus. The markers used in this study were developed based on the 8bp deletion located at the 7th exon of BAD2³. A previous study⁷ also reported that the 8bp deletion in *fgr* gene may not be the only cause for aroma and they suggested that at least one other mutation may drive the accumulation of 2-ACP. They found that several aromatic accessions containing 2-ACP, at the Genetic Resources Center of the International Rice Research Institute, collected from different countries, did not possess 8bp deletion in the BAD2. Accordingly, Sri Lanka has been mapped in the region having aromatic rice varieties lacking the *fgr* gene. Proving this information, later, a study¹¹ discovered another new fragrant allele causing the fragrant phenotype. The new fragrant allele has 7bp deletion in exon 2 of the same BAD2 homologue. It is not known whether the accessions that did not amplify the 257bp band in this study, but exhibited fragrance when smelling, contain null *fgr* allele in exon 2 or another mutated gene which increases the level of 2-ACP. Hence these accessions should be studied further to give some insight into the understanding of the aroma genes in *Oryza sativa* L.

In conclusion, all accessions that contained 8bp deletion at the 7th exon of BAD2 or *fgr* allele were positive for KOH treatment. Hence, breeders can be encouraged to utilize the discrimination procedure of fragrance and non-fragrance using ESP, IFAP, INSP and EAP markers. They can easily incorporate the *fgr* gene when improving rice varieties by MAS by selecting the fragrant lines at early breeding cycles. One Suwandal accession, one Suwanda Samba accession and two Heenati accessions, possessing *fgr* gene were identified among the Sri Lankan traditional rice varieties. Now breeders can utilize these accessions in their breeding programmes where Basmati varieties are difficult to be used. Moreover, these traditional varieties suitable for the native environment may have other beneficial characters that Basmati varieties do not possess.

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