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ANALYSIS AND COMPARISON OF FRAGRANT GENE SEQUENCE IN SOME RICE CULTIVARS

Noushafarin KARAMI[A], Ali AALAMI[B], Habibollah Samizadeh LAHIJI[C], Babak RABIEI[D] and Mehrzad ALAHGHOLIPOUR[E]

[a,b,c] and [d] Department of Agronomy and Plant Breeding, Faculty of Agricultural sciences, University of Guilan, Rasht, Iran
[e] Department of Plant Breeding, Rice Research Institute of Iran (RRII), Rasht/Iran

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It is known that the fragrant trait in rice (*Oryza sativa* L.) is largely controlled by *fgr* gene on chromosome 8 and it has been specified that the existence of an 8 bp deletion and three single nucleotide polymorphism (SNP) in exon 7 is effective on this trait. In this study, sequence alignment analysis of *fgr* exon7 on chromosome 8 for 11 different fragrant and non-fragrant cultivars revealed that 5 aromatic rice cultivars carried 3 SNPs and 8 bp deletion in exon7 which terminates prematurely at a TAA stop codon. However, 5 of the non-aromatics showed a sequence identical to the published Nipponbare, being non-fragrant Japonica variety sequence. An exception among them was Bejar, which had 8 bp deletion and 3SNPs but it was non-aromatic. Sequencing can determine nucleotide alignment of a gene and give beneficial information about gene function. *In silico* prediction showed proteins sequences alignment of *fgr* gene for Khazar and Domsiah genotypes were different. Betaine aldehyde dehydrogenase complete enzyme belongs to Khazar non-fragrant genotype that has complete length and 503 amino acids while non-functional BADH2 enzyme for Domsiah fragrant genotype has 251 amino acids that result in accumulate 2-acetyl-1-pyrroline (2AP) and produces aroma in fragrant genotypes.

*Key words***:** 2-acetyl-1-pyrroline (2AP), Betaine aldehyde dehydrogenase (BADH2), Exon 7, *Fgr* gene, Sequencing

INTRODUCTION

Fragrance is an important constituent for high-quality rice (*Oryza sativa* L.) varieties (BHATTACHARJEE *et al.*, 2002). Genetic analysis shows that a single recessive gene (*fgr*) on chromosome 8 is associated with rice fragrance while the dominant *Fgr* allele is associated with

Corresponding author: Ali Aalami, Department of Agronomy and Plant Breeding, Faculty of Agricultural sciences, University of Guilan, Iran. P.O. Box: 41635-1314/ Rasht/ Iran, E-mail address: ali_aalami@guilan.ac.ir

lack of fragrance. Also, betaine aldehyde dehydrogenase gene, *BADH2*, as the candidate locus responsible for aroma, presented exactly the same mutation as that identified in Basmati and Jasmine-like rice. This gene is a defective allele of a gene encoding betaine aldehyde dehydrogenase (BADH2). The deletion observed in exon 7 of this (*BADH2)* gene generates a premature stop codon and presumably results in loss of activity. It was hypothesized that loss of BADH2 activity would cause 2-acetyl-1-pyrroline (2AP) accumulation and produce aroma in rice (BOURGIS *et al*., 2008). After genetic mapping and sequence analysis, it was suggested that a gene encoding putative BADH2 would most likely be the *fgr* gene due to its sequence divergence between fragrant and non-fragrant rice varieties. Furthermore, the *BADH2* alleles from fragrant rice varieties all having common insertions/deletions and single nucleotide polymorphisms compared with those from non-fragrant genotypes demonstrate a common ancestor for all fragrant genotypes (BRADBURY *et al.,* 2005b). The availability of rice genome sequences provided an opportunity to discover the gene responsible by comparing the sequences of aromatic and non-aromatic genotypes (GOFF *et al.,* 2002; IRGSP 2005; LANG & BUU**,** 2008; JIN *et al.,* 2003; SARHADI *et al*., 2007; KIBRIA *et al*., 2008), it allows us to target resequencing of genes and genome sequencing in fragrant genotypes to the most likely regions of chromosome 8 (SARHADI *et al*., 2007; BRADBURY *et al*., 2005b). Sequencing of *BADH2* in all cases have indicated an 8 bp deletion within the gene that resulting in a loss of function. The aromatic compound, 2-acetyl-1-pyrroline was discovered in rice in 1983. Whereas, the gene controlling the accumulation of 2AP has only recently been identified by map-based cloning (VANAVICHIT and YOSHIHASHI, 2010).

SHI *et al.,* (2008) reported the discovery of a new *badh2* allele and the development of functional markers for the *badh2* locus. A total of 24 fragrant and 10 non-fragrant rice varieties were studied and sequenced for their *Badh2/badh2* loci. Of the 24 fragrant rice varieties, 12 were found to have the known *badh2* allele (*badh2-E7*), which has an 8bp deletion and three SNPs in exon 7; the others had a novel null *badh2* allele (*badh2-E2*), which has a sequence identical to that of the *Badh2* allele in exon 7, but with a 7 bp deletion in exon 2. They suggested that both null *badh2* alleles are responsible for rice fragrance. KUO *et al*., (2005) by using 19 aroma and 7 non-aroma rice varieties cloned and sequenced the *BADH2* gene. Their sequencing results were diverse from most of the published varieties in which the aroma traits were recessively controlled. They indicated that deletion in the *BADH2* gene for mechanism of aroma synthesis was not universal to all aroma rice varieties and aroma rice of different origins may have various regulations or mechanism. CHEN *et al.,* (2008) reported the presence of a dominant *BADH2* allele encoding BADH2 inhibits the synthesis of 2-acetyl-1-pyrroline. They are two recessive alleles, *badh2-E2* and *badh2-E7* inducing 2AP formation. Multiple *BADH2* transcript lengths were detected and the complete, full-length *BADH2* transcript was much less abundant than partial *BADH2* transcripts. These results indicated that the full-length BADH2 protein encoded by *BADH2* rendered rice non-fragrant by inhibiting 2AP biosynthesis. In summary, these data supported the hypothesis that *BADH2* inhibits 2AP biosynthesis.

In a prior study SRIVONG *et al*., (2008) with sequence analysis of exon 7 of *BADH2* gene of the Thai aromatic rice cultivars, revealed significant sequence polymorphisms containing a total of 3 SNPs and 8bp deletion, whereas, Thai non-aromatic rice did not. This kinetic analysis indirectly suggested loss of *BADH2* function in aromatic rice, which played important role in aroma synthesis in rice.

With the availability of molecular maps and genome sequences, a major gene for fragrance (*BADH2*) was identified on chromosome 8. An 8bp deletion in the exon 7 of this gene

was reported to result in truncation of BADH2 enzyme whose loss of function led to the accumulation of a major aromatic compound, 2AP in fragrant rice. However, several studies have reported exceptions to this mutation and indicated the involvement of other genetic loci in controlling fragrance trait. These studies emphasized the need to characterize the fragrance and its underlying factors in a wide range of genetic resources available for this trait (SAKTHIVEL *et al*., 2009; SARHADI *et al*., 2007; SHI *et al*., 2008; BRADBURY *et al*., 2005b). According to above sentences, in this study we analyzed the sequence of major and key gene controlling aroma in rice which is *fgr* on chromosome 8. Also, analysis of its amino acid sequence alignment in some fragrant and non-fragrant rice cultivars was done. The alignment was produced using the bio software and compared with its published sequence.

MATERIALS AND METHODS

Based on the existence of an 8 bp deletion (5'- GATTATGG -3') and three SNPs in the exon 7 of *fgr* gene on chromosome 8 and its introduction as main reason of aroma in fragrant genotypes, the fragrant gene sequence and also its exon 7 sequence were obtained from the NCBI web site (www.ncbi.nlm.nih.gov) Gen Bank accession number – NC008401. A primer pairs with sequence of 5'- TTTTTCCACCAAGTTCCAGTG -3' and 5'- TGAGAATCATGTTCGGGATG -3' were designed using Primer 3 Version 4.0(htpp://frodo. wi. mit. edu/ Primer 3/)to amplify fragment of genome which contains the above polymorphisms. Eleven Iranian rice cultivars consist: Taromdailamani, Alikazemi, Domsiah, Binam, Fajr, Khazar, Nemat, Sefidroud, Kadus, Sahel and Bejar (The genotypes are available in IRRI as well) were sown in pot and Genomic DNA was extracted from rice fresh young leaves using cetyltrimethylammonium bromide (CTAB) method of MURRAY and THOMPSON (1980), with minor modifications. PCR reactions using each genomic DNAas templates were carried out to amplify partial *BADH2* sequences using primers. In detail, a total of 10µl PCR reaction mixture was composed of 1X PCR buffer, 0.4 mM dNTPs mix, 2 mMMgCl, 0.4 µM of each primer, l U of Taq DNA polymerase (Bioflux Biotech), and 50 ng of template DNA. PCR amplification was carried out in the following conditions: one cycle at94°C for 4 min, 35 cycles (94°C for 40 s, 62°C for 40 s and 72°C for 2 min) and one cycle at 72°C for 5 min. PCR reactions were carried out on thermocycler (Astec, Japan). PCR products were analyzed by 1%agarose gel electrophoresis and visualized by Electrophoresis Gel Photo documentation System (Bio-Rad 2000, Germany).

The PCR products were sequenced (Faza biotech Co.) and sequences were aligned using Clustal W (http://www.genome.jp/tools/clustalw/) and Multalin (http://multalin. toulouse. inra. fr/multalin. p1), SNPs were identified by visual inspection of the alignments. Also, aroma in leaves was determined according to the method described by SOOD and SIDDIQ (1978), When the plants were forming side-shoots (*i.e.*, at the tillering stage) and grains aroma was evaluated with KOH method (BOURGIS *et al.*, 2008).

RESULTS AND DISCUSSION

Aroma phenotypic evaluation with KOH method divided the cultivars into aromatic and non aromatic groups. Taromdailamani, Alikazemi, Domsiah, Binam and Fajr were aromatic while Khazar, Nemat, Sefidroud, Kadus, Sahel and Bejar were non- aromatic. The sequence of target gene (*BADH2*) on chromosome 8 of *japonica* rice (*Oryza sativa japonica* cultivar Nipponbare) was obtained from the NCBI web site (http:// www. ncbi. nlm. nih. gov, gene = LOC_

Os08g0424500) Gen Bank accession number - NC008401. On the basis of the rice physical genome map, size of this gene sequence on chromosome 8 is 6153 bp and resides between 20372582 bp and 20378734 bp. This gene has 15 exons and 14 introns (Fig. 1) and encodes BADH2 protein with 503 amino acids (accession number NP001061833). The site-specific primer pair for exon 7 was designed to amplify the *BADH2* polymorphic region reported by BRADBURY *et al.*, (2005b) produced a single DNA fragment of approximately 752 base pairs (Fig. 2).The PCR products sequencing and japonica rice cv. Nipponbare as non aromatic genotype reference were aligned using Multalin (Fig. 3).

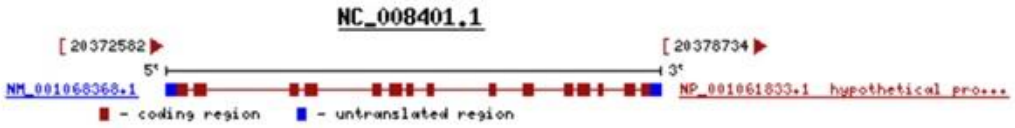

Fig. 1. Characterization of rice aroma gene (*Oryza sativa japonica* cultivar Nipponbare) from the NCBI web site

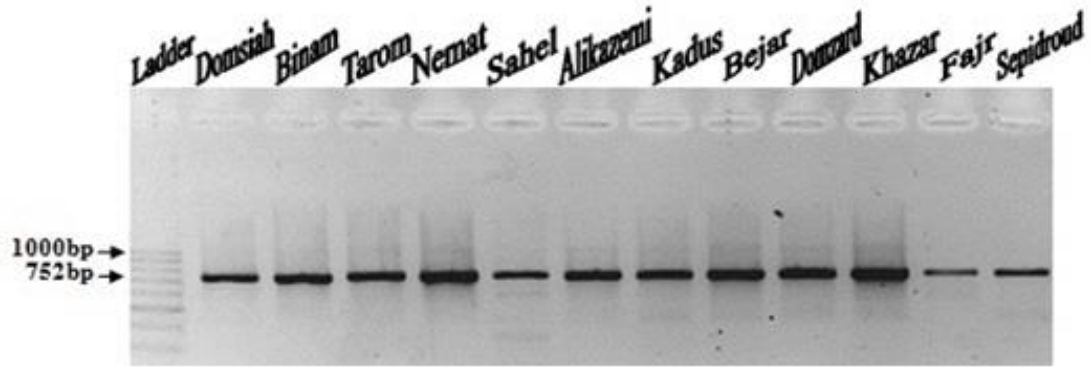

Fig.2. Agarose gel electrophoresis of partial *BADH2* gene fragments amplified by PCR with Exon7 primers and studied cultivars. 100 bp DNA ladder.

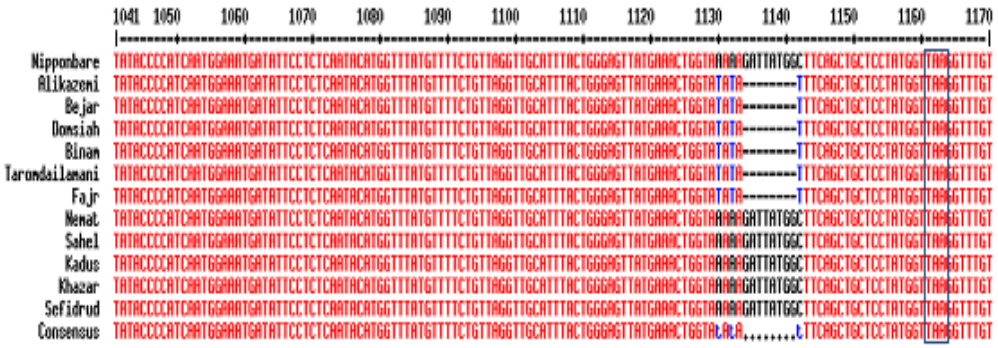

Fig.3. Partial of the betaine aldehyde dehydrogenase 2 gene (*BADH2*) sequence alignments in studied genotypes. The rectangle indicates the TAA stop codon.

Variation of the *BADH2* gene sequence in aromatic and non-aromatic rice

The sequences alignment from Khazar, Nemat, Sefidroud, Kadus and Sahel revealed an identical sequence polymorphism pattern with the non-aromatic variety Nipponbare sequence. Our data showed that the five aromatic varieties contain a large deletion and one single nucleotide polymorphism (SNP) in exon 7 which terminated prematurely at a TAA stop codon. Our alignment findings were in agreement with that published by BRADBURY *et al.,* (2005b). This implies that the aromatic trait in fragrant rice is caused by the loss of function of *BADH2*, although further complementary tests are essential to make the final conclusion. There was an exceptional in sequencing results, thereby Bejar which in phenotypic analysis had been identified as non-aromatic genotype, in sequencing process of *fgr* exon 7 contained 8 bp deletion. BOURGIS *et al.,* (2008) explained that although many convergent data accumulate to give a major role to the *BADH2* locus in the presence/absence of 2AP, this trait remains at a phenotypic level, a quantitative character that is largely dependent on environmental conditions and genetic background. Thus they suggested that 2AP synthesis had a polygenic aspect. SUN *et al.,* (2008) designed a primer pair SF6 to amplify and sequence the *BADH2* polymorphic region reported by BRADBURY *et al.,* (2005b). The results of PCR products sequencing of non-aromatic varieties revealed an identical sequence to the published Nipponbare sequence but it was different from aromatic ones. Three aromatic varieties indicated 8 bp deletion and one SNP in exon 7 which terminated prematurely at a TAA stop codon. This polymorphism agreed with all cultivars using in our study except for Bejar. SAKTHIVEL *et al*., (2009) stated that the results show the genotypes that do not carry the mutation and have low 2AP indicate neither 2AP nor *BADH2* could be the cause of fragrance but they indicate the involvement of different gene(s)/loci altogether. It is also possible that the presence of modifiers and the genetic background of the variety may play a role in producing different or modified aroma from the typical popcorn-like aroma produced by 2AP.

BRADBURY *et al.,* (2005b) suggested that the different flavours perceived in some genotypes may be due to the modifying influence of other secondary metabolic consequences of 8bp mutation in different genetic backgrounds. This reason can be in agreement with Bejar genotype. However, this explanation cannot be applied to all or at least to these 'exceptional' fragrant varieties, as they do not have the mutation at all and are still fragrant.

The diversity of this gene has been studied in a large collection of varieties and the results showed that an 8 bp deletion in the seventh exon of BADH2 causing a reading frame shift was present in most aromatic accessions, but some other less frequent mutations associated with aroma were also detected (BRADBURY *et al*., 2005b; BOURGIS *et al*., 2008; SHI *et al*., 2008; KOVACH *et al*., 2009; SAKTHIVEL *et al*., 2009).

The consequences of function loss of an enzyme

The functional *BADH2* gene consists of 15 exons and 14 introns with start codon (ATG) in exon 1 and stop codon (ATT) in exon 15 (SHI *et al.,* 2008). It was reported that the 8bp deletion and 3 SNPs in exon 7 of *BADH2* have led to the introduction of premature stop codon to produce a truncated protein which result in abrogation of the function of the enzyme BADH2 consequently accumulate substrate 2AP in fragrant varieties, while the functional *BADH2* gene codes for a 503 amino acid mature protein which consumes the substrate in non-fragrant varieties (SAKTHIVEL *et al.,* 2009). Several mapping studies have independently identified *BADH2* as the candidate gene responsible for fragrance (BRADBURY *et al.,* 2005a, b; VANAVICHIT *et al.,* 2006; AMARAWATHI *et al.,* 2008; SHI *et al.,* 2008) and sequencing of *BADH2* in each case found a deletion within the gene which would render the gene non-functional.

As shown in Fig. 3, the deletion of 8 bp in exon 7 is a couple deletion in the number of nucleotides, whereas the genetic codes are nucleotide triplets; it's natural this deletion at upstream of sequence results in frame shifts and changes in translation procedure. Therefore, after 18 base pairs it disrupts reading frame into TAA which is a premature stop codon and leads to truncate BADH2 protein and loses its function. Hence, for analysis of the probable protein and the effect of this large deletion, sequences of two fragrant and non-fragrant cultivars exon 7, Domsiah and Khazar respectively translated to protein using Expasy biosoftware. The proteins encoded by the exon 7 of *BADH2* gene which contains the deletion like Domsiah and the complete ones, Khazar are shown in Fig. 4.

After assembling of sequences for two normal and non-functional genes, protein sequences were aligned using Clustalw program. The intact 503amino acid BADH2 protein encoded by the complete *BADH2* gene inhibits 2AP synthesis and thus renders rice non-fragrant but non-functional enzyme of BADH2 has 251 amino acids that results in 2AP accumulation and generates aroma in fragrant cultivars. In fig. 5 sequence of this protein in non-fragrant, Khazar and fragrant cultivars, Domsiah is indicated.

BRADBURY *et al*., (2005b) with sequence analysis of a 25 bp region in 14 diverse fragrant and 64 non-fragrant rice varieties found that the 14 fragrant varieties would show identical sequence polymorphism; also they offered this large polymorphism contained a total of six SNPs and eight deletions within a 25 bp region causing the introduction of a stop codon. It is very likely that this mutation would render the protein non-functional. It was hypothesized that loss of BADH2 activity would cause 2AP accumulation while the 64 non-fragrant varieties

showed a sequence identical to the published Nipponbare sequence. The present results in our study are in agreement with these findings.

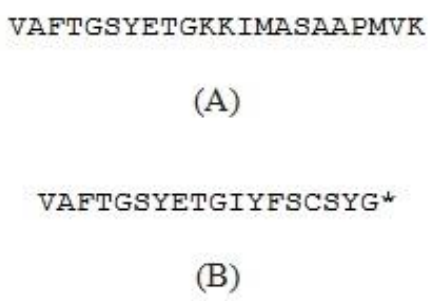

Fig. 4.Difference of amino acid sequences in deletion and SNP position for Domsiah (A) and Khazar (B) cultivars, Aromatic and non aromatic cultivars respectively.

For discussion and final analysis, we compared utilized phenotypic aroma evaluation and sequencing methods about 12 cultivars. It was recorded (+) for aromatic and (-) for non-aromatic rice (table 1).

Table 1. The comparison of aroma characteristics of 12 rice cultivars based on phenotypic analysis and sequencing results

No.	Cultivars name	phenotypic analysis†	sequencing result‡
1	Taromdailamani	+	+
2	Alikazemi	+	+
3	Domsiah	+	+
4	Binam	+	+
5	Fajr	+	+
6	Khazar	-	-
7	Nemat	-	-
8	sepidroud	-	-
9	Kadus	-	-
10	Sahel	-	-
11	Bejar	-	+

†&‡: Fragrant = +, Non-fragrant = -

SARHADI *et al.,* (2007) used conventional methods (tasting kernel and cooking test), 1.7% KOH sensory test and molecular marker analysis to distinguish between aromatic and non-aromatic rice cultivars from Afghanistan, Iran and Uzbekistan. Two of seven Iranian cultivars such as Fajr and Qaserdashti were found to be as aromatics, whereas Nemat, Kodus, Poya, Shiroudi and Doroudzan were non-aromatic. They found that the results of the 1.7% KOH test

were similar to those of the PCR analysis which will be described later. Cultivars classified as aromatic or non-aromatic rice by the molecular method and 1.7% KOH test well agreed with the conventional methods.

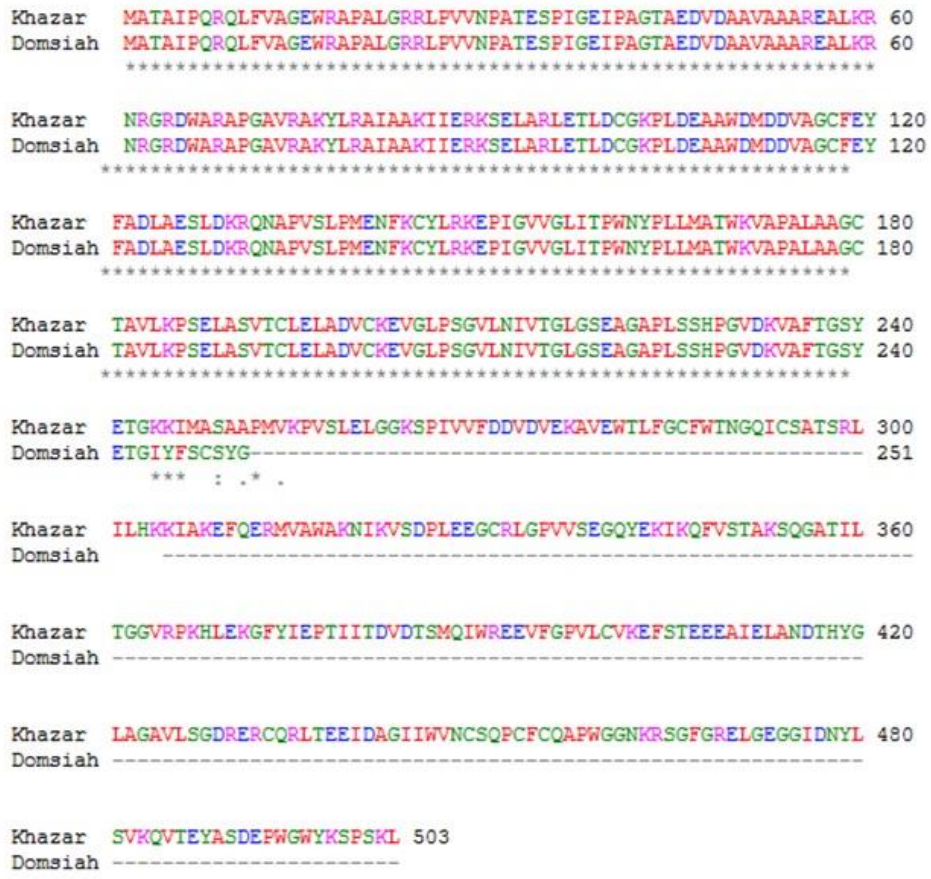

Fig. 5.ClustalW alignment of the amino acid sequences of betaine aldehyde dehydrogenase 2 (BADH2) protein from non-fragrant rice cultivar, Khazar, encoded on rice chromosome 8, and the predicted amino acid sequence of the truncated BADH2 protein from fragrant one, Domsiah.

CONCLUSION

By comparison of above methods we observed that phenotypic analysis included smelling leaf tissue in tillering stage (SOOD & SIDDIQ, 1978) and grains (BOURGIS *et al.*, 2008) that had been reacted with 1.7% KOH solution was confirmed by sequencing results. But in some cases it's possible the results of sensory tests do not agree with molecular methods because of its subjective nature and saturation of the analyst's sense over time in identity of fragrant or non-fragrant genotypes which it seems natural. An objective method of 2AP evaluation using gas

chromatography is available but the assay requires large quantities of tissue samples derived from more than one plant and is time consuming (LORIEUX *et al.,* 1996; WIDJAJA *et al.,* 1996). The GC–MS method, due to being expensive and time consuming, is only used to process a small number of samples to give an accurate evaluation of rice fragrance. It means that it can only be used in long term breeding programs. Given the limitations of these methods, molecular methods offers the advantages of being both objective and requiring small quantities of sample tissue which in turn allows accurate analysis of large numbers of individual plants in short term breeding program. Thus sensory and particularly molecular evaluations can be good complementary and even replaceable methods. Also primer designing on the base of effective SNPs for aroma trait (STS markers) can be valuable for easy and rapid screening of populations.

Using sequencing of target locus and comparison of that by aligning with published sequences in genome databases is an accurate method that can give beneficial information about nucleotide alignment in the target gene and also provides an opportunity to discover the association of a mutation with certain phenotype.

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ANALIZA I POREĐENJE FRAGRANT SEKVENCE GENA KOD NEKIH KULTIVARA PIRINAČA

Noushafarin KARAMI[a], Ali AALAMI[b], Habibollah Samizadeh LAHIJI[C], Babak RABIEI[D] and Mehrzad ALAHGHOLIPOUR[E]

[a,b,c] and [d] Department of Agronomy and Plant Breeding, Faculty of Agricultural sciences,

University of Guilan, Rasht, Iran

[e] Department of Plant Breeding, Rice Research Institute of Iran (RRII), Rasht/Iran

Izvod

Poznato je da je osobina slatkog mirisa kod pirinča (*Oryza sativa* L.) velikim delom pod kontrolom *fgr* gena na hromozomu 8. Specificirano je da postojanje delecije veličine 8 bp i tri polimorfna nukleotida (SNP) u egzonu 7 utiču na tu osobinu. Korišćenjem sekvencioniranja ciljnog lokusa i poređenjem sa poblikovanim sekvencama u bazi podataka je pokazano da je metod pouzdan i da može da da korisne informacije o pravolinijskom rasporedu nukleotida u ciljnom genu kao i da obezbedi mogućnost otkrivanja asocijacija mutacija sa određenim fenotipom.

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