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ALLELIC VARIATIONS IN AROMA GENE IN CULTIVATED RICE VARIETIES

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ABSTRACT

Germplasm is a valuable source of genetic diversity that supports crop improvement efforts in any breeding programme but it must first be fully characterised for economically valuable traits before it can be effectively utilised. In rice (*Oryza sativa*), the development of new varieties with improved aroma requires correct phenotyping and prior knowledge of the available genes and alleles governing the aroma trait in the gene pool. Correct phenotyping and genotyping can be achieved using sensory methods and functional markers associated with polymorphisms that define the aroma genes. The objective of this study was to evaluate the aroma status of rice accessions and to assess for the various alleles of *badh2* gene using functional markers. A total of 56 rice accessions were evaluated at National Crops Resources Research Institute (NaCRRI) in Uganda for their aroma using sensory methods and a molecular marker to differentiate between aromatic and non- aromatic accessions. The aromatic accessions were then evaluated for variations within the betaine aldehyde dehydrogenase2 (*badh2)* gene responsible for aroma in rice using functional markers. Sensory evaluation of aroma identified 23 accessions to be aromatic; while 33 were non-aromatic. Molecular results identified 20 accessions as aromatic; while 36 accessions were non-aromatic. Functional marker analysis indicated the presence of *badh2-E7* allele in 20 aromatic accessions within this collection that could be employed in the breeding programme for the rice aromatic trait.

Key Words: *badh2 alleles*, functional markers, *Oryza sativa*

RÉSUMÉ

Le germoplasme est une source précieuse de diversité génétique qui soutient les efforts d'amélioration des cultures dans tout programme de sélection, mais il doit d'abord être entièrement caractérisé pour ses traits économiquement précieux avant de pouvoir être utilisé efficacement. Dans le riz (*Oryza sativa*), le développement de nouvelles variétés avec un arôme amélioré nécessite un phénotypage correct et une connaissance préalable des gènes et allèles disponibles régissant le caractère aromatique

dans le pool génétique. Un phénotypage et un génotypage corrects peuvent être obtenus en utilisant des méthodes sensorielles et des marqueurs fonctionnels associés à des polymorphismes qui définissent les gènes aromatiques. L'objectif de cette étude était d'évaluer le statut aromatique des accessions de riz et d'évaluer les différents allèles du gène *badh2* à l'aide de marqueurs fonctionnels. Un total de 56 accessions de riz ont été évaluées au National Crops Resources Research Institute (NaCRRI) en Ouganda pour leur arôme en utilisant des méthodes sensorielles et un marqueur moléculaire pour différencier les accessions aromatiques et non aromatiques. Les accessions aromatiques ont ensuite été évaluées pour les variations au sein du gène de la bétaïne aldéhyde déshydrogénase2 (*badh2*) responsable de l'arôme du riz à l'aide de marqueurs fonctionnels. L'évaluation sensorielle de l'arôme a identifié 23 accessions comme aromatiques; tandis que 33 étaient non aromatiques. Les résultats moléculaires ont identifié 20 accessions comme aromatiques; tandis que 36 accessions n'étaient pas aromatiques. L'analyse des marqueurs fonctionnels a indiqué la présence d'allèles *badh2-E7* dans 20 accessions aromatiques de cette collection qui pourraient être utilisées dans le programme de sélection pour le trait aromatique du riz.

Mots Clés: *badh2 alleles*, Marqueurs fonctionnels, *Oryza sativa*

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple crop and major source of income for many farmers in sub-Saharan Africa. Aromatic rice is preferred by consumers compared to non-aromatic rice (Asante*,* 2017) and it fetches a premium price, both locally and internationally (Calingacion *et al.,* 2014; Diagne *et al.,* 2017). Production of aromatic rice in Uganda is far below the market demand, hence traders have resorted to the adulteration of aromatic rice with non-aromatic rice so as to maximise profits (Vemireddy *et al.,* 2015). Given the increasing demand for aromatic rice, the proportion of imported aromatic rice is likely to increase unless local production of aromatic rice is increased (Masette *et al*., 2013). Breeding rice varieties with enhanced aroma by introducing aroma into non-aromatic elite varieties that are already well adapted by farmers, will augment the production of aromatic rice in Uganda.

Conventional breeding methods combined with selection of DNA markers, so called Marker Assisted Selection (MAS), is a fast and efficient strategy for the introgression of aroma trait (Golestan Hashemi *et al*., 2015). But this requires correct phenotyping of the breeding materials for aroma and understanding the genetic basis of aroma in these materials.

Aromatic rice emits a popcorn-like aroma from the leaves, grains and flowering organs at various stages of growth (Hinge *et al.,* 2016). Three approaches are commonly used by researchers to determine the presence or absence of aroma in rice. Firstly, sensory evaluation of aroma from leaves or grains using dilute KOH (Sood and Siddq, 1978). The sensory test for aroma from leaf tissue or grain after reacting with KOH is a cheap, convenient, rapid and reliable method for identification of aromatic and non-aromatic genotypes by breeding programmes (Yeap *et al.,* 2013).

Secondly, analysis of aroma in the grain using gas chromatography by measuring the amount of 2-Acetyl-1-Pyrroline (2AP), a compound that is responsible for aroma (Maravel *et al*., 2010; Daygon *et al*., 2017; Ocan *et al*., 2019). Finally, using DNA markers linked to aroma trait and Functional markers that target the polymorphism that define the functional and non-functional alleles of the aroma gene (Bradbury *et al*., 2005b). Functional markers provide a more efficient selection of desired genotypes compared to DNA markers located nearby but at functionally irrelevant site (Andersen and Lübberstedt, 2003). Functional markers are diagnostic of the desired trait allele (Varshney *et al.,* 2005)

and can be used for validation of cultivar identity, selection of parental materials to build segregation populations and subsequent selection of lines (Lübberstedt *et al.,* 2005).

Aroma in rice is caused by loss of function of the Betaine Aldehyde Dehydrogenase2 (BADH2) enzyme that is coded by the *badh2* gene found on Chromosome 8 (Bradbury *et al.*, 2005b). Loss of function of BADH2 enzyme leads to accumulation of 2AP. The *badh2* gene has 15 exons and 14 introns and mutations within this gene have been associated with the accumulation of 2AP which determines the aroma of rice (Table 1).

Initially an eight base pair (bp) deletion and a three Single Nucleotide Polymorphism (SNP) was found in exon 7 and named the *badh2-E7* allele (Bradbury *et al.*, 2005a). Evidence of the existence of additional mutations within the *badh2* gene was found in a study of several accessions from Asia that had elevated levels of 2AP, but did not carry the *badh2-E7* allele (Fitzgerald *et al.,* 2008), implying the existence of other alleles. A seven bp deletion in exon 2 was discovered (Shi *et al.*, 2008) and eight other mutations were also found to be associated with fragrance (Kovach *et al.*, 2009). An 803-bp deletion between exons 4 and 5 (designated as *badh2.E4-5*) was later discovered (Shao *et al.,* 2011). Another new allele (*badh2-p-5*′*UTR*) that has a 3-bp deletion in the*5*′untranslated region (UTR) and an 8bp insertion in the promoter (-1,314 site upstream from the initiation codon) was also discovered by Shi *et al*. (2014). In addition, 3bp deletion in exon 12 and an 8bp insertion in the promoter region of the *badh2* gene have also been associated with fragrance (He *et al.,* 2015; Bindusree *et al.,* 2017). Nineteen alleles of *badh2* gene have so far been reported, which account for the unique spectrum of fragrance found in aromatic rice varieties.

There is a wide collection of rice germplasm that have been introduced from various rice breeding centers around the world and are currently held at the National Crops Resources

TABLE 1. Summary of *badh2* alleles reported to be associated with aroma in rice

Alleles	Location	Sequence Divergence	Reference
badh2-E7	Exon 7	8bp deletion	Bradbury *et al.* (2005a)
badh2-E8	Exon 8	7bp insertion	Amarawathi *et al.* (2007)
badh2-E2.1	Exon 2	7bp deletion	Shi *et al.* (2008)
badh2-E1.1	Exon 1	2bp deletion	Kovach *et al.* (2009)
badh2-E10.1	Exon 10	1bp insertion	Kovach *et al.* (2009)
badh2-E10.2	Exon 10	1bp deletion	Kovach *et al.* (2009)
badh2-E10.3	Exon 10	G/T SNP	Kovach *et al.* (2009)
badh2-E13.1	Exon 13	3bp insertion	Kovach *et al.* (2009)
badh2-E13.2	Exon 13	C/T SNP	Kovach *et al.* (2009)
badh2-E14.1	Exon 14	1bp insertion	Kovach *et al.* (2009)
badh2-E14.2	Exon 14	G/T SNP	Kovach *et al.* (2009)
badh2-E4-5.2	Exon 4-5	803bp deletion	Shao *et al.* (2011)
badh2-E4-5.1	Exon 4-5	806bp deletion	Shao *et al.* (2013)
badh2-E2.2	Exon 2	75bp deletion	Shao *et al.* (2013)
badh2-E10.4	Exon 10	G/A SNP	Shao *et al.* (2013)
badh2-p-5'UTR	*5'UTR*	3bp deletion	Shi *et al.* (2014)
badh2-E1.2	Exon 1–intron 1 junction	G/A SNP	Ootsuka *et al.* (2014)
badh2-E12	Exon 12	3bp deletion	He and Park (2015)
badh2-p	Promoter region	8 bp insertion	Bindusree *et al.* (2017)

Research Institute (NaCRRI) in Uganda to support aromatic rice-breeding efforts. However, utilisation of this germplasm for rice improvement is constrained by limited knowledge of the phenotypic and genotypic diversity within this collection. For a breeding programme to be successful, it is important to have prior knowledge of the phenotypic and genotypic characteristics of important traits in the available germplasm. Attempts have been made to assess the aroma panel of some accessions using sensory methods and gas chromatography (Kanaabi *et al.,* 2018; Ocan *et al.,* 2019), but the genetic basis of aroma in these and other accessions have not been comprehensively established. The objective of this study was thus to evaluate the aroma status of 56 rice accessions and to assess for the various alleles of *badh2* gene using functional markers.

MATERIALS AND METHODS

Source of rice germplasm. The study was based on a collection of 56 rice accessions which includes upland and lowland rice of *O. glaberrima*, *O. sativa* and interspecific crosses between *O. sativa* and *O. barthii* acquired from the National Crops Resources Research Institute, Namulonge (NaCRRI-Uganda) (Table 2).

Experimental setup. Individual rice accessions were planted in 15liter capacity pots with nine plants per pot in a complete randomised design at NaCRRI in Uganda. At two weeks post emergence, one leaf per plant was picked from all nine plants and stored at -80 °C for further analysis.

Assessment of aroma using sensory method. The presence or absence of aroma was determined by sensory testing using the procedure of Sood and Siddiq (1978). Fresh leaf samples (10 g) of rice plants at tillering growth stage were obtained and cut into 3 mm pieces using a pair of scissor; and placed in a ventilation vial containing 10 ml of 1.7% KOH and, thereafter, incubated at room temperature (27 °C) for 10 minutes. The samples were then smelled and rated one at a time for aroma by a panel of six evaluators, who were selected based on their experience as technicians with the rice breeding programme at NaCRRI and their ability to distinguish aromatic rice from non-aromatic rice, using blind samples. The evaluation of aroma was done on a scale of 1-3, where 1 was non-aromatic, 2 slightly aromatic and 3 highly aromatic (IRRI, 2013). The order of scoring of 56 accessions was randomised within each individual panelist.

Assessment of aroma and allele diversity using functional markers. Genomic DNA was extracted from the young leaves that were earlier collected and stored at -80 °C, using the CTAB method (Doyle, 1991) with minor modifications. The modification included addition of ice cold isopropanol to the aqueous layer and mixed thoroughly. This was incubated at -20 °C for 30 minutes, then added 10 mM ammonium acetate and incubated for another 30 minutes at -20 °C before spinning at 13000 rpm for 10 minutes to precipitate the DNA).

The quality of DNA was checked using a ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusettes, USA). Polymerase chain reactions (PCR) amplifications were performed using five primers (Table 3) in ArkTik thermal Cycler (Thermal Scientific, Finland). Each reaction contained 5 µl of AccuPower PCR Premix (Bioneer, Daejeon, Korea), 1 µM of each primer and 50 ng of template DNA. The final reaction volume was completed to 10 µl using sterile distilled water.

For the ASA (ESP, EAP, INT, IFAP, and ISP) the reaction was programmed for an initial 5 min cycle at 95 °C; followed by 35 cycles of 30 s at 95 °C, 40 s at 55 °C, and 1 min at 72 °C; with a final extension of 5 min at 72 °C. For the rest of primers, the cycling conditions followed a touch-down programme

TABLE 2. Description of genotypes used in the study

Entry no	Name Pedigree	Origin	Ecology	Species	Entry no	Name / Pedigree	Origin	Ecology	Species
1	Tog5674	ARC	Upland	Glaberrima	29	SUPA6	IRRI	Lowland	Sativa /indica
2	Tog5672	ARC	Upland	Glaberrima	30	Gigante	IRRI	Lowland	Sativa /indica
3	Tog5681	ARC	Upland	Glaberrima	31	Komboka	IRRI	Lowland	Sativa/ indica
4	MET3	ARC	Upland	ISC: O.S x O. B	32	Basmati370	IRRI	Lowland	Sativa/ indica
5	MET 4	ARC	Upland	ISC: O.S x O. B	33	WITA9	IRRI	Lowland	Sativa /indica
6	MET 6	ARC	Upland	ISC: O.S x O. B	34	Supa Local	NaCRRI	Lowland	Sativa /indica
7	MET12	ARC	Upland	ISC: O.S x O. B	35	AGRA41	unknown	Upland	Sativa /japonica
8	MET 13	ARC	Upland	ISC: O.S x O. B	36	AGRA60	unknown	Upland	Sativa /japonica
9	MET14	ARC	Upland	ISC: O.S x O. B	37	AGRA65	unknown	Upland	Sativa /japonica
10	MET15	ARC	Upland	ISC: O.S x O. B	38	NamChe1	NaCRRI	Upland	Sativa /japonica
11	MET16	ARC	Upland	ISC: O.S x O. B	39	NamChe2	NaCRRI	Upland	Sativa /japonica
12	MET30	ARC	Upland	ISC: O.S x O. B	40	NamChe3	NaCRRI	Upland	Sativa /japonica
13	MET40	ARC	Upland	ISC: O.S x O. B	41	NamChe4	NaCRRI	Upland	Sativa /japonica
14	MET60	ARC	Upland	ISC: O.S x O. B	42	NamChe5	NaCRRI	Upland	Sativa /japonica
15	MET70	ARC	Upland	ISC: O.S x O. B	43	NamChe6	NaCRRI	Upland	Sativa /japonica
16	ARC1	ARC	Lowland	Sativa /indica	44	E20	NaCRRI	Upland	Sativa /japonica
17	ARC2	ARC	Lowland	Sativa /indica	45	E22	NaCRRI	Upland	Sativa /japonica
18	ARC3	ARC	Lowland	Sativa /indica	46	NERICA1	ARC	Upland	Sativa /japonica
19	ARC4	ARC	Lowland	Sativa /indica	47	NERICA4	ARC	Upland	Sativa japonica
20	ARC5	ARC	Lowland	Sativa /indica	48	NERICA8	ARC	Upland	Sativa japonica
21	ARS11	ARC	Lowland	Sativa/indica	49	NERICA10	ARC	Upland	Sativa /japonica
22	IR64	IRRI	Lowland	Sativa /indica	50	1189 line	ARC	Upland	Sativa /japonica
23	SUPA1052	IRRI	Lowland	Sativa/indica	51	1190 line	ARC	Upland	Sativa /japonica
24	SUPA1	IRRI	Lowland	Sativa /indica	52	1191 line	ARC	Upland	Sativa /japonica
25	SUPA2	IRRI	Lowland	Sativa /indica	53	326104 line	KOREA	Lowland	Sativa /indica
26	SUPA3	IRRI	Lowland	Sativa /indica	54	Sande	TZ	Lowland	Sativa /indica
27	SUPA4	IRRI	Lowland	Sativa/ indica	55	TXD306	TZ	Lowland	Sativa /indica
28	SUPA5	IRRI	Lowland	Sativa /indica	56	Jaribu	TZ	Lowland	Sativa /indica

Africa Rice Center (ARC), International Rice Research Institute (IRRI), Tanzania (TZ), National Crops Resources Research Institute (NaCRRI), Interspecific Cross between *Oryza sativa* and *Oryza barthii* (ISC: O.S x O.B)

TABLE 3. Primers for the analysis of aroma and the allelic variations in *badh2* gene in rice

Primer name	Primer sequence (5'->3')	PCR Product Size	Source
FMBADH2-E2A	F: CCTCTGCTTCTGCCTCTGAT R:GATTGCGCGGAGGTACTTG	200/207bp (*badh2-E2*/*badh2*)	Shi *et al.* (2008)
FMBADH2-E2B	F: CTTCTGCCTCTGATTAGCCT R: GCCGTGAGCCATATACACTT	643/650bp (*badh2-E2*/*badh2*)	Shi *et al.* (2008)
FMBADH2-E7	F: GGTTGCATTTACTGGGAGTT R:CAGTGAAACAGGCTGTCAAG	260/268 bp (*badh2-E7*/*badh2*)	Shi *et al.* (2008)
FMBADH2-E-4-5	F: TGCTGGATGCTTTGAGTA R:GTTTAGACACCTGAAGGAAGACCA	321/1123 bp (*badh2-E4-5*/*badh2*)	Shao *et al.* (2011)
*External Sense Primer (ESP)	TTGTTTGGAGCTTGCTGATG	580bp	
External Antisense Primer (EAP)	AGTGCTTTACAAAGTCCCGC		Bradbury *et al*. (2005a)
*Internal Fragrant Antisense Primer (IFAP)	CATAGGAGCAGCTGAAATATATACC	257/355bp	
Internal Non-fragrant Sense Primer (INSP)	CTGGTAAAAAGATTATGGCTTCA	(Aromatic/Non-aromatic)	Bradbury *et al*. (2005a)

*ESP, EAP, IFAP and INSP are multiplexed in a single PCR tube for amplification and named Allele Specific Assay (ASA) hereafter. ASA and FMBADH2-E7 were designed to track the 8bp deletion and 3SNP on exon 7

FMBADH2-E2A and FMBADH2-E2B were designed to track the 7bp deletion on exon 2 while FMBADH2-E4-5 was designed to track the 803 bp deletion between exons 4 and 5

starting at an annealing temperature of 64 °C with 1 °C reduction in every cycle gradually until 56 °C (eight cycles) and then maintained at a constant annealing temperature of 56°C for the remaining 27 cycles. The reaction was, thus programmed for an initial 5 min cycle at 95 °C. This was followed by 8 cycles of 30 s at 95 °C, 40 s at 64 °C - 56 °C, and 1 min at 72 °C; followed by 27 cycles at similar conditions, except for annealing temperature maintained at 56 °C with a final extension of 5 min at 72 °C.

All PCR products were segregated on a 1.2% (w/v) agarose gel, except for primers FMBADH2-E2A and FMBADH2-E7 which were segregated on 3% (w/v) agarose gel. All gel were prepared in 1XTBE buffer and stained with EZ (0.8 ug^{-1} mL) (AMRESCO, Ohio, USA) and visualised using a BioDoc-It Imaging System (Thermo Fisher Scientific, Waltham, Massachusettes, USA). A 100 bp ladder (Bioneer, Daejeon, Korea) was used to estimate the PCR fragment sizes.

PCR products were then shipped to Macrogen Laboratory in the Netherlands for sequencing. PCR products were sequenced in the reverse direction using primers EAP, FMBADH2-E2A(R) and FMBADH2-E7(R). Each fragment was sequenced at least four times and high quality consensus sequences were used for data analysis.

Data analyses. Both descriptive and inferential data analyses were performed using R Statistical software (R Core Team 2017) and Microsoft excel (MS Excel 2017). Frequencies were generated using Excel. Friedman Sum Rank Test (Hollander and Wolfe, 1973) was used to determine differences in sensory scores among the 56 genotypes.

To determine whether a given genotype was aromatic or not, a Friedman Sum rank test was performed using a median score from the six evaluators while taking each individual assessor as a block.

To test for association between aroma and *badh2* alleles, Chi-square test of association was performed. Sequences were manually edited and aligned using Clustal W and MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar *et al.* 2016). Multiple sequence alignment was done to identify the presence of deletions and SNPs in the lines.

RESULTS

Sensory analysis for aroma. Friedman Sum Rank test showed significant (Friedman chi-squared = 229.82, df = 55 P-value < 2.2 x 10^{-16}) differences in sensory scores among the 56 genotypes (Table 4). Out of the 56 accessions, 23 were classified as aromatic by at least one evaluator; and 33 classified as non-aromatic based on median of sensory evaluation scores. Out of the 23 aromatic accessions, nine accessions were classified as highly aromatic and 14 classified as moderately aromatic (Table 4).

Sensory evaluation revealed 23 accessions to be aromatic (9 highly aromatic, plus 14 moderately aromatic) and 33 accessions were non-aromatic; thus grouping the accessions according to the strength of aroma emitted. MET40, SUPA1, SUPA5, AGRA41, AGRA60, MET16, MET3, MET4 and MET13 were highly aromatic (Median Score >2) while Basmati370, Komboka, SUPA6, Jaribu, MET12, MET14, MET15, MET6, NamChe1, Namche5, Sande, SUPA1052, SUPA2, and Supa Local were moderately aromatic (median Score ranging from 1 to 2).

Considering the ecology of the rice accessions, 13 upland accessions were aromatic while 20 were non-aromatic. Similarly, 10 lowland accessions were aromatic; while 13 were non-aromatic. A Chi-square test of association between aroma and ecology (upland or lowland) was not significant (Chi-square: 0.0543, P-value: 3.841)

Aroma and allelic variations in the *badh2* gene. Molecular marker analysis was used to validate the aroma status of rice accessions obtained in the previous section (sensory

TABLE 4. Summary of sensory evaluation of the 56 rice accessions as depicted by the median sensory score

Accessions	Number of accessions	Median score	Aromatic group
MET40, SUPA1, SUPA5, AGRA41, AGRA60, MET16, MET3, MET4, MET13 Basmati370, Komboka, SUPA6, Jaribu, MET12, MET14, MET15, MET6,	9	⩾2	Highly aromatic
NamChe1, NamChe5, Sande, SUPA1052. SUPA2, Supa local 1189, 1190, 1191, AGRA65, ARC1, ARC2, ARC3, ARC4, ARC5, ARS11, E20, E22, Gigante, IR64, MET30, NamChe2, NamChe4, NamChe6, NamChe3, NERICA1, NERICA4, NERICA10, NERICA8, SUPA3, SUPA4, Tog5674, Tog5672, Tog5681,	14	1 to 2	Moderately aromatic
TXD306, WITA9, MET60, 326104 line, MET70	33	0	Non-aromatic

Friedman Chi-squared=229.82, df = 55, P-value <2.2x10^{-16}

analysis of aroma) and to determine the allelic variations within the aroma gene in the accessions (Table 5).

Functional Marker ASA revealed that 21 accessions were aromatic, and thus carry *the badh2-E7 allele* of which three were heterozygous aromatic as they carried both the functional and non functional alleles. FMBADH2-E7 revealed only 19 accessions to be aromatic, and thus carry the *badh2-E7 allele;* but two accessions (SUPA1 and Jaribu) which were initially scored as aromatic using the sensory method and found to be non-aromatic because they did not carry the *badh2-E7 allele*. Similarly, three accessions (NamChe1, NamChe5 and MET14) were initially scored as aromatic using sensory test but both the ASA and FMBADH2-E7 functional marker indicated that they were non-aromatic and thus did not carry the *badh2-E7 allele*. In another twist, one accession (AGRA65) was found to be aromatic using ASA and FMBADH2-E7 molecular markers and thus carrying the eight bp deletion yet it was initially scored as non-aromatic, using sensory analysis. Sequencing results confirmed the presence of an eight bp deletion and three SNPs in AGRA65; and the absence of the eight bp deletion in SUPA1, Jaribu, NamChe1, NamChe5 and MET14.(Fig. 1).

The functional markers FMBADH2.E2A and FMBADH2.E2B designed to detect the seven bp deletion on exon two, designated as the *badh2-E2* allele, gave inconsistent results. Using FMBADH2.E2A, non aromatic accession was found to contain the *badh2-E2* allele. Surprisingly, four non-aromatic accessions (ARC3, E20, Tog5672 and TXD306) were positive for the *badh2-E2* allele. Functional marker FMBADH2-E2A, therefore, gave contradicting results indicating that four accessions were aromatic yet the sensory test indicated that they were not. FMBADH2-E2B identified nine accessions as aromatic and 26 accessions as non aromatic, similar to results revealed by the sensory test. In addition, 14 accessions were found to be non-aromatic contrary to the sensory test;

TABLE 5. Validation of sensory results and determination of allelic variations in the *badh2* gene using functional markers

Entry no.	Accession	Aroma status	Functional markers				
			ASA	FMBADH2 -E7	FMBADH2 -E2A	FMBADH2 -E2B	FMBADH2 -E4-5
			8bp deletion	8bp deletion	7bp deletion	7bp deletion	803 deletion
1	Tog5674	1	-	-	-	-	-
2	Tog5672	1	-	-	+	-	-
3	Tog5681	1	-	-	-	-	-
4	MET3	3	+	+	-	+	-
5	MET 4	3	+	+	-	+	-
6	MET 6	2	+	+	-	+	-
7	MET12	2	+	+	-	-	-
8	MET 13	3	+	+	-	-	-
9	MET14	2	-	-	-	-	-
10	MET15	2	+	+	-	+	-
11	MET16	3	+	+	-	+	-
12	MET30	1	-	-	-	-	-
13	MET40	3	+	+	-	-	-
14	MET60	1	-	-	-	+	-
15	MET70	1	-	-	-	-	-
16	ARC1	1	-	-	-	-	-
17	ARC2	1	-	-	-	-	-
18	ARC3	1	-	-	+	-	-
19	ARC4	1	-	-	-	-	-
20	ARC5	1	-	-	-	-	-
21	ARS11	1	-	-	-	-	-
22	IR64	1	-	-	-	-	-
23	SUPA1052	2	+	+	-	-	-
24	SUPA1	3	-+	-	-	-	-
25	SUPA2	2	+	+	-	-	-
26	SUPA3	1	-	-	-	-	-
27	SUPA4	1	-	-	-	-	-
28	SUPA5	3	+	+	-	-	-
29	SUPA6	2	-+	+	-	-	-
30	Gigante	1	-	-	-	-	-
31	komboka	2	-+	+	-	-	-
32	Basmati370	2	+	+	-	-	-
33	WITA9	1	-	-	-	-	-
34	Supa Local	2	+	+	-	-	-
35	AGRA41	3	+	+	-	-	-
36	AGRA60	3	+	+	-	+	-
37	AGRA65	1	+	+	-	-	-
38	NamChe1	2	-	-	-	+	-

TABLE 5. Contd.

Entry no.	Accession	Aroma status	Functional markers				
			ASA	FMBADH2-E7	FMBADH2-E2A	FMBADH2-E2B	FMBADH2-E4-5
			8bp deletion	8bp deletion	7bp deletion	7bp deletion	803 deletion
39	NamChe2	1	-	-	-	+	-
40	NamChe3	1	-	-	-	-	-
41	NamChe4	1	-	-	-	-	-
42	NamChe5	2	-	-	-	+	-
43	NamChe6	1	-	-	-	-	-
44	E20	1	-	-	+	-	-
45	E22	1	-	-	-	-	-
46	NERICA1	1	-	-	-	+	-
47	NERICA4	1	-	-	-	+	-
48	NERICA8	1	-	-	-	-	-
49	NERICA10	1	-	-	-	+	-
50	1189 line	1	-	-	-	-	-
51	1190 line	1	-	-	-	-	-
52	1191 line	1	-	-	-	-	
53	326104 line	1	-	-	-	+	-
54	Sande	2	+	+	-	+	-
55	TXD306	1	-	-	+	+	-
56	Jaribu	2	+	-	-	-	-

(+) = presence of deletion; (-) = absence of deletion; (-+) = heterozygotes using molecular marker method. (1) = non-aromatic; (2) = moderately aromatic and (3) = highly aromatic using sensory evaluation

while seven accessions were found to be aromatic using this marker even after the sensory test scored them as non-aromatic.

There was no direct correlation between the *badh2-E2* allele and aroma, based on the molecular marker data for these two primers, thus did not correlate with the sensory score. A Chi-square test of association between aroma and *badh2-E2* allele was not significant (Chi-square=3.00; *P*=0.2819). Sequencing of the PCR product of these primers did not reveal a seven bp deletion in any accession.

FMBADH2-E4-5 indicated that all accessions were non-aromatic as none of the accessions carried the *badh2-E4-5* allele.

DISCUSSION

Sensory evaluation revealed that 23 accessions were aromatic (Table 4); while 33 accessions were non-aromatic, and further subdivided the accessions into three distinct groups of accessions depending on the strength of the aroma perceived by the panelist (Table 4). This result is in agreement with Ocan *et al.* (2019) who also found differences in the strength of aroma among aromatic rice varieties. The strength of aroma is known to be influenced by cultivation condition such as temperature (Prodhan *et al.,* 2017), Light intensity (Mo *et al.,* 2015), plant nutrition (Lei *et al.,* 2018)

Figure 1. Sequence analysis showing the presence or absence of the 8bp deletion and three SNPs on exon two of the *badh2* gene in selected accessions.

and soil salinity (Poonlapdechal *et al.,* 2012). In the present study, there was no control for cultivation conditions; therefore variations in the strength of aroma is attributed to the genetic makeup of individual accessions.

There was no association between aroma and the ecology of the accession being either upland or lowland.

While 23 accessions were found to be aromatic using sensory evaluation, only 20 accessions were aromatic using molecular marker linked to the *badh2* gene that confers aroma in rice. Four accessions (NamChe1, NamChe5, Jaribu and MET14) were scored as aromatic but did not contain the *badh2-E7* allele characterised by an eight bp deletion and three SNPs. Two possible explanations; firstly, these accessions may have been scored as aromatic by mistake as each of those accessions were scored as aromatic by only one panelist. The ability to distinguish between aromatic and non-aromatic accessions diminishes with successive analysis either because the senses become saturated or the nasal passages may become damaged after inhaling KOH solution. Moreover, the ability to discriminate between samples varies between age, gender and cultural background.

Secondly, these findings indicate the presence of already reported or unknown mutations in a different part of the *badh2* gene or in the promoter region (Bindusree *et al.,* 2017). In order to obtain more conclusive results, we further evaluated the accessions using functional markers. The advantage of functional markers within the *badh*2 gene is that they permit direct selection of accessions that contain the gene that controls the aromatic phenotype, hence overcoming the limitations of sensory analysis. Molecular marker analysis, once combined with sensory methods, was able to represent the true aroma spectra of all the rice accessions in this study.

Twenty aromatic accessions had the *badh2-E7* allele, characterised by an eight base pair deletion and three SNPs. A significant Chi-square test implies existence of a relationship between the *badh2-E7* allele and aroma, thus confirming the genetic basis of aroma in these accessions that this allele is responsible for

the aroma in theses accessions (Fitzgerald *et al.* 2008).The *badh2-E7* allele is the most abundant allele in aromatic rice accessions, as nearly all aromatic rice accessions carry this allele (Bradbury *et al.* 2005a, Bourgis *et al.* 2008, Shoa *et al.* 2013, Bindusree *et al.* 2017). A whole genome sequencing of 76 aromatic accessions from the 3000 Rice Genome Project revealed the presence of *badh2-E7* in 39 of the genotypes (Bindusree *et al.,* 2017).

The 18 homozygous aromatic accessions for the *badh2-E7* allele will breed true for aroma even in subsequent generations, making selection for the aroma trait more effective to conduct and thus are valuable resources for breeding and or be released as new aromatic varieties. The three heterozygous aromatic accessions confirm the existing genetic underpinning of the aroma trait in rice (Bradbury *et al.* 2005b).

CONCLUSION

Continuous increase in demand for aromatic rice in the global market has attracted attention of rice breeders to include grain aroma among the major breeding objectives for rice improvement. Sensory analysis combined with use of functional markers was able to accurately select twenty rice accessions that can be included as breeding materials for development of highly aromatic varieties. Other alleles were not reported in this study indicating the existence of a narrow genetic base for improving aroma among the germplasm collection. The number of accessions used in this study was low and may not be representative of the overall allelic diversity within the *badh2* gene.

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