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Marker-Assisted Backcross Screening for Vietnamese Aromatic Rice Lines Derived from TLR7/Khaodawkmali105//TLR7

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Abstract

This research focused on genotyping via SSR molecular marker. Backcrossing populations of the combination of TLR7/KhaoDawkMali105//TLR7 was generated to stabilize aromatic genes of donors into rice varieties as acceptors by using an advanced backcrossing method (ABC) with molecular marker assistance. Fifty BC3F2 rice lines were used to evaluate aroma detection through the sensory test, genotypic analysis using microsatellite markers, agronomic characteristics, and other grain quality traits. Sixteen lines having aromatic gene (*fgr*) locus were found using marker RG28 as homozygous condition in 50 rice lines. The marker RM223 detected lowest number of *fgr* locus. Two lines (BC3F4-1-8 and BC3F4-1-48) were identified well-defined target for aroma genes having high yield and good quality traits. Thus, these lines were utilized SSR marker for demonstrating marker-assisted selection (MAS) breeding program.

Keywords: Microsatellite Marker; Aroma; Polymorphism; Codominant

Introduction

The quality of rice is considered based on milling quality, grain size, shape, appearance, aroma and other cooking characteristics [1]. Aromatic rice, also known as fragrant rice, is very popular in Asia. The biochemical basis of aroma was identified as 2-acetyl-1-pyrroline which a fragrant compound being discovered in KhaoDawkmli105, Basmati and Jasmine-type aromatic rice [2,3]. Also, there are pentanol, hexanol, benzaldehyde involving fragrance on rice. Application of results to introgressive a major gene into a high-yielding rice cultivar by continued backcrossing, with assistance of flanking marker. Ahn *et al.* determined *fgr* gene positioned on chromosome 8 via RFLP, controlling aroma while Lorieux, *et al.* observed gene of *fgr* closely linked with marker RG28 with genetic distance of 4.5cM [3,4]. Another marker RM223 can be possibly used for polymorphism at both of salt gene and aromatic gene.

Recently, molecular markers have been developed for the selection of aromatic plants in rice; they have the advantages of being inexpensive, simple, rapid, requiring small amounts of tissue and highly reliable [5]. Cornell University (USA) researched the original of aromatic rice varieties on genetic base via determined gene of betaine aldehyde dehydrogenase (BADH2) controlled aroma [6]. Yoshihashi, *et al.* identified a microsatellite marker RM17 that is closely linked to the *fgr* gene in Khao DawkMali105, a famous aromatic rice cultivar of Thailand [7]. Then a functional BADH2 enzyme inhibits 2AP biosynthesis which is a major component of aroma was reported by Bradbury, *et al.* [5]. Therefore, in this study, the development of PCR marker for aroma characteristic use in breeding program has made great advance, which made it possible to select rice lines having aroma with good quality and high yield in a backcross population derived from a cross between the aromatic and nonaromatic cultivars.

Materials and Methods

Plant materials

The cross between high yielding rice TLR7 (a high yield variety) and KhaoDawkMali105 determined aromatic gene as donor were obtained from the High Agricultural Technology Research Institute for Mekong delta, Vietnam (HATRI).

A field experiment was transplanted to an irrigated lowland field in a randomized complete block design in three replications in the field of HATRI. Fifty lines of BC3F2 with their parents were used to evaluate agronomic characteristics and aroma detection through sensory test and genotypic analysis using SSR markers in lab of HATRI. Data on important agronomic traits like plant height, panicle length, filled grains/panicle, unfiled grain/panicle, 1000-grain weight, harvest index and yield were recorded.

Phenotype analysis

Ten randomly selected plants of each genotype were used for agronomic data analysis. Data on plant height (cm), number of effective tillers/plants, panicle length (cm), number of filled grains/panicle, 1000-grain weight (g), days to maturity and grain yield/plant (g) were recorded and subjected to statistical analyses using SAS software. After harvesting, the seeds of each genotype were dehulled for evaluation of the grain quality and aroma. The grains were classified into different types based on their dimension according to Dela Cruz and Khush [8]. Ten seeds of each cultivar removed rice hull and mashed by hand. Take rice powder of each cultivar and put on each experimental tube or petri dish. Add 5ml KOH 1.7% into each petri dish and cover. Store the samples in room temperature for 30 minutes. The samples were scored, corresponding to absence of aroma, slight aroma, moderate aroma and strong aroma, respectively.

Molecular marker analysis

DNA isolation was carried out using the mini preparation CTAB method [9]. Two markers RM223 and RG28 (linked to aroma) were used to confirm the presence of fgr gene as described by Garland, *et al.* and Ahn *et al.* [10,4]. The details of the primers are given in Table 1. The amplified products were electrophoretic ally resolved on a 3% agarose gel in 0.5xTBE and stained with ethidium bromide 0.5mg/ml and analyzing by Alpha Imager 1220 (Alpha Innotech, CA, USA). The bands representing particular alleles at the microsatellite loci were scored manually on the basis of parental bands like non-aromatic type (TLR7), aromatic type band (KhaoDawMali105), and heterozygotes type band.

SSR marker, which is RG28 and RM223 on chromosome 8 closely associated with aromatic genes, detected these target genes from short-term high-yielding rice varieties and a number of rice varieties carrying aromatic gene.

Primer name	Chromosome locus	Sequence
RM233	8	Rev. GAAGGCAAGTCTTGGCACTG Fwd. GAGTGAGCTTGGGCTGAAAC
RG28	8	Rev. GATCTCACTCCAAGTAAACTCTGAC Fwd. ACTGCCATTGCTTCTGTTCTC

Table 1: The markers for analysis of fragrance in rice

Data Analysis

The agro-morphological data were initially analyzed through examining variance to verify genetic variation in the traits measured. The few traits with insignificant genetic variation, based on the F-test, were not considered for further analyses

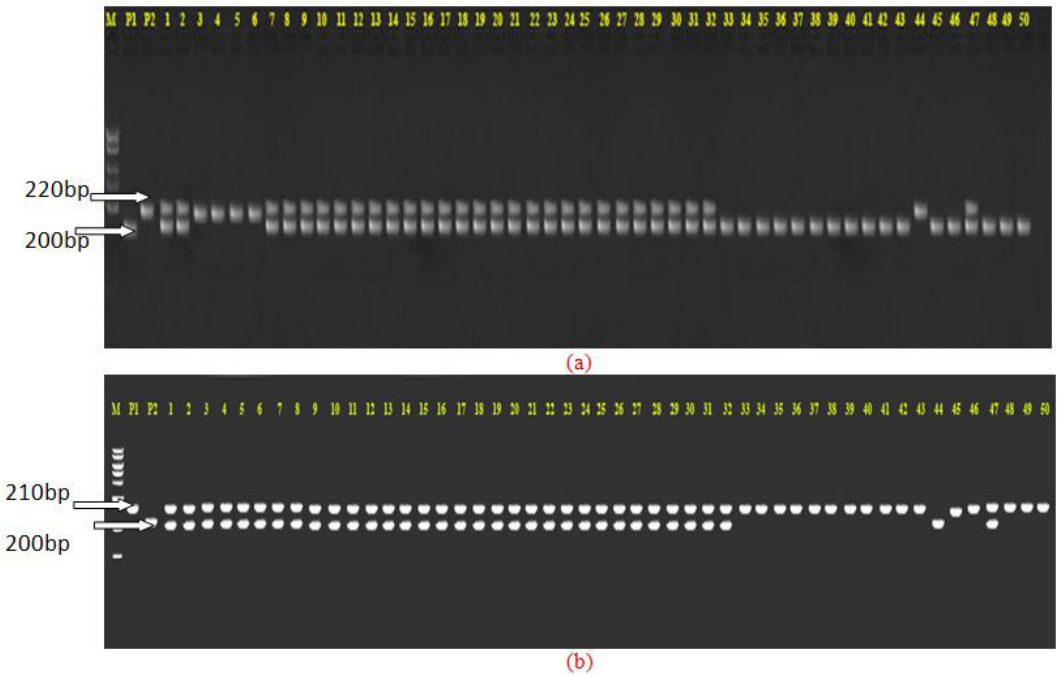
Results and Discussion

Developing advanced backcrossing (BC3F2) with molecular marker assistance to select individuals carrying aromatic gene in order to cross back with female lines (recurrent lines) and to stabilize aromatic gene in homozygosity was rapid, compared with conventional method. Molecular markers expressed polymorphism clearly, linking to aromatic gene which was recorded on backcrossing populations.

Analysis of BC2F2 showed that primer RG28 confirmed the presence of fragrance gene with size of 200bp to 220bp in 4 lines where the fragrance gene was similar to that of KhaoDawkMali105 (aromatic type) at homozygous and in 16 lines similar to that of TLR7 (non-aromatic type) as homozygous alllele. Rest of the genotypes showed heterozygous condition (Figure 1a and 1b). In case of RM233, the amplification of DNA was 100% and there were two alleles with size of 200bp to 210bp. Only one line gave aromatic banding pattern similar to that of KhaoDawkMali105. Most of the genotypes showed heterozygous allele.

For primer RG28, the amplification of DNA was 100% and there were two alleles with size from 200bp to 220bp. However, when recorded BC3F2, these lines had gradual decrease of heterozygote by 14%. There were 16 lines that carried aromatic gene: 6, 7, 8, 9, 10, 11, 23, 24, 25, 26, 27, 35, 36, 46, 47, and 48 (Figure 2a and 2b).

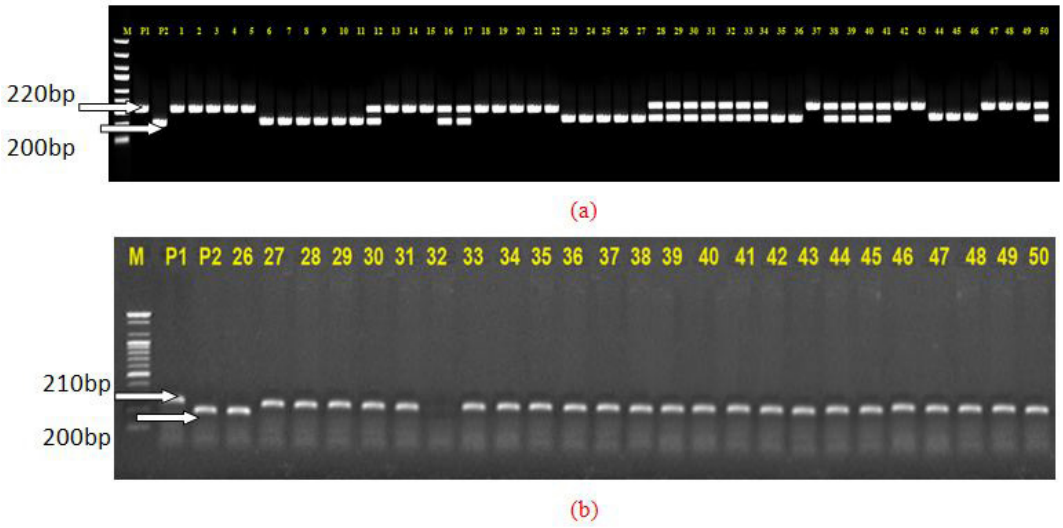
For molecular marker of RM 223, DNA amplification from population BC3F2 accounted for 100% presented bands. This result showed that samples had different bands with two alleles which were commonly clear at position of molecular marker. Difference in quantity and position of bands might show difference in size: 210 bp -220bp. Also, Buu, *et al.* applied marker RM223 associated with *fgr* on chromosome 8 with genetic distance of 1.6 cM in breeding high-yielding rice varieties carrying aroma and successfully developed rice varieties of OM4900, OM6161, and OM6162 [11]. The primer RM223 responded best in all the 26 rice genotypes, because RM223 primer could be able to identify aromatic and non-aromatic germplasm effectively which supported the phenotypic results. It can also be said that the RM223 responded best in all the 26 rice lines and it could be readily used in breeding program for releasing aromatic rice variety with considerable yield [12].



a) RG28 (200bp - 220bp): single band like lane 2 non-aromatic (TLR7); single band like lane 3 aromatic (KhaoDawkMali105) and double band indicated heterozygous allele

b) RM233 (200bp - 210bp)

Figure 1: PCR product on population of BC2F2 from cross TLR7/ KhaoDawMali105//TLR7



a) RG28 (200bp - 220bp): single band like lane 2 non-aromatic (TLR7); single band like lane 3 aromatic (KhaoDawkMali105) and double band indicated heterozygous allele;

b) RM233 (200bp - 210bp)

Figure 2: PCR product on population of BC3F2 from cross TLR7/ KhaoDawMali105//TLR7

Relationship between conventional breeding and maker assisted breeding

Conventional breeding such as genealogy or recurrent selection is to select phenotype between inbred lines or parental lines crossed on basis of test-cross. With such condition, MAS for individuals via using selective index combined molecular evaluation and phenotyping can increase selective effect higher than conventional method. MAS make it possible to enhance selection strength in first generations of genealogy while conventional method is nearly unselective and visible selection to remove the worst lines. Combination between position of molecular marker and QTL will help us to review drought tolerance or pests and diseases at F2 generation. In subsequent generation, selecting can be completed in young plant (seedlings stage) or mature plant which was planted in normal environment reducing the number of trial lines in problemed environment. In this examination, after phenotypic and genotypic observation, it was found that thirteen lines were aromatic gene compared with phenotype (Table 2). In other case, line BC3F2-50-18 had aroma alleles but it still presented non-aromatic in phenotype.

N_0	Lines	RG28	RM233	Aromatic evaluation	Phenotype and genotype
P1	TLR7	A	A	No aromatic	No aromatic
P2	KhaoDowMali105	B	B	Aromatic	Aromatic
1	BC3F2-2-10	A	H	No aromatic	No aromatic
2	BC3F2-2-14	A	H	No aromatic	No aromatic
3	BC3F2-1-33	A	H	No aromatic	No aromatic
4	BC3F2-9-4	A	H	No aromatic	No aromatic
5	BC3F2-9-9	A	H	No aromatic	No aromatic
6	BC3F2-1-6	B	H	Aromatic	Aromatic
7	BC3F2-15-7	B	H	Aromatic	Aromatic
8	BC3F2-1-8	B	H	Aromatic	Aromatic
9	BC3F2-1-47	B	H	Aromatic	Aromatic
10	BC3F2-2-10	B	H	Aromatic	Aromatic
11	BC3F2-47-111	B	H	Aromatic	Aromatic
12	BC3F2-1-112	H	H	No aromatic	No aromatic
13	BC3F2-47-113	A	H	No aromatic	No aromatic
14	BC3F2-7-142	A	H	No aromatic	No aromatic
15	BC3F2-2-150	A	H	No aromatic	No aromatic
16	BC3F2-8-16	H	H	No aromatic	No aromatic
17	BC3F2-1-170	H	H	No aromatic	No aromatic
18	BC3F2-2-11	A	H	No aromatic	No aromatic
19	BC3F2-7-1-19	A	H	No aromatic	No aromatic
20	BC3F2-6-20	A	H	No aromatic	No aromatic
21	BC3F2-6-3-21	A	H	No aromatic	No aromatic
22	BC3F2-51-22	A	H	No aromatic	No aromatic
23	BC3F2 -19--23	B	H	Aromatic	No aromatic
24	BC3F2-8-27	B	H	Aromatic	No aromatic
25	BC3F2-10-22	B	H	Aromatic	No aromatic
26	BC3F2-12-29	B	H	Aromatic	No aromatic
27	BC3F2-20-29	B	H	No aromatic	No aromatic
28	BC3F2-59-17	H	H	No aromatic	No aromatic
29	BC3F2-78-1-21	H	H	No aromatic	No aromatic
30	BC3F2-78-10	H	H	No aromatic	No aromatic
31	BC3F2-15-1	H	H	No aromatic	No aromatic
32	BC3F2-32-10	H	H	No aromatic	No aromatic
33	BC3F2-33-17	H	A	No aromatic	No aromatic
34	BC3F2-34-32	H	A	Aromatic	Aromatic
35	BC3F2-35-18	B	A	Aromatic	Aromatic
36	BC3F2-36-1	B	A	Aromatic	Aromatic
37	BC3F2-37-6	A	A	Aromatic	Aromatic
38	BC3F2-38-7	H	A	No aromatic	No aromatic
39	BC3F2-39-25	H	A	No aromatic	No aromatic
40	BC3F2-40-45	H	A	No aromatic	No aromatic
41	BC3F2-41-4	H	A	Aromatic	No aromatic
42	BC3F2-42-13	A	A	Aromatic	No aromatic
43	BC3F2-43-17	A	A	Aromatic	No aromatic
44	BC3F2-44-14	B	B	No aromatic	No aromatic
45	BC3F2-45-52	B	A	No aromatic	No aromatic
46	BC3F2-46-36	B	A	No aromatic	No aromatic
47	BC3F2-47-58	A	H	Aromatic	Aromatic

N_0	Lines	RG28	RM233	Aromatic evaluation	Phenotype and genotype
48	BC3F2-48-96	A	A	Aromatic	Aromatic
49	BC3F2-49-47	A	A	Aromatic	Aromatic
50	BC3F2-50-18	H	A	No aromatic	No aromatic

Note: A = homozygous recipient allele; **B** = homozygous donor allele; **H** = heterozygous allele

Table 2: Synthesis of PCR result with RG28 and RM233 evaluated and determined aromatic gene on combination of BC3F2 of TLR7/ KhaoDawkMali 105//TLR7

Phenotypic evaluation of aromatic rice lines: The results on agronomic performance in Table 3 showed that the plant height of the aromatic lines ranged from 95 cm (BC3F4-448-96) to 115 cm (BC3F4-1-8). In comparison to P2, there was considerable reduction in plant height in most of the lines. Days to maturity of the aromatic rice lines ranged from 90-105. The effective panicle length varied from 25.6 cm (BC3F4-2-10) to 30.1 (BC3F4-49-47). The number of filled grains/panicle was highest in BC3F4-1-47 (232) and lowest in P2 (165). The 1000-grain weight was highest in BC3F4-49-47 (28.5g) and lowest in BC3F4-2-10 (26.3 g). The HI of the aromatic rice lines ranged from 0.53 to 0.59. Six lines showed higher yield than both the parents (BC3F4-1-6, BC3F4-1-8, BC3F4-1-47, BC3F4-2-10, BC3F4-47-58, and BC3F4-48-96) (Table 3).

No.	Lines	Durations (day)	PH (cm)	FG	UFG	PL (cm)	1000-GW (g)	HI	Yield (ton/ha)
1	BC3F4-1-6	102a	105ab	210ab	11.0de	28.2bcd	27.2b	0.58ab	8.9a
2	BC3F4-15-7	98ab	110ab	211ab	10.0ef	27.0cde	26.0d	0.59a	8.2a
3	BC3F4-1-8	99ab	115a	200abc	10.0ef	26.9de	26.0d	0.56cd	9.1a
4	BC3F4-1-47	95ab	112ab	232a	9.0fg	29.6ab	27.4b	0.57bc	9.5a
5	BC3F4-2-10	95ab	105ab	195abc	12.0cd	25.6e	26.3cd	0.55de	8.4a
6	BC3F4-47-111	98ab	100ab	185bc	9.0fg	27.2cde	26.5c	0.58ab	8.2a
7	BC3F4-47-58	90b	100ab	196abc	8.0g	27.3cde	27.4b	0.53f	8.4a
8	BC3F4-48-96	90b	95b	195abc	8.0g	26,5de	27,4b	0.54ef	9,1a
9	BC3F4-49-47	100ab	109ab	200abc	13.0bc	30.1a	28,5a	0.56cd	8,1a
10	P1	95ab	105ab	210ab	14.0b	28.7abc	27,4b	0.57bc	8.2a
11	P2	105a	110ab	165c	18.2a	26.9de	26.3cd	0.54ef	4.5b
CV (%)		5.95	10.81	6.73	3.30	0.72	1.54	10.11	10.02
F		ns	ns	**	**	**	**	*	*

Means followed with the same letter in each column are not significantly different; *and ** = significant at 5 and 1% probability levels, and **ns:** no significant, respectively; **CV**: coefficient of variation; **PH:** plant height; **FG**: filled grain; **UFG**: unfilled grain, **PL:** panicle length; 1000-GW; **HI:** Harvest index. **P1** = TLR7; **P2** = Khaodawmali105

Table 3: Yield and yield components of the aromatic rice lines

Nine advanced lines along with their parents were subjected to quality analysis. Grain quality of rice consists of several components: the milling quality such as head rice and nutritional quality such as protein [13,14]. Cooking and eating qualities are mostly determined by amylose content (AC), gelatinization temperature (GT), and gel consistency (GC) of the grain starch [15]. Appearance quality is mainly specified by grain shape as defined by grain length, grain width, the length–width ratio, and the translucency or chalkiness of the endosperm [16]. As mentioned above, some lines were the first IR64 variety to have both intermediate amylose content and intermediate GT. These traits are considered important for the ideal texture of cooked rice, especially for many rice consumers in South and Southeast Asia [17]. Brown rice percentage varied from 80-84%. The head rice percentage of lines ranged from 41-55%.

Most of the studied lines were found to give non-chalkiness. Four lines were found to give moderate aroma (BC3F4-1-6, BC3F4-1-8, BC3F4-2-10, and BC3F4-48-96), compared to P2. The GL of BC3F4-1-47 recorded highest value of 7.23 mm and BC3F4-47-111 recorded lowest value of 7.05 mm among the parents. Most consumers prefer rice with intermediate amylose content ranged between 20-25%. Amylose content ranged from 16.2% (BC3F4-1-6) to 24% (BC3F4-49-47). Gel consistency (GC) is another major character responsible for the texture of cooked rice. Most of lines had higher GC than the GC of parent's varieties. The protein content was highest in BC3F4-49-47 (8.7%) while the lowest in BC3F4-1-8 (8.0%) (Table 4).

This presents a real obstacle for DNA extraction and analysis of marker. Using MAS more widely in the first generation will make the procedure less expensive. The use of MAS is ongoing in rice, which is mainly for pyramiding resistant gene and MAB. Increase in the use of the method is expected, especially for MAB [18]. The wider use of MAS is expected with the improvement of methods for analyzing genetic markers and identify candidates for economic traits.

N_o	Lines	BR (%)	WR (%)	HR (%)	GL (mm)	GW (mm)	AC (%)	GC (mm)	GT (Score)	chalk	PC (%)	aroma
1	BC3F4-1-6	81cd	72.0cd	55.0a	7.14b	3.2a	16.2e	78a	5	0	8.5bc	1
2	BC3F4-15-7	80d	74.0ab	51.0d	7.12bc	3.0c	16.9e	79a	5	0	8.6ab	0
3	BC3F4-1-8	84a	71.0d	50.3d	7.14b	2.9d	19.0cd	76a	5	0	8.0e	1
4	BC3F4-1-47	82bc	72,.cd	50.1d	7.23a	3.0c	18.5d	77a	5	1	8.4cd	0
5	BC3F4-2-10	83ab	75.0a	46.3e	7.10cd	3.0c	20.1c	78a	5	0	8.3de	1
6	BC3F4-47-111	80d	73.0bc	55.0a	7.05e	3.2a	20.0c	77a	5	0	8.4cd	0
7	BC3F4-47-58	80d	70.6d	51.2cd	7.10cd	3.1b	18.6cd	77a	5	0	8.2d	0
8	BC3F4-48-96	80d	71.2d	53.6b	7.08d	3.0c	18.7cd	77a	5	0	8.6ab	1
9	BC3F4-49-47	80d	74.0ab	52.4bc	7.12bc	3.0c	24.0ab	65b	3	0	8.7a	0
10	P1	80d	71.2d	50.1d	7.10cd	3.2a	23.0b	64b	3	0	8.6ab	0
11	P2	80d	72.0cd	41.0f	7.05e	3.1b	18.5a	62b	3	0	8.5bc	1
CV (%)	1.11	1.16	1.50	0.21	0.60	4,28	2.73	-	-	1.09	-	
F	**	**	**	**	**	**	**	-	-	**	-	

Means followed with the same letter in each column are not significantly different; ** = significant at 1 %; **CV**: coefficient of variation; **BR**: Brown rice; **WR**: White rice; **HR**: Head rice; **GL**: grain length; **GW**: grain wide; **AC**: Amylose content; **GC**: Gel consistency; **GT**: Gel temperature; **PC**: Protein content; **P1** = TLR7; **P2** = Khaodawkmali105

Table 4: Evaluated grain quality in rice

Conclusion

Two molecular markers of RG28 and RM 223 were evaluated with population of BC3 F2 and BC3F4 of TLR7/KhaoDawkMali105//TLR7. Important agronomic characteristics of selected aromatic plants were recorded. Percentage of evaluating trait compared genotype and phenotype showed that percentage of RG28 was higher than RM223. It was found that the aroma trait selected from lines in TLR7/KhaoDawkMali105//TLR7 (lines 3 (BC3F2-1-8) and lines 8 (BC3F2-48-96) had good aroma and superior agronomic. These lines continued to evaluate yield and yield components in the field and also evaluated quality.

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