Omonrice 16: 16-23 (2008)

DEVELOPMENT OF PCR-BASED MARKERS FOR AROMA (*fgr)* GENE IN RICE (*Oryza sativaI* L.)

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ABSTRACT

PCR-based DNA markers were developed for precise and efficient transfer of the fgr gene into new elite improved lines. This was done with an informative RFLP marker RG28, and RM223, which showed the closest linkage to fgr gene in rice, provides means to perform marker-assisted selection in rice breeding program. The objective of this study was to investigate the possibility of generating a polymerase chain reaction PCR–based polymorphic marker that can distinguish aroma and non-aroma. Based on the sequence tagged site data (STSs) of RG28FL-RL and microsatellite RM223, pairwise primers were designed to amplify genomic DNA from C53/Jasmine 85 and C51/Jasmine 85 to identify polymorphic amplified products between the two rice lines OM4900 from C53/Jasmine 85 and OM6161 from C51/Jasmine 85. Two polymorphic markers, amplified with RG28 FL-RL and RM223 were identified. To examine the power of the identified specific amplicon polymorphic marker in predicting the genotypes of the F_2 individuals. These results demonstrate the utility of STS and microsatellite markers for use in marker-assisted selection and breeding within cultivated rice.

Key words: *fgr* gene**,** marker-assisted selection, RFLP, SSR, STS.

INTRODUCTION

The aromatic rice is preferred over non-aromatic rice due to special occasions and for export, and thus they command a higher market price. Based on the presence of aroma, rice cultivars are classified as aromatic and non-aromatic genotypes. The term "aroma" is derived from the fact that resine, oils and balsams were distinguished as aromatic compounds by a market odor. The aroma or fine flavor of cooked rice has been shown to be composed mainly of formaldehydes, ammonia and hydrogen sulfide. Some research reported that an increase of propanol, pentanal, and hexanal during storage seemed to be responsible for the stale flavor of cooked rice. As many as 100 volatile flavor components such as hydrocarbons, alcohols, aldehydes, ketones, acids, esters, phenols, pyridines, pyrazines, and other compounds have been identified in cooked rice.

Genetic studies out on the inheritance of aroma in rice revealed that a recessive nuclear gene controls aroma in rice (Dong et al. 2000). Molecular marker that is closely linked to the aroma gene can be used to facilitate early selection for the presence or absence of scent, and to identify the nature of the locus (homozygous or heterozygous condition), it may also be useful for the rapid incorporation of the scent character into breeding lines. Ahn et al. (1992) reported a DNA marker closely linked to *fgr* gene for aroma on rice chromosome 8. The chromosome segments introgressed from the donor (Della) genome were distinguished by RFLPs, among the NILs (nearly isogenic lines). Linkage association of the clone with the gene was verified using F_3 segregating data for aroma. RFLP analysis showed that the gene is linked to a single copy DNA clone, RG28, on chromosome 8 at a distance of 4.5 cM. Thereby, it is possibly providing an opportunity to initiate marker-assisted selection. The present article aims at reporting a development of PCR-based DNA markers designed from RG28 clone to facilitate an appropriate marker-assisted selection in rice breeding for aroma.

MATERIALS AND METHODS

Plant materials

Two BC_2F_2 populations were developed from two indica crosses C53/Jasmine 85 and C51/Jasmine 85. Jasmine 85 has always been the breeder's

choice as donor for aroma with high yielding plant type. C51 and C53 are improved varieties developed at CLRRI and non-aromatic genotypes. One hundred twenty BC_1F_1 seeds were produced from the cross of C53/Jasmine 85. They were planted in a protected field to produce BC_2F_2 seeds. About 1,000 BC_2F_2 seeds were collected with one panicle per plant and two selected seeds per panicle. They were developed BC_2F_3 generation.

Aroma assessment

The seeds from BC_1F_1 plants (means BC_2F_2 population) were manually dehulled. The seeds from each BC_2F_2 (BC_2F_3 population) were treated by Satake dehuller. They were milled by test miller for one hour. Ten seeds from each BC_2F_2 plant were individually ground for 10 seconds with a medium speed by Wil grinder. Rice powder of each grain was placed in an individual 5x5 cm plastic box. To each box, 500µl of diluted alkali (1.7%) was added and covered immediately. The treated samples were placed at room temperature for 30 minutes. The boxes opened one by one and aroma was scored by smelling. The heterozygotes were recognized based on the presence of aromatic and non- aromatic grains in BC_2F_2 progeny test. When all ten seeds of individual BC_2F_2 plant were aromatic, the individual was considered as homozygous for aroma. If the ten seeds of individual BC_2F_2 plant were non-aromatic, otherwise, the individual would be considered as homozygous for non-aroma. Presence of aromatic and non-aromatic seeds in BC_2F_2 progenies indicated heterozygous nature of plant. Due to importance and accuracy of the phenotyping in mapping process, particularly in bulk-segregant analysis, additional 30 seeds from each homozygous aromatic and homozygous non-aromatic plants were analyzed. It was done to assure the accuracy of phenotyping.

Due to importance and accuracy of phenotying in mapping process, rice leaves were also evaluated at tillering stage. Ten leaves were sampled from individual plants at tillering and cut into 5mm long pieces. They were put into a capped glassware, and stored at - 20°C before aroma evaluation. One hour was measured from each frozen leaf sample, by putting into a capped test tube, and mixed with 5ml of 1.7 % KOH solution for 10min at 50°C. Four to five panelists were asked to classify the samples as either aromatic or non-aromatic by their own smell.

DNA extraction

DNA suitable for PCR analysis was prepared using a simplified miniscale procedure. A piece of young rice leaf (2 cm) was collected and placed in a labeled 1.5 ml centrifuge tube in ice. The leaf was ground using a polished glass rod in a well of a Spot Test Plate (Thomas Scientific) after adding 400 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 25mM EDTA, 300mM NaCl and 1% SDS). Grinding was done until the buffer turned green, which is an indication of cell breakage and release of chloroplasts and cell contents. Another 400 µl of the extraction buffer was added and mixed into the well by pipetting. Amount 400 µl of the lysate was transferred to the original tube of the leaf sample. The lysate was deproteinized using 400 µl of chloroform. The aqueous supernatant was transferred to a new 1.5 ml tube and DNA precipitated using absolute ethanol. DNA was air-dried and resuspended in 50 µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). An aliquot of 1 µl is sufficient for PCR analysis. The remaining DNA was stored in -20°C for any later use.

Primers

One marker derived from the RG28 sequence (Ricegenes). The STS primers was designed as RG28LF 5'-GATCTCACTCCAAGTAAACTCTGAC-3'

RG28LR5'-ACTGCCATTGCTTCTGTTCTC-3'

One microsatellite marker on chromosome 8 as RM223 was designed by Cornell University: RM223 F 5'-GAGTGAGCTTGGGCTGAAAC-3'

RM223 R 5'-GAAGGCAAGTCTTGGCACTG-3'

Microsatellite assay

PCR amplification was performed in 10mM Tris-HCl (pH 8), 50mM KCl, 1.5mM $MgCl_2$.1 unit of TAKARA *Taq*, 4 nmol dNTP, 10 pmol primer and 50ng genomic DNA . The PCR reactions were denatured at 95°C for 5 min, followed by 35 cycles of 94°C for 60 seconds, 55°C for 30 seconds and

72°C for 60 seconds. The final extension was at 72°C for 5 min. After PCR, 13µl of loading buffer (98% formamide, 10mm EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) were added. Polymorphism in PCR products were detected by ethidium bromide staining after electrophoresis on 5% agarose gels

Microsatellite marker scoring and linkage analysis

The marker was scored for presence or absence of the corresponding band among two extremes: pool segregant with good aroma and non-aroma type along with the parents. Segregating bands were scored as either 1 (aroma) or 2.

RESULTS

Initial aromatic score among breeding materials was emphasized that Khao Dawk Mali 105 obtained the highest one. Jasmine 85 and Nang Thom Cho Dao, a traditional cultivar from Southern Vietnam obtained score 2

Table 1: Aroma scoring among different rice genotypes

Variety	Aromatic Score
Nang Thom Cho Dao	2
Khao Dawk Mali 105	4
Jasmine 85	2
C51	0
C53	0

Phenotypic variation

C53, Jasmine 85, and Khao Dawk Mali 105 (control), and 400 BC_2F_2 of C53/Jasmine 85 were evaluated for aroma. A segregation of 99 aromatic and 301 non-aromatic lines indicated the presence of a recessive aroma gene with goodness of fit for 1:3 ratio (χ^2 = 0.013333). The frequency distribution of aroma and non-aroma phenotypes among the BC_2F_2 was continuous. About 33.0% of BC_2F_2 segregants were aromatic as well as Jasmine 85. Otherwise, 67.0 % was non-aromatic like C53. This showed a good recombination for aromatic segregants in the population.

Analysis of BC_2F_3 of C51/Jasmine 85 was done based on four categories unlike analysis of BC_2F_2 population (only two categories as aroma and non-aroma). 150 non- aromatic, 38 slightly aromatic, 17 moderately aromatic, and 7 aromatic individuals were recognized. Aroma in BC_2F_2 and BC_2F_3 populations was evaluated based on the score of 1, 2, 3 and 4 for non-aroma, slight aroma, moderate aroma, and real aroma, respectively.

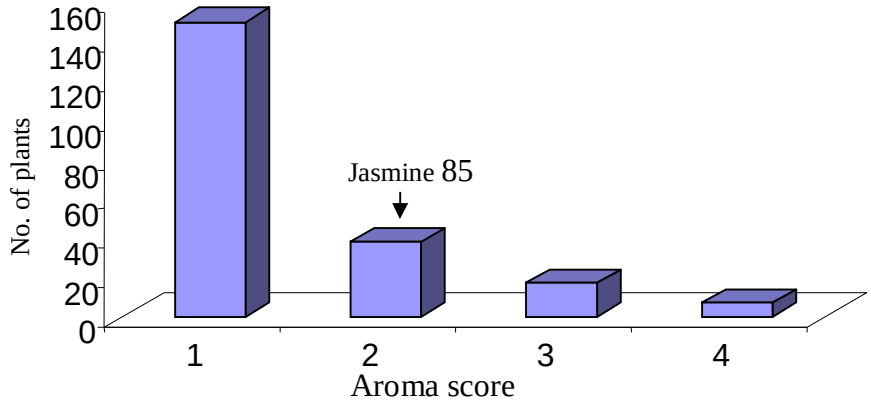

Figure 1: Distribution of BC_2F_3 population in cross between C53/Jasmine 85 based on aromatic expression

DNA survey and polymorphic markers

Thirty primers (SSRs) were screened for DNA polymorphism among parents. The primer was successfully amplifying the target locus of RM223 from C53 and Jasmine 85 (Figure 2). It revealed that the DNA polymorphism between the parents was 33.3 %.

For selective genotype analysis with amplified RG28, extreme lines with aroma of Jasmine type and C53 were selected.

Analysis of the BC_2F_2 population

The phenotype of aroma and genotype of 12 markers for the BC_2F_2 population was evaluated. The two extremes of the population were identified for selective genotype. 50 of the most aromatic ones, 50 of the most non-aromatic ones were identified by DNA into two clusters. Twelve markers showed polymorphism in parental survey were selected to evaluate the polymorphism between the two bulked samples.

Marker RM223 F-R showed polymorphism between the BC_2F_2 of C53/Jasmine 85 and generated to distinguish aromatic from non-aromatic genotypes. The fragments amplified ranged from 90 to 190bp. This polymorphism can, therefore be used as a marker to distinguish between the *fgr* gene and its allelomorph conferring non-aromatic property. The 120 bp band corresponds to an allele from parent Jasmine 85 and the 160- bp band represent the parent C53

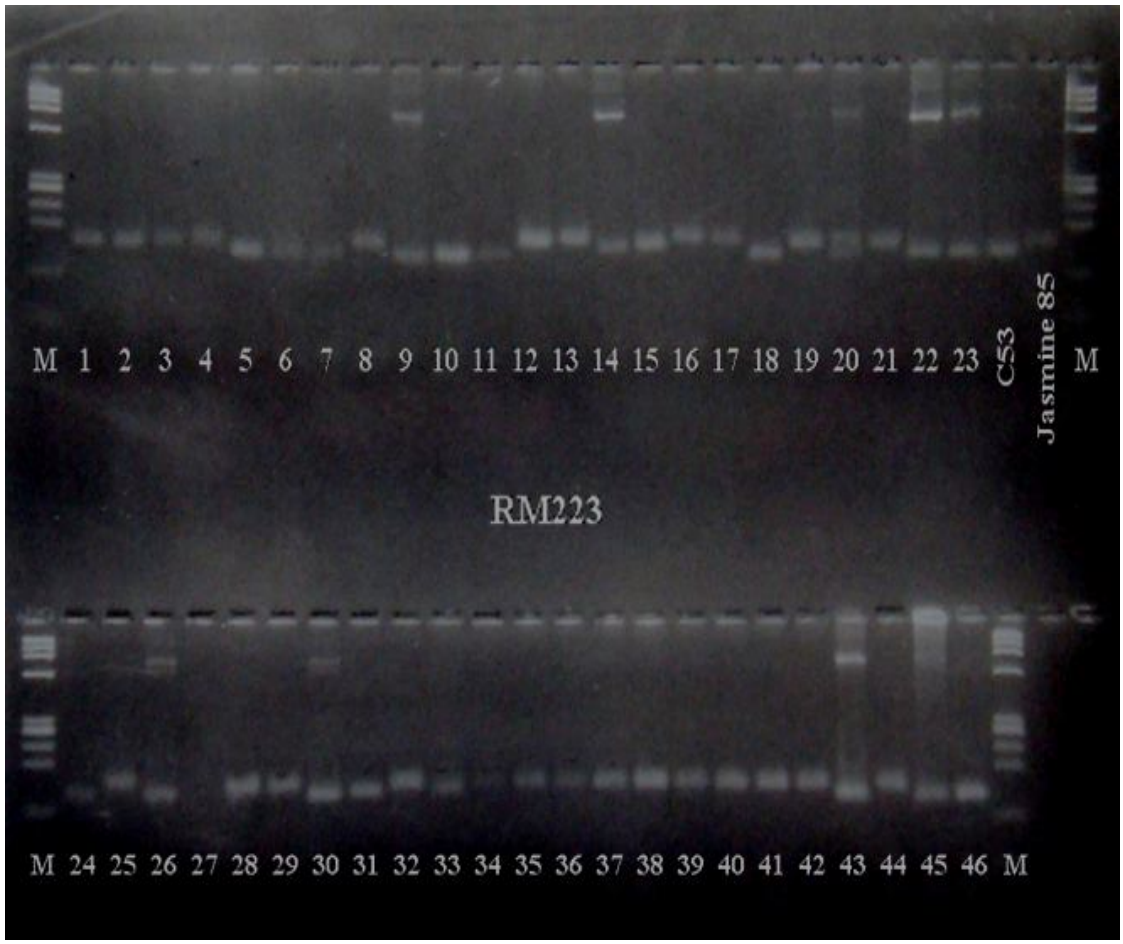

Figure 2: PCR products of BC_2F_2 population from C53/Jasmine 85 with primer RM223

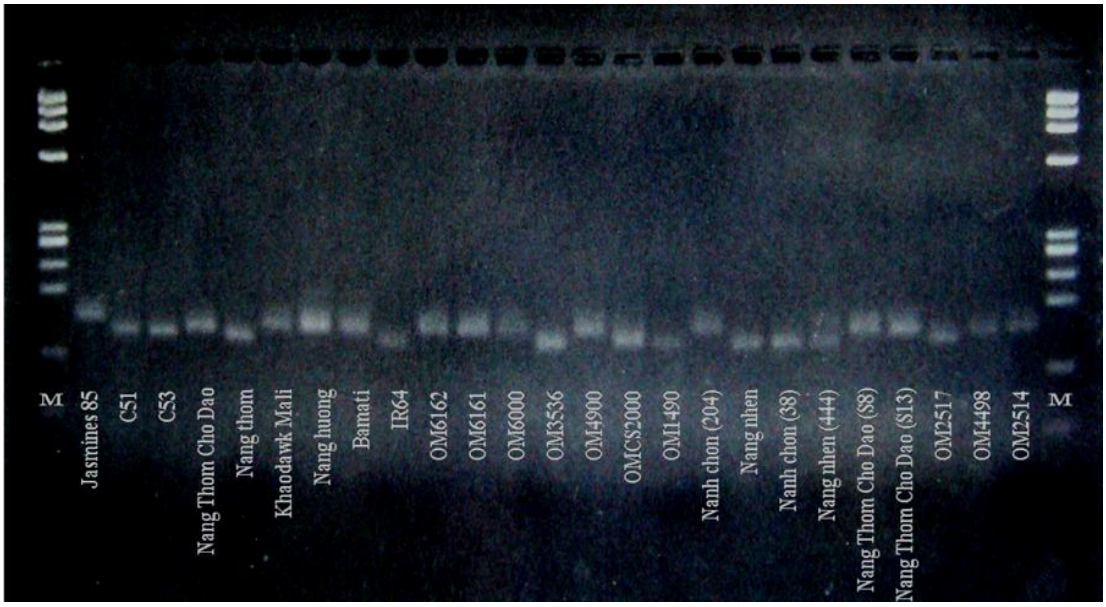

Figure 3: DNA survey on rice germplasm at locus RG28FL-RL

DNA marker associated with aroma gene

To examine the validity of microsatellite as genetic markers; genomic DNAs from the 50 F_3 individual of the cross between the non-aroma parent (OM 1490) and aroma (Jasmine 85) were PCR-amplified using primers RG28F-R and RM223, to compare this prediction with the data derived from the aroma scoring. Of the 50 in individual plants that were scored as homozygous aromatic lines at the locus RG28F-R; 7 plants and 43 plants were found to be homozygous non-aromatic and homozygous aromatic lines, respectively. This gives an accuracy of 84%. Of the 50 plants that were scored as homozygous susceptible based on the RG28 marker; 46 plants and 4 plants were found to be homozygous non-aromatic and homozygous aromatic lines, respectively. This gives an accuracy of 92%. However, the error in scoring phenotype that cannot be avoided with effect of environment, such as aroma.

Applicability of RG28F-R, RM223 as markers for aroma was implemented with 90 BC_1F_1 lines of C51/Jasmine 85. The markers (RG28F-R, RM223) gave a signal of the correct size in rice aromatic genotypes.

There are small discrepancies between scoring based on the marker genotypes for homozygous and heterozygous aroma and non-aroma classes.

Germplasm analysis

If the RG28LF-LR and RM223 markers could distinguish genotypes with closely related to aromatic cultivars in germplasm (Nang thom Cho Dao, Nho Thom, Jasmine 85, Basmati), the evaluation would be compared to non-aromatic genotype as C51 and C53. The diversity of alleles in aromatic genotypes was measured at RG28F-R and RM223 loci. This indicated that the landraces were not heterogeneous for these loci except Nang Nhen 444, a rainfed genotype. The results indicate a very large number of polymorphic bands detected per primer and suggest very high genetic variation among varieties.

Table 2: Allele variation for one microsatellite and one STS marker loci in *fgr*

Designation	Gene	RG28FR	RM233	Phenotype *
Jasmine 85	*fgr*	A	A	3
C51	no	B	B	1
C53	no	B	B	1
NTCD	*fgr*	A	A	2
Nho Thom	*fgr*	B	B	4
KhaoDawk Mali 105	*fgr*	A	A	4
Nang Huong	*fgr*	A	A	3
Basmati	*fgr*	A	A	2
IR64		B	B	1
OM6161	*fgr*	A	A	2
OM6162	*fgr*	A	A	2
OM6000	*fgr*	A	A	2
OM3536	*fgr*	B	B	2
OM4900	*fgr*	A	A	2
OMCS2000	no	B	B	1
OM1490	no	B	B	1
Nanh chon 205	*fgr*	A	A	2
Nang Nhen	no	B	B	2
Nang Nhen 444	*fgr*	A, B	B	2
NTCD 8	*fgr*	A	A	3
NTCD 13	*fgr*	A	A	3
OM2517	no	B	B	1
OM4498	no	B	B	1
OM 2514	*fgr*	A	A	1

* Non-aromatic = 1, slightly aromatic = 2, moderately aromatic=3 and strongly aromatic = 4

Breeding for aroma rice

The line OM4900 derived from C53/Jasmine 85//Jasmine 85 can gain 6-7 ton/ha under wet season and dry season of Mekong Delta. Its growth duration of 95 days, plant height of 100 cm make it adapted to the delta rice production. It was noticed to be suitable to acid sulfate soils and moderately tolerant to salinity. A total of 1,105 lines was evaluated for aroma: 97.8% and 2.1% were found to be non-aromatic and slightly aromatic, respectively. These lines were shuttled and entered into the observational nursery, selected and released in 2008. Beside aroma trait, OM4490 expressed intermediate amylose content, intermediate gelatinization temperature and good kernel elongation. These are preferred by most of the consumers in the world. Another line from C51/Jasmine 85 such as OM6161 were found light aroma. It will be selected in BC_2F_2 populations

Table 3: Yield and yield components of promising lines in 2008 dry season at CLRRI

Designation	Panicle/m^2	Filled grains/panicle	1000-grain weight (g)	Yield (t/ha)
OM 2488	326 b	113 bc	29.4 c	7.50 a
OM 4498	299 bcd	153 a	27.9 de	7.36 a
OM 6162	308 bcd	149 a	29.1 c	7.30 a
OM 6161	**380 a**	**111 c**	**29.3 c**	**7.23 a**
OM 6073	310 bcd	143 ab	31.0 a	7.23 a
OM 4900	**324 bc**	**139 abc**	**28.3 d**	**7.06 ab**
VN 124	313 bcd	168 a	27.4 ef	7.03 ab
OM 5239	392 a	115 bc	28.9 c	7.00 ab
MTL 425	322 bc	112 c	30.0 ab	6.90 ab
OM 5798	316 bc	141 abc	30.0 b	6.80 ab
OM 5900	275 cd	135 abc	27.0 f	5.53 abc
OM 5936	302 bcd	131 abc	27.5 ef	5.40 bc
OMCS2000	264 d	136 abc	27.6 ef	4.90 c
CV%	8.06	7.06	1.06	8.37

DISCUSSION

Plant breeding, in its conventional form, is based on phenotypic selection of superior genotypes within segregating progenies obtained from crosses. Application of this methodology often encountered difficulties related principally to genotype x environment (G x E) interactions. In addition, several phenotyping procedures are often expensive, time consuming or sometimes unreliable for particular traits (*i.e.* for some traits related to abiotic stress tolerance). Molecular marker-assisted selection (MAS) is an approach that has been developed to avoid the problems connected with conventional plant breeding changing the selection criteria from selection of phenotypes towards selection of genes, either directly or indirectly. Molecular markers are clearly not environmentally regulated and are unaffected by the conditions in which the plants are grown and are detectable in all stages of plant growth. With the availability of an array of molecular markers and genetic maps, usefulness of a given molecular marker is dependent from its capability in revealing polymorphisms in the nucleotide sequence. This is allowing microsatellite to be possible to exploit this information to trace the flow of genes or quantitative trait loci of interest in rice and make prediction about crossing and selection that will increase the efficiency of variety development. In addition, microsatellite marker analysis can be automated and this feature is attractive for marker assisted selection program. This study reports the localization of RG28F-R, RM223 on chromosome 8, which can serve as a starting point for the positional cloning RG 28F-R, RM223. For this purpose and for fine mapping, saturation of the genetic map by increasing the amount of markers at the chromosomal region of interest is in progress. All two markers showed a positive signal in breeding lines (C51/Jasmine 85 and C53/Jasmine 85). Thus, selection for progeny with the gene of interest is not always possible based on aromatic and non-aromatic. Molecular markers linked to the gene of interest are required. Compared to the phenotype, marker-assisted selection for aroma can improve the cost effectiveness and significantly speed up the introgression of aroma gene to rice

REFERENCES

Ahn SW, CN Bollich, and SD Tanksley. 1992. RFLP tagging of a gene for aroma in rice. Theor. Appl. Genet. 87:27-32

Dong JY, E Tsuzuki, and H Terao. 2000. Inheritance of aroma in four rice cultivars (*Oryza sativa* L.). IRRI. International Rice Research Notes 25:2

Lander ES, D Botstein. 1989. Mapping Mendelian factors underlying quantitative traits using RFLP linkage map. Genetics 121:185-199.

Nguyen Thi Lang and Bui Chi Buu. 2002. Identification and fine mapping of SSR marker linked to *fgr* gene. OMonRice 10: 16-22

Phát hiện gen lặn *fgr* điều khiển mùi thơm cây lúa bằng PCR markers

DNA marker trên cơ sở PCR đã được áp dụng trong trường hợp tìm kiếm gen điều khiển mùi thơm trong genome cây lúa (*fgr*). Từ những thông tin đầu tiên về RFLP marker (RG28) và microsatellite marker (RM223), Viện Lúa đã tìm thấy gen *fgr* định vị trên nhiễm sắc thể số 8, điều này đã mở ra triển vọng tìm kiếm gen lặn chịu ảnh hưởng bởi yếu tố môi trường, rất khó tìm thấy trong liểu hình bên ngòai. Viện thực hiện các cặp lai C53/Jasmine 85 và C51/Jasmine 85 nhằm xác định sản phẩm đa hình, thực hiện fine mapping, trên 2 dòng OM4900 của cặp lai C53/Jasmine 85 và OM6161 của cặp lai C51/Jasmine 85. Hai chỉ thị phân tử giúp cho nhà chọn giống phát hiện con lai của thế hệ đang phân ly có chứa gen *fgr* trong các cá thể F_2.