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Identification and fine mapping of SSR marker linked to *fgr* gene of rice

Nguyen Thi Lang and Bui Chi Buu

ABSTRACT

The genomic clone RG28, which is tighly linked to fgr gene in rice, provides to perform marker-aided selection in rice breeding program. This study aims at identifying and making fine mapping of SSR marker linked to fgr gene in rice which controls aromatic property. RG28 marker can be converted by sequencing into SSR and used as primers for PCR amplification of genomic DNA from rice varities differing in the aromatic responsiveness. Fine genetic mapping was conducted at initial steps with an effort to apply marker-assisted selection in plant breeding. We used 12 selected DNA markers and an F_2 population included 250 plants, fgr was fine mapped to a genomic region <2 cM on the chromosome 8. Two markers RG28, RM223 closely linked to extremely aromatic phenotypes. DNA marker-assisted selection was used to detect fgr gene. To examine the power of the identified SSR markers in predicting the phenotype of the fgr locus, we determined the genotypes of the F_2's individuals at this locus by performing progeny testing for fgr in the F_3 generation. The results indicated an accuracy of more than 84% in identifying the resistant plants which was similar to that using RG28F-R. The results of the germplasm survey will be useful for the selection of parents in breeding program aimed at transferring this gene from one variety background to another using marker-assisted selection.

Key words: Molecular marker, aroma, SSR (simple sequence repeat), marker-assisted selection (MAS), fragrant rice, *fgr* gene.

INTRODUCTION

The aromatic rice is preferred over non-aromatic rice during special occasions and for export, and thus they command a higher market price. Based on the presence of aroma, the rice varieties are classified as aromatic and non-aromatic. 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 fineflavor of cooked rice has been shown to be composed mainly of formaldehydes, ammonia and hydrogen sulfide. Some researches 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 inrice (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 a gene for aroma in rice. Chromosome segments introgressed from the donor (Della) genome were distinguished by RFLP, among the NILs. 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 4.5cM. Thereby, providing an opportunity to initiate marker-aided selection. In the present article, we report the development of a PCR-based DNA marker based on the RG28 clone.

MATERIALS & METHODS

Plant materials

F_2 population was developed from an indica Khao Dawk Mali / OM1490. Khao Dawk Mali has always been considered as a donor for aroma. OM1490 is an improved variety developed at CLRRI without aroma. One hundred twenty F_1 seeds were produced and planted in a protected field to produce F_2 seeds. About 1000 F_2 seeds were planted in the field. One panicle per plant was harvested and two seeds per harvested panicle were planted for F_3 generation.

Assessment of aroma

The seeds from F_1 plant (F_2 individuals) were dehulled manually and the seeds from each F_2 (F_3 population) were dehulled using Satake dehuller and milled by test miller for one hour. A total of ten seeds from each F_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 treaed samples were placed at room temperature for 30 minutes. The boxes opened one by one and were scored for aroma (smelling). The heterozygotes were recognized based on the presence of aromatic and non-aromatic grains in F_2 progeny test. When all ten seeds of individual F_2 plant were aromatic, the F_2 individual was considered as homozygous for aroma. If the ten seeds of individual F_2 plant were non-aromatic then plant was considered as homozygous for non-aroma. Presence of aromatic and non-aromatic seeds in F_2 progeny indicated heterozygous nature of plant. Due to importance and accuracy of the phenotying in mapping process, particularly in bulk segregant analysis additionally 30 seeds from each homozygous aromatic and homozygous non-aromatic plant were analysed. It was done to assure the accuracy of phenotyping.

Due to importance and accuracy of the phenotying in mapping process, the leaf sample was used to evaluate at tillering. Ten leaves were sampled from individual plants at tillering and cut into 5mm long pieces, put into a capped glassware, and stored at - 20^{o}C before aroma evaluation. Every one hour, they were measured from each frozen leaf sample, put into a capped test tube, and mixed with 5ml of 1.7 % KOH solution for 10 min at 50^{o}C. Four to five panelists were asked to classify the samples as either aromatic or non-aromatic by their 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 ^{o}C for any later use.

The primers use

One marker derived from the RG28 sequence (Ricegenes). The primers was converted from RFLP as:

RG28.F
5'-GATCTCACTCCAAGTAAACTCTGAC-3'

RG28.R
5'-ACTGCCATTGCTTCTGTTCTC-3'

and 11 marker microsatellite on chromosome 8 as:

RM134, RM118, OSR34, RM38, RM25, RM310, RM344, RM42, RM223, RM284 and RM308 from Cornell University.

For the 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, 10pmol 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 type. The linkage between SSR marker and the gene was estimated, then linkage map was generated by MAPMARKER (Lander 1989)

RESULTS

Development of population

Khao Dawk Mali and Hoa Lai used as aromatic donors in target crosses, IR64, OM1490 genotypes were used as non-aromatic. The F_1 plants were grown and selfed to produce the F_2 populations for fine mapping.

Designation	Aroma (score)
IR64	0
Hoa Lai	1
KhaoDawkMali 105	4
Jasmine	1
OM1490	0

Phenotypic variation

Khaw Dawk Mali, OM1490, Jasmine (aroma control), and the 250-F_2 family were used to evaluate aroma. A segregation of 71 aromatic and 179 non-aromatic in F_2 was observed, indicating the presence of a recessive *fgr* gene with goodness of fit for 1:3 ration. The results of the evaluation were showed in the table 1. Among the 250-F_2 family of the cross Khao Dawk Mali x OM1490, there was wide variation observed for aroma. The frequency distribution of aroma and non-aroma reaction among the F_2 was continuous. The aroma of Khao Dawk Mali obtained score 2 while OM1490 obtained score 0. About 39.66% of F_2 individuals were as aromatic as Khao Dawk Mali and 61.30% as non-aromatice as OM1490. This showed a good recombination for aroma in the population.

Analysis of F_3 seeds was implemented based on four categories unlike analysis of F_2 population that was based on only two categories of aromatic and non-aromatic property. The results showed 118 non-aromatic individuals, 33 slightly aromatic, 37 moderately aromatic, and 12 strongly aromatic. Evaluation of aroma in F_2 and F_3 populations based on quality value (non-aroma = 1, slight aroma = 2, moderate aroma = 3 and strong aroma = 4), showed that aroma is a complex trait. (figure 1)

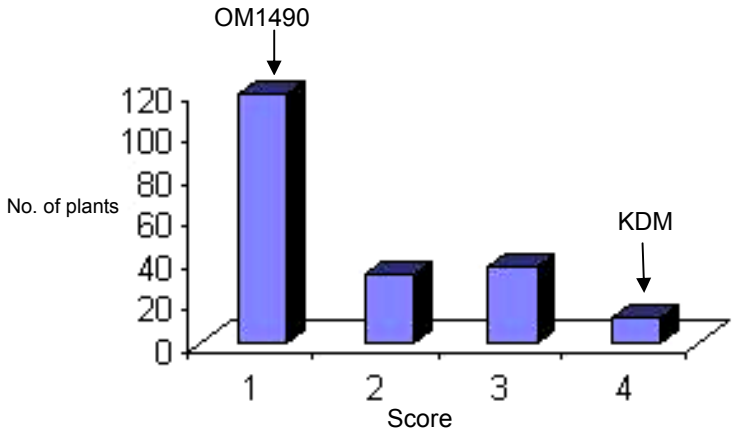

Figure 1: Distribution of aroma in F_3 population in cross between Khao Dawk Mali 105 and OM1490

Molecular marker polymorphism of the parents

Electrophoretic analysis of PCR products derived from OM1490 and Khao Dawk Mali was carried out. Thrity primers were screened for DNA polymorphism between parent. Thirty primers were successful in amplify locus of Khao Dawk Mali and OM1490. Twelve markers in chromosome 8 such as RM134, RM118. OSR34, RM38, RM25, RM310, RM344, RM42, RM223, RM284 and RM308 were observed their polymorphic levels (Figure 2). It revealed that the DNA polymorphism between the parents was 33.3% for microsatellites. For selective genotype analysis with amplified RG28, extreme lines with type 1 of aroma as Khao Dawk Mali and type 2 as OM1490 were select. As compared with Jasmine, Khao Dawk Mali was higher for selective genotype analysis with amplified and codominant primer RM223 (figure 3)

Analysis of the F_2 population

Based on phenotyping aroma and genotyping with 12 markers in the F_2 population, the two extremes of the population were identified for selective genotype, 50 of the most aromatic, 50 of the most non-aromatic lines were identified. DNAs of these two groups of 50 individuals each were samples. Twelve polymorphic markers in parental survey were selected to evaluate the polymorphism between the two bulked samples.

To establish linkage between aroma gene and linked marker, SSR data are pooled together and analyzed with MAPMARKER. A linkage map was established (figure 4). The *fgr* gene is linked with two markers RG28 and RM223 of chromosome 8 at a distance of 1.6 and 1.8 cM, respectivity. Marker RG28 showed polymorphism between the two bulks and generated to distinguish aroma from non-aroma. The fragments amplified ranged from 90-190 bp. This polymorphism can, therefore be used as a marker to distinguish between the aroma gene and it allelomorph conferring non-aroma. The 160 bp band corresponds to an allele from parent Khao Dawk Mali and the 120 bp band represent the parent OM1490

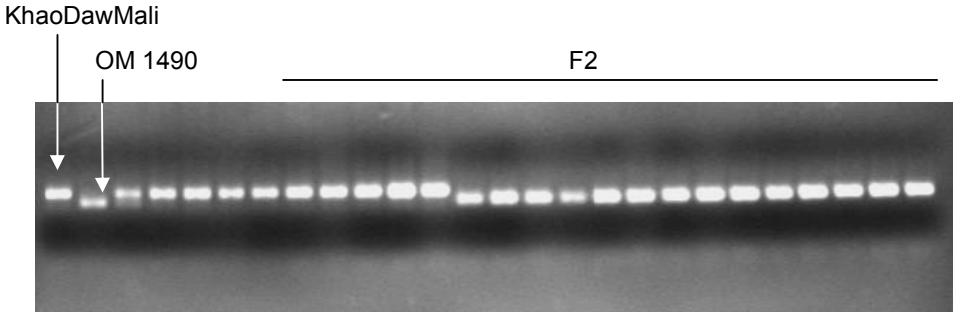

Figure 2: PCR products of F_2 population from KhaoDawmali 105/OM1490, with primer RG28F-R

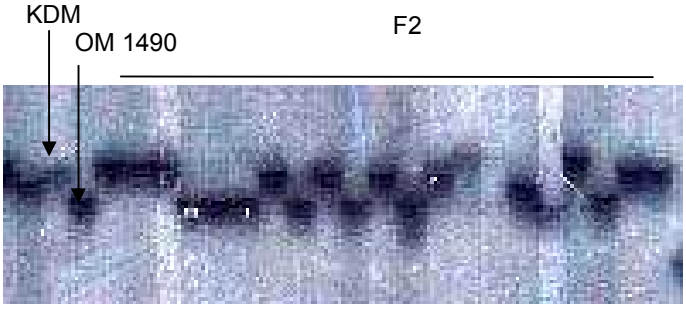

Figure 3: PCR products of F_2 population from Khao Dawk Mali 105 / OM1490, with primer RM223

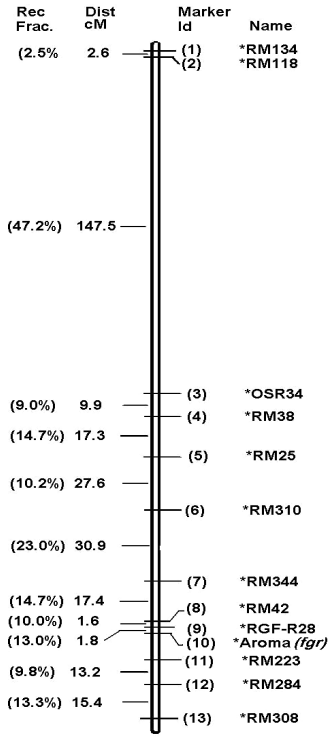

Figure 4: The molecular map of the *fgr* region in rice chromosome 8. Map was derivered from 250 F_2 plants of the cross betwwen Khao Dawk Mali / OM1490

Table 3. *fgr* gene analysis of F_2 through progeny test and PCR from KhaoDawk Mali / OM1490

Genotype	Number of plant	RG 28		Accuracy (%)
		AA	aa	
AA	50	43	07	84
aa	50	04	46	92

DNA marker associated with *fgr* gene

To examine the validity of microsatellites as genetic markers, genomic DNAs from the 50 F_3 individuals of the cross were PCR-amplified using primer RG28F-R and RM223 (Table 1). To compare this prediction with the data derived from the aroma scoring. Of the 50 individual plants were scored as homozygosity on the RG28F-R, seven plants were found to be homozygous for non-aroma and 43 plants were found to be homozygous fro aroma. This gives an accuracy of 84%. Of the 50 plants that were scored as homozygous aroma based on the RG28 marker and 46 plants were found to be homozygous for non-aroma, and 4 plant was found to be be homozygous for aroma. This gives accuracy 92%. However, the error in scoring phenotype that cannot be avoided with the effect of environment, especially aroma .

Applicability of RG28F-R, RM223, as marker for aroma was tested in the population. Marker-assisted selection was used in 90 BC_1F_1 plants of IR64 / Hoa Lai. All markers (RG28F-R, RM223) gave a signal of the correct size in the rice genotype which carries aromatic property. In contrast, no signal band of the correct size was detected by any marker RM42 in this population.

There are small discrepancies between scoring based on the marker genotypes for homozygous and heterozygous aroma and non-aroma classes and based on the progeny testing could be due to recombination events between the RG28F-R and RM223 marker and aroma.

Germplasm analysis

To examine if the RG28F-R and RM223 could distinguish genotypes with closely related to aromatic landraces (Hoa Lai, Nang thom Cho Dao, Nho Thom) and Jasmine variety from non-aromatic IR64. The diversity of alleles in aromatic genotypes was measured for RG28F-R and RM223. DNAs of some varieties were tested, indicating that the landraces were not heterozygous at the locus except Jasmine. The results indicate a very large number of polymorphic bands detected per primer and suggest very high genetic variation among varieties.

DISCUSSION

Microsatellite should, therefore, 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 localicazation of RG28F-R, RM223 in chromsome 8, which can serve as a starting point for the positional cloning RG28F-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 (IR64 / Hoa Lai and KhaoDawk Mali/OM1490). Thus, selection for progeny with the gene of interest is not always possible based on aroma and non-aroma, and molercular markers linked to the gene of interest are required. As compared to phenotyping, marker-assisted selection for aroma can improve the cost effectiveness and significantly speed up the introgression of aroma gene to rice

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SUMMARY IN VIETNAMESE

Xác định gen *fgr* điều khiển tính trạng mùi thơm bằng phương pháp Fine Mapping với microsatellites

Gen *fgr* điều khiển tính trạng mùi thơm là một gen lặn định vị trên nhiễm thể số 8, bị ảnh hưởng bởi môi trường. Thiết kế cặp mồi từ RG28

RG28.F 5'-GATCTCACTCCAAGTAAACTCTGAC-3'
RG28.R 5'-ACTGCCATTGCTTCTGTTCTC-3'

và sử dụng các microsatellites để thực hiện "fine mapping". Kết qủa cho thấy RG28F-R và RM223 có thể được sử dụng trong chương trình chọn giống nhờ marker (MAS). Băng thể hiển mùi thơm xuất hiện ở độ lớn 190 bp và không thơm thể hiện ở độ lớn 90 bp trong trường hợp RG28F-R. Băng thể hiện mùi thơm 160 bp, và không thơm là 120 bp trong trường hợp RM223.