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Identification of fragrance gene in some elite advance lines of rice cultivated in foothills of the Himalayas

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Abstract

A molecular survey was conducted for the screening of fragrance (*fgr*) gene in some elite advance lines of rice developed by Dr. Fida Muhammad Abbasi, Professor at Department of Genetics Hazara University Mansehra. Sequence Tag Site (STS) marker RG 28L was used in this study that amplified 140 and 120 bp fragment in fragrant and non-fragrant genotypes, respectively. Among the cultivated varieties Basmati-385 and Swat-1 showed the presence of *fgr* gene (140 bp amplicon) while IRBB59, JP-5, Fakhre Malakand, and IR24 were lacking this gene. Among the advance lines 12 genotypes showed the presence of *fgr* gene (140), two genotypes (NPT-86 and Line 36) were segregating while the remaining 16 genotypes were lacking this gene. Grain length of genotypes was also measured that ranges from 4.67 to 8.10 mm. On the basis of grain length the genotypes were categorized into short, medium, long and extra-long. In this study 4 genotypes possessed extra-long, 17 long, 13 medium and only 2 have short grains.

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Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population (Marathi *et al.,* 2012). In rice physical grain quality plays an important role in consumer preference. Juliano and Duff (1991) concluded that improvement of physical grain quality is the second major objective of rice breeding programs after yield in many rice producing countries of the world. The physical grain quality of rice is a complex trait that is composed of many components such as appearance quality, cooking quality, eating quality and nutritional quality. Each one of these components also consists of many attributes whose values are determined not only by their physio-chemical properties but also by the history and cultural traditions of the people in the human communities who consume the rice (Tan *et al.*, 1999). One of the most valuable traits in high-quality rice is aroma or fragrance, which is important for consumer preference and global trade (Singh *et al.*, 2000). Fragrant rice emits specific scent in the fields at the time of flowering, at harvesting, in storage, during milling, cooking and eating. Economically, it possesses an extraordinary position in the global business sector because of its pronounced, pleasant and unique scent and mouth feeling taste after cooking. Fragrant rice is preferred over non-fragrant rice due to special occasions and for export, and thus they command a higher market price. A better understanding of the factors that contribute to the overall grain quality of rice will lay the foundation for developing new breeding and selection strategies for combining high quality, with high yield. This is necessary to meet the growing global demand for high quality rice while offering producing countries additional opportunities for generating higher export revenues.

Pakistan is famous for exporting high quality basmati rice but rice yield is stagnant from the previous few decades. Dr. Fida Muhammad Abbasi Professor at Department of Genetics, Hazara University Mansehra Pakistan has developed advance lines of rice in order to break yield stagnation. These lines are high yielding but their physical grain quality has not been properly assessed, therefore the present study was being proposed with the aims to identify fragrance (*fgr*) gene in these advance lines.

Materials and methods

Plant material

Plant material was comprised of 6 cultivated varieties viz. Basmati-385, IRBB59, JP-5, Fakhre Malakand and 30 elite advance lines of rice. Seeds of cultivated varieties were obtained from the Gene Bank of Plant Genetic Resource Institution (PGRI), NARC (National Agriculture Research Centre), Islamabad, Pakistan while the advance lines were provided by Dr. Fida Muhammad Abbasi that have been developed at Hazara University, Mansehra, Pakistan. These lines were planted at National Tea and High value crops Research Institute (NTHRI), Shinkiari Mansehra Pakistan (Fig. 1).

DNA extraction and PCR analysis of advance lines of rice for the presence of (fgr) gene

Five seeds of each genotype were taken in 1.5 ml Eppendorf tubes and 500µl of 2x CTAB buffer (50mM Tris-HCl, pH 8.0, 25mM EDTA, 300mM NaCl and 2% CTAB) was added to it, incubated at 65° C for 1 hour and then crushed with squashing needles. 500µl Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) was added and leave at room temperature for 30 minutes. Centrifuge the tubes at 8000 rpm for 15 minutes. 400µl clear supernatant was transfer to new tubes and equal volumes of 2-propanol was added and incubated at – 20° C for 2 to 3 hours. Spin at 8000 rpm for 10 minutes to form DNA pellet. Supernatant was discarded and pellet was washed with 70% ethanol. The tubes were kept invert for few hours to become dried. Then 50µl TE buffer was added to each tube. 5µl DNA sample of each genotype was checked on electrophoresis on 1% agarose gel and stained with ethidium bromide. The concentration of extracted genomic DNA was measured by Spectrophotometer and was adjusted from 20 to 50 ng/µl by using sterilized distilled water and stored in Eppendorf tubes at 4°C for further use.

Amplification of *fgr gene* was carried out using allele

specific primers RG 28L (Table 1). Amplification reactions was carried out in 16 ul reaction volumes containing 1µl genomic DNA (20 – 50 ng/µl), 0.5µl each of forward and reverse primers (10 µM / µl), 1.2µl of dNTPs (25 mM each) , 0.4 µl of Taq DNA Polymerase (2 units, Enzynomix), 1X Taq Buffer and 1.6µl $MgCl_2$ (2.5 mM).

PCR amplification was carried out in DNA Thermal Cycler (Applied Bio System) set at: an initial denaturation of 5 min at 94°C; 32 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1:30 sec. One additional cycle of 7 min at 72°C was used for final extension. Amplification products were resolved by electrophoresis on 3% agarose gel run in 1 X TAE buffer. The amplified products were observed under UV light after staining with ethidium bromide (10 ug/ml). The data was scored for the presence or absence of *fgr* linked DNA fragments.

Determination of grain size

Physical grain quality including grain size was determined by measuring the grain length of ten unbroken milled kernels. On the basis of average length, the grains were classified by using the scale as reported by Khush *et al.*, 1979 (Table 2).

Results and discussions

DNA Extraction

Genomic DNA was extracted from fresh seeds of cultivated varieties and advance lines of rice using CTAB method as reported by Delaporta *et al.*, 1983, with few modifications. High quality genomic DNA was obtained as shown in Fig. 2. The concentration of DNA samples were adjusted from 20 to 50 ng/µl with the help of Spectrophotometer.

Table 1. Primer sequence of RG28L used in this study.

Forward	5´-GATCTCACTCCAAGTAAACTCTGAC-3'
Reverse	5´-ACTGCCATTGCTTCTGTTCTC-3´

Table 2. Scale for measurement of grain size of rice.

Scale	Size category	Length in mm
1	Extra-long	More than 7.5
3	Long	6.61 to 7.5
5	Medium	5.51 to 6.6
7	Short	less than 5.5

PCR analysis of cultivated varieties and advance lines of rice for the presence of fgr gene

A molecular survey was conducted for the identification of *fgr* gene in advance lines of rice. STS marker RG 28L was used in this study that amplified 140 and 120 bp fragment in aromatic and non-aromatic genotypes, respectively. Among the cultivated varieties Basmati-385 and Swat-1 showed the presence of *fgr* gene (140 bp amplicon) while IRBB59, JP-5, Fakhre Malakand, and IR24 were lacking this gene (120 bp). Among the advance lines 12 genotypes showed the presence of *fgr* gene (140), two genotypes (NPT-86 and Line 36) were segregating and the rest were non aromatic. Genotypes that possessed *fgr* gene in homozygous condition include line-5, line-34, line-18, line-39, line-59, line-71, line 87, line-88, line-109, line-111, line-130 and line-136.

The rest of advance lines such as line-35, line-38, line-42, line-67, line-77, line-82, line-86, line-87, line-93, line-101, line-103, line-107, line-108, line-154, and line-158 were observed to be non-aromatic as lacking *fgr* gene.

The data was scored using "+" sign for presence of gene (*fgr*) and "–" sign for absence of gene (Table 3 and Fig. 3).

Table 3. Screening of cultivated varieties and advance lines of rice for the presence of fragrance (*fgr) gene.*

S/No	Varieties/ Lines	Target gene (*fgr*)	S/No	Varieties/ Lines	Target gene (*fgr*)
1	Bas-385	+	19	Line-77	-
2	IRBB-59	-	20	Line-79	-
3	JP-5	-	21	Line-81	-
4	F. Malakand	-	22	Line-82	-
5	Swat-1	+	23	Line-86	-
6	IR-24	-	24	Line-87	+
7	Line-5	+	25	Line-88	+
8	Line-34	+	26	Line-93	-
9	Line-18	+	27	Line-101	-
10	NPT-86	±	28	Line-103	-
11	Line-35	-	29	Line-107	-
12	Line-36	±	30	Line-108	-
13	Line-38	-	31	Line109	+
14	Line-39	+	32	Line-111	+
15	Line-42	-	33	Line-130	+
16	Line-59	+	34	Line-136	+
17	Line-67	-	35	Line-154	-
18	Line-71	+	36	Line-158	-

Table 4. Grain length of cultivated varieties and advance lines of rice (*Oryza sativa* L.).

S/No	Genotypes	Grain Length (mm)	S/No	Genotypes	Grain Length (mm)
1	Bas-385	6.87	19	Line-77	7.16
2	IRBB-59	6.66	20	Line-79	7.40
3	JP-5	4.67	21	Line-81	6.57
4	F. M	6.06	22	Line-82	6.13
5	Swat-1	6.40	23	Line-86	7.20
6	IR-24	6.80	24	Line-87	7.57
7	Line-5	6.60	25	Line-88	7.57
8	Line-34	5.73	26	Line-93	7.10
9	Line-18	8.10	27	Line-101	6.70
10	NPT-86	7.00	28	Line-103	7.07
11	Line-35	6.07	29	Line-107	6.47
12	Line-36	6.60	30	Line-108	7.10
13	Line-38	6.30	31	Line109	7.13
14	Line-39	6.60	32	Line-111	7.17
15	Line-42	6.57	33	Line-130	5.80
16	Line-59	6.77	34	Line-136	7.00
17	Line-67	8.10	35	Line-154	5.00
18	Line-71	7.43	36	Line-158	7.47

Fragrant rice is preferred over non-fragrant rice and in this way they summon a higher business sector cost. Fragrance or fine flavor of cooked rice has been shown to be composed mainly of formaldehydes, ammonia and hydrogen sulfide. Some researchers reported that an increase of propanol, pentanal, and hexanal during storage seemed to be responsible for fragrance in rice. As many as 100 volatile aromatic components such as hydrocarbons, alcohols, aldehydes, ketones, acids, esters, phenols, pyridines, pyrazines and other compounds have been identified in cooked rice. Aroma development in rice grain is influenced by both genetic and environmental factors. The biochemical basis of aroma was identified as 2-acetyl-1-pyrroline (Tanchotikul & Hsieh 1991). The conventional methods of plant selection for aroma are not easy because of the high effects of the environment and the very low narrow sense

heritability of fragrance. Molecular approaches such as PCR and RFLP analysis using single nucleotide polymorphism (SNPs), simple sequence repeats (SSRs) and sequence tag site (STS), which are tightly linked to aroma and have the advantage of being simple, rapid and only requiring small amounts of tissue, have been developed for the selection of fragrant rice (Cordeiro *et al.*, 2002). In addition, an allele specific amplification assay (ASA) allows us to distinguish fragrant and non-fragrant genotypes and identify homozygous and heterozygous individuals in a population segregating for aroma (Louis *et al.,* 2005).

Fig. 1. Advance lines of rice grown at National Tea and High value crop Research Institute (NTHRI), Mansehra, Pakistan.

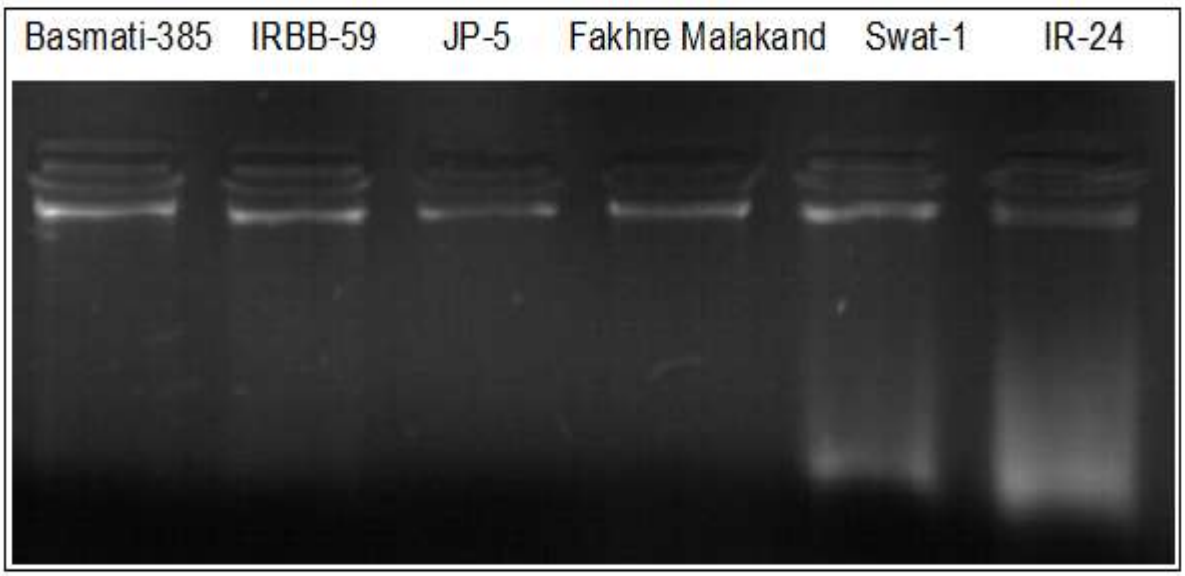

Fig. 2. Genomic DNA extracted from fresh seeds of cultivated varieties of rice using CTAB method and resolved on 1% agarose gel.

Genetic studies carried out on the inheritance of aroma in rice revealed that a recessive nuclear gene controls fragrance in rice (Dong *et al.,* 2001). Molecular markers that are closely linked to aroma has facilitated early selection for the presence or absence of scent, and to identify the nature of locus (homozygous or heterozygous condition). It has proved very useful for the rapid incorporation of scent

character into rice breeding lines. Ahn *et al.*, (1992) reported a DNA marker that is closely linked to fragrance in rice and located on chromosome 8. The chromosome segments introgressed from the donor genome were distinguished by RFLPs, among NILs (nearly isogenic lines). Linkage association of the clone with the gene was verified using F_3 segregating data. 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 (Fig. 4).

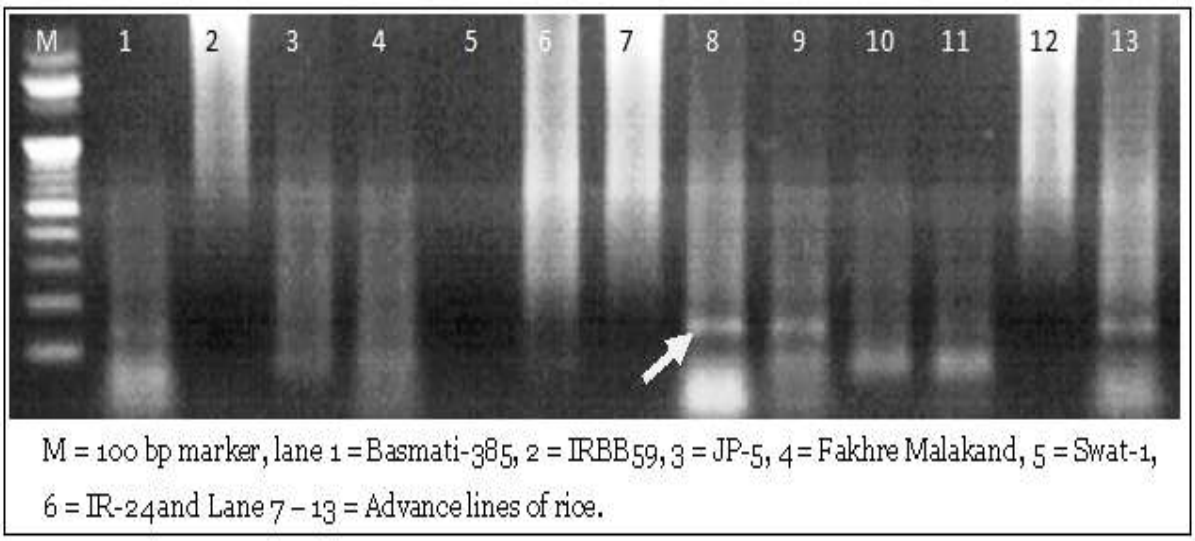

Fig. 3. PCR analysis of cultivated varieties and advance lines of rice for the presence of fgr gene. (Arrow showing 140 bp bands linked to *fgr* gene).

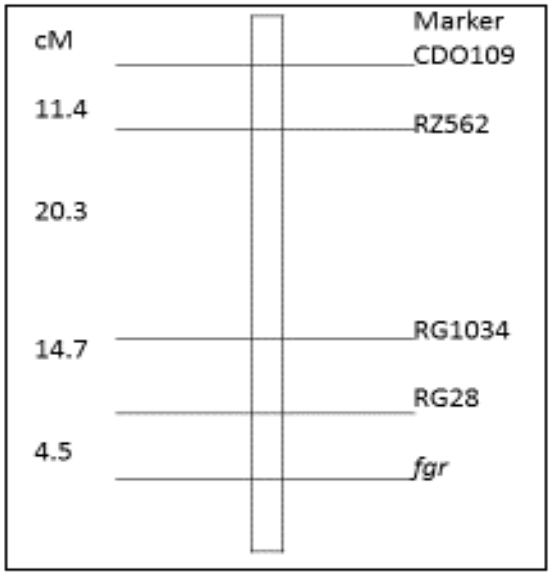

Fig. 4. RFLP map of chromosome 8 of rice showing the location of fgr and linked markers. A map of chromosome 8 was developed by segregation of RFLPs of rice genomic (RG), cDNA (RZ), and oat cDNA (CDO) clones based on F3 population derived from a cross of aromatic and non-aromatic genotypes. CDO109, RZ562 and RG1034 were not polymorphic in the NIL survey but were polymorphic between the mapping parents (Ahn *et al.*, 1992).

Grain Size

Grain length of advance lines and cultivated varieties of rice used in this study was also measured that ranges from 4.6 to 8.1 mm. Maximum grain length was possessed by line-18 and line-67 (8.10 mm), followed by line-87 and line-88 (7.57 mm). Minimum grain length was recorded for cultivated variety JP-5 (4.67 mm) followed by line-154 which was 5 mm. Among the cultivated varieties three genotypes viz., Basmati-385, IRBB59 and IR24 possessed long grains; two genotypes Fakhre Malakand and Swat-1 medium size grains while only JP-5 showed short grains. Among the 30 Advance lines 2 possessed extra- long grains, 16 long, 11 medium and only one (line 154) short grains (Table 4 and Fig. 5).

Grain length is an important agronomic trait for artificial selection in rice breeding. Breeders tend to select plants with large seed size for high yield and appropriate grain size for milling yield and market preferences. However, it is difficult for breeders to improve grain size efficiently by phenotypes, since the traits are quantitatively inherited (McKenzie and

Rutger, 1983). Many quantitative trait loci (QTLs) for grain size have been detected, of which four genes, grain size on chromosome 3 (GS3), grain weight on chromosome 2 (GW2), grain incomplete filling on chromosome 1 (GIF1), and seed width on chromosome 5 (qSW5/GW5), have been isolated and characterized recently (Song *et al.*, 2007; Shomura *et al.*, 2008; Wang *et al.*, 2008; Weng *et al.*, 2008). Among them GS3, is a major QTL for grain length and weight and minor QTL for grain width and thickness in rice. A causal C–A mutation in GS3 is highly associated with rice grain length (Fan *et al.*, 2009).

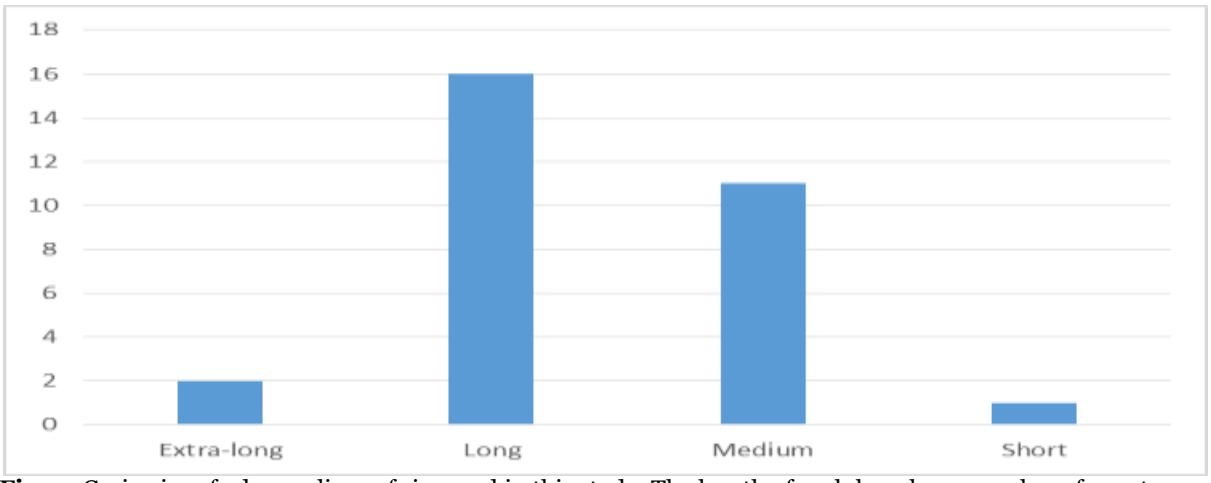

Fig. 5. Grain size of advance lines of rice used in this study. The length of each bar shows number of genotypes.

Conclusions

Most of the advance lines used in this study showed fragrance gene (*fgr*) and possessed long grains. These lines can be released as new varieties and could be used for further improvement in breeding programs. However, we recommend these lines for further evaluation of other quality traits and replicated yield trials.

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