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# Fragrance Analysis among Recombinant Inbred Lines of Rice

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**Abstract:** The aroma or fragrance of Basmati rice is associated with the presence of 2-acetyl-1-pyrroline. Aroma in Basmati is reported to be monogenic recessive. Several PCR-based co-dominant markers based on RG28 locus were developed which can differentiate between fragrant and non-fragrant rice cultivars. For analysis of aroma among RILs derived from a diverse cross between CSR 10 and Taraori Basmati through SSD method both molecular as well as biochemical methods were used to clearly distinguish aromatic and non-aromatic RILs of rice RG28 locus specific markers. Recombinant Inbred Lines (RILs) among various mapping populations provide a noble material for linkage of marker and trait. RILs were analyzed for polymorphism using RG28 locus specific primers (BAD2, BADEX7-5, SCUSSR1). Biochemical analysis of aroma was performed with the 1.7% KOH solution and molecular analysis of aroma was carried out with microsatellite markers present on chromosome 8 to determine the extent of association between trait, marker and chromosome 8. Out of these, BAD 2 amplified aroma specific alleles having 256 bp in 72 lines, BADEX7-5 with 95 bp in 74 lines and SCUSSR1 with 129 bp in 79 lines. Mental test of significance detected by biochemical analysis of RILs (with 1.7% KOH) and molecular marker study revealed 97 , 95 and 90.1% of association of aroma with the markers, respectively. Some of the $F_{10}$ lines amplified the heterozygous alleles by using the 2 sets of specific markers but did not show the presence of aroma as analyzed by chemical test. In Basmati, it seems that aroma is a complex trait and not as simple monogenic (3:1) as reported earlier. Aromatic and non-aromatic lines were almost common in three markers indicating association of markers with the trait and chromosome 8. The results revealed that these markers could be used for marker assisted selection and RIL population for mapping of aroma QTLs/genes.

**Key words:** Rice, recombinant inbred lines, variation, grain quality, aroma

## INTRODUCTION

Aroma in rice is considered as a special trait with huge economic importance that determines the premium price in global trade. Rice possessing aroma in plant parts and grains are called aromatic rices as aroma in them is retained after storage and on cooking (Huang *et al*., 1999). Aroma occurs in cultivated species only and conspicuously absent in wild rice. About 4% of world stock possesses scent. Different flavors or aromas occur in different aromatic genotypes arising from diverse origins and there is no consensus as yet on the nature of rice aroma. Previous studies have reported varying non-aromatic to aromatic $F_2$ segregation patterns, such as 15:1 (Pinson, 1994), 37:27 (Reddy and Sathyanarayaniah, 1980) and 175:81 (Dhulappanavar, 1976), 3:1 (Sood, 1978). At least six chromosomes have been implicated in the mapping process for aroma. Lorieux *et al*. (1996) confirmed close linkage between RG28 and fgr (5.8 cM) on chromosome 8 and identified two quantitative trait loci for fragrance, one on chromosome 4 and the other on chromosome 12. Then Bradbury *et al*. (2005a) identifies that functional BADH2 enzyme inhibits 2AP biosynthesis which is major component of aroma. Non fragrant varieties possess fully functional copy of the gene encoding BAD2 while fragrant varieties possess a copy of the gene containing eight base

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pair deletion resulting in a frame shift mutation disabling the BAD2 enzyme activity.

Though some progress has been made towards the identification of molecular markers linked to the genes/QTLs for grain quality traits including aroma (Ahn *et al*., 1993; Bradbury *et al*., 2005b) and kernel elongation (Ahn *et al*., 1993; Jain *et al*., 2006), but reports on linkage mapping of Basmati rice traits are few. Several PCR-based co-dominant markers based on RG28 locus were developed which can differentiate between fragrant and non-fragrant rice cultivars (Garland *et al*., 2000; Cordeiro *et al*., 2002; Jin *et al*., 2003). Recently, Singh *et al*. (2011) concluded that marker assisted breeding has been successfully employed for the development of Improved Pusa Basmati-1 and the improved versions of PRR78 and has become an integral component in the Basmati rice breeding program.

A number of sensory methods have been utilized to assist breeders in selecting fragrant rice but there are limitations when processing large numbers of samples and results become unreliable. These include chewing and cooking grains or plant parts in boiling water, reacting with solutions of KOH and smelling the samples (Sood, 1978). Therefore, microsatellite (SSR or SSLP) markers are advocated to test for varietal difference between modern varieties of aromatic and non-aromatic rice (Jain *et al*., 2006). Several PCR-based co-dominant markers based on RG28 locus were developed which can differentiate between fragrant and non-fragrant rice cultivars. Thus, for the purpose of linkage mapping $F_{10}$ generation of CSR 10 (non-aromatic, high yielding)×Taraori basmati (aromatic, poor yielder) was used for aroma using biochemical test and molecular markers, a quality trait of Basmati rice.

## MATERIALS AND METHODS

**Plant material:** The experimental materials comprised of 208 RILs derived from a cross between CSR 10×Taraori Basmati. CSR 10 (non-aromatic) is a selection from CSR1/Jaya developed and released by CSSRI, Karnal (India) for cultivation in saline soil Mishra *et al*. (1992) and HBC19 (aromatic) a pure line selection from Taraori Basmati. Thus, RILs were grown in Augmented Design (using checks after every 20 lines) during Kharif season at CCS HAU Rice Research Station, Kaul (Kaithal). Each RIL and parental lines were planted in a single row of 3.5-meter length. Seedlings were transplanted with plant-to-plant spacing of 15 cm and row-to-row spacing of 20 cm. All recommended agronomic practices were followed for raising crop.

**Biochemical analysis of aroma:** Three sets of 5 plants each i.e., total of fifteen plants were randomly selected from each line. Plants were thrashed, grain obtained were hulled, milled and then a sample of 10 milled grains from each line in triplicate were evaluated for aroma by 1.7% KOH (Sood, 1978).

**Molecular analysis of aroma:** Genomic DNA was isolated from one month old plant leaf samples (~100 mg each) using CTAB method (Saghai-Maroof *et al*., 1984) from each $F_{10}$ RIL and parents. Then RILs were analyzed for polymorphism using RG28 locus specific primers (BAD2, BADEX7-5, SCUSSR1,). A description of molecular markers and their amplified product size in parental rice genotype is shown in Table 1.

PCR amplifications were performed using PTC-100$^{TM}$ 96V thermocycler (MJ Research, Inc., Watertown, MA, USA) and *Taq* DNA polymerase. The PCR reaction was conducted in a reaction volume of 25 μL containing 10X PCR buffer, 1 μL dNTPs, 2.5 μL of each primer (Forward and reverse), *Taq* DNA polymerase and 2 μL template DNA. PCR amplification was performed with initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and final extension at 72°C for 7 min before cooling at 4°C. Amplification products were stored at -20°C till further use. PCR products were analyzed by electrophoresis in ethidium bromide stained (0.5 μg mL$^{-1}$) 1.5% agarose gel and for SCUSSR-1 PAGE (Polyacrylamide Agarose Gel Electrophoresis) was used. A 100 bp ladder molecular weight standard were used to estimate PCR fragment size. The molecular marker data generated was used to assess the similarity coefficient and linkage between aroma and RG28 locus.

Table 1: Molecular markers used for aroma analysis among RILs

| Markers | Clone No. | Forward Primer 5'-3' | Reverse Primer 5'-3' | Size range in CSR 10 (bp) | Size range in HBC19 (bp) |
|---|---|---|---|---|---|
| BAD2 | 8 | TTGTTTGGAGCTTGCTGATG | CATAGGAGCAGCTGAAATATATACC | 355,585 | 257,585 |
| — | | CTGGTAAAAAGATTATGGCTTCA | AGTGCTTTACAAAGTCCCGC | 355,585 | 257,585 |
| BADEX7-5 | 8 | TGTTTTCTGTTAGGTTGCATT | ATCCACAGAAATTTGGAAAC | 103 | 95 |
| SCUSSR1 | 8 | GATCTCACTCCAAGTAAACTCTGAC | ACTGCCATTGCTTCTGTTCTC | 129 | 130 |

## RESULTS AND DISCUSSION

**Aroma detection by KOH:** Using 1.7% KOH test in $F_{10}$ generation 74 lines were having aroma of varying intensity and 134 lines were found to be non- aromatic.

**Aroma detection by molecular marker (genetic diversity analysis):** A microsatellite DNA fingerprint database was prepared for $F_{10}$ generation using markers. BAD2 (Fig. 1), BADEX7-5 (Fig. 2) and SCUSSR-1 (Fig. 3) displaying allelic polymorphism among $F_{10}$ plants and are easily able to distinguish aromatic and non aromatic lines as well as BAD2 and SCUSSR-1 also able to distinguish heterozygotes among the population and percent distribution of alleles among RILs using aroma specific markers is given in Table 2.

Similarity matrices for the 208 $F_{10}$ lines were generated using 'Simqual' sub-program of software NTSYS-PC. Genetic relationship in CSR 10×HBC19 $F_{10}$ genotypes on the basis of 3 primers linked to RG28 aroma locus has been determined by cluster tree analysis (NTSYS-PC)

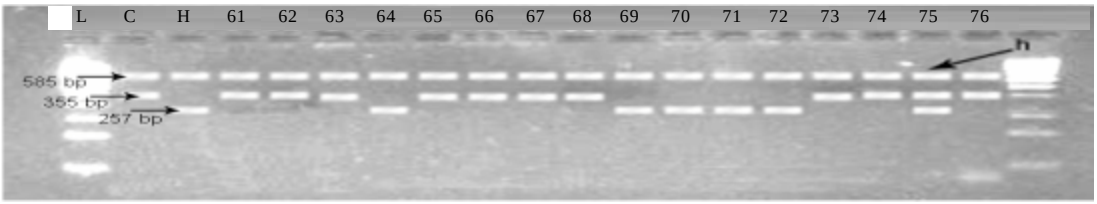


Fig. 1: Agarose gel showing allelic polymorphism among CSR 10×HBC19 F10 lines at BAD2 locus, h: Heterozygous band, L: 100 bp ladder, C: CSR 10 and H: HBC19

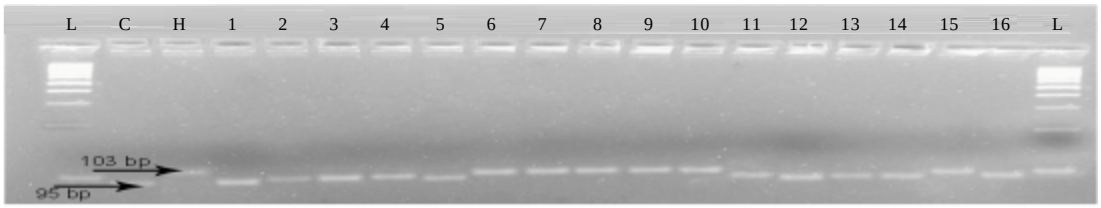


Fig. 2: Agarose gel showing allelic polymorphism among CSR 10×HBC19 F10 lines at BADEX7-5 locus, L: 100 bp ladder, C: CSR 10 and H: HBC19

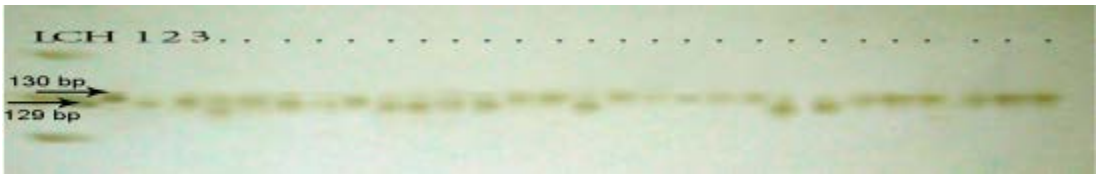


Fig. 3: Silver stained gel showing allelic polymorphism among CSR 10×HBC19 F10 lines at SCUSSR1 locus, L: 10 bp ladder, C: CSR 10 and H: HBC19 and lines 1-28

Table 2: Percent distribution of alleles in 208 lines ($F_{10}$ generation) from cross CSR 10×HBC19 using aroma specific primers

| S. No. | Primers | CSR 10 | HBC19 | Heterozygote | Recombinant alleles | Percent distribution of alleles* |
|---|---|---|---|---|---|---|
| 1 | BAD2 | 130 | 72 | 6 | 0 | 62:34:3:0 |
| 2 | BADEX7-5 | 134 | 74 | 0 | 0 | 63:35:0:0 |
| 3 | SCUSSR1 | 126 | 79 | 3 | 0 | 60:38:1:0 |

*Percent distribution of alleles: Ratio of the $F_{10}$ plants with alleles from CSR 10, HBC19, both the parents (CSR 10 as well as HBC19) and new/recombinant alleles

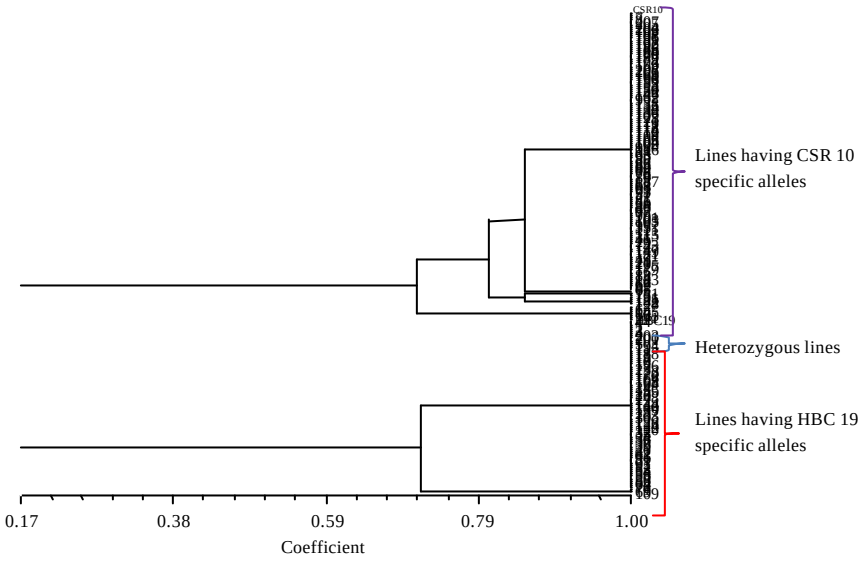


Fig. 4: Dendrogram showing genetic relationship among 208 derived ($F_{10}$ generation) lines of cross CSR 10×Taraori Basmati using 3 RG28 locus specific markers present on chromosome No. 8

(Fig. 4). All the lines were clustered into two major groups at a similarity coefficient of 0.17 using 3 RG28 locus specific primers.

These three RG28 locus specific primers identify the fragrance trait with great efficiency. BAD2 primer combination amplified the fragrant specific allele (257 bp) in 72 lines and non-fragrant (355 bp) allele in 128 lines and 6 lines showed amplification at both fragrant and non-fragrant locus i.e., they were heterozygous. SCUSSR1 is also reported to be closely linked to the fragrance gene (Garland *et al*., 2000). This primer led to amplification of fragrant specific allele (130 bp) in 79 of the 208 $F_{10}$ lines and 129 bp (non-fragrant) in 129 lines whereas 3 lines amplified both 130 and 129 bp alleles. BADEX7-5: amplified a fragrant specific allele (95 bp) in 74 of the $F_{10}$ lines. Of the remaining 134 lines had 103 bp amplified (non-fragrant) and there were no heterozygous lines.

The Mantel test of significance was used to compare fragrance trait detected by the 1.7% KOH test and the fragrance specific alleles BAD2 which is present on RG28 locus on chromosome 8. The marker specific to BAD2 locus was able to identify the fragrance trait with 97% accuracy. Correlations (r = 0.97) were observed between the fragrance trait detected by the chemical test and the fragrance specific alleles genotyped by BAD2 markers. BADEX7-5 and SCUSSR1 exhibited 95 and 90.1% correlation with fragrance trait detected by KOH solution. Bradbury *et al*. (2005a, b) reported significant polymorphisms in the coding region of fragrant rice genotypes relative to non-fragrant genotypes for a gene encoding betaine aldehyde dehydrogenase 2 (BAD2). Similarly, Jain *et al*. (2006) also evaluated the levels of genetic diversity within and among Basmati and non-Basmati rice varieties using 26 SSR markers surrounding the aroma and kernel elongation loci.

However, some of the $F_{10}$ lines amplified the heterozygous alleles by using the 2 sets of specific markers but did not show the presence of aroma as analyzed by chemical test. Similar results were observed by Lang and Buu (2002) when they studied $F_2$ and $F_3$ population (derived from cross *indica* Khao Dawk Mali×OM1490) for fragrance trait and they observed that in both the generations' ratio of fragrant: non fragrant was slightly different. So, they concluded that aroma is a complex trait. Similarly, in Basmati it seems that aroma is a complex trait and not as simple monogenic (3:1) as reported earlier by Sood (1978).

With BAD2 analysis 6 of the lines were heterozygous out them 5 were non aromatic and one was found aromatic by KOH test. These discrepancies could be due to several reasons: (i) Unlike other traits controlled by major genes, rice fragrance is easier to be influenced by many elements such as, genetic background, environmental condition and storage time (Chen *et al*., 2006; Itani *et al*., 2004), (ii) Fragrance trait is governed by a recessive gene and heterozygosity can lead to abolition of aroma specific trait which is observed in case of BAD2 and SCUSSR1, (iii) Apart from recessive fgr gene present on chromosome 8, two QTLs located on chromosome 4 and 12 also affect the fragrance (Lorieux *et al*., 1996), (iv) chemical method to

assess aroma is a sensory test and ability to distinguish between mildly aromatic and non-aromatic samples is limited. So, the chances of error by any analyst cannot be ruled out, (v) Position effects due to active crossing over/recombination at the RG28 locus cannot be ruled out. It shall be worthwhile to examine the RILs for 2-acetyl-1-pyrroline content and to analyze for aroma using more specific and sensitive method like gas chromatography/mass-spectrometry (Itani *et al*., 2004).

## CONCLUSION

From the above experimental study it is concluded that BAD2, BADEX7-5 and SCUSSR1 markers can be used for aroma detection among recombinant inbred lines and there is a strong correlation among aroma, BAD2 and chromosome 8. It seems that aroma is a complex trait and not simple monogenic (3:1). Results obtained using these markers are interesting but further investigation is required for better explanation towards understanding of the fragrance a complex trait in rice genotypes.

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