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Full Length Research Paper

Fragrance analysis using molecular and biochemical methods in recombinant inbred lines of rice

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The aroma or fragrance of Basmati rice is associated with the presence and content of the chemical compound, 2-acetyl-1-pyrroline and the trait is monogenic recessive. Several polymerase chain reaction (PCR)-based co-dominant markers based on RG28 locus were developed, which can differentiate between fragrant and non-fragrant rice cultivars. For molecular and biochemical analysis of aroma, a mapping population comprising 208 recombinant inbred lines (RILs) derived from a diverse cross between CSR10 and Taraori Basmati through Single seed descent (SSD) method was used. RILs are among the best mapping populations, which provide a novel material for linkage mapping of genes/QTLs marker for various traits. 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 (BAD2, BADEX7-5, SCUSSR1) to determine the extent of association between trait, marker and chromosome 8. Among these markers, BAD2 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. Mantel test of significance detected by biochemical analysis of RILs (with 1.7% KOH) and molecular marker study revealed high degree (>90%) of association of aroma with the above mentioned markers, respectively. Some of the F_{10} lines amplified the heterozygous alleles for two sets of specific markers (BAD2 and SCUSSR-1) but did not show the presence of aroma as analyzed by chemical test. Aromatic and non-aromatic lines were almost common in three markers, indicating association of markers with the trait and chromosome 8. The results reveal that these markers could be used for marker assisted selection and RIL population for mapping of aroma QTLs/genes.

Key words: Basmati, recombinant inbred lines, fragrance, association.

INTRODUCTION

Aromatic rice varieties constitute a small but special group of rice and have gained greater importance with the worldwide increase in the demand for fine quality rice (Sun et al., 2008). Lack of aroma in wild *Oryza* rice implies that the aroma associated with some domesticated rice varieties may have arisen from a gene mutation during evolution or is the outcome of a separate domestication event (Bradbury et al., 2008). Different flavors or aromas occur in different aromatic genotypes arising from diverse origins and there is no consensus 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), 175:81 (Dhulappanavr, 1976) and 3:1 (Sood and Siddiq, 1978). At least four chromosomes (3,

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4, 8 and 12) have been implicated through mapping with trait aroma, chromosome 4, 8, 12 by Loriex et al. (1996) and 3, 4, 8 by Amarawathi et al. (2008). 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) showed that a functional BADH2 enzyme inhibits 2AP biosynthesis which is a major component of aroma. Non-fragrant varieties possess a fully functional copy of the gene encoding BAD2 while fragrant varieties possess a copy of the gene containing eight base pair deletion resulting in a frame shift mutation disabling the BAD2 enzyme. Recent studies by Kovach et al. (2009) also confirmed that BADH2 is the major genetic determinant of fragrance in rice.

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., 1992; Bradbury et al., 2005b) and kernel elongation (Ahn et al., 1993; Jain et al., 2006), reports on linkage mapping for traits specific for Basmati rice are few. Several polymerase chain reaction (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; Sakthivel et al., 2009). 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.

In any molecular breeding program, development of suitable permanent mapping population for linkage studies is a prerequisite. Controlled crosses of diverse parents are made to obtain a mapping population. Recombinant inbred lines (RILs) among various mapping populations provide a noble material for linkage of marker and trait. These are one of the most widely used populations for gene/QTL mapping (Burr and Burr, 1991). Linkage maps thus obtained are permanent and suitable for genetic studies. Keeping this in view, a F_{10} RIL mapping population was developed by single seed descent method from a cross of diverse parents CSR10 (non-aromatic, high yielding) x Taraori basmati (aromatic, poor yielder) for purpose of linkage mapping. The genotyping and phenotyping of this population was done for aroma using molecular markers and biochemical test.

MATERIALS AND METHODS

The experimental materials comprised of 208 RILs derived from a cross between CSR-10 x Taraori Basmati. CSR10 (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 m 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, that is, total of 15 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 (Bradbury et al., 2005b), BADEX7-5 (Sakthivel et al., 2009) and SCUSSR1 (Garland et al., 2000). 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 10XPCR 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) 1.5% agarose gel and for SCUSSR-1, PAGE (Polyacrylamide Agarose Gel Electrophoresis) was used. A 100 bp ladder molecular weight standard was 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.

RESULTS AND DISCUSSION

Aroma detection by KOH

Using 1.7% KOH test in F_{10} generation, 74 lines had 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. Agarose gels and silver stained gel displaying allelic polymorphism among F_{10} plants for some of the markers is shown in Figures 1 to 3. Among RILs, CSR10 specific alleles were present at a higher frequency (0.61) as compared to the HBC19 specific alleles (0.35) (Table 2). In a F_{10} generation, population was expected to achieve a homozygosity of 99.8%. The average homo-zygosity achieved in F_{10} generation was 98.6%.

Table 1. Molecular markers used for aroma analysis among RILs.

Marker	Reference	Clone number	Forward Primer 5'-3'	Reverse Primer 5'-3'	Size range in CSR10 (bp)	Size range in HBC19 (bp)
BAD2	Bradbury et al., 2005b	8	TTGTTTGGAGCTTGCTGATG	CATAGGAGCAGCTGAAATATATACC	355, 585	257, 585
—			CTGGTAAAAAGATTATGGCTTCA	AGTGCTTTACAAAGTCCCGC	355, 585	257, 585
BADEX7-5	Sakthivel et al., 2009	8	TGTTTTCTGTTAGGTTGCATT	ATCCACAGAAATTTGGAAAC	103	95
SCUSSR1	Garland et al., 2000	8	GATCTCACTCCAAGTAAACTCTGAC	ACTGCCATTGCTTCTGTTCTC	129	130

Table 2. Percent distribution of alleles in 208 lines (F_{10} generation) from cross CSR10 x HBC19 using aroma specific primers.

Primer	CSR10	HBC19	Heterozygote	Recombinant alleles	Percent distribution of alleles*
BAD2	130	72	6	0	62:34:3:0
BADEX7-5	134	74	0	0	63:35:0:0
SCUSSR1	126	79	3	0	60:38:1:0

*Percent distribution of alleles: Ratio of the F_{10} plants with alleles from CSR10, HBC19, both parents (CSR10 as well as HBC19) and new/recombinant alleles.

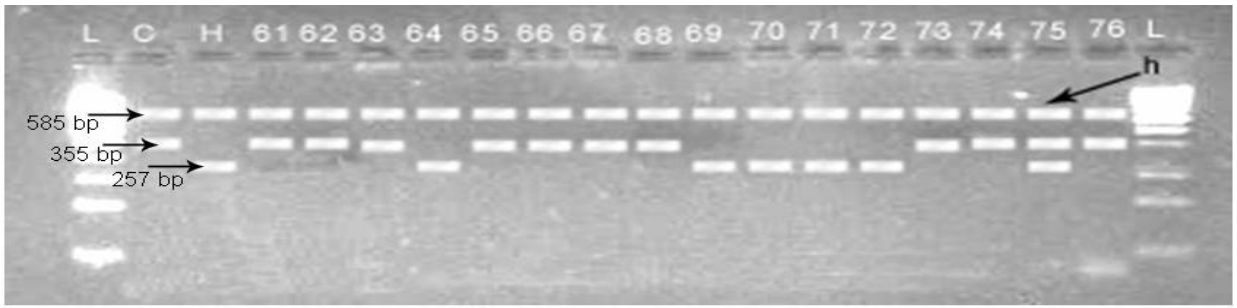

Figure 1. Agarose gel showing allelic polymorphism among CSR10 x HBC10 F_{10} lines at BAD2 locus, where H represent heterozygous band, L = 100 bp ladder, C = CSR10 and H = HBC19.

three primers linked to RG28 aroma locus was deter-mined by cluster tree analysis (NTSYS-PC) (Figure 4). All the lines were clustered into two major groups at a similarity coefficient of 0.17 using three RG28 locus specific primers (BAD2, BADEX-7 and SCUSSR-1).

Similarity matrices for the 208 F_{10} lines were generated using 'Simqual' sub-program of soft-ware NTSYS-PC. Genetic relationships in CSR10 x HBC19 F10 genotypes on the basis of these three RG28 locus specific primers were used to identify the fragrance trait with a very high great efficiency. BAD2 primer combination amplified the fra-grant specific allele (257 bp) in 72 lines and non-fragrant (355 bp) allele in 128 lines and six

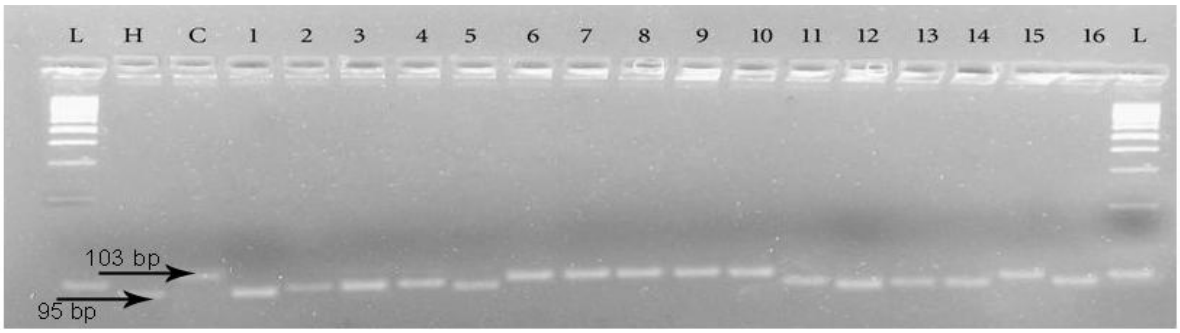

Figure 2. Agarose gel showing allelic polymorphism among CSR10 x HBC10 F_{10} lines at BADEX7-5 locus. Where L = 100 bp ladder, C = CSR10 and H = HBC19.

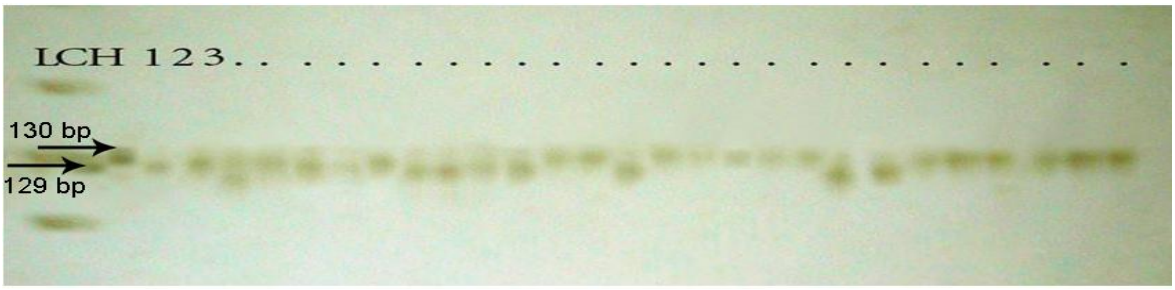

Figure 3. Silver stained gel showing allelic polymorphism among CSR10 x HBC19 F_{10} lines at SCUSSR1 locus. Where L = 10 bp ladder, C = CSR10 and H = HBC19.

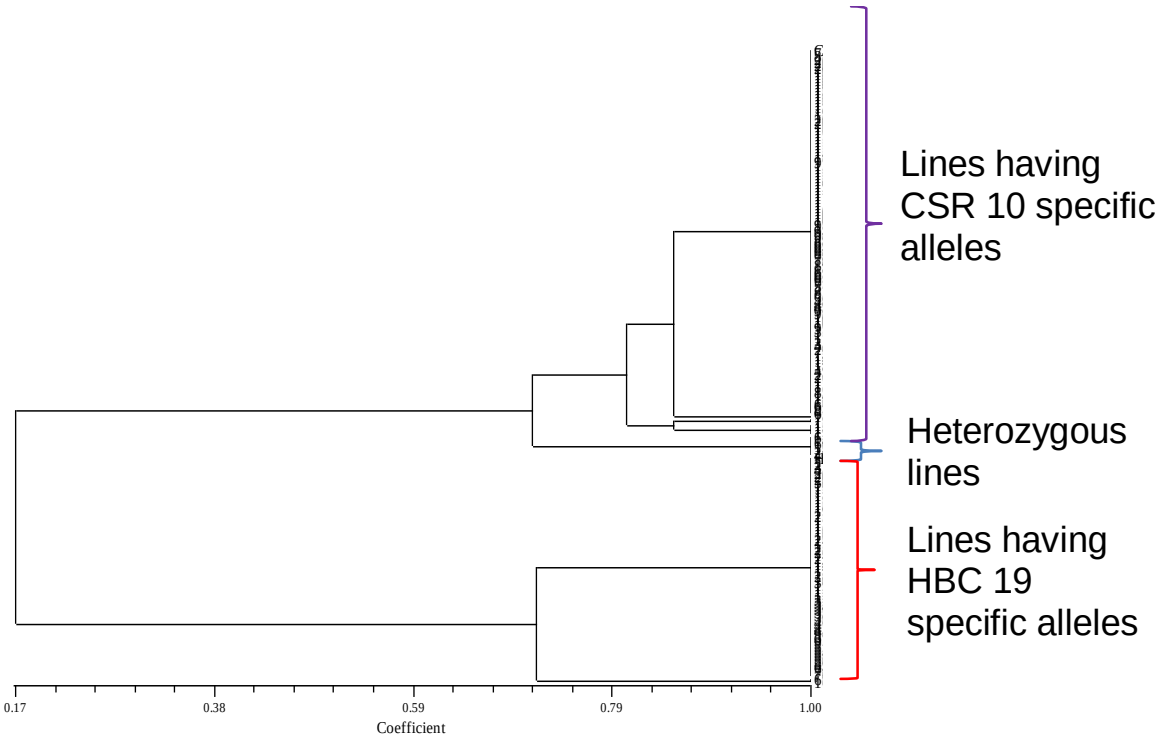

Figure 4. Dendrogram showing genetic relationship among 208 derived (F_{10} generation) lines of cross CSR10 x Taraori Basmati using 3 RG28 locus specific markers present on chromosome No. 8.

lines showed amplification at both fragrant and non-fragrant locus, that is, 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. From 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 in 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 two 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 x OM1490) for fragrance trait and they observed that in both generations' ratio of fragrant, non fragrant was slightly different. So they concluded that aroma is a complex trait.

With BAD2 analysis, six of the lines were heterozygous, out them, five were non aromatic and one was found to be 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, environ-mental 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 the case of BAD2 and SCUSSR1; iii) apart from recessive *fgr* gene present on chromosome 8, two QTLs located on chromosomes 4 and 12 also affect the fragrance (Lorieux et al., 1996); iv) KOH method used to assess aroma is a crude method thus the ability to distinguish between mildly aromatic and non-aromatic samples is limited. So, the chances of error by any analyst cannot be ruled out, thus it shall be worthwhile to examine the RILs for 2-acetyl-1-pyrolline 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 between aroma, BAD2 and chromosome 8. It seems that aroma is a complex trait. Results obtained using these markers are interesting but further investigation is required for better explanation towards understanding the fragrance complex trait in rice genotypes.

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