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Research Article

Genetic analysis and gene fine mapping of aroma in rice (*Oryza sativa* L. Cyperales, Poaceae)

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Abstract

We investigated inheritance and carried out gene fine mapping of aroma in crosses between the aromatic elite hybrid rice *Oryza sativa indica* variety Chuanxiang-29B (Ch-29B) and the non-aromatic rice *O. sativa indica* variety R2 and *O. sativa japonica* Lemont (Le). The F_1 grains and leaves were non-aromatic while the F_2 non-aroma to aroma segregation pattern was 3:1. The F_3 segregation ratio was consistent with the expected 1:2:1 for a single recessive aroma gene in Ch-29B. Linkage analysis between simple sequence repeat (SSR) markers and the aroma locus for the aromatic F_2 plants mapped the Ch-29B aroma gene to a chromosome 8 region flanked by SSR markers RM23120 at 0.52 cM and RM3459 at 1.23 cM, a replicate F_2 population confirming these results. Three bacterial artificial chromosome (BAC) clones cover chromosome 8 markers RM23120 and RM3459. Our molecular mapping data from the two populations indicated that the aroma locus occurs in a 142.85 kb interval on BAC clones AP005301 or AP005537, implying that it might be the same gene reported by Bradbury *et al* (2005a; Plant Biotec J. 3:363-370). The flanking markers Aro7, RM23120 and RM3459 identified by us could greatly accelerate the efficiency and precision of aromatic rice breeding programs.

Key words: aroma, genetic analysis, gene fine mapping, rice (*Oryza sativa* L.).

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Introduction

Rice (*Oryza sativa* L.) is a primary food crop and has an important role both economically and in terms of food security. Aromatic rice varieties constitute a small but special group of rice and have gained greater importance with the worldwide increase in the demand for super rice. Not only is aroma one of the most important characteristics for determining good quality rice but aromatic varieties have comparable or superior nutritional values and better amino acid profiles, with, for example, Basmati-370 rice having a higher lysine, phenylalanine, leucine and methionine content than non-aromatic varieties (Sekhar and Reddy 1982). Consumers will pay higher prices for fine-grained aromatic rice, because of which the sensory evaluation of grain quality has become an important consideration in rice breeding.

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 be the outcome of a separate domestication event (Bradbury *et al.* 2005a). 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 (Dhulappanavr, 1976). And at least six chromosomes have been implicated in the mapping process for aroma, Lorieux *et al.* (1996) having confirmed the 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. A dominant aroma gene was located on chromosome 11 in Baspatri, an Indian rice landrace, by Tomar and Prassad (1997), while Siddiq *et al.* (1986) located two recessive aroma genes on the chromosome 5 and 9 in the Indian rice variety T3. These reports suggest that varying numbers of dominant or recessive genes are involved in the aroma trait. Recently, Bradbury *et al.* (2005a) reported significant polymorphisms between aromatic rice genotypes and non-aromatic geno-

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types in regard to the coding region of a gene with homology to the betaine aldehyde dehydrogenase 2 gene *BAD2*. However, *BAD2* appears not to be responsible for aroma in all aromatic rice varieties, since this gene is not responsible for a dominant aroma trait of an aroma mutant SA0420 (Kuo *et al.* 2005). Even so, it has been established that in most rice varieties aroma is controlled by a single recessive gene mapped to chromosome 8 of *O. sativa* (Li *et al.* 2006).

Molecular fine mapping for aroma and the subsequent use of this information in the selection process would greatly accelerate the efficiency of aromatic rice breeding programs. In addition, physical mapping of aroma could be useful for understanding rearrangement in the rice genome and exploring candidate genes for aroma. Three-line hybrid rice varieties were first used in China, but, however, because the grain quality of hybrid rice is generally not as good as that of common rice varieties the improvement of the grain quality of these strains has become a continual challenging for rice breeders.

The market importance of aromatic rice stimulated our group to introduce the aromatic trait into hybrid rice to improve hybrid rice grain quality. In order to achieve this it is necessary either to introduce a dominant fragrant gene into one of the three types of rice lines normally used in breeding (the cytoplasmic male sterile (CMS) line, the maintainer line or the restorer line) or introduce a recessive aromatic gene into all three lines. In our hybrid rice breeding program, we introduced the aromatic trait from Xiangsimiao-2, an aromatic rice landrace native to southern China, into a CMS maintainer line II-32B by crossing. From the progenies we obtained an elite CMS maintainer named Chuanxiang-29B. Subsequently, a series of hybrid rice varieties was released for rice production. However, all the hybrid rice varieties were not aromatic, which largely limited their exploitation. In order to understand the molecular nature of aromatic trait in Chuanxiang-29B and to develop molecular markers for aromatic trait selection in future breeding programs it is necessary to verify whether or not the aromatic gene in Chuanxiang-29B is one of the genes previously mapped or identified.

In this paper we report the genetic analysis and fine mapping of aroma in rice variety Chuanxiang-29B and provide a detailed analysis of its physical mapping based on the rice Gramene database. The identified markers linked with aroma provide a useful avenue for molecular marker-assisted selection of hybrid rice in future breeding programs.

Materials and Methods

Materials

Crosses were made between the aromatic *Oryza sativa indica* variety Chuanxiang-29B (Ch-29B) and the leading American non-aromatic *O. sativa japonica* variety Lemont (Le) and between Ch-29B and the non-aromatic *O. sativa indica* variety R2 (derived from progenies of the cross *O. sativa* Mianhui-502 Le). In the winters of 2004 and 2005 field-grown F_1 plants from the two crosses were grown in paddy fields of the experimental farm of Sichuan Academy of Agricultural Sciences with sandy type soil in the Chinese province of Hainan, all normal agricultural practices being applied to the rice. Two F_2 mapping populations, each containing about 281 and 1182 plants, were established and segregation for aroma was investigated in the Ch-29B/Le F_2 population in 2005 and tested repeatedly in 2006, the Ch-29B/R2 F_2 about 2400 plants being likewise studied in summer of 2006. In the summers of 2005 and 2006 each F_2 plant was self-crossed to obtain the corresponding F_3 families in Chengdu China with clayish soil. Two additional *O. sativa* aromatic rice varieties, *O. sativa japonica* Della (De) and *O. sativa indica* Bastmati-370 (Ba) were used as controls.

Aroma evaluation

Aroma in leaves was determined according to the method described by Sood and Siddiq (1978). When the plants were forming side-shoots (*i.e.*, at the tillering stage) we excised 2 g of two or three leaves from each F_1,F_2 or parent individual, cut the tissue into pieces and placed these in petri dishes containing 10 mL of 1.7% (w/v) potassium hydroxide (KOH). The petri dishes were kept at room temperature (≈25 °C) for about 10 min and then opened one by one and the samples smelled and rated for the presence or absence of aroma.

The tasting of individual rice grains has traditionally been the preferred method for aroma evaluation so we also assessed aroma by chewing 12 single grains, one at a time, from each F_2 plant and rating each plant assessed as homozygous aromatic if all 12 F_3 grains were aromatic or as non-aromatic if some of the grains were non-aromatic (Ahn *et al.* 1992). The F_3 grains, obtained by self-crossing the F_2 plants, were chewed to evaluate the aroma of the F_3 families and the genotype of each F_2 plant was inferred from the phenotype of the corresponding F_3 family. The F_3 grains from heterozygous F_2 plants were expected to contain both aromatic and non-aromatic seeds. The rating panel consisted of three to four people selected for their ability to differentiate between aroma (Ch-29B) and non-aroma (Le or R2) plants, and the process being repeated if there was no consensus on the aroma. The observed segregation ratio of aromatic:segregating:non-aromatic in F_3 grains from self-crossed F_2 plants was tested by chi-squared (P^2) analysis against the expected ratio for a single gene.

DNA extraction and simple sequence repeat (SSR) analysis

Total genomic DNA was extracted from the fresh leaves of the five parents including two controls and F_2 offsprings from the two crosses (Ch-29B/Le and Ch-29B/R2) using the cetyltrimethylammonium bromide (CTAB)

method of Murray and Thompson (1980), with minor modifications. To quantify the DNA in each sample the DNA was separated on 1.5% (w/v) 1 TAE (Tris base, glacial acetic acid and 0.5 M EDTA pH 8.0) agarose gel, stained with ethidium bromide and viewed under UV light, the quatity of plant DNA present being calculated by comparison with lambda DNA standards (TakaRa, Dalian, China) run at the same time and under the same conditions. For use in the SSR analysis we standardized each sample to a DNA concentration of 50~100 ng μL^{-1}. We sampled all the plants for each parent and each F_2 individuals.

To map the aroma locus, we screened 700 SSR markers for polymorphism between Ch-29B and Le, these markers being well-scattered on rice 12 chromosomes. The other SSR markers (microsatellites) were designed with the aid of SSRHunter 1.3 (Li and Wan, 2005), a local searching software for SSR sites, and Primer Premier Version 5.0 (Premier Biosoft International, Palo Alto, CA). The sequences and bacterial artificial chromosome (BAC) clones of the relative region covering the gene of interest were obtained from the rice Gramene database (www.gramene.org/Oryza_sativa/geneview?gene = LOC_Os08g32870), and selected based on their proximity to the SSR markers linked with the aroma locus. Gene-specific primers were designed according to the DNA sequence of the betaine-aldehyde dehydrogenase-2 gene (*BAD*2) gene (GenBank ID: J023088C02) and a site specific primer pair was also developed for the *Bad*2 exon 7 region. The primers used were Aro1 (SSR motif (AG)9, annealing temperature (*Ta*) = 56.8 °C, expected product size (S) = 173 bp, forward primer (F) = 5'-CATCTATCCTCCTCGGGCAACA-3' and reverse primer (R) = 5'-GGCGGCGTCATATCCA ACA-3') and Aro7 (SSR motif (AGG)9, *Ta* = 54.4 °C, S = 302 bp, F = 5'-ATTTGCCTCCTGAGTCTG-3' and R = 5'-GAGGATGGGGAAGATAAA-3').

To ascertain the linkage relationship between the aroma locus and the molecular marker we carried out bulked segregant analysis (BSA; Michelmore *et al.* 1991). In each F_2 population from cross Ch-29B/Le or Ch-29B/R2, 20 DNA samples from aromatic F_2 individuals were mixed with same amount to form the aroma bulk and 20 non-aromatic F_2 DNAs were mixed similarly to form the non-aroma bulk. All PCR reactions were performed in a MyCycler Thermal Cycler (BIO-RAD, USA) using a basic 25 μL reaction mixture consisting of 2.5 μL of 10 buffer, 1.5 μL $MgCl_2$ (TaKaRa, Dalian, China), 0.1 mM of each of dNTP, 1.5 units of Taq polymerase (TaKaRa, Dalian, China), 50 ng of sample DNA as template and 50 ng of each primer, the final volume being made up to 25 μL with distilled water and overlaid with one drop of mineral oil. The amplification reactions were carried out at an initial 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C ~65 °C and 1 min at 72 °C, with a 10 min extension at 72 °C. The amplification products were separated on a 6% (w/v) polyacrylamide-gel electrophoreses (PAGE) sequencing gel run at 80 w for 2 h following a pre-electrophoresis for 20 min. The gel was removed from the apparatus and stained by the silver-staining method (Xu *et al.* 2002).

Data analysis and DNA sequencing of the *BAD2* polymorphic region

The parent-specific band was designated as either (A) or (B), or heterozygous (H) when two bands were present. The data were analyzed with Mapmaker/Exp 3.0 b program (Lincoln *et al.* 1992) to construct a local genetic map for the aroma genomic region.

We used our genetic and physical mapping data to develop a site-specific primer pair SF6 (Forward: 5'-GCC GGTGCTCCTTTGTCATCA-3'; Reverse: 5'-TGTACCA TCCCCACGGCTCAT-3') to amplify and sequence the *BAD2* polymorphic region reported by Bradbury *et al.* (2005a). The DNA fragments covering the target region of *BAD2* from the aromatic (Ch-29B, De and Ba) and non-aromatic (Le and R2) rice varieties were amplified with high fidelity using LA-Taq (TakaRa, Dalian, China). The PCR reaction conditions and amplification protocol were the same as those described above. The PCR products were purified and sequenced using a model 373 ABI automatic sequencer (Applied Biosystems, Shanghai, China). The alignment was produced using the biosoftware Vector NTI Suit 8.0 program (Invitrogen, Carlsbad, CA) and associated molecular information was analyzed using the DNAMAN (Lynnon Biosoft, USA) and Clustal W (http://align.genome.jp/) programs.

Results

Aroma inheritance

The F_1 grains and leaves from the two crosses of Ch-29B/Lemont and Ch-29B/R2 were non-aromatic. The aroma data for the two populations of F_2 plants is presented in Table 1. The segregating ratio of F_2 non-aromatic to aromatic plants was 3:1, which indicated the inheritance of a single recessive gene in aromatic rice variety Ch-29B.

Mapping the aroma locus in the Ch-29B/Le F_2 population

Polymorphism between the *O. sativa indica* variety Ch-29B and the *O. sativa japonica* variety Le was assessed with 700 SSR markers, 268 (38.29%) of which were polymorphic. The PCR reactions carried out on the DNA from the two parents, the aroma bulk and the non-aroma bulk with the polymorphic SSR markers showed that the chromosome 8 markers RM515 and RM8264 co-segregated with the aroma locus. We genotyped 281 Ch-29B/Le F_2 plants for the RM515 and RM8264 markers and used the P^2 test to compare the observed segregation ratios of these markers with the expected ratio for a Mendelian segregation model of 1:2:1. Recombinant plants cannot be properly identified in gene mapping with dominant plants (Pan *et al.*

Table 1 - The segregation of aroma in F_2 populations of a cross between the aromatic rice cultivar *Oryza sativa indica* variety Chuanxiang-29B (Ch-29B) and the non-aromatic *O. sativa japonica* variety Lemont (Le) and the non-aromatic *O. sativa indica* variety R2.

F_2	Year	Total	Non-aroma	Aroma	χ^2 (3:1)*	p-value
Ch-29B/Le	2005	281	211	70	0.019	0.95 to 0. 99
	2006	1182	902	280	1.084	0.20 to 0.30
Ch-29B/R2	2006	2400	1823	577	1.176	0.20 to 0.30

*The limiting Chi squared value was $\chi^2_{0.05} = 3.84$.

2003; Chen *et al.* 2006), because of which we used aroma-recessive plants as the mapping population in our study. In the 2005 experiment there were 2 recombinant plants both at marker RM515 (one a double exchange recombinant) and RM8264. Based on the Mapmaker data obtained from the SSR marker analysis we initially mapped the aroma gene as being located between the RM515 marker at a genetic distance of 2.0 cM and the RM8264 marker at 1.3 cM. In the repeated experiment carried out in 2006 we selected 350 aroma-recessive plants (70 plants obtained in 2005 and 280 in 2006) for fine mapping and further analysis narrowed the location of the aroma locus to a 2 cM interval flanking RM515 and RM8264. Other SSR markers linked with the aroma locus were RM7049, RM7356 and RM7556. The relative orders of the SSR markers obtained in Ch-29B/Le were found to be identical both years and, furthermore, the arrangement of the aroma-linked SSR markers agrees with that published by the International Rice Research Institute (IRRI) and by Connell (2003), indicating that our mapping was successful.

The number of polymorphic SSR markers in the aroma locus region is limited and our initial attempts at primer design were successful for only eight SSR markers. When we tested these SSR markers on aromatic and non-aromatic parents we found that in Ch-29B and Le the Aro1 and Aro7 primer pairs were polymorphic while in Ch-29B and R2 only the Aro7 primer pair was polymorphic. The Aro1 and Aro7 primer pairs, described in the Materials and Methods, produced a product close to the expected size, 173 for Aro1 and 302 bp for Aro7. In addition, we also identified a novel SSR marker, RM23097, linked with aroma locus at a genetic distance of 0.71 cM. The fragrant gene (*fgr*) was mapped to the interval between SSR marker Aro7 (0.57 cM) and RM515 (0.71 cM), based on which a molecular linkage map and corresponding BAC clones covering this region of chromosome 8 were constructed (Figures 1 b and 1 c).

Mapping the aroma locus in the Ch-29B/R2 F_2 population

We also mapped the aroma locus in the Ch-29B/R2 F_2 population but, since the parents of this cross were both indica varieties, the level of polymorphism was not as high as that of the Ch-29B/Le indica/japonica cross. In the fine mapping population of 577 F_2 recessive plants, 10 plants

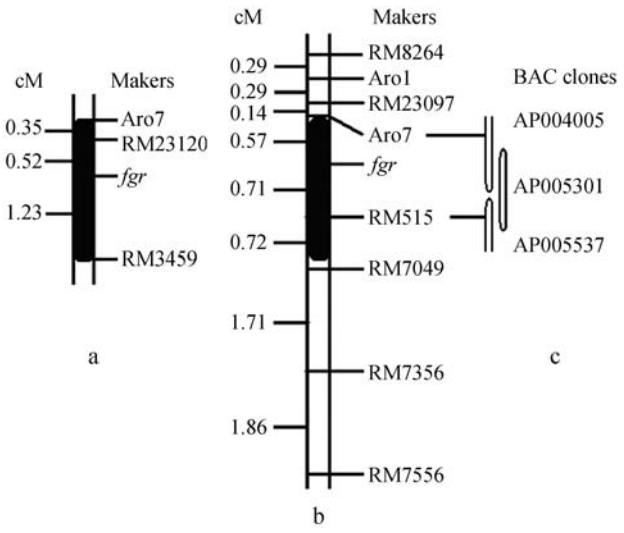

Figure 1 - Location of the fragrance gene in chromosome 8. (a) Linkage map of the Ch-29B/R2 F_2. (b) Linkage map of the Ch-29B/Le F_2. (c) The bacterial artificial chromosome (BAC) clones covering Aro7 and RM515.

were found to be recombinant at the Aro7 (0.87 cM) SSR marker, this marker being a common polymorphic SSR marker between Ch-29B and the two non-aromatic parents Le and R2 (Figure 2). The markers linked with the aroma locus in the 29B/Le population were detected in the Ch-29B and R2 parents but were all monomorphic, because of which we used the SSR markers flanking the aroma locus to investigate the relationship between the aroma gene and molecular markers and found that the SSRs markers RM23120 and RM3459 (Figure 3) were linked with the fragrance locus at a genetic distance of 0.52 cM for RM23120 and 1.23 cM for RM3459 (Figure 1 a). On the basis of the rice physical genome map, the RM23120 marker was flanked by the Aro7 and RM515 markers while the RM3459 marker was flanked by the RM515 and RM7049 markers and the Ch-29B fragrance locus was restricted to the interval between RM23120 and RM3459. Upon careful examination of the two mapping datum, we calculated that the Ch-29B aroma gene was most likely to reside on the physical map of chromosome 8 between 20117016 bp and about 20259866 bp, with bacterial artificial chromosome (BAC) clones AP005301 and AP005537 being most likely to contain the gene. Since *fgr* has already been mapped in this region and the candidate gene was identified to be the

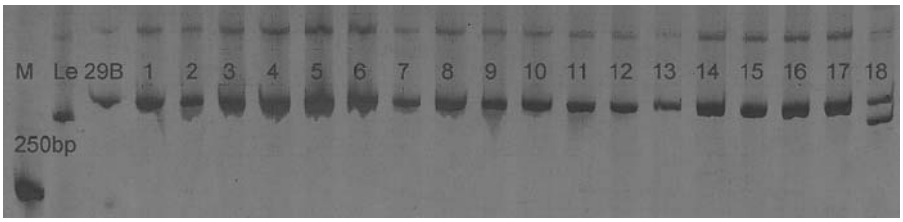

Figure 2 - Polymerase chain reaction profiles produced by the simple sequence repeat (SSR) marker Aro7 for Ch-29B/Le F_2 recessive plants.

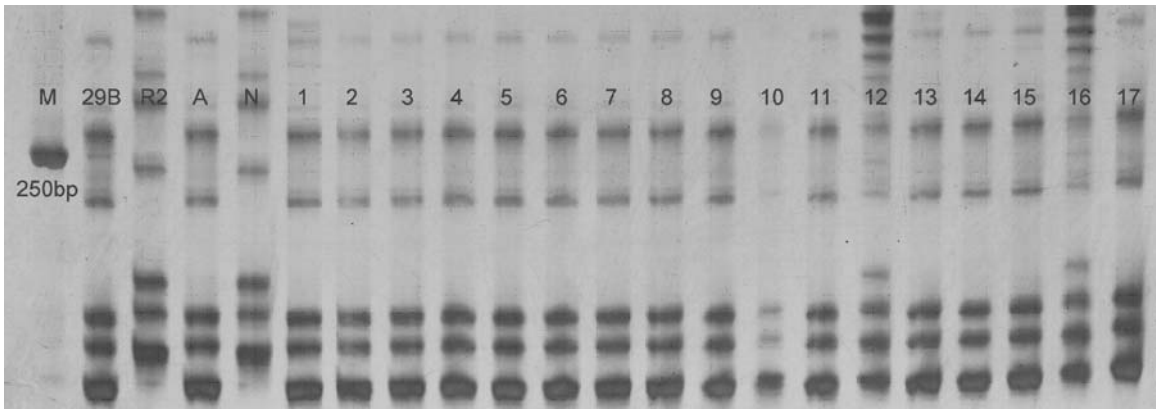

Figure 3 - Polymerase chain reaction profiles produced by the simple sequence repeat (SSR) marker RM3459 in Ch-29B, R2, two bulks A (aromatic) and N (non-aromatic) and F_2 recessive plants.

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29B_SF6F        TAGGTTGCATTTACTGGGAGTTATGAAACTGCTATATA--------TTTCAGCTGCTGCT
Le__SF6F        TAGGTTGCATTTACTGGGAGTTATGAAACTGCTAAAAAGCATTATGGCTTCAGCTGCTGCT
De__SF6F        TAGGTTGCATTTACTGGGAGTTATGAAACTGCTATATA--------TTTCAGCTGCTGCT
Ba__SF6F        TAGGTTGCATTTACTGGGAGTTATGAAACTGCTATATA--------TTTCAGCTGCTCCT
SF6_Genomic     TAGGTTGCATTTACTGGGAGTTATGAAACTGCTAAAAAGCATTATGGCTTCAGCTGCTCCT
                ************************************ * *       ************

29B_SF6F        ATGGTTAAGGTTTGTTTCCAAATTTCTGTGGATATTTTTTTGTTCTCTTTCTACTAACTCT
Le__SF6F        ATGGTTAAGGTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCT
De__SF6F        ATGGTTAAGGTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCT
Ba__SF6F        ATGGTTAAGGTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCT
SF6_Genomic     ATGGTTAAGGTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCT
                ************************************************************

29B_SF6F        CTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAAAATTGTGA
Le__SF6F        CTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAGAATTGTGA
De__SF6F        CTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAGAATTGTGA
Ba__SF6F        CTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAGAATTGTGA
SF6_Genomic     CTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAGAATTGTGA
                ************************************************ ********

29B_SF6F        TCAAGACACTTTGAGCATCATTCTAGTACCCAGTTCTATCCTGTTTCTTACCTTTTTATG
Le__SF6F        TCAAGACACTTTGAGCATCATTCTAGTAGCCAGTTCTATCCTGTTTCTTACCTTTTTATG
De__SF6F        TCAAGACACTTTGAGCATCATTCTAGTAGCCAGTTCTATCCTGTTTCTTACCTTTTTATG
Ba__SF6F        TCAAGACACTTTGAGCATCATTCTAGTAGCCACTTCTATCCTGTTTCTTACCTTTTTATG
SF6_Genomic     TCAAGACACTTTGAGCATCATTCTAGTAGCCACTTCTATCCTGTTTCTTACCTTTTTATG
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Figure 4 - Part of the betaine aldehyde dehydrogenase 2 gene (*BAD2*) sequence (SF6_Genomic) alignments in the Ch-29B, Le, De and Ba rice varieties. Identical nucleotides are marked with asterisks (*) while mutated nucleotides are blank. The rectangle indicates the TAA stop codon.

BAD2 loss-off-function mutant (Bradbury *et al.* 2005a) the aroma gene detected by us in Ch-29B might be the same gene.

Variation of the *BAD2* sequence in aromatic and non-aromatic rice

From our genetic and physical mapping data, the aroma gene in Ch-29B is presumably the same *fgr* gene encoding BAD2. The site-specific primer pair SF6 designed by us to amplify and sequence the *BAD2* polymorphic region reported by Bradbury *et al.* (2005a) produced a single DNA fragment of 3106-bp covering the region of exon 7. The banding pattern obtained from Ch-29B, Le, De and Ba showed no differences and the DNA sequencing of the PCR products form 29B, De and Ba revealed an identical sequence polymorphism pattern compared to that from the non-aromatic variety Le which shares identical sequence to the published Nipponbare sequence (Figure 4). Our data shows that the three aromatic varieties contain a large exon 7 deletion and one exon 7 single nucleotide polymorphism (SNP), which terminates prematurely at a TAA stop codon, along with two SNP nonsense mutations in the central section of intron 8. Our alignment agrees with that published by Bradbury *et al.* (2005a) and implies that the aromatic trait in fragrant rice is caused by the loss-off-function of BAD2, although further complementary tests are essential to make the final conclusion.

Discussion

It is well known that aroma in rice is genetically controlled by cell nuclear genes and independent of cytoplasmic heredity, inheritance probably involving modifier genes without maternal effect. Hang *et al.* (1995) reported that the aroma of rice leaves was controlled by the sporophyte (2n) genotype while in rice grains aroma was controlled by the endosperm (3n), non-aroma was dominant character just as the behavior of unscented parent. Aromatic rice varieties emit aroma from their leaves, grains and flowering organs at various stages of maturity and several

chemical constituents have been shown to be important for rice aroma, 2- acetyl -1- pyrroline (2AP) being a key component of rice scent (Wongpornchai *et al.* 2003). The accumulation of 2AP in fragrant genotypes may be explained by the presence of mutations resulting in a loss of function of the *fgr* gene product (Bradbury *et al.* 2005a).

Aroma in rice is a sensory character, and in the study by Paule and Powers (1989) the judges used terms with which they were familiar to describe the odor of scented rice. It is essential for rice breeders to develop reliable, fast and cost-effective evaluation for rice aroma in breeding programs. As sensory quality has always been an important consideration in rice improvement, we used sensory methods including eluting aroma from leaves with dilute KOH and chewing grains for aroma. However, the detection of aroma by sensory or chemical methods is by no means practical when processing large numbers of samples. In order to minimize the influence of environmental condition and other factors, identification of aroma in our study was performed on two F_2 populations produced in different years and at different sites. The F_2 plants with high aroma (*i.e.*, similar to Ch-29B) identified by the two sensory methods were selected for genetic analysis and gene fine mapping, with our results supporting those of most other studies.

To clone the recessive aroma gene we employed a map-based cloning approach in which we used two F_2 populations to construct two fine genetic maps which restricted the fragrance locus to a region flanked by BAC clone AP005301 (20008614 bp to about 20153950 bp) and BAC clone AP005537 (20126652 bp to about 20279561 bp). In addition, the fragrance *fgr* gene identified in Kyeema rice (*O. sativa indica*) by Bradbury *et al.* (2005a) was located on BAC clone AP004463 at 20246981 bp to about 20253219 bp. Given the shared location of physical map and *BAD2* sequence variation in Ch-29B and Le the fragrance character in Ch-29B and Kyeema may be encoded by the same gene. As the biochemical pathway leading to fragrance in rice is not fully understood, and *BAD2* has significant polymorphisms in the coding region of fragrant genotypes relative to non-fragrant genotypes, it is necessary to analyze the biological function of *BAD2*. Now genetic transformation of rice and certification of transformants will be the next major step towards determining the role of this gene in the Ch-29B aroma trait.

Molecular mapping for the aroma trait and its subsequent use in selection processes would greatly accelerate the efficiency and precision of aromatic rice breeding programs. Some progress has been made towards the identification of molecular markers linked to fragrance genes, including the SCU015RM (Cordeiro *et al.* 2002) SNP marker (Jin *et al.* 2003) and a single tube allele specific amplification (ASA) assay (Bradbury *et al.* 2005b). However, these assays cannot work on a broad range of any one rice sample and predictions made from the results are not of high accuracy. Moreover, with some genotypes segregation distortion would be generated by the genes that reduce the viability of zygote (Schimenti, 2000; van Boven and Weissing, 2001). Previous studies on genetic analysis have shown that the gene or quantitative trait loci (QTL) for aroma and cooked kernel elongation (CKE) are linked and present on chromosome 8 (Ahn *et al.* 1993). Jain *et al.* (2006) 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, including the PCR-based co-dominant markers SCU-Rice-SSR-1 which amplified three alleles (128 bp, 129 bp and 130 bp). It thus seems necessary to identify specific molecular markers for special populations or novel aromatic rice varieties. However, samples from breeding programs could be assessed for aroma in the early stages of the development of varieties by using marker-assisted selection to keep the target gene intact. Based on our results that the aroma gene in Ch-29B is a single recessive gene and the fact that the introgression of gene in good quality rice is available by successive backcrosses in different populations it will be easy to raise aromatic restorer lines to exploit the aroma trait in hybrid rice with the aid of our newly discovered flanking markers Aro7, RM23120 and RM3459.

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Internet Resources

Gramene database (www.gramene.org/Oryza_sativa/geneview?gene = LOC_Os08g32870).

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