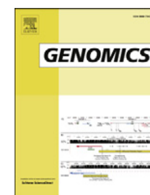
Genomics 101 (2013) 157–162

Contents lists available at SciVerse ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno



Haplotype variation at *Badh2*, the gene determining fragrance in rice

Gaoneng Shao [a,1], Shaoqing Tang [a,1], Mingliang Chen [b,1], Xiangjin Wei [a], Jiwai He [a], Ju Luo [a], Guiai Jiao [a], Yichao Hu [c], Lihong Xie [a], Peisong Hu [a,*]

[a] *State Key Laboratory of Rice Biology, China National Rice Research Institute, Hangzhou 310006, China*
[b] *Jiangxi academy of agricultural sciences, Nanchang 330200, China*
[c] *Hunan agricultural university, Changsha 410128, China*

A R T I C L E I N F O

Article history:
Received 1 May 2012
Accepted 30 November 2012
Available online 6 December 2012

Keywords:
Rice fragrance
Badh2/badh2
Gene evolution
Functional nucleotide polymorphisms

A B S T R A C T

Fragrance is an important component of end-use quality in rice. A set of 516 fragrant rice accessions were genotyped and over 80% of them carried the *badh2.7* allele. A subset of 144 mostly fragrant accessions, including nine of *Oryza rufipogon*, was then subjected to a detailed diversity and haplotype analysis. The level of linkage disequilibrium in the *Badh2* region was higher among the fragrant accessions. Re-sequencing in the *Badh2* region showed that *badh2.7*, *badh2.2* and *badh2.4–5* all arose in the *japonica* genepool, and spread later into the *indica* genepool as a result of deliberate crossing. However, loss-of-function alleles of *Badh2* are also found in the *indica* genepools, and then transferred into *japonica*. Evidence for three new possible FNPs was obtained from the *Badh2* sequence of 62 fragrant accessions. Based on these data, we have elaborated a model for the evolution of *Badh2* and its participation in the rice domestication process.

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1. Introduction

Rice feeds about one third of the world's population. The two domesticated forms of rice are *Oryza sativa* and *Oryza glaberrima*; the former was domesticated from its wild ancestor *O. rufipogon* ~9000 years ago [1,2]. Genomic analysis has demonstrated that the two major subspecies of *O. sativa* (*japonica* and *indica*) arose from distinct *O. rufipogon* populations [3–7]. A small number of genes have been associated with the domestication process in the major crop species [8–12], while in rice, the domestication syndrome genes *GS3*, *rc*, *wx*, *sh4*, *qSH1* and *sd1* have all been isolated and extensively characterized [13,14]. The aroma of the rice grain ("fragrance") is an important consumer trait, and is largely controlled by allelic variation at *Badh2*, a gene which comprises 15 exons [15]. The full length BADH2 protein is associated with non-fragrance [16]. *Badh2* sequence variants associated with fragrance include an 8 bp deletion and three single nucleotide polymorphisms (SNPs) in exon 7, and a 7 bp deletion in exon 2 [15,17]; these polymorphisms are referred to as "functional nucleotide polymorphisms" or FNPs. The wild type BADH2 protein catalyzes the oxidation of a 2-acetyl-1-pyrroline-aminobutyraldehyde (2AP) precursor, so that a non-functional allele results in the enhanced synthesis of 2AP [18]. Fragrant accessions have been identified in various subspecies of rice, including notably the "Basmati" types, and within both the *indica* and the *japonica* genepools, and the fragrant allele has long been thought to have arisen from within the *indica* genepool.

An increasing number of fragrant rice cultivars lacking any of the known *Badh2* FNPs have been identified, suggesting the possibility that the trait may be in some cases controlled by gene(s) other than, or in addition to *Badh2*. Here, we have analyzed the *Badh2* genotype of a panel of 144 rice accessions (including a representation of *O. rufipogon*), some of which are fragrant and others not. Our objective was to catalogue the range of haplotype variation present at *Badh2*, with a view to shedding new light on the domestication of *indica* and *japonica* rice.

2. Materials and methods

2.1. Germplasm and the determination of fragrance

The entries making up the full collection of 549 accessions originated from 15 countries, and comprised 516 (405 listed in Table S1 and 111 in Table S2) classified previously as fragrant and 33 as non-fragrant (Table S2). Nine of the accessions were *O. rufipogon* (Table S2). The full set of material was field-grown at Hangzhou (Zhejiang Province) during 2009. The leaf fragrance of each entry was determined following [19]. About 2 g of green leaf harvested from plants at the tillering stage was sliced and immersed for 10 min in 10 mL 1.7% KOH at room temperature, after which the fragrance was graded independently by three operators. The determination of grain fragrance followed the protocol described in ref. [20]. Out of a random sample of 16 mature grains per line, if none were fragrant, the entry was deemed to be non-fragrant; if five consecutive sampled grains were all fragrant, the entry was deemed to be

* Corresponding author. Fax: +86 571 63370080.
E-mail addresses: hupeisong@yahoo.com.cn, riceh@caas.net.cn (P. Hu).
[1] These authors contributed equally to this paper.

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http://dx.doi.org/10.1016/j.ygeno.2012.11.010

fragrant; and if some of the 16 grains were fragrant and others not, then the entry was deemed to be heterozygous.

2.2. DNA extraction, SSR analysis, primer synthesis and re-sequencing

Template DNA for the PCRs was extracted from leaf tissue using the CTAB method. Each 20 μL reaction contained 2 μL 10× PCR buffer (25 mM $MgCl_2$), 1.6 μL 2 mM dNTP, 2 μL of each SSR primer (5 μM), 1 U *Taq* DNA polymerase and 2 μL genomic DNA. A set of 48 SSR assays was applied to a subset of 144 accessions (Table S3). For primer sequences, refer to www.gramene.org. The amplification regime consisted of an initial denaturation of 94 °C/2 min, followed by 35 cycles of 94 °C/45 s, 55 °C/45 s, 72 °C/60s, and ending with a 72 °C/8 min final extension. Amplicons were electrophoresed through non-denaturing polyacrylamide gels, and visualized by silver staining. Details of the 12 primer pairs used to amplify segments of *Badh2* for the purpose of re-sequencing [17,18], along with those for the three pairs each targeting a functional region of the gene, as described in ref. [21]. The amplicons in this case were electrophoresed through 1.2% w/v agarose gels, recovered using a TIAN gel Midi Purification Kit (TIANGEN), inserted into pGEM-T Easy Vector (Promega), and subsequently transformed into competent DH5α *Escherichia coli* cells. DNA sequencing was performed by a commercial company (Invitrogen, Shanghai).

2.3. Determination of genotypic diversity and population structure

The SSR allelic data for the 144 accessions (Table S2) was analyzed using MEGA v4.1 software in combination with PowerMarker v3.25 to generate an UPGMA dendrogram based on neighbor-joining [22]. The admixture model and the observed allele frequencies were adopted to obtain a representation of the population structure present using STRUCTURE v2.2 software [23]. Both the "length of burn-in period" and the "number of MCMC reps after burn-in" were set to 10,000.

2.4. Blast, haplotype and genetic diversity analysis of the Badh2 gene

Various regions of the *Badh2* were re-sequenced for the 144 accessions, including a segment ~75 K bp upstream of the gene, the promoter, the 3′-UTR and a segment ~46 K bp downstream of the gene. The sequences were spliced by software contig and the resulting contigs were used as BLAST queries using ClustalW v1.8. The aligned sequences were then imported into TASSEL software to identify the polymorphisms present at a frequency >3%, leading to the recognition of *Badh2* haplotypes [24]. The polymorphism data were used to estimate linkage disequilibrium (LD) using the software package DnaSP v4.52 [25].

3. Results

3.1. Allelic variation in Badh2

When the 516 fragrant rice accessions were classified at each of the three known *Badh2* FNPs [17,21], four alleles were recognized: *badh2.7* carries an 8 bp deletion in exon 7 (similar to *badh2.1* reported in the ref. [18]), *badh2.2* a 7 bp deletion in exon2 (the same to *badh2.2* reported in the ref. [18]), *badh2.4–5* an 806 bp deletion involving intron 4 and parts of exons 4–5, and the wild type (*badh-wt*) (Table 1). The bulk (~80%) of the fragrant rice varieties were of the *badh2.7* type.

3.2. Determination of and allelism test for fragrance

516 accessions classified as fragrant (Tables 1, S1–2) were verified as having fragrance by means of the leaf and grain tests. Of these, 62 possessed none of the three known *Badh2* FNPs, and so were crossed to both cv. Zhong2A and cv. Zhong3A to allow for an allelism test for fragrance; both these lines are male sterile, but while the former is fragrant, the latter is not. The F_1 seedling leaves and the F_1 hybrid involving cv. Zhong2A as the parent were all fragrant (data not shown), but those having cv. Zhong3A as the parent were all non-fragrant (data not shown). This result was consistent with the fragrant trait in all of the 62 accessions being determined by a *Badh2* allele.

Table 1
Descriptors of 549 rice accessions. Fragrance determination, allelism test and functional marker analysis are integrated to assess the *Badh2*/*badh2* of those materials.

Allele	No.	Frg(+)/non-frg(−)
badh2.7	416	+
badh2.2	30	+
badh2.4–5	8	+
badh2-wt	62	+
Badh2	24	−
O. rufipogon	9	−

3.3. SSR diversity and genetic structure analysis

The phylogeny of the 144 accessions based on SSR genotype demonstrated the presence of three major groups, corresponding to *indica* types, *japonica* types and *O. rufipogon* accessions. Some admixture between *indica* and *japonica* has clearly occurred (Fig. 1). The *badh2.7* allele was distributed among both the *indica* and the *japonica* groups, while both *badh2.2* and *badh2.4–5* were restricted to the *japonica* group. Thus both *badh2.2* and *badh2.4–5* acted as the mutant genes for *Badh2*, and fixed in the fragrant rice varieties. The structure-based analysis provided evidence for a significant population structure in the germplasm set, with three being the most likely number of distinct groups (the *K* parameter). The three clusters corresponded to the *indica* and the *japonica* types, and the *O. rufipogon* accessions (Fig. 2).

3.4. LD around the Badh2 locus

When the squared allele frequency correlations were related to the position of the various SNPs and indels, the resulting regressions demonstrated that the rate of LD decay among the non-fragrant accessions was less than among the fragrant ones, while the wild types were more stable across the whole gene *Badh2* (Fig. 3). The expected value of r^2 for the non-fragrant rice varieties declined to 0.1 at a distance of >5 K bp, whereas the level remained >0.1 for at least 7.5 K bp in the fragrant varieties. This difference in LD can arise as a result of variable genome organization and/or of population structure. These results also indicated that the *Badh2* gene was an evolution gene and differential between the fragrance and non-fragrance rice varieties.

3.5. Haplotype analysis of Badh2

The re-sequenced segments of *Badh2* covered 5.86 K b of the reference cv. Nipponbare sequence. The analysis revealed the presence of >100 variable sites, including SNPs, indels and a TA microsatellite; of these, 37 present at a frequency of >3% were used to define 27 haplotypes of *Badh2* (Fig. 4). Ten of the haplotypes were restricted to a group of accessions dominated by *japonica* types, and the other 17 to a group composed of *indica* types and *O. rufipogon* accessions. Haplotype H1 was identical to *badh2.7* defined the presence of an 8 bp deletion and three SNPs in exon 7, and represented ~20% of the accessions (in 144 rice accessions). It is found in both *japonica* and *indica* types, although is thought to have originated in the *japonica* genepool [18], a notion confirmed by the observation that the polymorphic sites in the key exon 7 region were mostly of *japonica* origin (Figs. 4–5; Tables S4–5). Twelve fragrant rice varieties

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