The origin and evolution of fragrance in rice (*Oryza sativa* L.)

Michael J. Kovach[a], Mariafe N. Calingacion[b], Melissa A. Fitzgerald[b], and Susan R. McCouch[a,1]

[a]Cornell University, Department of Plant Breeding and Genetics, Ithaca, NY 14853; and [b]Grain Quality Nutrition and Postharvest Centre, International Rice Research Institute, Los Baños, Philippines

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Fragrance in the grain is one of the most highly valued grain quality traits in rice, yet the origin and evolution of the betaine aldehyde dehydrogenase gene (*BADH2*) underlying this trait remains unclear. In this study, we identify eight putatively nonfunctional alleles of the *BADH2* gene and show that these alleles have distinct geographic and genetic origins. Despite multiple origins of the fragrance trait, a single allele, *badh2.1*, is the predominant allele in virtually all fragrant rice varieties today, including the widely recognized Basmati and Jasmine types. Haplotype analysis allowed us to establish a single origin of the *badh2.1* allele within the *Japonica* varietal group and demonstrate the introgression of this allele from *Japonica* to *Indica*. Basmati-like accessions were nearly identical to the ancestral *Japonica* haplotype across a 5.3-Mb region flanking *BADH2* regardless of their fragrance phenotype, demonstrating a close evolutionary relationship between Basmati varieties and the *Japonica* gene pool. These results clarify the relationships among fragrant rice varieties and challenge the traditional assumption that the fragrance trait arose in the *Indica* varietal group.

BADH2 | Basmati | Jasmine

Recent findings suggest that although Asian cultivated rice (*Oryza sativa*) is comprised of several genetically distinct groups, a number of the alleles responsible for key domestication and grain quality traits are shared among these groups. Given the complex evolutionary history of rice, the origin of these genetic alterations and how they came to exist across the highly divergent subpopulations of *O. sativa* remain central questions in rice evolutionary biology.

Fragrance is considered one of the most important grain quality traits in rice, as it is a key factor in determining market price and is related to both local and national identity (1, 2). Investigations into the genetic basis of fragrance in rice led to the identification of a single locus on chromosome 8 (*fgr*) associated with fragrance (3, 4). Fine mapping (5–7) and subsequent sequence analysis identified a betaine aldehyde dehydrogenase gene, *BADH2*, associated with the fragrant phenotype [nomenclature follows (8)]. The functional mutation creating the recessive *badh2.1* allele was first described as three single nucleotide polymorphisms (SNPs) and an 8-bp deletion in the seventh exon of the gene that resulted in a premature stop codon and putatively truncated the BADH2 protein (9). Other sequence alignments have been used to describe this complex mutation (10, 11), and so the mutation in *badh2.1* will hereafter be referred to as the functional nucleotide polymorphism (FNP). Recent surveys of diverse fragrant germplasm support the association of *badh2.1* with fragrance (10, 12, 13), and transformation of a fragrant variety with the dominant nonfragrant allele has been shown to abolish fragrance (14), confirming that *BADH2* is the major genetic determinant of fragrance in rice.

Over 100 volatile compounds have been detected in fragrant rice varieties, but the major compound responsible for the characteristic aroma is 2-acetyl-1-pyrroline (2AP) (15, 16). This compound, which is produced in all parts of the rice plant except the roots, has a very low odor threshold, allowing humans to detect it at minute concentrations in field-grown plants or crushed leaf tissue, as well as in the grain before, during, and after cooking (17). While the biochemical pathway leading to 2AP synthesis has not been fully resolved, it is believed the BADH2 protein catalyzes the oxidation of γ-aminobutyraldehyde (AB-ald; a 2AP precursor), so that a nonfunctional allele results in the accumulation of both AB-ald and its cyclic form, Δ^1pyrroline, resulting in enhanced 2AP synthesis (14, 18).

O. sativa consists of two major varietal groups, *Indica* (*Hsien*) and *Japonica* (*Keng*) (capitalized when referring to varietal groups), that have been recognized in China since ancient times (19, 20). A set of 15 isozyme markers was able to further subdivide the two major varietal groups into six genetically differentiated subpopulations corresponding to well-recognized ecotypes (21). Subsequent studies using SSRs (22) and SNPs (23) distinguished five genetically defined groups that roughly corresponded to the isozyme groups identified by Glaszmann (21): *indica*, *aus*, *temperate japonica*, *tropical japonica*, and *aromatic* (lowercase when referring to subpopulations) (Fig. 1). Phylogenetic analysis and F_{ST} values demonstrate a close evolutionary relationship between the *aromatic*, *temperate japonica*, and *tropical japonica* subpopulations, which comprise the *Japonica* varietal group, while the *indica* and *aus* subpopulations have a distinct ancestry and are recognized as members of the *Indica* varietal group (22, 23). Despite its name, the *aromatic* subpopulation is phenotypically diverse and includes both fragrant and nonfragrant varieties. To avoid confusion, we will hereafter refer to the *aromatic* subpopulation by its isozyme name, *Group V* (24).

Fragrant accessions have been identified within at least three of the distinct genetic subpopulations of rice, including *Group V* (i.e., "Basmati" and "Sadri" varieties), *indica* (i.e., "Jasmine" varieties), and *tropical japonica*. In surveys of diverse fragrant rice accessions, nearly all have been shown to carry the *badh2.1* allele, suggesting that this allele is common by descent in fragrant rice varieties (9, 10, 12). Recently, a second mutation in the *BADH2* gene, *badh2.2*, was found to be associated with fragrance within a limited set of germplasm from China (13). Evidence that there may be additional mutations in the pathway leading to 2AP synthesis comes from a rigorous study involving a diverse panel of fragrant germplasm that identified several accessions, mostly from Southeast Asia, that had elevated levels of 2AP but did not carry the *badh2.1* allele (12). Given that a single allele is largely responsible for fragrance in rice, the goal of this study was to investigate the origin of this allele and trace its ancestry among the genetically divergent subpopulations of rice. We also set out to identify additional functional mutations in the *BADH2* gene that may be responsible for independent, local origins of the fragrant phenotype.

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[1]To whom correspondence should be addressed. E-mail: srm4@cornell.edu.

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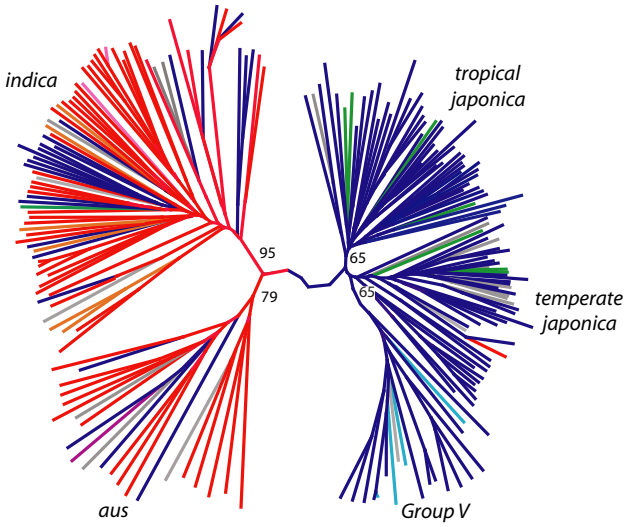

Fig. 1. Subpopulation structure in *O. sativa*. Unrooted neighbor-joining tree constructed from 169 nuclear SSRs (22). Branch color corresponds to chloroplast haplotype. Bootstrap values (out of 100) are indicated at the branch points. This tree clearly illustrates the major division between the two varietal groups (*Indica* and *Japonica*), which are further subdivided into the five rice subpopulations: *indica, aus, tropical japonica, temperate japonica*, and *Group V* (*aromatic*). Reproduced and modified, with permission (22).

Results

Frequency of the *badh2.1* Allele in Diverse Rice Germplasm. We examined the occurrence of the *badh2.1* allele in 280 accessions of wild rice (*Oryza rufipogon*/*Oryza nivara*) and found that it was absent from all wild genotypes, except for a single accession that was heterozygous for the allele (Table 1). This wild accession exhibited several traits characteristic of domesticated rice, including white pericarp, suggesting it was the result of a recent hybridization event with a fragrant accession of *O. sativa*. A diverse collection of 176 *O. sativa* accessions was also surveyed, and the subpopulation identity of these cultivars was determined using a set of genome-wide SSR and SNP markers (22, 23). Overall, the *badh2.1* allele was detected in 17 (10%) of these accessions, with the fragrant allele detected at the highest frequency in *Group V* and at the lowest frequency in *temperate japonica* and *aus* (Table 1).

Origin of the *badh2.1* Allele. Given that an identical *badh2.1* allele was detected in both the *Japonica* and *Indica* varietal groups, it was our goal to determine in which group it had originated. To address this question, we sequenced across the *BADH2* gene in a panel of 242 *O. sativa* accessions, which included the original panel and additional accessions known to possess the *badh2.1* allele (12) (Table S1). In ≈5 kb of aligned sequence, we detected 106 SNP, insertion-deletion (indel) and SSR polymorphisms, 54 of which were present at a frequency >5%. These polymorphisms were used to construct eight gene haplotypes (GH), and these haplotypes were clearly differentiated into two distinct clusters (Fig. 2*A* and Table S2). Within the first cluster, all accessions carrying the wild-type allele were from the *Japonica* varietal group (Jap_GH), while the majority of accessions from the second cluster (74%) were from the *Indica* varietal group (Ind_GH). Every accession carrying the *badh2.1* allele fell within the Jap_GH cluster, regardless of subpopulation identity. Within the Jap_GH cluster, fragrant accessions differed from the ancestral group of nonfragrant accessions only at the FNP that defines the *badh2.1* allele (highlighted in yellow in Fig. 2*A*). These data therefore support a single origin of the *badh2.1* allele in a *Japonica*-like genetic background.

Table 1. Frequency of *badh2.1* allele in wild and cultivated rice

	No. individuals		
	Total	*badh2.1*	% *badh2.1*
O. rufipogon/*O. nivara*	280	0.5	0.2
O. sativa	176	17	10.0
indica	54	3	6.0
aus	23	0	0
Group V (aromatic)	10	6	60.0
temperate japonica	36	0	0
tropical japonica	53	8	15.0

All fragrant varieties from the *Indica* varietal group that carried the *badh2.1* allele clustered with Jap_GH, creating an apparent contradiction between the gene and genome phylogenies of these accessions. This incongruence can be explained if the fragrant *indica* accessions are found to carry a defined region of *Japonica*-like DNA around the *BADH2* gene within an *Indica* genetic background. We therefore broadened the scope of our haplotype analysis by sequencing 24 amplicons in a genomic region spanning 3.2 Mb upstream and 2.1 Mb downstream of *BADH2* in our panel of 242 *O. sativa* accessions. In this 5.3-Mb region flanking *BADH2*, a total of 426 SNP, indel, and SSR polymorphisms were identified across ≈13 kb of aligned sequence, with 271 polymorphisms present at a frequency >5%. From these, 78 ancestrally informative polymorphisms (AIPs) were identified and used to create extended haplotypes (see *Materials and Methods* and Table S3). These extended haplotypes were then summarized into "consensus" extended haplotypes representing six major haplotype classes (Fig. 2*B*). The extended haplotypes were consistent with the gene haplotypes, with all *badh2.1*-containing accessions having an extended region of *Japonica* DNA around the *BADH2* gene. In the 24 *indica* accessions carrying the *badh2.1* allele, the *Japonica* region was bordered by recombination breakpoints ≈650 kb upstream and ≈330 kb downstream, with the flanking regions identical to the ancestral *Indica* extended haplotype (Fig. 2*B*). This supports the hypothesis that the *badh2.1* allele was transferred via introgression into *indica*.

Group V accessions carrying the *badh2.1* allele possessed an extended haplotype identical to ancestral *Japonica*, with the exception of the FNP and three unique polymorphisms at sites 14, 30, and 69 (highlighted in green; Fig. 2*B*). These same three signature polymorphisms flanking the FNP were detected within the *Japonica*-like region found in all *indica* accessions carrying the *badh2.1* allele. The single wild accession from Myanmar that was heterozygous for the *badh2.1* allele segregated 1:2:1 in the next generation, and haplotype analysis confirmed the chromosome carrying the *badh2.1* allele also contained the same three *Group V*-specific polymorphisms described above. We were therefore able to trace the ancestry of the *badh2.1* allele in the heterozygous wild accession and all fragrant *indica* accessions to a *Group V* ancestor.

There were several nonfragrant *Japonica* accessions that grouped with the Ind_GH cluster, which was otherwise made up of nonfragrant *Indica* accessions (Fig. 2*A*). These nonfragrant accessions from the *Japonica* varietal group contained an *Indica*-like genomic region around the *BADH2* gene, and all showed recombination back to ancestral *Japonica* in the flanking regions (Fig. 2*B*; "Recombinant *Japonica*"). This provides an explanation for the number of *Japonica* accessions having a *BADH2* gene haplotype that clustered with Ind_GH.

Reduced Nucleotide Diversity and Elevated Linkage Disequilibrium at the *badh2.1* Allele. To examine evidence of selection around the *badh2.1* allele, we analyzed the nucleotide diversity across the *BADH2* gene in our panel of 242 accessions. We compared the total nucleotide diversity (θ_π) in the ≈5 kb of aligned sequence across the *BADH2* gene between accessions carrying the wild-type and *badh2.1* alleles. Fragrant accessions carrying the *badh2.1* allele

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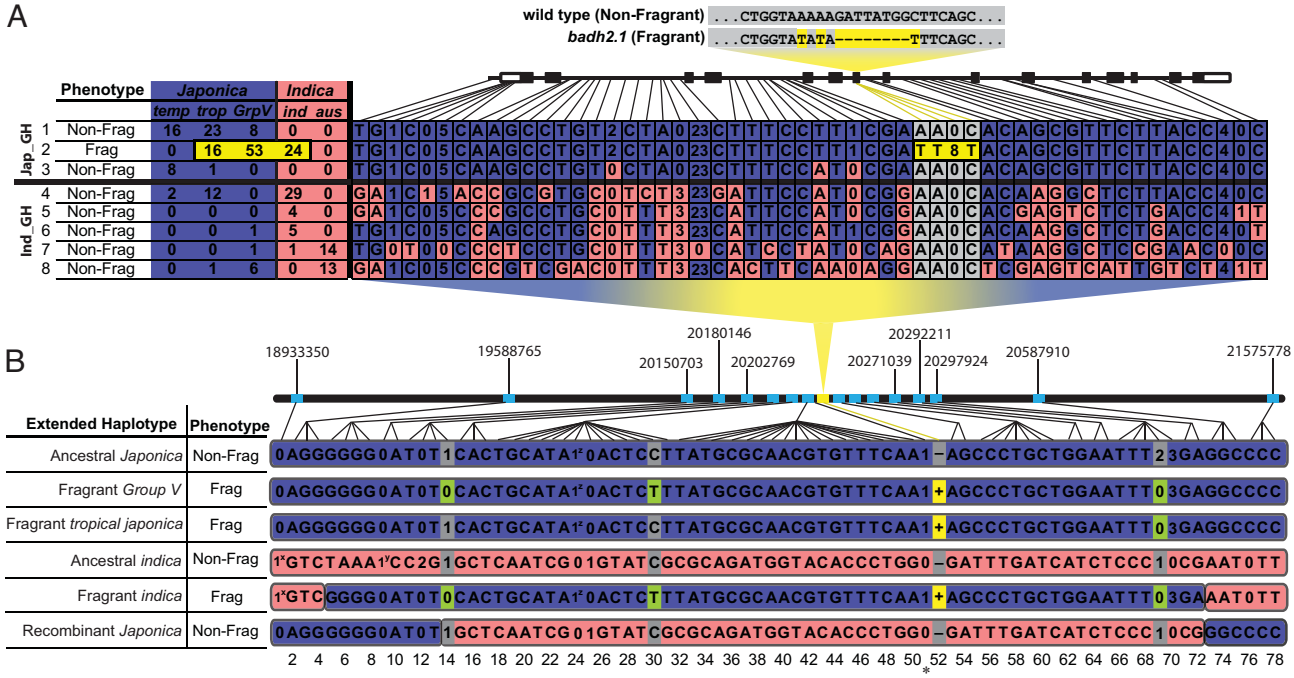


Fig. 2. Haplotype analysis of the *BADH2* gene region. (*A*) *BADH2* gene haplotypes. Sequence reads across the *BADH2* gene were aligned for 242 *O. sativa* accessions and all polymorphisms (frequency >5%) were concatenated and used to create eight gene haplotypes. Letters in each haplotype represent alternative nucleotides at a SNP site; numbers indicate the size of a deletion (0 = no deletion), with the relative position of each polymorphism indicated along the *BADH2* gene model. Haplotypes are numbered 1 through 8 followed by the corresponding fragrance phenotype, and the number of accessions from each subpopulation possessing that haplotype. Two gene haplotype clusters were identified: The *Japonica* Gene Haplotype Cluster (Jap_GH) and *Indica* Gene Haplotype Cluster (Ind_GH). Blue cells represent polymorphisms characteristic of Jap_GH, while red cells represent those characteristic of Ind_GH. The *badh2.1* FNP is depicted in gray/yellow, with yellow representing the fragrant allele. (*B*) Extended haplotypes. Out of 17 total extended haplotypes for *BADH2*, six ''consensus'' haplotypes are depicted with the phenotype of each indicated. Letter/number designations and color coding are as in Fig. 2*A* (with the following abbreviated deletions: $1^x = 28$, $1^y = 12$, $1^z = 48$). Breaks in coloration indicate positions where recombination was detected. Position 51, marked with a star, represents the MITE polymorphism described previously (10). The *badh2.1* FNP is highlighted in yellow at site 52, with ''+'' referring to the derived (fragrant) allele and ''−'' referring to the wild-type (nonfragrant) allele. The polymorphisms highlighted in green at positions 14, 30, and 69 are those that were fixed within all fragrant *Group V* and *indica* accessions carrying the *badh2.1* allele. The relative positions and locations (on the TIGR v.5 pseudomolecule) of the 16 markers that provided AIPs are depicted as light blue dots along a black bar, representing the stretch of chromosome 8 that was sample-sequenced.

exhibited a 97.4% reduction in diversity on average compared to the nonfragrant accessions (Table 2).

Extended haplotype homozygosity (EHH) estimates the probability that two randomly chosen genomic regions are identical by descent, and allows the measurement of linkage disequilibrium (LD) decay with increasing distance from a functional mutation (25). We calculated the EHH for the *BADH2* gene in our germplasm panel by comparing the extended haplotypes of the fragrant (*badh2.1*) and nonfragrant accessions. The fragrant accessions carrying the *badh2.1* allele exhibited a large block of extended LD around the FNP, while LD around the wild-type allele declined rapidly (Fig. 3). This same pattern was observed when the extent of LD was compared between accessions containing the wild-type or the *badh2.1* allele within individual subpopulations (Fig. S1). The dramatically reduced nucleotide diversity at *badh2.1*, coupled with the extended region of linkage disequilibrium surrounding this allele, provide evidence for strong positive selection for the *badh2.1* FNP.

Additional Mutations in the *BADH2* Gene. To detect additional mutations in *BADH2* that may be responsible for enhanced 2AP synthesis, the *BADH2* gene was sequenced in 26 fragrant accessions that lacked either of the previously described derived alleles conferring fragrance (12). Sequence alignments from these 26 accessions with the 242 previously sequenced accessions revealed eight nonsynonymous polymorphisms, four of which were frameshift-inducing indels and one of which was a SNP creating a premature stop codon, all putatively resulting in a truncated BADH2 protein (Fig. 4*A* and Table S4). The other three potentially functional polymorphisms included a 3-bp insertion and two different SNPs in the coding region. While several of these polymorphisms were found and confirmed in only a single accession, four were found in multiple fragrant accessions, and these polymorphisms appear to have strong geographic associations (Fig. 4*B*). There were two accessions for which we do not, as yet, have candidate functional mutations that could explain the presence of elevated 2AP levels.

Discussion

A *Japonica* Origin of Fragrance in Rice. This study presents an in-depth survey of the genetic diversity of the *BADH2* gene in a

Table 2. Average Nucleotide Diversity across *BADH2* gene ($\theta\pi$ per kb)

	indica	*aus*	*Group V (aromatic)*	*temperate japonica*	*tropical japonica*	All
Badh2 (non-fragrant) (n = 151)	3.79	5.25	6.75	2.13	4.50	6.64
badh2.1 (fragrant) (n = 93)	0.03	None	0.06	None	0.29	0.17

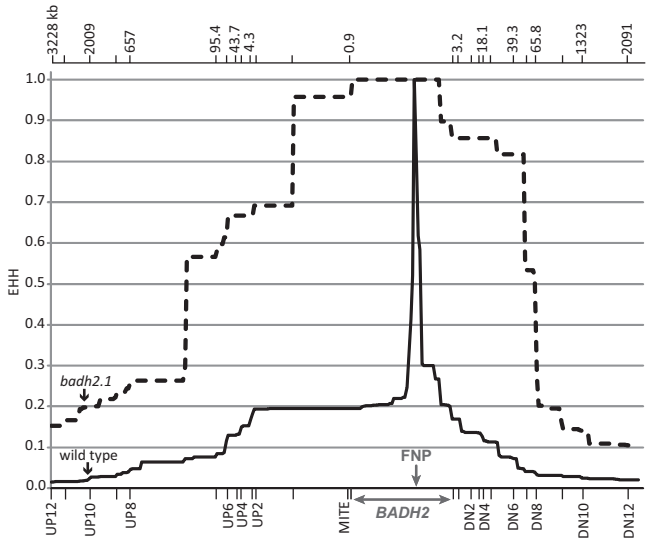
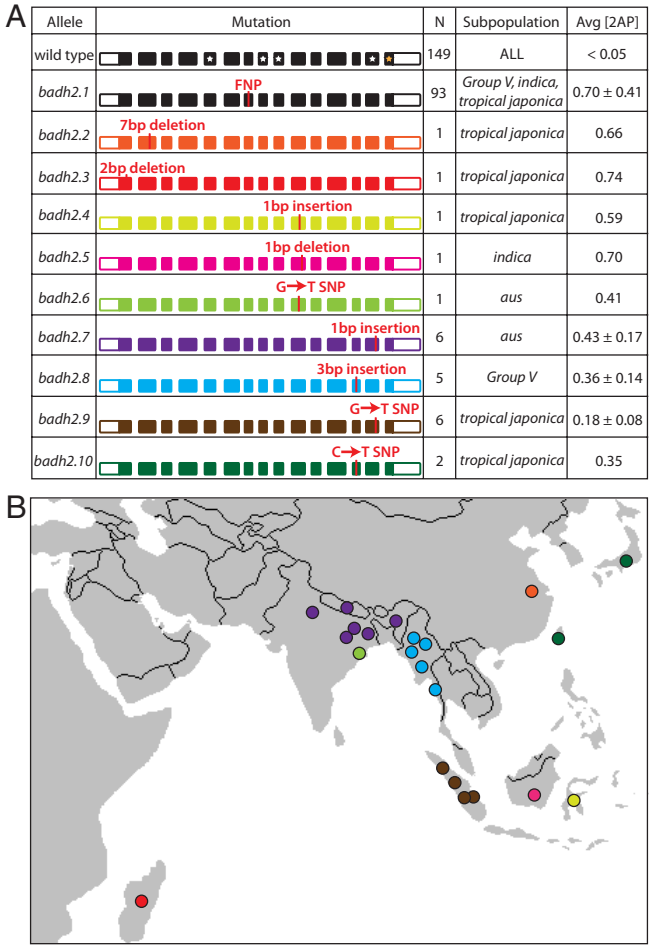


Fig. 3. EHH across the *BADH2* genomic region. EHH values were calculated for the 242 *O. sativa* accessions examined in this study based on haplotype data across a 5.3-Mb genomic region surrounding the *BADH2* gene. Solid and dashed lines indicate the combined EHH values of accessions having the wild-type and *badh2.1* alleles, respectively. The position of the *BADH2* gene is indicated, and *badh2.1* FNP is indicated with an arrow. Dashes along the x-axis represent the locations of each amplicon used to obtain haplotype data (Table S5). The physical distances of selected amplicons from the *BADH2* gene (in kb) are depicted across the top.



Allele	Mutation	N	Subpopulation	Avg [2AP]
wild type		149	ALL	< 0.05
badh2.1	FNP	93	*Group V, indica, tropical japonica*	0.70 ± 0.41
badh2.2	7bp deletion	1	*tropical japonica*	0.66
badh2.3	2bp deletion	1	*tropical japonica*	0.74
badh2.4	1bp insertion	1	*tropical japonica*	0.59
badh2.5	1bp deletion	1	*indica*	0.70
badh2.6	G→T SNP	1	*aus*	0.41
badh2.7	1bp insertion	6	*aus*	0.43 ± 0.17
badh2.8	3bp insertion	5	*Group V*	0.36 ± 0.14
badh2.9	G→T SNP	6	*tropical japonica*	0.18 ± 0.08
badh2.10	C→T SNP	2	*tropical japonica*	0.35

Fig. 4. *BADH2* allelic diversity. (*A*) Coding Mutations in the *BADH2* gene. Sequencing across 26 fragrant accessions that lacked either known mutation in *BADH2* identified eight mutations in the coding region of the gene that are putatively responsible for the fragrance phenotype. The gene model for *BADH2*, with 15 exons and 14 introns, is depicted for each allele along with the location and identity of the coding mutation. Four white stars in the gene model of the wild-type allele illustrate the locations of critical catalytic or substrate-binding domains, while the orange star indicates the location of an oligimerization domain (14). For each allele, the number (N) and genetic subpopulation(s) of accessions having that allele are shown, along with the average 2AP concentration of the accessions possessing that allele (with standard deviations). We obtained accurate 2AP measurements for only one of the accessions carrying the *badh2.10* allele, so no standard deviation is indicated. (*B*) Geographic locations of accessions possessing mutations in *BADH2*. Each accession having a mutation in *BADH2* that is predicted to cause fragrance was placed on a map of Asia using all available collection/passport data for the accessions. The clustering of accessions with a given mutation within the same geographical region suggests that the fragrance trait was selected on multiple occasions, but these alleles remained isolated within local rice gene pools.

large panel of genetically and geographically diverse rice germplasm from across Asia. Fragrant accessions carrying the *badh2.1* allele exhibited a dramatic reduction in nucleotide diversity (97%) and elevated linkage disequilibrium around the gene compared to nonfragrant accessions, consistent with strong positive selection for the *badh2.1* allele. The intensity of selection on the *badh2.1* allele was similar to reports from other rice genes controlling grain morphology traits (i.e., *wx*, *rc*, *gs3*) (26–28).

Haplotype analysis allowed us to demonstrate that the *badh2.1* allele arose in the genetic background of the *Japonica* varietal group. Extended haplotype analysis revealed a clear introgression of a *Japonica* genomic region encompassing the *badh2.1* allele in all fragrant *indica* accessions, including Jasmine varieties. All fragrant *indica* also possessed three polymorphisms flanking the *badh2.1* allele that were diagnostic of the *Group V* subpopulation, demonstrating that this important grain quality allele originated in the *Group V* subpopulation (i.e., Basmati) and was introgressed into *indica* (i.e., Jasmine).

The derived polymorphism at site 69 that was ancestrally informative and fixed within fragrant *Group V* accessions was also fixed within all *tropical japonica* accessions carrying the *badh2.1* allele. This suggests that: (*i*) The *badh2.1* allele may have arisen in an ancestor common to both the *Group V* and *tropical japonica* lineages, and the two *Group V*-specific polymorphisms (sites 14 and 30; Fig. 2*B*) arose in the *Group V* lineage following the divergence of these groups or (*ii*) that a genomic region containing the *badh2.1* allele and one downstream polymorphism (site 69) may have been introgressed from *Group V* to *tropical japonica*. The extended region of identical sequence flanking the *BADH2* gene between fragrant *Group V* and *tropical japonica* accessions precludes the detection of recombination breakpoints that would define an introgression from *Group V* to *tropical japonica* (or vice versa).

Evidence from this study suggests that the *badh2.1* allele was selected as a de novo mutation in *O. sativa* after domestication from its wild progenitor, and presumably after the divergence of the *Japonica* subpopulations, given the high frequency of the allele in *Group V* and vanishingly low frequency in *temperate japonica*. In our collection of 280 diverse accessions of *O. rufipogon*/*O. nivara* from across Asia, we found only a single accession from Myanmar that was heterozygous for the *badh2.1* allele. Sequence analysis of this accession demonstrated that the chromosome carrying the *badh2.1* allele contained the three signature polymorphisms fixed in all fragrant *Group V* accessions bordered by DNA sequence characteristic of *Indica*. While *badh2.1* has been identified in other wild rice germplasm from Southeast Asia (29–31), it is likely that these represent introgressions of the derived allele into wild and weedy rice populations growing in the vicinity of fragrant cultivars (10). Gene flow of alleles from *O. sativa* into wild rice populations has been reported on multiple occasions and is now thought to be ubiquitous (32, 33).

EVOLUTION

This study improves our understanding of the genetic relationships between *O. sativa* subpopulations and further clarifies the evolutionary history of Asian cultivated rice. Basmati varieties from the *Group V* subpopulation are often erroneously referred to as members of the *Indica* varietal group (34–36). While varieties from the *indica* and *Group V* subpopulations are widely grown in South and Central Asia and both may exhibit long, slender grain morphology, researchers have long recognized the high levels of hybrid sterility in crosses between these two groups (37–40). Glaszmann postulated an explanation for these hybrid incompatibilities by demonstrating with isozyme markers that *Group V* was clearly distinct from *Indica* and that it grouped more closely with *Japonica* (21, 24). Additional examinations of the genetic diversity in *O. sativa* using chloroplast markers, nuclear SSRs, and SNPs independently determined that the *Group V* subpopulation is a unique genetic entity closely related to the *Japonica* varietal group (22, 23, 41). Our haplotype analysis in this study demonstrated that the *Group V* accessions, both fragrant and nonfragrant, cluster with the ancestral *Japonica* accessions both across the *BADH2* gene and across the entire 5.3-Mb region of chromosome 8 surveyed. This provides further evidence that *Group V*, despite its morphological similarities and geographical distribution that overlaps with *Indica*, is genetically a member of the *Japonica* varietal group. Interestingly, the overlapping distributions of *Group V* and *Indica* varieties in South Asia may have provided a corridor for the transfer of the major fragrance allele to *Indica*.

Independent Origins of Fragrance in Rice. The presence of rice varieties exhibiting elevated 2AP levels, but lacking any known nonfunctional allele of *BADH2*, raised the possibility that there might be additional fragrance-causing alleles of *BADH2* (12). Analysis of 26 fragrant accessions that lacked any known fragrance allele identified eight polymorphisms in the coding region of the gene that are predicted to alter the BADH2 protein. It had been previously shown that only the full-length *BADH2* transcript, resulting in an intact 503-aa protein, was capable of inhibiting 2AP production (14). Four of the eight coding polymorphisms identified in this study (creating alleles *badh2.3-2.6*) are predicted to cause premature transcript termination, which would putatively abolish protein function and result in fragrance. These mutations all result in truncation of the *BADH2* protein before critical residues that form the catalytic and/or substrate binding domains (14). Additionally, the *badh2.7* allele, which was shared by six *aus* accessions, is also predicted to result in a shortened transcript that would eliminate an oligomerization domain of the protein. The other three alleles (*badh2.8–2.10*) either result in an additional in-frame amino acid (*badh2.8*) or amino acid substitutions (*badh2.9–2.10*). Despite the association of fragrance with these coding mutations in the *BADH2* gene, future work is needed to confirm the effect of these mutations on the degree of 2AP accumulation.

The geographic association between accessions carrying the same mutant alleles at *BADH2* suggests fragrance was selected independently on multiple occasions in different geographic regions. Interestingly, while we now know the predominant allele responsible for enhanced 2AP production in rice originated within the *Japonica* varietal group, it appears that other fragrant alleles of *BADH2* were identified within the *Indica* varietal group, exemplified by the *badh2.7* allele, which was found in several *aus* accessions. These and other findings suggest that the *Group V* and *aus* lineages harbor useful alleles that have yet to be fully exploited in rice improvement (22, 27, 47, 48).

Out of the 26 accessions exhibiting fragrance but lacking any of the previously identified fragrance alleles, there were two accessions for which we could find no mutation in the coding or promoter regions that would be predicted to alter the BADH2 protein or its expression. It is therefore possible that these accessions contain genetic lesions elsewhere in the metabolic pathway controlling 2AP synthesis; mapping experiments are underway to test this hypothesis. Identifying additional genes responsible for fragrance would provide opportunities to tailor rice grain quality to suit diverse cultural preferences (1).

***BADH2* in the Broader Context of Rice Domestication and Varietal Differentiation.** A variety of functional alleles at genes associated with rice domestication and grain quality enhancement have been described in rice. Alleles at some of these loci appear to have been selected from standing variation in the wild, while others have been selected de novo from within a cultivated gene pool (42, 43). Regardless of their origin, these alleles either remained isolated or became widely disseminated among the subpopulations of *O. sativa*. Many of the derived alleles characterized thus far in rice show evidence of a single origin within one subpopulation, but are shared by both the *Indica* and *Japonica* varietal groups, consistent with the process of introgression. The *rc* allele, which is responsible for white pericarp in 98% of modern *O. sativa* varieties, offers an example of a domestication-related gene that arose in a *Japonica* genetic background followed by dissemination into other, genetically divergent subpopulations (27). The average size of the *rc*-containing introgression in the *Indica* varietal group was around 1 Mb, similar to the size of the *badh2.1* introgression in *indica* as reported in this study. Similarly, a mutation in the *GS3* gene conferring long grain originated in a *Japonica* ancestor, with subsequent introgression into *indica* cultivars (26). In this study, we demonstrate that the evolutionary history of the *badh2.1* allele is also consistent with this pattern. These alleles would have been transferred between the rice subpopulations through the process of introgressive hybridization, facilitated by the higher outcrossing rates among early rice cultivars and the physical proximity of divergent (*Indica* and *Japonica*) cultivars as a result of population expansion and human migration throughout Asia (42, 44–46). It is noteworthy that despite the apparent importance of hybridization and gene flow during rice evolution, opposing forces have maintained the genetic divergence among the subpopulations of *O. sativa*. Solving this paradox will require future research to identify the key factors that contribute to subpopulation isolation, as well as to provide insight into the dynamics of genetic exchange among these groups.

Materials and Methods

Plant Materials. Our germplasm panel consisted of a total of 280 *O. rufipogon* and 242 *O. sativa* accessions collected from 38 countries across Asia. We also obtained 26 fragrant accessions lacking the *badh2.1* allele from a previous study (12). A complete list of the accessions used in this study can be found in Table S1. All *O. sativa* accessions not previously reported were analyzed with diagnostic SSR markers to determine their subpopulation identities.

DNA Extraction, PCR, and Sequencing. DNA extraction was performed using a potassium acetate-SDS protocol for leaf tissue (49) and a modified protocol for milled seeds (50). The functional marker for *badh2.1* (51) was used to genotype our germplasm panel. For gene haplotype analysis, eight ≈700-bp amplicons were sequenced across the coding region of the *BADH2* gene, resulting in ≈5 kb of aligned sequence. For extended haplotype analysis, 24 regions were sequenced, spanning 3.2 Mb upstream and 2.1 Mb downstream of *BADH2*, and resulting in over 13 kb of aligned sequence. A previously described MITE polymorphism (10) was also genotyped in the *O. sativa* panel. A complete list of the primers for the 32 sequenced regions can be found in Table S5. PCR products were purified and sequencing was performed on ABI Prism 3700/3100 DNA analyzers (Applied Biosystems) at the Cornell Life Sciences Core Laboratories Center. Sequences were aligned using the CodonCode Aligner program (CodonCode), and the ends of amplicons were trimmed to remove low quality sequences. Singletons and ambiguous sites were resequenced as necessary. The nonsynonymous polymorphisms found in the fragrant accessions lacking any known nonfunctional allele at *BADH2* were resequenced several times for confirmation.

2AP Phenotyping. Extraction of 2AP with dichloromethane was performed using a modified method (52). Chemically synthesized 2AP was provided by Dr. T. Yoshihashi (Japan International Research Centre for Agricultural Sciences, Ibaraki, Japan) and was used to quantify 2AP in the samples. Each sample was

extracted and analyzed on six different occasions and from at least three biological replicates.

Haplotype and Genetic Diversity Analysis Across the *BADH2* Region. Aligned sequences for the eight amplicons across the coding region of the *BADH2* gene in the 242 *O. sativa* accessions were imported into the TASSEL program to extract all polymorphisms present at a frequency above 5% in the sample for constructing gene haplotypes (Table S1). The highly polymorphic SSR at position 20249280 was either long (TA_{11-14}) or short (TA_{6-8}), which corresponded to the *Japonica* and *Indica* varietal groups, respectively, and so was coded as ''1'' if the allele had greater than eight repeats and ''0'' if the allele had eight or fewer repeats. A total of eight gene haplotypes were inferred using 238 accessions, with four recombinant haplotypes not included in Fig. 2*A*. The Bayesian clustering program STRUCTURE was used to analyze the gene haplotypes and the highest likelihood was obtained at K = 2 clusters, which were then labeled *Japonica* Gene Haplotype Cluster (Jap_GH) and *Indica* Gene Haplotype Cluster (Ind_GH).

For the extended haplotype analysis, we sequenced 24 regions flanking *BADH2* in the same 242 *O. sativa* accessions as described above (Table S5). TASSEL was used to extract the 216 polymorphisms present at a frequency above 5% in the sample. To reduce the number of haplotypes, we applied the following criteria to select AIPs: A polymorphism must (*i*) have a significant frequency difference between the *Japonica* varietal group and the *indica* subpopulation ($P < 0.00003$) and (*ii*) be present at a frequency above 20% in the population. A total of 78 polymorphisms from 16 of the sequenced amplicons, the FNP, and the MITE polymorphism met these criteria, and were used in Fig. 2*B* and Table S3.

Polymorphism data from the 24 sequenced regions flanking *BADH2* was used to calculate EHH across the region in the 242 *O. sativa* accessions, following the method described previously (25).

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