

The *badh2* Allele of the Fragrance (*fgr/BADH2*) Gene Is Present in the Gene Population of Weedy Rice (*Oryza sativa* f. *spontanea*) from Thailand

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Abstract: The gene population of weedy rice (*Oryza sativa* f. *spontanea*) is expected to play important role in increasing the genetic diversity of cultivated rice (*Oryza sativa* L.). A candidate gene (*fgr/BADH2*) homologous to *betaine aldehyde dehydrogenase* is responsible for aroma metabolism in fragrant rice varieties. The presence of a dominant *BADH2* allele encoding betaine aldehyde dehydrogenase inhibits the synthesis of 2-acetyl-1-pyrroline (2AP), a potent flavor component in rice fragrance. By contrast, its recessive alleles, *badh2*, induce 2AP formation. The present study was carried out to determine the presence of the recessive allele of the fragrance gene of the weedy rice population in an important rice growing area of Northeastern Thailand. Among the 215 weedy rice plants examined, three genotypes, *BADH2/BADH2*, *BADH2/badh2* and *badh2/badh2* were detected. Frequencies of the *badh2* allele showed a high value of 0.547. A test of goodness-of-fit was performed to assess the evolutionary processes influencing this gene locus. The test indicate that the evolutionary process did not play a significant role in influencing the gene locus ($\chi^2 = 3.5$, $P > 0.05$). The *badh2* allele was not found in the wild rice populations used in this study. This finding strongly supports the hypothesis that the *badh 2* allele in the weedy rice population originated from fragrant rice cultivars.

Key words: Crop-to-weed gene flow • Weedy rice • *Oryza sativa* f. *spontanea* • Fragrance gene

INTRODUCTION

Weedy rice (*Oryza sativa* f. *spontanea*) has been reported in the northeastern region of Thailand and it might have originated from the introgression between cultivated rice and wild rice, *Oryza rufipogon* which often takes place in nature mostly in one way from cultivated rice to its wild relative [1].

Weedy rice has negative influences in all tropical and subtropical rice growing regions of the world [2]. In Thailand this weed is a major problem for commercial *Thai Hom Mali rice* production in the northeastern region of the country. *Thai Hom Mali rice* is categorized into two rice varieties, Khao Dawk Mali 105 (KDML 105) and RD 15. These two rice varieties are recognized as premium grade in the world rice market because their grain contains a potent flavor component (2-acetyl-1-pyrroline (2AP)). The retail price of these jasmine rice varieties produced in Thailand is higher than that of non-fragrant rice [3].

Bradbury *et al.* [4] reported that the *badh 2* gene was most likely to be the *fgr* gene in rice (*Oryza sativa*). The

presence of null *badh 2* (or recessive allele) of the *fgr* gene enhanced 2AP biosynthesis [5]. Historically, traditional rural practices and modern scientific methods have been employed to determine aromatic traits. These methods have included chewing individual seeds, smelling KOH-treated leaf tissue, and measuring 2AP content using the GC-MS method [6]. The *fgr* locus has been proven to be responsible for rice fragrance because of an eight-base-pair (8-bp) deletion in the *bad 2* allele. Consequently, molecular markers linked to the aromatic trait have been extensively exploited to assist breeders in selecting individuals with this trait. An allele-specific perfect marker was developed with which both *BADH 2* and *badh 2* alleles could be simultaneously detected in a single PCR amplification [7]. More recently, an additional null *fgr* allele of the *fragrance* locus in fragrant rice was developed [5],[8]. In addition, by determining the fragrance genotype and phenotype in a wide range of traditional varieties from 23 counties, Fitzgerald *et al.* [9] suggested that the 8-bp deletion in the *fgr* gene is not the only cause of 2AP synthesis; there is at least one other

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mutation at a second gene locus. Recently, the *qSH1* gene, a major quantitative trait locus of seed shattering in rice, encodes a BEL1-type homeobox gene and demonstrated that a single-nucleotide polymorphism (SNP) in the 5' regulatory region of the *qSH1* gene caused loss of seed shattering owing to the absence of abscission layer formation [10]. In addition, *Rc* is a domestication-related gene required for red pericarp in rice (*Oryza sativa*). The red grain color is ubiquitous among the wild ancestors of *O. sativa*, in which it is closely associated with seed shattering and dormancy. *Rc* encodes a basic helix-loop-helix (bHLH) protein that was located on chromosome 7 of rice. The dominant red allele differed from the recessive white allele by a 14-bp deletion within exon 6 that knocked out the bHLH domain of the protein [11].

Wild rice (*O. rufipogon* Griff.) populations from Thailand occur along roadsides and rice fields in many locations [12]. *Oryza rufipogon* is thought to be the wild progenitor of *O. sativa* L. The two species hybridize freely, resulting in a conglomerate of hybrids (*O. sativa* f. *spontanea*) or weedy rice [13]. This weed species is an aggressive weed of rice in many regions of the world [2]. In Thailand, weedy rice infests *Thai Hom Mali rice* production fields in the northeastern region of the country [1]. Rice cultivars cross easily with their related weed form found in direct-seeded paddy fields and produce viable and fertile hybrids; nevertheless, cross-pollination is possible and does indeed take place to some extent, the amount depending largely on climatic and varietal differences. The degree of out-crossing is generally higher in *indica* cultivars and wild species than in *japonica* cultivars [14]. Cross-pollination between wild species of the *Oryza* genus and *O. sativa* cultivars has been reported to occur in natural habitats [13,15-17]. Annually, most local Thai farmers in this area used their own self-supplied seed for domestication in the next growing season. These farmer saved seed lots may have dramatically contaminated seed of *Thai Hom Mali rice* with genotype heterozygous, resulting from gene flow between *Thai Hom Mali rice* and weedy rice. In addition, gene flow between cultivated rice and weedy rice, possibly lead to the origin of new weedy rice forms. This event raises the question of the maintenance of cooking quality, i.e. rice grain with fragrance, in *Thai Hom Mali rice* populations despite their co-existence with cultivated weedy forms. The results from a previous assessment to determine whether weedy and wild species can hybridize with rice cultivars to produce fertile offspring of weedy rice in natural conditions of the northeastern region of

Thailand, show that gene flow occurred predominantly from *Thai Hom Mali rice* into wild rice [1]. A previous study indicated that some weedy rice is closely related to *O. sativa* while others are related to *O. rufipogon* [18]. In addition Watanabe *et al.* [19] suggested that different rice-growing locations often show different patterns of genetic diversity, depending on the specific combination of germplasm from which weedy rice emerges. In this paper, the weedy rice populations resulted from crop-to-wild gene flow grown in the Thung Kula Ronghai areas, northeastern Thailand were collected and determine the frequencies of fragrance gene (*fgr/BADH2*) by using a PCR assay. Knowledge of the status of the fragrance gene in weedy rice populations in the *Hom Mali rice* fields of northeastern Thailand is useful to in-paddy field management particularly with respect to maintaining cooking quality based on the aromatic characteristic of the *Thai Hom Mali rice* that share the same growing area in fields infested with weedy rice that carry the dominant allele of the fragrance gene. While tracing the origin of the *badh 2* allele in the gene population of weedy rice, an abundant number of samples of the Asian wild rice species *O. rufipogon*, which are the original populations grown in swamps in the Thung Kula Ronghai areas, were collected and examined for the *badh2* allele which is responsible for the aromatic characteristic of the fragrance gene.

MATERIALS AND METHODS

Plant Materials: In 2007, plant samples were randomly collected by cutting 215 flag leaves from 6 natural populations of weedy rice (*Oryza sativa* f. *spontanea*) co-occurring within *Thai Hom Mali rice* fields in Northeastern Thailand (Fig. 1). Each flag leaf was placed in a paper bag and kept in a refrigerator (-20°C) until used. During the field survey, 125 individuals of *O. rufipogon* were randomly chosen, and their leaves were collected from the wild rice species. These populations were typical of *O. rufipogon* in the Thung Kula Ronghai areas because they were collected from numerous sites throughout the area of its distribution (Fig. 2).

DNA Extraction, PCR Amplification and Electrophoresis: Genomic DNA was extracted from the leaves of individual plants of the samples using a modified CTAB (cetyltrimethyl ammonium bromide) protocol of Doyle and Doyle [20]. To characterize genotypes of each sample, oligonucleotide primers: F: 5'-TGCTCCTTTGTCAT CACACC-3' and R: 5'-TTCCACCAAGTTCCAGTGA-3')



(A)



(B)

Fig. 1: Paddy field of Thai Hom Mali rice in the Thung Kula Ronghai, northeastern Thailand (A) and weedy rice plant co-occurring in Thai Hom Mali rice fields (B)



Fig. 2: A typical population of *O. rufipogon* in the Thung Kula Ronghai area, northeastern Thailand

were used to amplify the fragrance gene located on chromosome 8 as previously reported by Prathepha [21]. Amplifications were performed in a 20 μ l reaction mixture containing 2 μ l of DNA solution, 50 pmol each of the primer pairs, 2.0 mM $MgCl_2$, 2 units *Taq* polymerase

(Promega), 0.1 mM dNTPs. Cycling conditions were 94°C (5 min); then 40 cycles of 94°C (1 min), 60°C (1 min), 72°C (1.5 min), and a final extension of 72°C (5 min). In this experiment, the DNA template from a fragrant rice, Khao Dawk Mali 105 (KDML 105) and a non-fragrant rice, Chai-nart 1 (CN1), were used as positive and negative controls, respectively, for comparison of bands resulting from PCR between fragrant and non-fragrant rice. The PCR products were separated in 4.5% polyacrylamide denaturing gels of 200x125x1 mm (length x width x thickness). After electrophoresis, the bands were stained with silver-stain. The PCR product of approximately 396 bp obtained from KDML 105 was present in every sample with the recessive allele (the 8 bp deletion), whereas the dominant allele gave a product of approximately 404 bp from the cultivar CN 1. From the PCR assay, heterozygote can be discriminated by the presence of both PCR products.

For identification of genotypes for the fragrance gene in the rice samples, the non-fragrance allele (*BADH2*) of the fragrance gene was used as molecular marker. The genotypic and allelic frequencies of weedy rice plant samples were computed based on Hardy-Weinberg formulations. Goodness-of-fit statistics were calculated for the figure observed compared to values expected using the Hardy-Weinberg equilibrium.

RESULTS

Among the 215 weedy rice plants examined for the *badh 2* allele of the fragrance gene, three genotypes, namely *BADH2/BADH2* (homozygous non-fragrant), *BADH2/badh2* (heterozygote) and *badh2/badh2* (homozygous fragrant) were detected (Fig. 3). Frequencies of the *badh2* allele showed a high value of 0.547. Test of goodness-of-fit was performed to assess the evolutionary processes influencing this gene locus. The test indicated the evolutionary processes did not have a significant influence ($\chi^2 = 3.5$, $P > 0.05$). This can be indicative that the fragrance locus of weedy rice population was in Hardy-Weinberg equilibrium (Table 1). On the other hand, the *badh2* allele of the fragrance gene was not found in the wild rice samples examined (Fig. 4).

Table 1 Statistical analysis of fragrance gene (*fgr/BADH2*) in sample of weedy rice from Thung Kula Ronghai, northeastern Thailand. Goodness-of-fit testing showed not significant difference suggesting that locus of the fragrance gene is conformed to Hardy-Weinberg equilibrium.

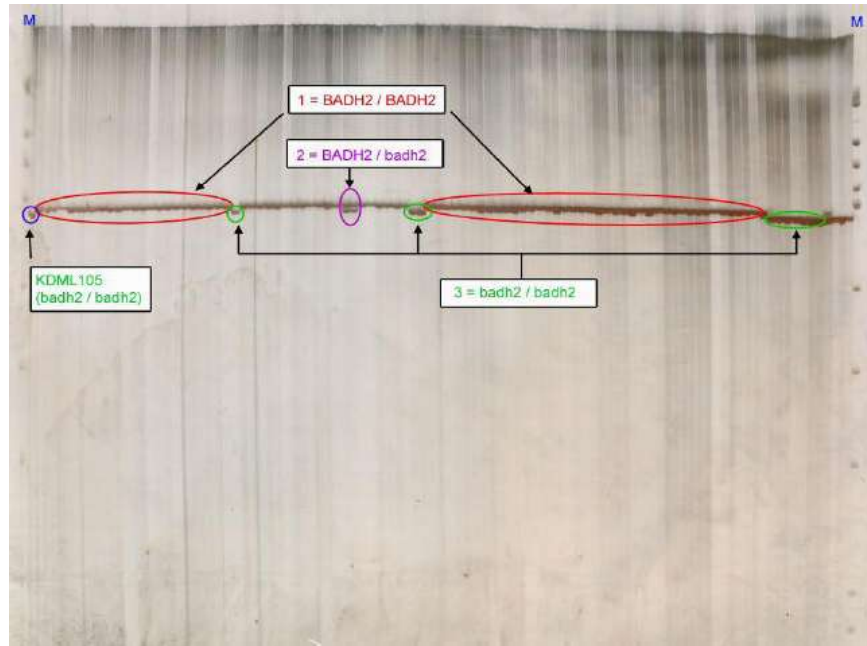


Fig. 3: Polyacrylamide gel electrophoresis of the BADH2 gene by using PCR of 99 accessions of weedy rice (*O. sativa* f. spontanea) and a fragrant rice cultivar (cv. KDML105) which used as positive control for fragrant homozygous (*badh2/badh2*)

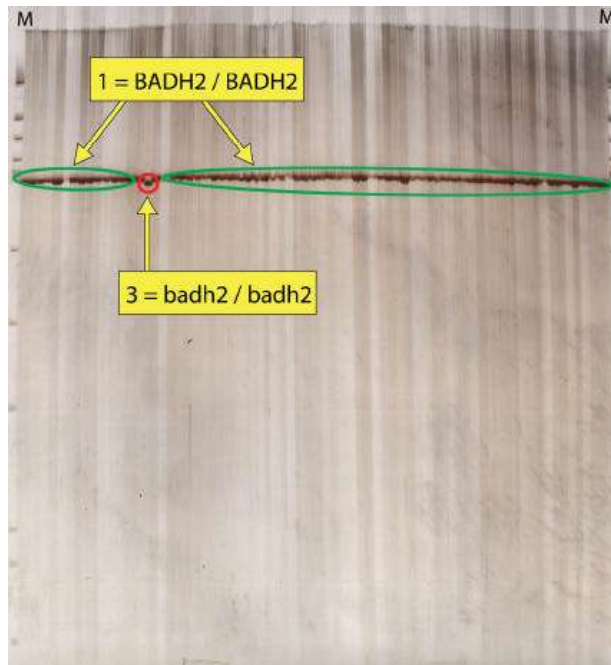


Fig. 4: Polyacrylamide gel electrophoresis of the BADH2 gene by using PCR of 99 accessions of wild rice (*O. rufipogon*) carried genotype of nonfragrant homozygous (1=BADH2/BADH2), and a fragrant rice cultivar (cv. Hom Nangh Nuan) carried fragrant homozygous (3=badh2/badh2) which used as positive control for fragrant homozygous (*badh2/badh2*)

Genotype frequencies			
BADH2/BADH2	BADH2/badh2	badh2/badh2	Total
51	93	71	215
Allele frequencies			
BADH2 allele = 0.453		badh2 allele = 0.547	
Goodness-of-fit $\chi^2 = 3.5$, d.f. = 1 (P > 0.05)			

DISCUSSION AND CONCLUSION

Gene flow can be estimated using direct or indirect methods. For flowering plants, pollen and seed are the two major vectors of gene flow. Much of the work on gene flow has been conducted using indirect methods, such as morphological traits [22] or DNA assay [14]; [23]. In this study DNA marker on the fragrance gene was analyzed and revealed that the cultivar allele (i.e., *badh 2* allele of the fragrance gene) persist at high frequencies in naturally occurring weedy rice populations in Thailand. The *badh2* allele was not found in the wild rice populations used in this study. This evidence strongly supports the hypothesis that the *badh 2* allele in weedy rice population originated from the fragrant rice cultivars. The occurrence of the crop-to-wild gene flow was detected by scoring a simple sequence repeat (SSR) molecular marker [14].

Weedy rice (*Oryza sativa* f. *spontanea*) is an important resource for breeding and studying the evolution of rice [24]; [25]. Several studies of their genetic characteristics showed that weedy rice strains also appear to be differentiated into *indica* and *japonica* types based on morphological and physiological traits, isozymes, and DNA markers [24], [26], [27]. Weedy rice possesses useful genes conferring tolerance to various biotic and abiotic stress [28]. One advantage of employing weedy rice in a breeding program is that hybrid sterility is not observed, when weedy rice is crossed with cultivars, due to their genetic similarity to cultivars [16]. In this respect, weedy rice is expected to play an important role in increasing the genetic diversity of cultivated rice [24]. However, most weedy rice strains possess seeds with red pericarps and are frequently referred to as red rice although some strains have white pericarps [1]. Morphologically, weedy rice is highly variable and appears to be an intermediate between wild and cultivated rice. Long-term sympatric distribution has led to similarities between weedy and cultivated rice through natural hybridization and introgression, making the control of weedy rice very difficult when compared with other weeds. In Northeastern Thailand weedy rice has re-emerged in the rice planting region of Thunk Kula Ronghai. This region produces rice with the best cooking quality based on the aroma grain characteristic of the two

fragrant rice varieties (i.e., Khao Dawk Mali 105 and RD 15). The occurrences of weedy rice in other areas of northeastern region of Thailand have already caused considerable problems for rice production in the region. If it continues to spread, larger production related weed problems may occur, becomes more frequent and propagate between regions while the manpower needed for weed control diminishes.

The knowledge of the genetics of Thailand's weedy rice hinders the design of effective practical tools and methods for weedy rice management. The results of this study will augment our knowledge and help fill the gaps in our understanding of the levels and distribution of the *badh 2* allele. The results will also provide guidance for effective control programs and assist in the exploration of the origins of weedy rice which may be useful in developing a breeding program.

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