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NEW FRAGRANCE ALLELE ACCORDING TO INSERTION/DELETION IN NON-CODING DNA SEQUENCE OF THE *FRAGRANCE* GENE FOUND IN ASIAN CULTIVATED RICE (*ORYZA SATIVA* L.), WILD AND WEEDY RELATIVES FROM INDOCHINA

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Abstract

Fragrance is one of the most important trait in cultivated rice. The fragrant allele in rice germplasm worldwide was investigated continuously. The purpose of this study was to investigate variation in non-coding sequences of the fragrance (*fgr*) gene in cultivated rice and its relatives from Indochina (Thailand, Laos and Cambodia) using PCR and re-sequencing techniques. Two new additional insertion/deletion were observed in intron 6 of the *fgr* gene in fragrant cultivated rice and its relatives from Thailand compared to fragrant and non-fragrant cultivated rice accessions from Myanmar, China and Japan. This finding may shed light on a wide range of genetic resources of rice available for aroma trait in breeding program and on the evolutionary process underlying differentiation of rice cultivars from progenitor lineage.

Key words: Asian cultivated rice, Fragrance gene, Mutation.

Introduction

Aroma grains is an important aspect of the end-use quality of fragrant rice. DNA polymorphism of the fragrance gene (*fgr*) in Asian cultivated rice has been analyzed with a view of shedding new light on many aspects, such as on the domestication of *indica* and *japonica* rice and on the creation of markers assisted in breeding program (Shao *et al*., 2013; Bradbury *et al*., 2005a,b). A mixture of volatile compounds was detected in the flavor of cooked rice (Yajima *et al*., 1978). Buttery *et al*., (1982) demonstrated that 2-acetyl-1-pyrroline (2AP) is a potent flavor component that gives their fragrance in both basmati and jasmine rice. Fragrance gene in rice comprises 15 exons and encodes *badh 2* (*betaine aldehyde dehydrogenasehomologue 2)* and an 8-bp deletion in exon 7 associated with 2AP production as the cause of fragrance in the fragrant rice including basmati and jasmine rice (Bradbury *et al*., 2005b).

A survey of literatures revealed that the fragrance gene exhibited allelic variants at this locus in fragrant rice varieties worldwide (research review paper of Sakthivel *et al*., 2009), including 8-bp deletion and three SNP in exon 7; a 7-bp insertion in exon 8; a 7-bp deletion in exon 2; absence of MITE (miniature interspersed transposable element) in promoter; two new SNPs in the central section of intron 8; a TT deletion in intron 2 and a repeated (AT)n insert in intron 4.

Therefore, investigations of the variation and genetic distribution of the fragrant alleles existing in rice will allow rice breeders to better understand the gene pool of fragrant rice and shed a light of perspective to conserve and germplasm management of rice germplasm. By tracing the variants of the alleles of domestication genes and the paths they traveled to achieve their current distribution will gain fresh insights into the history of human interactions, a history that has not been recorded but is written in the genomes of plants (Sweeney & McCouch, 2007).

In the present study, a collection of samples of fragrant, non-fragrant rice cultivars, weedy rice (*Oryza sativa* f. *spontanea*) and wild rice (*Oryza rufipogon*) were investigated for the DNA variation in intron 6 and exon 7 of the fragrance gene by using a polymerase chain reaction (PCR) assay and DNA re-sequencing techniques. Identification of fragrance gene to look for allelic variants at this locus in rice germplasm is likely an attempt to understand their evolutionary among fragrant rice worldwide. In addition, this investigation will likely support the progress of DNA markers for marker-assisted selection breeding programs on fragrant rice cultivars.

Materials and Methods

Rice samples: Traditional fragrant rice cultivars used in the present study were provided by local farmers in the northern, northeastern regions of Thailand, the Plain of Vientiane of the Lao PDR, and improved rice varieties based on KDML105 genetic background and japonica rice providing from Rice Research Station in northeastern region of the country. Sample of wild rice and weedy rice were also used in the experiment. The collection samples were listed in Table 1.

Determination of aroma grains: In this study, some rice cultivars which have no report on characteristic of grain (aroma or non-aroma) were determined for aroma grains by using a sensory test followed the protocol of the Rice Gene Discovery Unit, Kasetsart University, Thailand (Yi *et al*., 2009) as follows: five brown rice seeds were placed into 1.5 centrifuge tubes, and 200 µl of distilled water was added to the tubes and then incubated with closed the tube at 65°C for 30 min. The samples were allowed to cold by putting on ice, and then opened the tube individually, the samples were smelled and scored for fragrance by five panelists.

Table 1. List of 24 rice accessions used in this study.

Cultivar name /Code	Aroma (+)/ Non-aroma (-)	Endosperm type	Origin	In/Del in intron 6	Breeding method/ parents*
KDML105	+	Non-glutinous	Thailand	TC-/TAA	Selected from the original line of Khao Dawk Mali 4-2-105
RD15	+	Non-glutinous	Thailand	TC-/TA-	Gamma-ray irradiated KDML105
RD96	+	Non-glutinous	Thailand	TC-/TA-	Gamma-ray irradiated KDML105/ Sang Yod
Hawm Surin	+	Non-glutinous	Thailand	TC-/TA-	Selection from traditional rice cultivar (SRNC05053
RD12	+	Glutinous	NE, Thailand	TC-/TA-	Hahng Yi 71/ RD 6
Hawm Nahng Nuan	+	Glutinous	NE, Thailand	TC-/TA-	Traditional cultivar
Hawm Pamah	+	Glutinous	NE, Thailand	TC-/TA-	Traditional cultivar
Hawm Tung	+	Glutinous	NE, Thailand	TC-/TA-	Traditional cultivar
So Ma Li	+	Glutinous	NE, Thailand	TC-/TA-	Traditional cultivar
E Tia Kon Jod	+	Glutinous	NE, Thailand	TC-/TA-	Traditional cultivar
Kai Noi	+	Glutinous	NE. Thailand	TC-/TA-	Traditional cultivar
Khao Pong Krai	+	Non-glutinous	N, Thailand	TC-/TA-	Traditional cultivar
Chiang Pattalung	-	Non-glutinous	S, Thailand	TC-/TA-	Traditional cultivar
Peun Neon Yim	+	Non-glutinous	N, Thailand	TC-/TA-	Traditional cultivar
Hawm Mali Daeng	+	Non-glutinous	NE, Thailand	TC-/TA-	Recommended rice variety
Hawm Sa Ngiam	+	Non-glutinous	NE, Thailand	TC-/TA-	Traditional cultivar
SP-RE(purple)	-	Non-glutinous	NE, Thailand	TC-/TA-	Weedy rice
OR-SKN3-6	-	Non-glutinous	NE, Thailand	TCC/TAA	Wild rice
OR-NSM4	-	Non-glutinous	NE, Thailand	TC-/TA-	Wild rice
OR-Surin1-3	-	Non-glutinous	NE, Thailand	TC-/TA-	Wild rice
OR-CR22	-	Non-glutinous	N, Thailand	TC-/TAA	Wild rice
OR-TM	-	Non-glutinous	Vietiane, Laos	TC-/TA-	Wild rice
OR-LSK14	-	Non-glutinous	Savannakhet, Laos	TC-/TA-	Wild rice
OR-Siam Reap	-	Non-glutinous	Siam Reap, Cambodia	TC-/TA-	Wild rice

*According to Chitrakon & Somrith (2003); N, Northwestern; NE, Northeastern; S, Southern Thailand

DNA extraction and polymerase chain reaction: Genomic DNA was extracted from the one-month young leaves of each rice sample according to the protocol of Doyle & Doyle (1987). DNA samples of rice were examined for the allele of the *fgr* gene, which is responsible for the fragrance, by using a polymerase chain reaction (PCR) assay following a previous report by Prathepha (2009). The DNA sequences of oligonucleotide primers (i.e., Os2AP-exon7.1F: 5′-TGCTCCTTTGTCAT CACACC-3′ and Os2AP-exon7.1R: 5′-TTTCCACC AAGTTCC AGTGA-3′).The PCR reaction was performed in a 20 µl reaction mixture containing 2 µl 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). The PCR products were separated in 3% agrose gel. After electrophoresis, the bands were stained with ethydium bromide. The PCR products were further analyzed using the direct sequencing of the PCR products by DNA sequencing Services of 1st BASE, Science Park Road, The Gemini, Singapore Science Park II, Singapore.

DNA sequence analysis: The re-sequenced segments of the *fgr* gene covered intron 6 and exon 7 (approx. 396 bp) from rice samples used in this study were compared to each other and to previously reported DNA sequences of Myanmar fragrant and non-fragrant rice cultivars (Yi *et al*., 2012). Some rice accessions which were classified based on isozyme into group 5 were selected and along with other rice accessions (Genbank no. JQ308416.1-JQ308435.1) used in the analysis (Genbank accessions no. JQ308366.1, 308372.1). In addition, non-fragrant rice cultivar, Nanjing 11 from China (acc. no. EU770319) and fragrant rice cv. Suyuno from Japan (acc. no. EU770320) were included. These re-sequences were visually aligned. Insertion/deletion polymorphisms in the comparisons DNA sequences were identified by visualized inspection from chromatograms.

Results and Discussion

DNA sequence variation in coding and non-coding sequence of fragrance gene: A survey of previous reports revealed the two known *Badh2* functional nucleotide polymorphisms (FNPs), i.e. an eight base-pairs (8-bp) deletion and three SNP in exon 7 of the *fgr* gene which encodes *betaine aldehyde dehydrogenase 2 (BADH2)* on chromosome 8 of Asian cultivated rice (*Oryza sativa* L.). These FNPs are a primary cause of fragrance in Basmati and Jasmine rice (Bradbury *et al*., 2005b, Roy *et al*., 2012). Results from this study revealed that all rice samples were fragrant rice cultivars had DNA sequences with the two known FNPs (i.e., 8-bp deletion and three SNPs) that is characteristic of aroma grains. This mutation was recognized as *badh2.1* allele (Kovach *et al*., 2009).

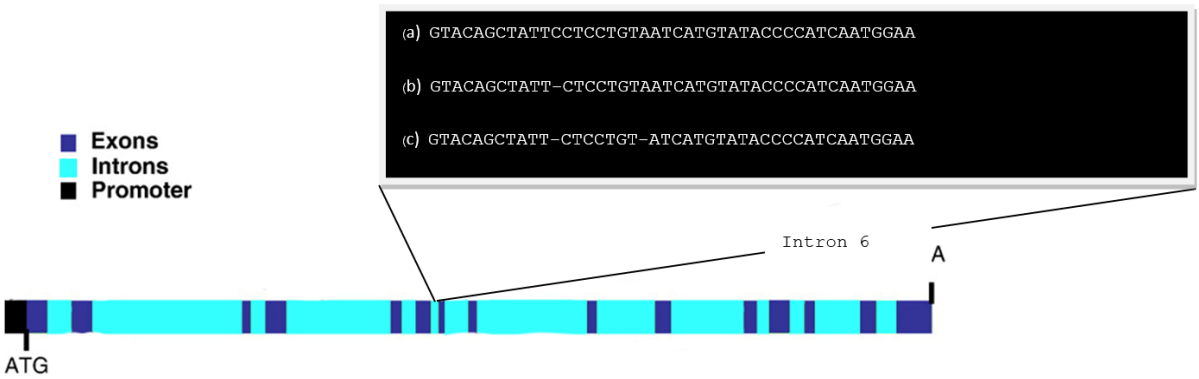

Fig. 1. The structure of fragrance gene shows insertion/deletion mutations in DNA sequences of intron 6. (a) intron 6 sequences of rice cultivars from Myanmar, China and Japan which deposits in GenBank (Yi et al. 2009; Chen et al. 2008). (b) Insertion/deletion in Thai jasmine rice cultivars, KDML 105 and wild rice from Chiangrai province, Thailand. (c) deletion of nucleotide A and C were commonly observed in traditional cultivars and rice varieties derived from KDML 105.

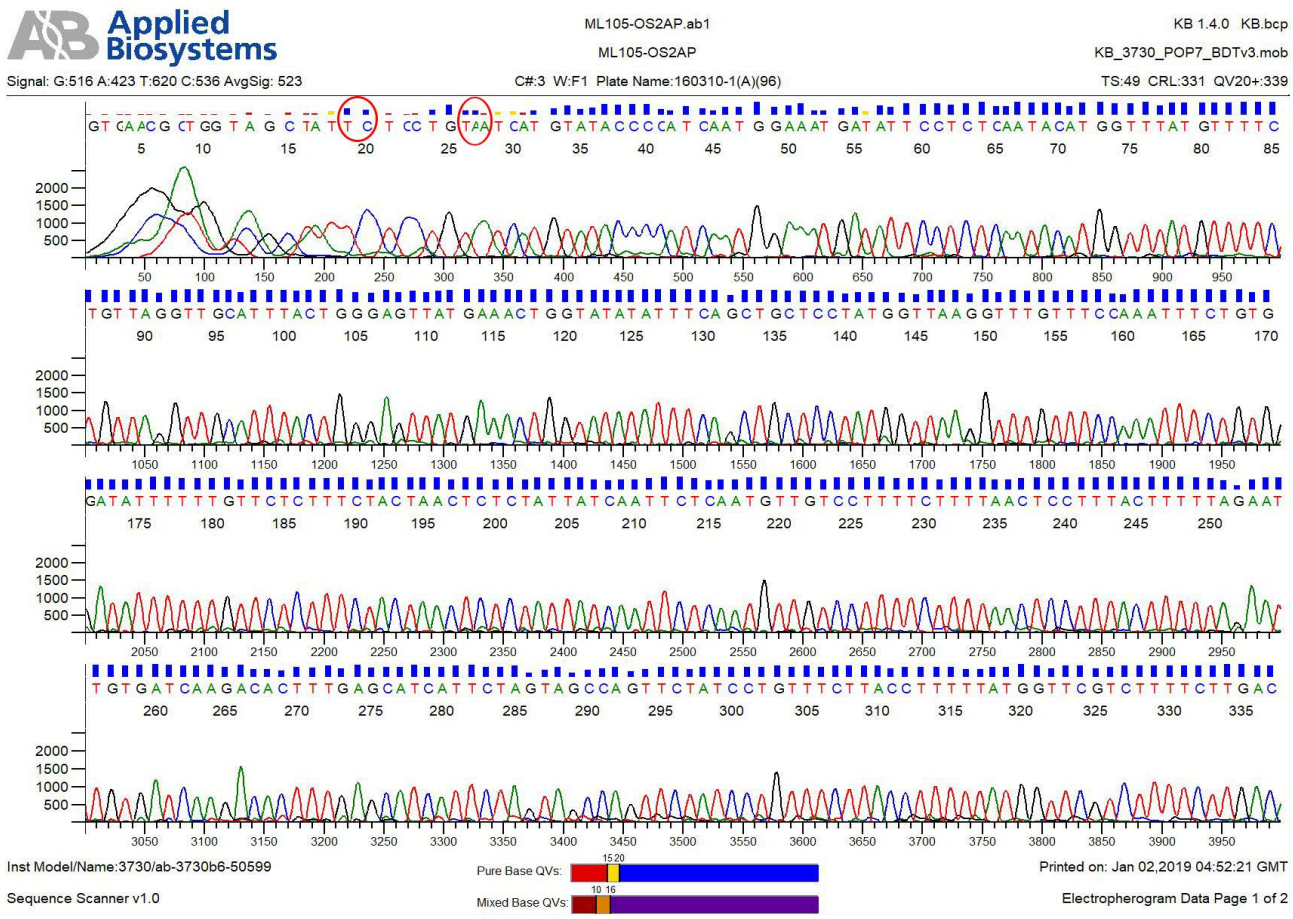

Fig. 2. DNA sequence of intron 6 and exon 7 of *fragrance gene* in cultivar KDML105 that exhibit deletion of C (TC-/TAA) as indicated in circle.

An additional insertion/deletion (In/Del) was observed in DNA sequences of intron 6 as follows: two TC-/TA- and TC-/TAA, in rice accessions used in this study. The seven represent wild rice accessions revealed three variants as follows: five accessions had TC-/TA-, that commonly found in traditional rice cultivars and weedy rice samples. One accession from Sakon Nakhon province showed TCC/TAA, whereas a variant with TC-/TAA was found in wild rice sample from Chiangrai province, northern Thailand and KDML 105, a premium jasmine rice (Fig. 2). In addition, rice cultivars derived from KDML 105 using gamma-ray irradiation induced mutation (i.e., RD 6, RD 12, RD 15 and RD 69) showed TC-/TA- in the DNA sequences of intron 6. This mutation may result from induced mutation (Morita *et al.*, 2009). DNA sequences revealed insertion/deletion for identification of traditional cultivars from Thailand and Laos, (TC-/TA-) and fragrant rice in Myanmar which have the same lineage with Basmati rice in India, Pakistan, Sri Lanka and Bangladesh, (TCC/TAA) as shown in Fig. 1. These evidences would shed light on the evolutionary process underlying differentiation of cultivar

from progenitor lineage (Glaszmann, 1987; Londo *et al*., 2006; Lawton-Rauh & Burgos, 2010).

Rice varieties grown in Thailand, Laos, Myanmar and Cambodia were recognized as *indica* or tropical *japonica* group (Garris *et al*., 2005). A represent cultivar 'Khao Kai Noi' grown in northern and northeastern Laos which was recognized as tropical *japonica* and non-fragrant accession (Prathepha *et al*., 2018) was included in this study. This rice cultivar showed the common mutation at the re-sequenced segment of intron 6 (Figs. 1&2). Thus, it might be used this mutation to distinguish temperate *japonica* and tropical *japonica*.

Conclusion

This study reports two additional insertion/deletion polymorphisms in intron 6 of *fgr* gene in wild relative, weedy rice and cultivated rice, these DNA markers may reflecting origin and domestication process of the Asian rice in Indochina (Thailand, Laos and Cambodia) and other region (Myanmar, China and Japan). In addition, the insertion/deletion polymorphism was also observed in fragrant rice cultivars which were derived and selection by gamma irradiation approach in KDML105. It is likely that the re-sequence DNA fragment of the *fgr* gene are applicable to other research fields, such as studies of genetic variation in rice germplasm, marker-assisted breeding and cultivar discrimination for aroma quality control in marketplace.

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