

# A PCR-based marker for a locus conferring the aroma in Myanmar rice (*Oryza sativa* L.)

Khin Myo Myint · Siwaret Arikrit · Samart Wanchana ·  
Tadashi Yoshihashi · Kiattawee Choowongkomon ·  
Apichart Vanavichit

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**Abstract** Aromatic rice is an important commodity for international trade, which has encouraged the interest of rice breeders to identify the genetic control of rice aroma. The recessive *Os2AP* gene, which is located on chromosome 8, has been reported to be associated with rice aroma. The 8-bp deletion in exon 7 is an aromatic allele that is present in most aromatic accessions, including the most popular aromatic rice varieties, Jasmine and Basmati. However, other mutations associated with aroma have been detected, but the other mutations are less frequent. In this study, we report an aromatic allele, a 3-bp insertion in exon

13 of *Os2AP*, as a major allele found in aromatic rice varieties from Myanmar. The insertion is in frame and causes an additional tyrosine (Y) in the amino acid sequence. However, the mutation does not affect the expression of the *Os2AP* gene. A functional marker for detecting this allele was developed and tested in an aroma-segregating  $F_2$  population. The aroma phenotypes and genotypes showed perfect co-segregation of this population. The marker was also used for screening a collection of aromatic rice varieties collected from different geographical sites of Myanmar. Twice as many aromatic Myanmar rice varieties containing the 3-bp insertion allele were found as the varieties containing the 8-bp deletion allele, which suggested that the 3-bp insertion allele originated in regions of Myanmar.

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K. M. Myint  
Tropical Agriculture Program, Kasetsart University,  
Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

K. M. Myint · S. Arikrit · A. Vanavichit  
Rice Science Center and Rice Gene Discovery, Kasetsart  
University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand

S. Wanchana  
International Rice Research Institute, Laguna, Los Baños,  
Philippines

T. Yoshihashi  
Postharvest Science and Technology Division,  
Japan International Research Center for Agricultural Sciences,  
1-1, Ohwashi, Ibaraki, Tsukuba 305-8686, Japan

K. Choowongkomon  
Department of Biochemistry, Faculty of Science,  
Kasetsart University, Bangkok 10900, Thailand

A. Vanavichit (✉)  
Department of Agronomy, Kasetsart University,  
Kamphaeng Saen, Nakhon Pathom 73140, Thailand  
e-mail: vanavichit@gmail.com

## Introduction

The flavor and palatability of aromatic rice are preferred by consumers all over the world. This type of rice is in high demand, and it obtains a premium price in both domestic and international markets. Although most of the trade is from India, Pakistan and Thailand, aromatic rice is cultivated and prized in many other countries of the world. Rice grain aroma results from the production of many biochemical compounds (Petrov et al. 1996), but the most important compound is 2-acetyl-1-pyrroline (2AP), which is considered to be a potent aroma constituent (Buttery et al. 1982). A single recessive gene located on chromosome 8 has been identified as the gene responsible for the aroma trait (Bradbury et al. 2005a; Vanavichit et al. 2008) because it has a key role in the synthesis of 2AP (Bradbury et al. 2005a). The identified gene has been named differently by different groups as *BAD2* (Bradbury et al. 2005a,

2008), *BADH2* (Niu et al. 2008) and *Os2AP* (Vanavichit et al. 2008); the latter name is used in the present study. The most important sequence variation of the gene was found in exon 7 as an 8-bp deletion. This sequence deletion generates a premature stop codon that presumably results in loss of function. The loss of *Os2AP* activity may cause 2AP accumulation. Although the unique genotype (8-bp deletion) determines the aromatic biosynthetic capability in most aromatic rice strains, the aroma content in different aromatic rice varieties does not always correlate with this deletion. The trait may be limited or regulated by other genes or other regions of the known aromatic gene. A previous study has shown that a number of aromatic varieties, primarily from South and Southeast Asia, do not contain the 8-bp deletion and that 2AP is identified in both raw and cooked rice of these varieties (Fitzgerald et al. 2008). Researchers have suggested that the 8-bp deletion in *Os2AP* is not the only sequence variation that causes aroma and that there are other mutations that drive the accumulation of 2AP. Studies on the diversity of the gene in a large collection of varieties have shown that the 8-bp deletion in exon 7 is present in most aromatic accessions but that other less frequent mutations associated with aroma are also detected (Bradbury et al. 2005a; Bourgis et al. 2008; Shi et al. 2008; Kovach et al. 2009).

The 2AP aromatic compound is present in various parts of the rice plant, such as the stems, leaves and grains, but not in the roots (Lorieux et al. 1996; Yoshihashi 2002). The following sensory methods have been applied to determine the aroma in rice: chewing several seeds or cooking a sample of seeds from individual plants (Ghose and Butany 1952; Dhulappanavar 1976), heating leaf tissue in water or eluting the aroma from leaf tissue with diluted potassium hydroxide (KOH) (Sood and Siddiq 1978; Hien et al. 2006) and heating several half-cut seeds in fresh water (Wanchana et al. 2005). However, these sensory evaluation methods are not consistent or reliable because the aroma is subjected to human preference. Alternatively, an objective gas chromatography/mass spectrometry (GC/MS) method that directly assays 2AP content has been introduced, but the assay is expensive and time consuming (Chen et al. 2006). Molecular markers, especially functional markers, linked to economically important traits have been extensively exploited to assist breeders in selecting individuals with favorite traits, and these markers are useful with respect to the aromatic trait in rice breeding programs. With these markers, the difficulty in evaluating the aroma phenotype is minimized, and the accuracy of selecting desired plants is increased. After the discovery of the aroma gene, the following functional molecular markers were developed: Aromarker (Vanavichit et al. 2008), allele-specific amplification (ASA) marker (Bradbury et al. 2005b), FMbadh2-E2A and FMbadh2-E2B (Shi et al.

2008). However, these markers cover only two types of aromatic alleles, including an 8-bp deletion in exon 7 (for the first two markers) and a 7-bp deletion in exon 2 (for the latter marker).

Nakagahra (1978) estimated that the center of diversity of Asian rice may be located in mountainous regions on the northern side of Southeast Asia, such as Myanmar, Thailand and Yunnan in China. Myanmar is rich in natural resources, and plant genetic diversity is one of the natural resources. Myanmar is well known as the primary center of plant genetic resource (PGR) diversity for rice and is the secondary center of PGR diversity for other crops. Rice is one of the most important food crops in Myanmar because it is an important part of the daily diet. Rice is cultivated all over the country in various agro-ecological conditions, such as in lowland, flooded, deep water, dry land and upland conditions. Rice is grown extensively, and it covers an area of 8.28 million hectares, which is approximately 60 % of the total cultivated area. The consumption of rice per capita in Myanmar is approximately 210 kg per person per year, and this amount is the highest consumption level in Asia. The rice preferred by Myanmar people varies depending on locations within the country. However, the majority of Myanmar people prefer intermediate amylose rice (20–25 %), which has a soft texture and is aromatic. Good-quality aromatic rice is grown in different regions of Myanmar to meet the high local demands. The price of these varieties in local markets is three times the price of non-aromatic rice varieties. In Myanmar, different places require a specific quality of rice for their cooking quality traits. Some of the preferred rice varieties include the popular aromatic varieties that have a soft texture, high aroma and high kernel elongation, and other rice varieties are locally adapted aromatic rice varieties, which are preferred by local consumers. Locally adapted varieties vary not only in terms of aroma degree, grain shape and grain size, but also with regard to morpho-agronomic characteristics. However, molecular information regarding the aromatic gene that is present in these aromatic rice varieties is still limited. Although an alternative mutated allele (a 3-bp insertion in exon 13) of the *Os2AP* gene has been identified in Myanmar aromatic rice accessions (Kovach et al. 2009), there are still a large number of aromatic rice accessions that remain uncharacterized. Screening the diverse aromatic rice varieties in Myanmar will allow for additional information regarding aromatic genes to be gathered. In this study, we chose aromatic rice varieties collected from different locations of Myanmar to identify the aroma gene and aromatic allele specific to Myanmar aromatic rice varieties and to develop the functional marker for breeding programs and genetic resource conservation programs.

## Materials and methods

### Plant materials

Two sets of rice samples were used in this study. The first set was a group of 52 Myanmar landrace rice varieties that belong to Isozyme group V (Khush et al. 2003); this group was kindly provided by the Plant Biotechnology Center at the Myanma Agriculture Service in Myanmar. The first set used the tracking system of the International Rice Germplasm Collection (IRGC) accession numbers. The second set consisted of 25 diverse aromatic rice varieties from unknown Isozyme groups that were collected from different geographical regions of Myanmar; this set of rice varieties was provided by the Seed Bank at the Department of Agricultural Research in Myanmar. Based on the variety names and passport data, all varieties in the second set were considered to be aromatic rice.

### PCR genotyping for *Os2AP* gene mutations

The 52 Myanmar varieties and the two control varieties, KDML105 (aromatic, *indica* group; Kovach et al. 2009) and ISL-10 (non-aromatic), were used to characterize the *Os2AP* gene mutations. All plants were grown in an experimental field at Kasetsart University (Nakhon Pathom, Thailand) in July of 2008. Leaf samples from all of the plants were collected from 3-week-old seedlings. Genomic DNA was extracted from 1 g of leaf tissue according to the DNA Trap method developed by the DNA Technology Laboratory at Kasetsart University (Nakhon Pathom, Thailand). Two PCR markers, Aromarker (Vanavichit et al. 2008) and FMBadh2-E2B (Shi et al. 2008), were developed for the detection of the 8-bp deletion in exon 7 and the 7-bp deletion in exon 2 of *Os2AP*, respectively. These markers were used to amplify genomic DNA from the 52 rice varieties. The amplified PCR products were resolved by 4.5 % polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining.

### Aroma evaluation by sensory test and 2AP quantification

Two sensory test methods were performed for aroma evaluation. For the first method, the aroma was evaluated from 1-month-old leaves by incubating cut leaves in a diluted KOH solution and noting the aroma according to the method of Sood and Siddiq (1978). For the second method, the aroma was evaluated by chewing premature and mature seeds. Both sensory methods were performed by five trained panels. The 2AP content was analyzed from two-month-old callus samples cultured on Murashige and Skoog media (1962) with a previously described GC–MS method (Wongpornchai et al. 2004) and from grain samples (5 g) using an improved GC–

MS method developed for detecting 2AP in a small-scale sample (as little as 0.5 g; Mahatheeranont et al. 2001).

### Reverse transcription PCR (RT-PCR)

Total RNA was extracted from callus, leaf and root samples from aromatic and non-aromatic varieties with a High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Roche Applied Science, Germany). Reverse transcription polymerase chain reaction (RT-PCR) was performed according to the manufacturer's protocol for the Superscript<sup>TM</sup> III First Strand Synthesis System for RT-PCR (Invitrogen<sup>TM</sup> Life Technologies, UK). AromarkerBig (5' TGCTCCTTTGTCATCACACC 3' and 5' CCATGCAACCATCCTTTCTT 3') was used as the *Os2AP* gene-specific primer. Actin primers (5' AGGGCTGTTTTCCCTAGTATCGTG 3' and 5' GATGGCATGAGGAGGGGCAT 3') were used as a positive internal control. The RT-PCR products were analyzed on a 1 % agarose gel in 1× TBE buffer.

### Sequencing of the *Os2AP* gene in selected aromatic rice varieties

The full-length *Os2AP* gene was sequenced in aromatic varieties from Pathein Nyunt, Yangon Saba and Kyet Paung. Primer pairs designed to amplify fragments covering the entire *Os2AP* gene were provided by the Rice Gene Discovery Unit at Kasetsart University in Thailand. Amplified PCR products were purified by a Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taiwan) and cloned into a pGEM<sup>®</sup>-T Easy cloning vector (Promega, Madison, WI). Plasmids from positive colonies were purified with a High Speed Plasmid Minikit QIAprep Spin Miniprep Kit (QIAGEN, USA) and then sequenced using ABI PRISM<sup>®</sup> BigDye<sup>TM</sup> Terminators (Applied Biosystem/Perkin-Elmer, San Jose, CA, USA). The sequences of each variety were assembled based on the identical overlapping regions with the CAP3 Sequence Assembly Program (Huang X and Madan A. 1999). The genomic sequence of the non-aroma allele of the *Os2AP* gene on chromosome 8 (GenBank Accession No. AP005537) of *japonica* rice (*Oryza sativa japonica* cultivar Nipponbare) was retrieved from the NCBI website (<http://www.ncbi.nlm.nih.gov>). Multiple sequence alignment of the *Os2AP* alleles among Myanmar aromatic rice varieties and the non-aromatic rice variety, Nipponbare, was performed using the CLUSTALW program (Thompson et al. 1994).

### Development of an aroma-specific functional marker for Myanmar aromatic rice

The genomic sequence of the aromatic variety, Pathein Nyunt, was used as a reference sequence for the aroma allele with the 3-bp insertion. The PCR marker, 3In2AP,

was developed to detect this sequence variation. The forward (5' GTCCTGTTCAATCTTGCAGC 3') and reverse (5' CTTGATGCAACCATGTCATA 3') primers were designed with Primer 3 (version 0.4; <http://frodo.wi.mit.edu/primer3>) by applying the constraints of 20 nucleotides in length and a GC content of 40–60 % to avoid primer self-complementarity. The PCR marker was verified by amplifying the genomic DNA extracted from the leaf, seed and callus samples of different aromatic and non-aromatic rice varieties. PCR was performed in a total volume of 10  $\mu$ l containing 2  $\mu$ l of genomic DNA (50 ng/ $\mu$ l), 1  $\mu$ l of 10 $\times$  buffer, 1  $\mu$ l of 25 mM MgCl<sub>2</sub>, 2  $\mu$ l of 1 mM dNTPs, 0.5  $\mu$ l of each primer (5  $\mu$ M) and 0.1  $\mu$ l of Taq DNA polymerase (Fermatas; Life Science, USA). The PCR analysis was initiated by denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. A final incubation at 72 °C for 7 min was allowed for the completion of primer extension. A portion (2  $\mu$ l) of the PCR products was resolved on a 4.5 % polyacrylamide gel and visually examined after silver staining.

#### Application of functional markers for genotyping F<sub>2</sub> populations for aroma

To evaluate the accuracy and reliability of the newly developed marker, 3In2AP, 160 F<sub>2</sub> plants derived from a cross of Pathein Nyunt (aromatic variety; 3-bp insertion) and ISL-10 (non-aromatic variety; isogenic line derived from KDML105  $\times$  CT9993 crosses) were genotyped. The PCR amplicons were resolved on a 4.5 % polyacrylamide gel, and the segregation ratio according to the banding patterns was observed. Phenotypic evaluation was also performed by a sensory test with the leaves of 160 F<sub>2</sub> plants according to the method of Sood and Siddiq (1978) and by chewing the F<sub>3</sub> seeds of individual F<sub>2</sub> lines.

#### Screening aromatic alleles in aromatic rice germplasms from Myanmar

To validate the utility of the 3In2AP marker and to screen the aromatic rice germplasms from Myanmar, 25 diverse aromatic rice varieties collected from different geographical regions of Myanmar were analyzed. Two markers for the different aroma alleles (Aromarker for the 8-bp deletion and 3In2AP for the 3-bp insertion) were used to amplify the target regions of the *Os2AP* gene. KDML105 (an aromatic variety with the 8-bp deletion), Pathein Nyunt (an aromatic variety with the 3-bp insertion) and ISL-10 (a non-aromatic variety) were used as positive and negative control varieties. PCR was performed in a total volume of 10  $\mu$ l containing 2  $\mu$ l of genomic DNA (50 ng/ $\mu$ l), 1  $\mu$ l of

10 $\times$  buffer, 0.4  $\mu$ l of 25 mM MgCl<sub>2</sub>, 2  $\mu$ l of 1 mM dNTPs, 0.4  $\mu$ l of each primer (5  $\mu$ M) and 0.1  $\mu$ l of Taq DNA polymerase. The PCR analysis was initialized by denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min followed by a 7-min incubation at 72 °C. A portion (2  $\mu$ l) of the PCR products was subjected to electrophoresis on a 4.5 % polyacrylamide gel, and the banding pattern was examined after silver staining.

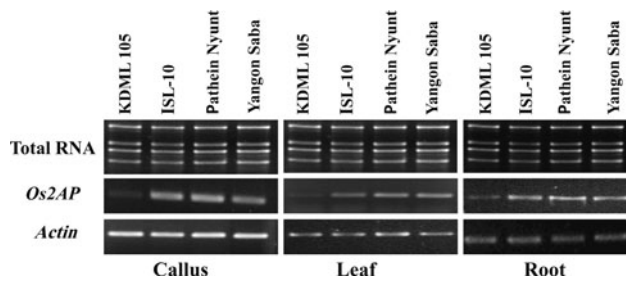
## Results

### Aroma evaluation and *Os2AP* genotyping of Myanmar rice varieties

A collection of 52 Myanmar landrace rice varieties that belong to Isozyme group V, which contains many aromatic rice varieties (Khush et al. 2003), was used for the characterization of *Os2AP* gene alleles. The aroma characteristics of the varieties were evaluated by two sensory test methods as follows: the incubation of cut leaves in diluted KOH and the chewing of several seeds. Only 3 Myanmar rice varieties (Pathein Nyunt, Yangon Saba and Kyet Paung) out of the 52 varieties were detected and confirmed by both sensory methods to be aromatic rice. As analyzed by GC–MS, the 2AP content was present at an intermediate level in the grains of the two aromatic varieties (0.92 ppm for Pathein Nyunt and 0.86 ppm for Yangon Saba) when compared to Thai Jasmine rice (1.3 ppm for KDML105) and another Myanmar rice (1.46 ppm for Kyet Paung). All of the 52 varieties were subsequently genotyped for the two aromatic gene-specific markers to detect the 8-bp deletion in exon 7 and the 7-bp deletion in exon 2. The deletions were not present in all of the non-aromatic varieties. Interestingly, out of the three aromatic rice varieties, only the Kyet Paung variety contained the 8-bp deletion in exon 7. The other two aromatic rice varieties, Pathein Nyunt and Yangon Saba, did not contain the 8-bp deletion or the 7-bp deletion.

### Expression of *Os2AP* in the two Myanmar aromatic rice varieties

The expression of *Os2AP* was analyzed by RT-PCR in the two Myanmar aromatic rice varieties, Pathein Nyunt and Yangon Saba (these two varieties did not contain gene mutations in exon 2 or exon 7), to investigate whether this gene had a role in determining the aromatic trait in these two varieties. RT-PCR analysis demonstrated that the expression level of the *Os2AP* gene in the two varieties did not differ from that of the non-aromatic rice, ISL-10, but the gene expression level in KDML105 (the aromatic



**Fig. 1** RT-PCR analysis of *Os2AP* gene expression in callus, leaf and root samples. KDML 105 is the aromatic variety with the 8-bp deletion, and ISL-10 is a non-aromatic variety. Pathein Nyunt and Yangon Saba are aromatic varieties with the 3-bp insertion. The actin gene was used as a control because it was expressed at the same level in all tissues

variety containing the 8-bp deletion) was highly reduced (Fig. 1).

Sequence variation in the *Os2AP* gene in the two aromatic Myanmar rice varieties

The *Os2AP* gene in the two aromatic rice varieties was sequenced further to investigate whether the other type of gene mutation was present in this gene, even though no effect on gene expression was observed. Gene sequencing revealed a 3-bp insertion (TAT) in exon 13 at the 1,257–1,259 position of the 1,512-bp cDNA sequence in both the Pathein Nyunt and Yangon Saba varieties. The *Os2AP* gene was also sequenced in the aromatic variety (Kyet Paung). The sequencing result confirmed the presence of an 8-bp deletion in this aromatic variety (Fig. 2a). The 3-bp insertion found in the two Myanmar aromatic rice varieties was an in-frame translation that caused an additional amino acid (tyrosine, Y) to be translated into the cDNA sequence of the *Os2AP* gene of the two aromatic varieties. The total amino acid sequence length encoded by the *Os2AP* gene in the two varieties was 504 amino acids (aa), and the normal length found in the non-aromatic varieties was 503 aa (Fig. 2b).

Development of a functional marker specific to the 3-bp insertion in exon 13 of *Os2AP*

A new PCR marker, 3In2AP, was developed to detect the 3-bp insertion (TAT) in exon 13 of the *Os2AP* gene (Fig. 3a). The primer was validated by amplifying the genomic DNA of the two aromatic varieties, Pathein Nyunt and Yangon Saba, and KDML105 and ISL-10, which were used as controls. As a result, different banding patterns were observed between the two Myanmar aromatic varieties compared to KDML105 and ISL-10. The sizes of the PCR products amplified by the 3In2AP marker were

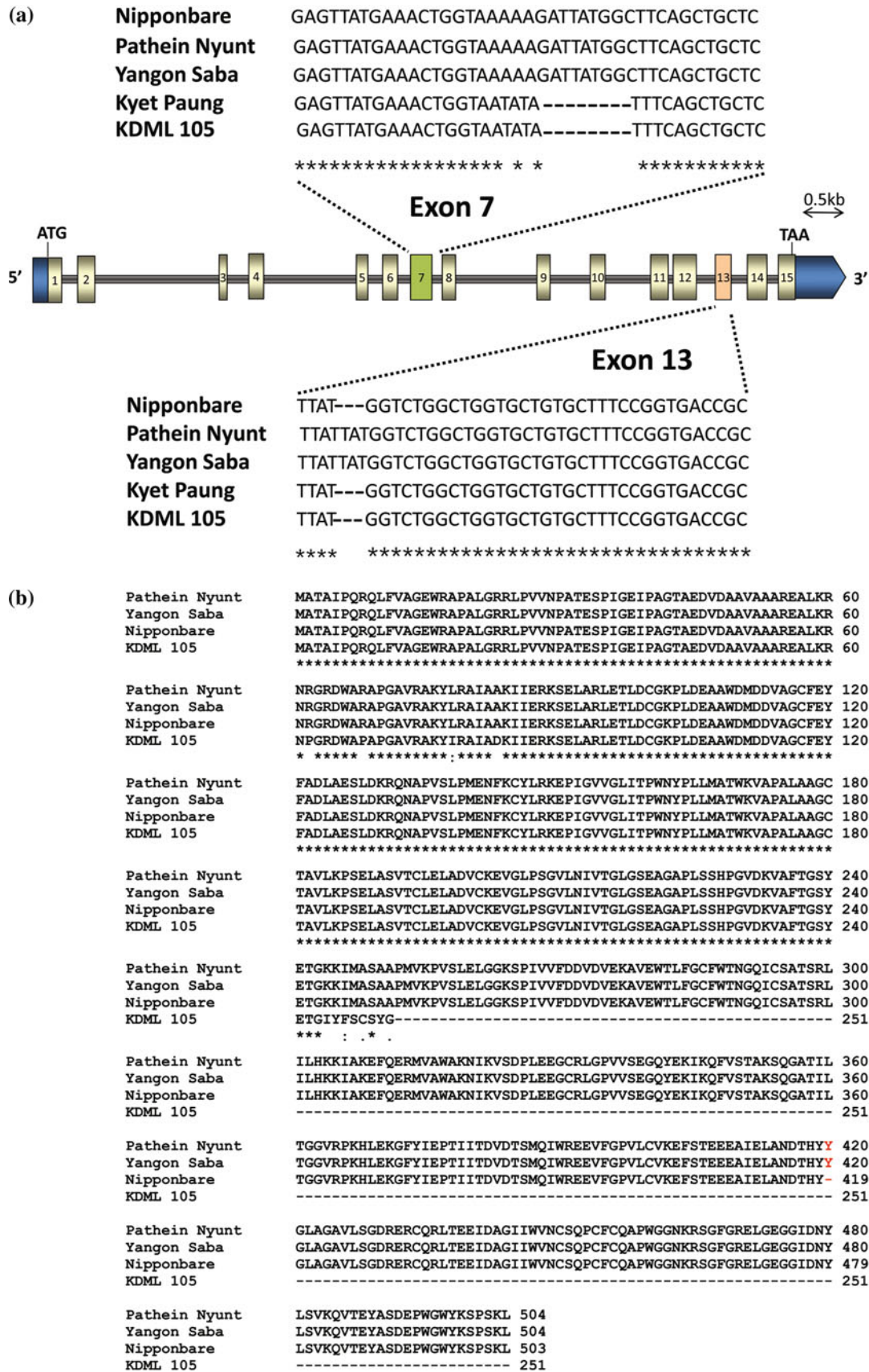
197 bp for aromatic varieties with the 3-bp insertion and 194 bp for KDML105 and ISL-10 (Fig. 3b).

Application of the 3In2AP marker for genotyping the  $F_2$  population

The aromatic variety, Pathein Nyunt, and non-aromatic variety, ISL-10, were used as parental lines to produce an  $F_2$  population that was segregated for aroma. The 3In2AP marker was used to genotype the parental lines and  $F_1$  plants. The polymorphic PCR bands were 197 bp in Pathein Nyunt and 194 bp in ISL-10, and both bands (197 and 194 bp) were observed in the  $F_1$  hybrid. The marker was then used to genotype 160 lines of the  $F_2$  population derived from a single  $F_1$  plant of the cross between Pathein Nyunt and ISL-10. As a result, 33 lines were identified as homozygous to the aromatic variety, Pathein Nyunt, and 80 lines were heterozygous. Moreover, 47 lines carried the allele of ISL-10 (Fig. 4). The segregation ratio of the three genotypes in the  $F_2$  population agreed with the expected ratio of 1:2:1 ( $\chi^2 = 2.450$ ;  $P = 0.2938$ ), which corresponded to the Mendelian segregation of a single gene (Table 1). The phenotypic evaluation performed by a sensory test with the leaf samples of  $F_2$  plants showed that 33 lines were aromatic and 127 lines were non-aromatic. The numbers of the aromatic and non-aromatic  $F_2$  lines had a ratio of 1:3 ( $\chi^2 = 1.633$ ;  $P = 0.2013$ ). The segregation of phenotypes also agreed with the Mendelian segregation ratio for a single recessive gene that controls a trait (Table 1). According to these results, the phenotypes and genotypes of all 160  $F_2$  progenies were correlated. All 33 aromatic lines contained the same 3-bp insertion allele, which suggests that this single recessive allele is associated with the aroma phenotype (Fig. 4).

Application of the 3In2AP marker to screen aromatic rice germplasm from Myanmar

To screen the aromatic rice germplasm from Myanmar, Aromarker and 3In2AP were used to detect the two different aroma alleles in 25 aromatic rice varieties. The KDML105 and Pathein Nyunt aromatic varieties were used as positive control varieties, and ISL-10 was used as a negative control variety. The functional markers clearly showed that this set of Myanmar aromatic varieties carries either the 8-bp deletion or the 3-bp insertion aroma alleles; three varieties, which were not detected by the two functional markers, were exceptions. Sixteen aromatic varieties carried the 3-bp insertion aromatic allele, and six carried the 8-bp deletion aromatic allele (Table 2). The distribution of the aromatic rice varieties that contain the two



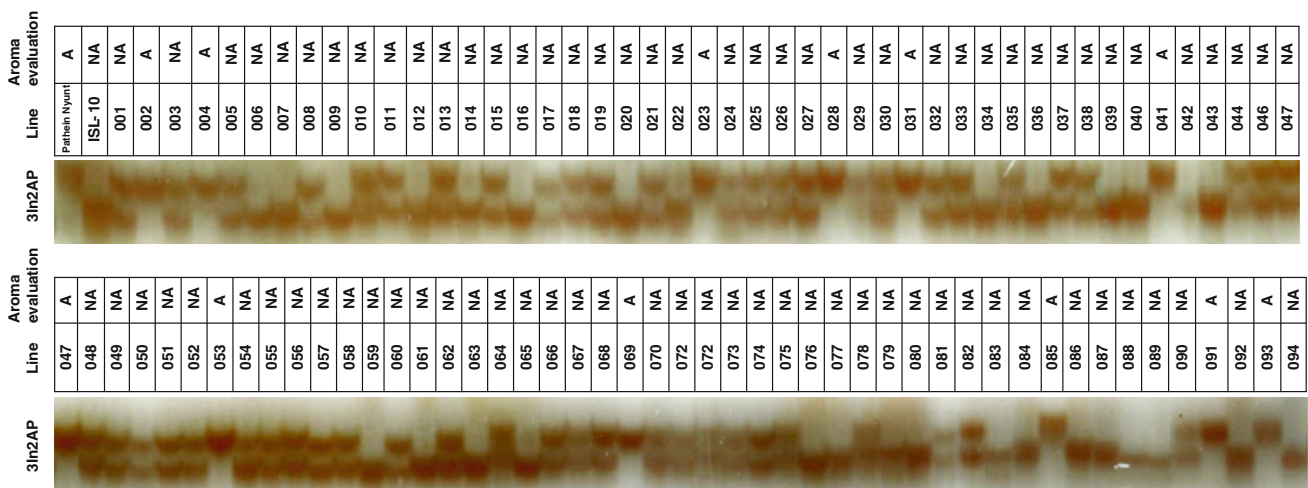
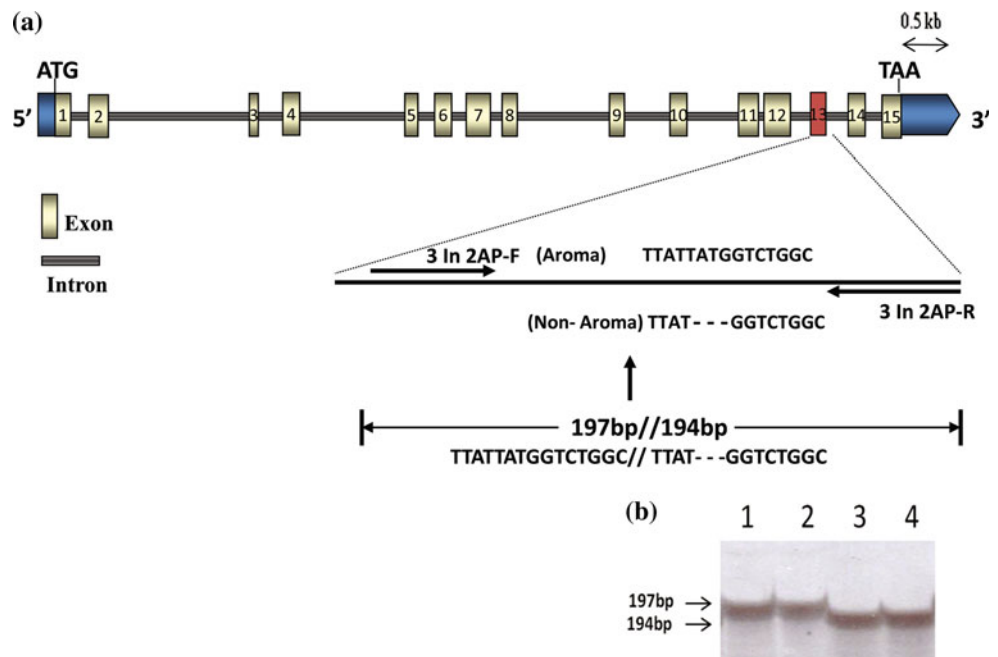
**Fig. 2** **a** The *Os2AP* gene structure. The functional gene consists of 15 exons and 14 introns with its start codon (ATG) in exon 1 and stop codon (TAA) in exon 15. The two aromatic alleles (the 8-bp deletion in exon 7 and the 3-bp insertion in exon 13) are shown in the nucleotide sequence alignments. Nipponbare is a non-aromatic rice variety. KDML105 is an aromatic rice variety containing the 8-bp deletion. Kyet Paung is a Myanmar aromatic rice variety containing the 8-bp deletion. Pathein Nyunt and Yangon Saba are Myanmar aromatic rice varieties containing the 3-bp insertion. **b** The multiple alignments of deduced protein sequences of *Os2AP* compared among aromatic and non-aromatic rice varieties. An additional amino acid, tyrosine (Y), in the Pathein Nyunt and Yangon Saba varieties is highlighted in red (color figure online)

different aromatic alleles in different geographical regions of Myanmar is shown in Fig. 5.

**Discussion**

A single recessive gene located on chromosome 8 controls the aroma trait in rice. After the gene was identified, many scientists have studied a large collection of aromatic rice varieties. The 8-bp deletion in exon 7, which leads to 2AP accumulation in aromatic rice, has been found to be the

**Fig. 3** **a** The schematic of the 3In2AP marker location in exon 13 of *Os2AP*. The sequence variation, 3-bp insertion (TAT) and expected sizes of the PCR products are shown. **b** The banding patterns of the 3In2AP marker. The following lanes are shown: lane 1 Pathein Nyunt (an aromatic rice with the 3-bp insertion), lane 2 Yangon Saba (an aromatic rice with the 3-bp insertion) and lane 3 KDML105 (an aromatic rice with the 8-bp deletion) and lane 4 ISL-10 (a non-aromatic rice). The PCR product sizes are indicated by arrows



**Fig. 4** The segregation pattern, as revealed by the 3In2AP marker, of the representative 94 F<sub>2</sub> plants from the cross between Pathein Nyunt and ISL-10. The aroma phenotypes are shown as A aromatic and NA non-aromatic

**Table 1** The Chi-squared test for phenotypic and genotypic segregation in the F<sub>2</sub> plants derived from the Pathein Nyunt and ISL-10 cross

	Number		$\chi^2$	P value
	Observed	Expected		
Phenotype (the expected ratio is 1:3)				
Aromatic	33	40	1.225	0.2013
Non-aromatic	127	120	0.408	
Total	160	160	1.633	
Genotype (the expected ratio is 1:2:1)				
Pathein Nyunt	33	40	1.225	0.2938
Pathein Nyunt/ISL-10	80	80	0.000	
ISL-10	47	40	1.225	
Total	160	160	2.450	

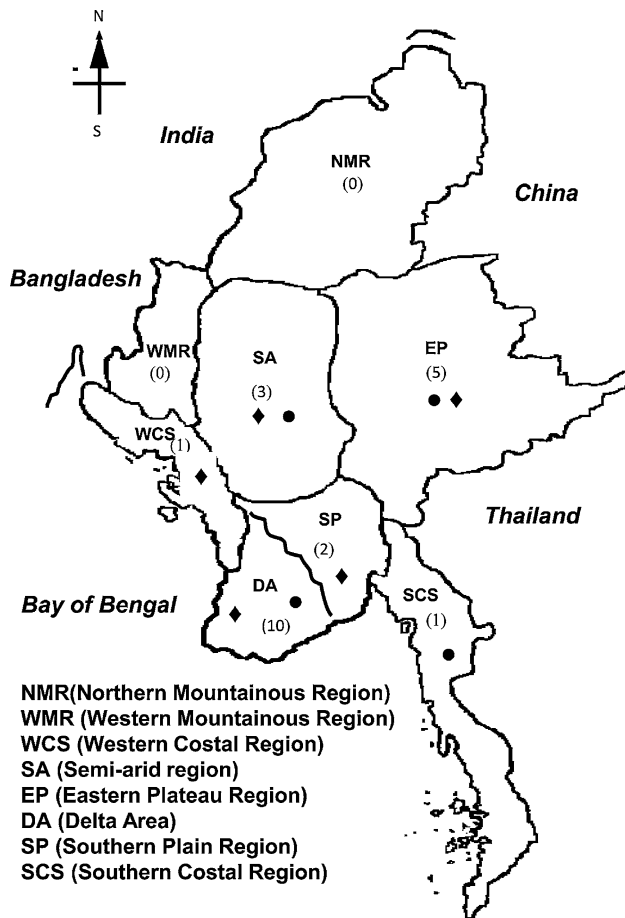
major allele in most aromatic accessions, and other types of mutations with less frequency have also been reported (Bradbury et al. 2005a; Bourgis et al. 2008; Shi et al. 2008; Kovach et al. 2009). Interestingly, the present study found that there were twice as many Myanmar aromatic rice varieties containing the 3-bp insertion allele as those

containing the 8-bp deletion allele. It is likely that the aromatic rice varieties with the 3-bp insertion in the *Os2AP* gene, which results in a tyrosine insertion into the protein at position 420 of the 503 amino acid protein, contain less 2AP in the grains compared to the varieties that contain the 8-bp deletion. The *Os2AP* expression level in the aromatic varieties with the 3-bp insertion was not reduced compared to the non-aromatic variety, which suggests that the *Os2AP* protein could be produced in these aromatic lines. However, a recent study has demonstrated that the enzyme encoded by *Os2AP* from aromatic rice containing the 3-bp insertion exhibits less catalytic efficiency toward 4-aminobutanal (Wongpanya et al. 2011). The tyrosine (Y) insertion at position 420 could alter the conformation of a loop located near the NAD<sup>+</sup> binding site and result in a slight reduction of the binding affinity for NAD<sup>+</sup> and substrates (Wongpanya et al. 2011). The mutation may account for the accumulation of 1-pyrroline, the immediate substrate of 2AP and the generation of aroma in the Myanmar aromatic rice. It is possible that the *Os2AP* gene with the 3-bp insertion can partially function and that the *Os2AP* gene with the 8-bp deletion cannot function at all, which may explain the lower 2AP level in the varieties

**Table 2** A list of Myanmar aromatic rice varieties with different aroma alleles on the *Os2AP* gene

No	Accession No.	Variety	Mutation	Region
1	MSB 682	Sabani Hmwe	8-bp deletion	Southern coastal region
2	MSB 804	Paw San Bay Kyar	3-bp insertion	Delta area
3	MSB 1128	Paw San Yin	3-bp insertion	Delta area
4	MSB 1158	Bahan Hmwe	8-bp deletion	Delta area
5	MSB 1207	Paw San Bay Kyar	8-bp deletion	Delta area
6	MSB 1382	Nga Bya Yin Kauk Hnyin Net	3-bp insertion	Semiarid region
7	MSB 1789	Nga Kywe Yin	3-bp insertion	Eastern plateau region
8	MSB 1791	Tyaung pyan Yin	3-bp deletion	Delta area
9	MSB 1863	Paw San Bay Kyar	8-bp deletion	Delta area
10	MSB 1871	Nga Pyar gyi	3-bp insertion	Semiarid region
11	MSB 2297	Kamar Kyi Saw	3-bp insertion	Eastern plateau region
12	MSB 2579	Paw San Bay Kyar	3-bp insertion	Delta area
13	MSB 2620	Paw San Hmwe	3-bp insertion	Delta area
14	MSB 3163	Paw San Bay Kyar	3-bp insertion	Delta area
15	MSB 3708	Bay Kyar Paw San	3-bp insertion	Southern plain region
16	MSB 6701	KaukHnyin Hmwe	8-bp deletion	Eastern plateau region
17	MSB 6097	Kauk Hnyin Phyu	3-bp insertion	Eastern plateau region
18	MSB 6801	Kauk Hnyin Net	3-bp insertion	Eastern plateau region
19	MSB 6973	Paw San Bay Kyar	3-bp insertion	Western coastal region
20	IRGC 33552	Pathein Nyunt	3-bp insertion	Delta area
21	IRGC 33858	Yangon Saba	3-bp insertion	Southern plain region
22	IRGC 58069	Kyet Paung	8-bp deletion	Semiarid region
23	MSB 2502	Paw San Hmwe	Neither	Delta area
24	MSB 2877	Paw San Bay Kyar	Neither	Delta area
25	MSB 1628	Taung Pyan Hmwe	Neither	Eastern plateau region





**Fig. 5** The geographical locations of the aromatic varieties collected in Myanmar. The number of varieties collected from each region is shown. (filled diamond) Collection site of aromatic rice varieties with the 3-bp insertion. (filled circle) Collection site of aromatic rice varieties with the 8-bp deletion

containing the 3-bp insertion compared with the variety containing the 8-bp deletion (KDML105).

The identification of multiple mutations for 2AP enables rice breeding programs to actively select for multiple genetic sources of 2AP in different aromatic rice varieties. The availability of a functional marker to detect the different mutations will facilitate marker-assisted selection aimed at developing the aromatic rice varieties. A functional marker to detect the 3-bp insertion was developed in this study. The validity of the newly developed marker, 3In2AP, was tested on  $F_2$  segregation lines of the Pathein Nyunt and ISL-10 cross. The 3In2AP marker predicted the phenotype of all the  $F_2$  plants segregated for aroma, and it clearly distinguished the homozygotes from heterozygous  $F_2$  plants. Due to its co-dominant nature, this marker can be potentially useful in testing large segregating materials of aromatic rice improvement programs through a marker-assisted breeding program. Moreover, the assay for genotyping rice aroma using the 3In2AP marker is a simple and

robust method for screening rice populations for aroma. The PCR products can be easily and inexpensively analyzed by polyacrylamide gel electrophoresis (PAGE) with silver staining or by sophisticated high-throughput experiments.

The screening of aromatic varieties from Myanmar demonstrated that the majority of aromatic rice from seven different Myanmar regions had the 3-bp insertion in exon 13 and that several varieties from three other regions had the 8-bp deletion in exon 7. Most of the prominent aromatic varieties, such as Paw San Hmwe (MSB 2620), Paw San Yin (MSB 1128) and Paw San Bay Kyar (MSB 804), and landrace varieties, such as Pathein Nyunt (IRGC 33552) and Yangon Saba (IRGC 33858), contained the 3-bp insertion. The collection site of these varieties with the 3-bp insertion aromatic allele was the delta region of Myanmar. Moreover, the Paw San Bay Kyar (MSB 1863) and Taung Pyan Yin (MSB 1791) varieties collected from the same delta region carried the aroma allele with the 8-bp deletion. The locally adapted aromatic rice varieties (sticky aromatic rice) in the Eastern Plateau region, Kauk Hnyin Phyu (MSB 6097) and Kauk Hnyin Net (MSB 6801), contained the 3-bp insertion, and the Kauk Hnyin Hmwe (MSB 6701) variety contained the 8-bp deletion. In a recent study on the origin and evolution of aroma in rice, Kovach et al. (2009) reported that five Myanmar aromatic varieties, Paw San Hmwe (IRGC 97793), Paw San Hmwe (N/A), Yangon Saba (IRGC 33858), Emahta Longyu (IRGC 33064) and Balugyun IRRI (IRGC 32960), contain the 3-bp insertion and that three varieties, Bokehmye (IRGC 32985), Ma Waine Ohn (IRGC 33357) and Paw San Hmwe (IRGC 33571), contain the 8-bp deletion in exon 7. The results of this study and previous studies demonstrate that the 3-bp insertion is the major aroma allele in Myanmar rice varieties and that most of the prominent aromatic rice varieties of Myanmar originated in the delta regions of Myanmar. In summary, the molecular information collected from Myanmar rice varieties can be used to study a large panel of aromatic rice varieties and their diversity at a whole genome level. Furthermore, the functional marker developed in this study can be used for aromatic rice breeding for improvement with respect to certain characteristics and also for the genetic resource conservation program.

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