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Development of an allele specific amplification (ASA) co-dominant marker for fragrance genotyping of rice cultivars

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Abstract

Fragrance is one of critical factors in rice eating quality in rice, is a recessive trait and it has been confirmed that an 8-bp deletion as well as some SNPs within *Os2AP* gene on rice chromosome eight results in production of fragrance. In this research we attempted to develop an allele specific amplification (ASA) marker based on the 8-bp deletion in the gene. Arm1 primer pair flanking the 8-bp deletion region, has produced a co-dominant polymorphic banding pattern, with band size in highly fragrant and most fragrant cultivars was of 103 bp length, while in non-fragrant ones was of 111 bp length. Results suggested the use of ASA co-dominant marker Arm1 instead of phenotyping via smelling for fragrance genotyping of rice cultivars.

Keywods: Rice, Fragrance, ASA marker.

Introduction

One of the most attractive characteristic of high quality rice is grain aroma that increasingly is demanded not only by the Asian market but also widely recognized in Europe and all over the world [1]. Cooked rice fragrance is composed of more than one hundred volatile compounds such as hydrocarbons, alcohols, aldehydes, ketones, acids, esters, phenols, pyridines, pyrazines, and other compounds [2, 3, 4, 5]. The “popcorn-like” aromatic compound, 2-acetyl-1-pyrroline (2AP), was discovered as the major potent flavor component of all aromatic rice, crust of bread wheat and rye bread [6, 7]. 2-acetyl-1-pyrroline is chiefly responsible for the characteristic fragrance of many aromatic rice varieties [8]. Surprisingly, this rice fragrance has also been isolated and identified from pandan leaves [7], bread flowers (*Vallaris Glabra* Ktze.) [9], wet millet [10], popcorn [11], Bacillus cereus [12] and fungi [13]. 2-acetyl-1-pyrroline is present in all parts of the aromatic rice plant (stems, leaves, grains) except roots [14]. While this fragrance is present in aromatic grains, it is not present in all grains.
The amount of 2-acetyl-1-pyrroline in rice can vary depending on harvest conditions and soil type. A non-aromatic variety, Nipponbare, has 2-acetyl-1-pyrroline levels in the range of 0 to 0.1

ppm (parts per million). In contrast an aromatic rice variety, Thai Hom Mali, has an amount of 2-acetyl-1-pyrroline in the range of 1 to 2.5 ppm [1].

The rice aroma trait had been previously mapped onto chromosome 8 based on both qualitative and quantitative aroma determination methods [14]. The aroma gene has been mapped at 4.5 cM away from RG28 [15] and within 12 cM in between RG28 and RG1 [14]. Recently, the single nucleotide polymorphism (SNP) marker RSP04 developed from the public rice genomic sequence has been mapped 2 cM away from the aroma gene [16]. Both expression studies and positional cloning support Os2AP as the regulator responsible for accumulation of grain aroma and the synthesis of 2-acetyl-1-pyrroline *in vivo* [1]. Both classical and molecular genetics supported that the mutation in exon 7 is the molecular mechanism regulating 2-acetyl-1-pyrroline accumulation in planta [17].

The first evidence linking the amino acid proline as the precursor synthesizing 2-acetyl-1-pyrroline was found in experiments in cell and callus culture [18, 19]. That conclusion was supported by experiments using isotopic labeling showing that the precursor of the grain 2-acetyl-1-pyrroline is most likely the amino acid proline in Thai Hom Mali (THM) rice [20] and probably other aromatic rice. However, the exact biosynthetic pathway of the compound 2-acetyl-1-pyrroline is not known, but it is a derivative of proline. It was hypothesized that proline can be converted either to 2-acetyl-1-pyrroline or to glutamic acid, so inhibition of the glutamic acid synthesis pathway will increase the availability of proline (or intermediates) for 2-acetyl-1-pyrroline synthesis [1]. A gene encoding a protein controlling aroma in rice, named Os2AP was identified as a member of the aldehyde dehydrogenase family that may play a key role in the conversion of proline to glutamic acid. All aromatic rice varieties tested have an eight nucleotide deletion in this gene. The deletion creates a premature stop codon that leads to nonsense mediated degradation against its own mRNA, leading to a loss-of-function phenotype. RNA interference (RNAi) studies showed that disruption of transcription of the Os2AP gene led to elevated levels of 2-acetyl-1-pyrroline in plants, along with increased aroma [17].

Recently molecular markers including SNPs and SSRs, which are genetically linked to fragrance and have the advantage of being inexpensive, simple, rapid and only requiring small amounts of tissue, have been developed for the selection of fragrant rice [21]. However, these markers are only linked with the fragrance gene and therefore do not allow prediction of the fragrant status of any one rice sample with 100% accuracy due to recombination events [22]. Recently, an eight base pair deletion (GATTAGGC) and three SNPs in exon 7 of the gene encoding betaine aldehyde dehydrogenase 2 (BAD2) on chromosome 8 of *Oryza sativa* was identified as the likely cause of fragrance in Jasmine and Basmati style rice [23]. This mutation caused a frameshift translation start at position 729 and created the premature stop codon starting at position 753. In Nipponbare, a non-aromatic rice, the Os2AP full-length cDNA is translated into 503 amino acids. However, the deletion created a truncated peptide of 252 amino acids [1, 17]. This premature stop codon may have significant effects on expression of Os2AP. This polymorphism provides an opportunity for the construction of a perfect marker system for fragrance in rice. We report here the development of a PCR assay, producing an allele specific amplification (ASA) co-dominant marker for fragrance genotyping in rice.

Material and Methods

Plant material

We used a diverse set of 42 aromatic and non-aromatic rice cultivars, in addition to a segregating backcross population (BC_1) derived from a cross between Sadri (aromatic landrace in Northern

Iran) x Neda (Non-aromatic, high-yielding cultivar). BC_1 individuals were generated by crossing the F_1 plants with parent Neda.

Fragrance scoring

Since the use of chemicals such as KOH or I_2KI [24] interfere with smelling and can cause damage to the nasal passages, we developed a simple, non-interfering method for fragrance scoring. Fragrance was evaluated with three replications (individuals smelling vapor) per cultivar. In each replication, three dehulled rice grains were put in a 1.5 ml eppendorf tube. 300 µl distilled water was added to each tube and cap-closed tubes were incubated in 50 ºC for 16 h or in 95ºC for 15 min. Then vapor was smelled by three individuals and samples were scored compared to check cultivars. In this study, IR24 and Domsiah were considered as non-fragrant and highly fragrant cultivars, respectively.

Marker validation

The phenotype of BC_1 individuals were classified as homozygous non-fragrant or heterozygous segregant by tasting dehulled BC_1F_2 seeds. At least 10 BC_1F_2 seeds from individual BC_1 plants were tested individually. BC_1F_2 plants were rated homozygous non-fragrant or heterozygous segregants if all 10 BC_1F_2 seeds were non-fragrant or segregating, respectively. BC_1F_2 seeds from heterozygous BC_1 plants were expected to contain both fragrant and non-fragrant seeds, therefore if the sample from a single BC_1 plant was a mixture of fragrant and non-fragrant, the BC_1 plant was considered heterozygous. The observed segregation ratio of non-fragrant: segregant was tested by $\chi 2$ analysis against the expected ratio of 1:1 for a single gene.

Genomic DNA Extraction

Young leaves were collected from samples. Total genomic DNA was extracted by a modified CTAB method [25]. Leaves were ground in liquid nitrogen using mortar and pestle to a very fine powder. It was then transferred to pre-warmed extraction buffer and incubated at 65°C for 45 min and vortexed for 60s, then added an equal amount of chloroform: isoamyl alcohol (24:1), mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.7 volume of cold isopropanol to precipitate DNA. After centrifugation, the pellet was dried and dissolved in water. DNA was quantified on the agarose gel, diluted and used in PCR.

Polymerase chain reaction (PCR) condition and electrophoresis

Polymerase chain reaction (PCR) was performed in 15 µl volumes containing 0.75 µM/l of each primer, 7.5 µl Master Mix (CinnaGen, Iran), 5µl H_2O and 1µl DNA. The PCR profile was 94 oC for 5 min (denaturation), followed by 35 cycles of 94 ^oC for 1 min, 50, 55 or 60 ^oC (depend on the melting temperature of the primer pairs) for 1 min, 72 ^oC for 2 min, and finally 72 ^oC for 7 min in the final extension.

The products from PCR reaction were resolved by horizontal electrophoresis in 2.5% agarose gel containing 0.5 µg/ml ethidum bromide or resolved by vertical electrophoresis in 6% polyacrilamide gel and subsequent silver staining.

Primer synthesis

Fragrance-specific primer pairs namely Arm1 (forward: 5'- TCCTCTCAATACATGGTTTATG-3', and reverse: 5'-TTGGAAACAAACCTTAACCATAG -3') were designed using Primer 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For non-fragrant varieties the sequence of the gene encoding BAD2 was obtained from the NCBI web site (www.ncbi.nlm.nih.gov) Gen Bank accession number AP004463 and for fragrant varieties the sequence of the gene encoding BAD2 reported by Bradbury et al (2005) [23] was used.

Results

Fragrance scoring of rice cultivars

On the basis of average fragrance scoring by three individuals, the cultivars were classified into three groups including highly fragrant, fragrant and non-fragrant (Table 1). Only a few cultivars including Champa, Domsaih, Deilamani, Sadri and etc were scored as highly fragrant, while most cultivars were ranked as fragrant or non-fragrant.

Table 1. Results of phenotyping of rice cultivars for fragrance

Cultivar	Phenotype
Champa, Domsiah, Deilamani, Hashemi, Binam, Hasan Saraei, Sang Jo, Sadri, IR68899	Highly fragrant
Rashti, Tarom Mahali, Ahlami Tarom, Sang Tarom, Ghashngeh, Salari, Mir Tarom, Dom Zard, Mohamadi Chaparsar, Darang, Abjiboji, Hasani, Bejar, Saleh, Anbarboo	Fragrant
IR60966, IR56, IR28, IR24, IR36, Nemat, Neda, Unda, Gedeh, Dasht, Dollar, Mousa Tarom, Usen, Kadus, Amol3, Gharib, Khazar	Non-fragrant

Determination of rice genotype by allele specific amplification (ASA) marker for fragrance

Two long primers were designed for genotyping rice cultivars in a single PCR reaction. The primers Arm1 might amplify fragments of 103 and 111 bp for fragrant and non-fragrant genotypes, respectively, in PCR as expected from their location in BAD2 sequences (Figure 1). All highly fragrant cultivars such as Domsiah, Champa, Sadri etc and a few number of fragrant cultivars produced 103-bp allele (Table 2), while all non-fragrant and some fragrant cultivars produced 111-bp allele (Figure 2).

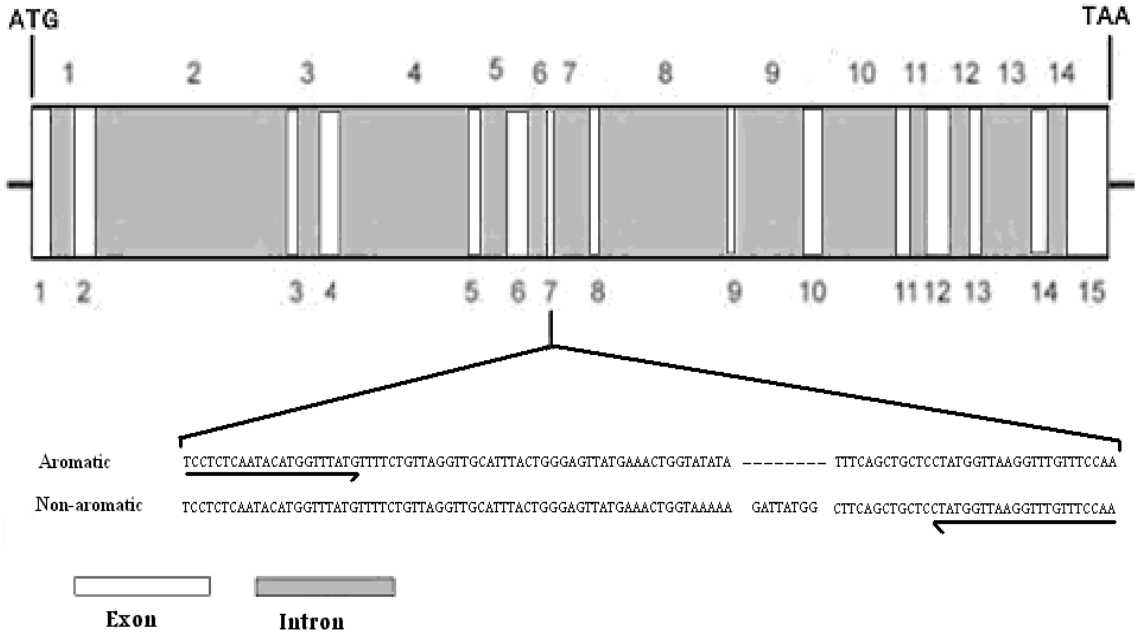

Figure 1. Location of designed primers flanking an 8-bp deletion on the DNA sequence of BAD2 gene

Table 2. Result of genotyping rice cultivars by Arm1 primers.

Cultivar	Allele size (bp)	Genotype
Domsiah	103	F
Champa	"	F
Sadri	"	F
Hashemi	"	F
Binam	"	F
Hasan Saraei	"	F
Sang Jo	"	F
Deilamani	"	F
IR68899	"	F
Rashti	"	F
Tarom Mahali	"	F
Ahlami Tarom	"	F
Sang Tarom	"	F
Ghashngeh	"	F
Salari	111	NF
Mir Tarom	"	NF
Dom Zard	"	NF
Mohamadi Chaparsar	"	NF
Darang	"	NF
Abjiboji	"	NF
Hasani	"	NF
Bejar	"	NF
Saleh	"	NF
Anbarboo	"	NF
IR24	"	NF
IR60966	"	NF
IR56	"	NF
IR28	"	NF
Nemat	"	NF
IR36	"	NF
Neda	"	NF
Unda	"	NF
Gedeh	"	NF
Dasht	"	NF
Dollar	"	NF
Mousa Tarom	"	NF
Kadus	"	NF
Amol3	"	NF
Gharib	"	NF
Usen	"	NF
Khazar	"	NF

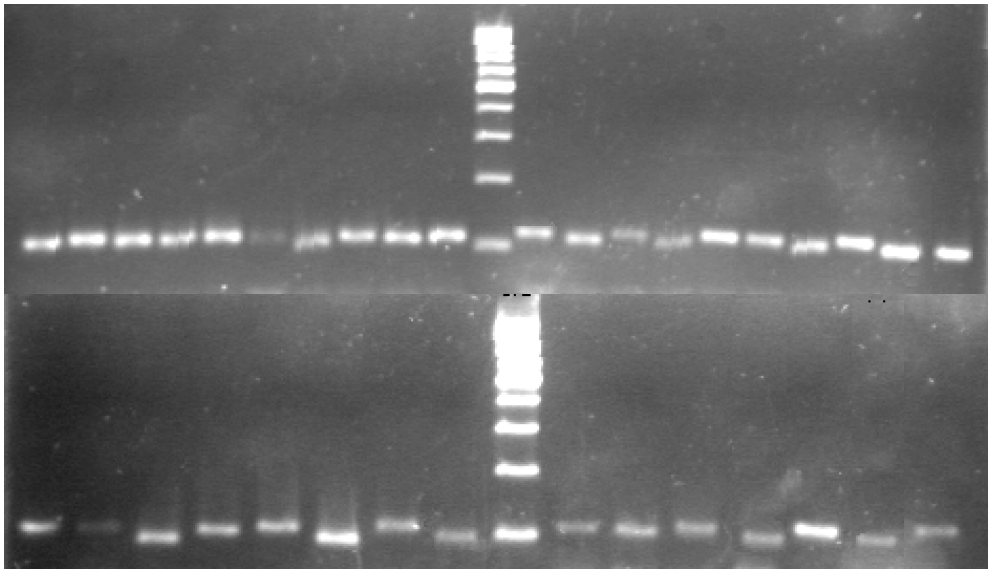

Figure 2. Banding pattern of Arm1 PCR primers on 2.5% agarose gel. Two alleles of 103- and 111-bp length are fragrance and non-fragrance specific

Genotyping BC_1 population using Arm1 primers

For estimating the efficiency of Arm1 primers in genotyping of segregating populations, we tested some BC_1 individuals derived from a cross between Sadri (highly fragrant cultivar) and Neda (non-fragrant cultivar). BC_1 population segregated into 1:1 ratio (homozygous non-fragrant: heterozygous non-fragrant). Arm1 marker could estimate with 100% accuracy the genotype of BC1 individuals (Figure 3).

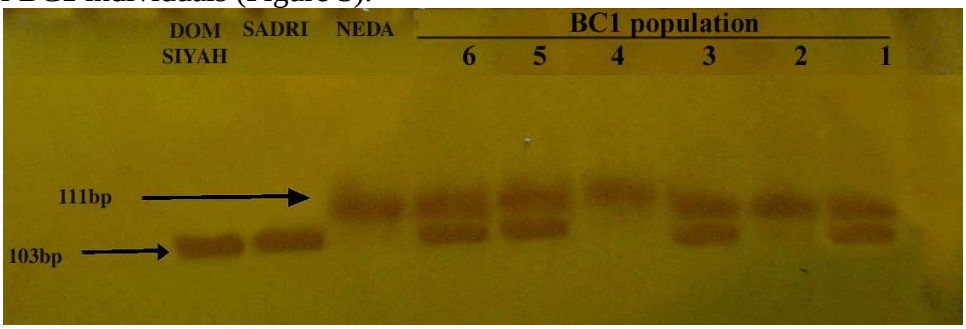

Figure 3. A sample of genotyping of BC_1 individuals using Arm1 primers

PCR products were resolved on a 6% polyacrylamide gel and subsequent silver staining. Domsiah (check fragrant cultivar) and Sadri produced fragrance specific allele and Neda produced non-fragrance specific allele. BC1 individuals showed non-fragrance allele or heterozygous status as expected.

Discussion

To develop new aromatic rice by conventional cross-breeding, one may cross an aromatic rice donor and a more productive recipient rice variety. The progeny from the cross will segregate for grain aroma among other traits. This situation confuses breeders and makes conventional breeding for better aromatic rice less successful. The discovery of the aroma gene [23, 26] will turn out to be a new paradigm for conventional breeders. Specific molecular markers can be developed to detect the aroma gene so that thousands of plants can be screened with the highest

accuracy. DNA marker technology can allow breeders to detect the aromatic allele of the Os2AP gene at an early stage with high sensitivity [1]. This leaves more opportunity for breeders to add preferred traits into aromatic plants at a later stage. The molecular basis of aroma identified in rice can possibly be found in other cereals and this will open ways to develop DNA markers for other cereals as well [1].

In this study we successfully developed an allele specific amplification (ASA) marker system for genotyping rice cultivars in fragrance. Designed primers Arm1 that flanked an 8-bp deletion in Os2AP (Figure 1) produced the alleles of expected size. Since the PCR products are of small size (103-111 bp), their difference (8 bp) is enough large to be detected even by using an agarose gel-based system. Bradbury et al (2005) [22] also developed an ASA marker system on this 8 bp deletion, involving four primers (2 external and 2 internal primers) to be used in a single tube, which produce 3 bands in PCR. However, our system is based on two primers that produce co-dominant bands. Considering the need for a marker system suitable for application in high-throughput marker-assisted selection (MAS) programs, the use of Arm1 marker system will save considerable time and reduce costs.

As seen from results, we could successfully identify highly fragrant and non-fragrant rice cultivars with 100% accuracy (Table 2). By scanning 95 landrace varieties using the "Aromarker" PCR primers, Vanavichit et al (2006) [1] also found that the allele with the 8 base pair deletion correlates exactly with increased aroma. They also sequenced exon 7 for the 8 base pair deletion among wild species including Oryza nivara and O. rufipogon and found that most aromatic varieties including Thai Jasmine, Basmati and Azucena have the deletion, and therefore share a common ancestor that may trace back to the ancient time. They identified the aromatic allele in aromatic wild rice as well. Therefore, a single mutation arose long before cultivation by humans to give rise to the aromatic rice we know today.

Results also show that only a part of fragrant samples carried fragrance-specific allele (including Rashti, Tarom Mahali, Ahlami Tarom, Sang Tarom and Ghashngeh) and the remaining fragrant samples amplified 111-bp non-fragrant specific fragment, suggesting it is probable that there is another gene encoding fragrance within the rice genome. This issue was supported by some researchers via the study of inheritance of fragrance in segregating populations derived from crosses between fragrant and non-fragrant cultivars [14, 15, 27, 28, 29]. However, some researchers associated two-gene inheritance to incorrect phenotyping of rice fragrance due to adverse effect of chemicals (such as KOH or I_2KI) on the nasal passages of individuals [24, 30] as well as due to different aroma-understanding by individuals in reception ability of aroma signal when smelling.

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