Molecular Breeding (2005) 16: 279–283
DOI 10.1007/s11032-005-0776-y

A perfect marker for fragrance genotyping in rice

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Received 22 April 2005; accepted in revised form 6 July 2005

Key words: Aromatic, Assay, Basmati, High-throughput, Jasmine, *Oryza sativa*

Abstract

Allele specific amplification (ASA) is a low-cost, robust technique that can be utilised to discriminate between alleles that differ by SNP's, insertions or deletions, within a single PCR tube. Fragrance in rice, a recessive trait, has been shown to be due to an eight bp deletion and three SNP's in a gene on chromosome 8 which encodes a putative betaine aldehyde dehydrogenase 2 (BAD2). Here we report a single tube ASA assay which allows discrimination between fragrant and non-fragrant rice varieties and identifies homozygous fragrant, homozygous non-fragrant and heterozygous non-fragrant individuals in a population segregating for fragrance. External primers generate a fragment of approximately 580 bp as a positive control for each sample. Internal and corresponding external primers produce a 355 bp fragment from a non-fragrant allele and a 257 bp fragment from a fragrant allele, allowing simple analysis on agarose gels.

Abbreviations: 2AP – 2-Acetyl-1-pyrroline; ASA – Allele specific amplification; BAD2 – Betaine aldehyde dehydrogenase 2; SNP – Single nucleotide polymorphism

Introduction

The demand for fragrant rice has increased markedly in recent years in both traditional and non-traditional rice growing countries to such an extent that consumers are willing to pay a premium price for fragrant rices. In order to assist in the development of fragrant rice varieties suited to particular local environmental conditions, rice breeders have an interest in gaining access to a simple and inexpensive method for distinguishing between fragrant and non-fragrant rice.

The flavour and fragrance of Basmati and Jasmine style rice have been associated with increased levels of 2-acetyl-1-pyrroline (2AP) (Buttery et al. 1983; Lorieux et al. 1996; Widjaja et al. 1996; Yoshihashi (2002). A number of sensory methods have been utilised to assist breeders in selecting fragrant rice but there are limitations when processing large numbers of samples. For example, tasting individual grains is one of the original methods for the quality selection of fragrant rice varieties within the Australian breeding program (Reinke et al. 1991) and is still the principal means of identifying fragrance in many breeding programs worldwide. However, the objective evaluation of fragrance using this method is labour intensive, difficult and unreliable. A panel of

analysts is required as the ability to detect fragrance varies between individuals. For any individual analyst, the ability to distinguish between fragrant and non-fragrant samples diminishes with each successive analysis because the senses become saturated or physical damage occurs from abrasions to the tongue which often result from chewing the hard grain. Chemical methods are available which involve smelling leaf tissue or grains after heating in water or reacting with solutions of KOH or I_2-KI (Sood 1978) but these can cause damage to the nasal passages. An objective method of 2AP identification using gas chromatography is available but the assay requires large tissue samples and is time consuming (Lorieux et al. 1996; Widjaja et al. 1996).

More recently molecular markers, such as SNPs and simple sequence repeats (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 (Cordeiro et al. 2002). 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.

Recently, an eight base pair deletion 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 (Bradbury et al. 2005). Non-fragrant rice varieties possess what appears to be a fully functional copy of the gene encoding BAD2 while fragrant varieties possess a copy of the gene encoding BAD2 which contains the deletion and SNPs, resulting in a frame shift that generates a premature stop codon that presumably disables the BAD2 enzyme. This polymorphism provides an opportunity for the construction of a perfect marker for fragrance in rice. We report here the construction of a PCR assay for fragrance genotyping in rice.

Materials and methods

Plant materials

All rice samples were supplied by Yanco Agricultural Institute, NSW Agriculture. A diverse collection of 14 fragrant and 74 non-fragrant varieties (Bradbury et al. 2005) in addition to a population of 168 field grown F_2 individuals derived from a cross between Kyeema (Pelde//Della/Kulu) (tall, Jasmine-style, long-grain, Australian cultivar) and Gulfmont (Lebonnet//CI9881/PI 331581) (early-maturing, semi-dwarf, non-aromatic long-grain USA cultivar) was used to validate the marker.

Genetic mapping

Fragrance was evaluated according to Berner and Hoff (1986). The phenotype of F_2 individuals were classified as fragrant, segregating or non-fragrant by tasting dehulled F_3 seed. At least 12 F_3 seeds from individual F_2 plants were chewed individually. F_2 plants were rated homozygous fragrant or non-fragrant if all 12 F_3 seeds were fragrant or non-fragrant, respectively. F_3 seeds from heterozygous F_2 plants were expected to contain both fragrant and non-fragrant seeds, therefore if the sample from a single F_2 plant was a mixture of fragrant and non-fragrant, the F_2 plant was considered heterozygous. The observed segregation ratio of fragrant:segregating:non-fragrant was tested by χ^2 analysis against the expected ratio for a single gene.

Primer design

Oligonucleotide primers were designed, using Primer Premier Version 5.0 (Premier Biosoft International, Palo Alto, CA). 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) was used.

DNA extraction, PCR and genotyping

Genomic DNA was extracted from leaf material using a Qiagen DNeasy® 96 Plant Kit (Qiagen GMbH, Germany) and from whole seeds as described by Bergman et al. (2001). Rough leaf DNA extractions were performed by boiling 0.1 g of leaf

material in 50 μl 10X PCR Buffer (Gibco BRL®) for 10 min. Oligonucleotide primers were synthesised by Proligo Australia Pty Ltd. PCR was performed using 0.2 μl Platinum® Taq DNA Polymerase (Gibco BRL®), 1 μl of genomic DNA 10 ng μl^{-1}, 2.5 μl of 10X buffer (Gibco BRL®), 1 μl of 50 mM $MgCl_2$ (Gibco BRL®), 1 μl of dNTPs [5 mM], 2.5 μl of each primer (ESP, IFAP, INSP and EAP – Table 1) [2 μM], in a total volume of 25 μl. PCR was performed using a Perkin Elmer, Gene Amp PCR system 9700. Cycling conditions were an initial denaturation of 94°C for 2 min followed by 30 cycles of 5 s at 94°C, 5 s at 58°C, 5 s at 72°C; concluding with a final extension of 72°C for 5 min.

PCR products were analysed by electrophoresis in ethidium bromide stained (0.5 ug ml^{-1}) 1.0% agarose gels. A 100 bp ladder molecular weight standard (Roche) was used to estimate PCR fragment size.

Results

Development of the single tube Allele Specific PCR fragrance assay

Four primers, two that anneal to sequences common to both fragrant and non-fragrant varieties and external to the area where the mutation occurs and two that are specific to one of the two possible alleles were designed and synthesised (Figure 1). The two external primers were designed to act as an internal positive control amplifying a region of approximately 580 bp in both fragrant (577 bp) and non-fragrant (585 bp) genotypes. Individually, these external primers also pair with internal primers to give products of varying size, depending upon the genotype of the DNA sample. The internal primers, IFAP and INSP (Table 1), will anneal only to their specified genotype producing DNA fragments with their corresponding external primer pair, ESP and EAP respectively. Using these four primers in a PCR results in three possible outcomes. In all cases a positive control band of approximately 580 bp is produced. In the first case a band of 355 bp is produced indicating a variety or individual is homozygous non-fragrant. In the second case a band of 257 bp is produced indicating a variety or individual is homozygous fragrant. In the third case both bands of sizes 355 bp and 257 bp are produced indicating an individual is heterozygous non-fragrant.

Determination of plant genotype using single tube ASA PCR fragrance assay

PCR products were easily separated on an agarose gel. The PCR product of approximately 580 bp serves as a positive control and is present in every

Table 1. Primers for analysis of fragrance in rice.

Primer name	Primer sequence
External Sense Primer (ESP)	TTGTTTGGAGCTTGCTGATG
Internal Fragrant Antisense Primer (IFAP)	CATAGGAGCAGCTGAAATATATACC
Internal Non-fragrant Sense Primer (INSP)	CTGGTAAAAAGATTATGGCTTCA
External Antisense Primer (EAP)	AGTGCTTTACAAAGTCCCGC

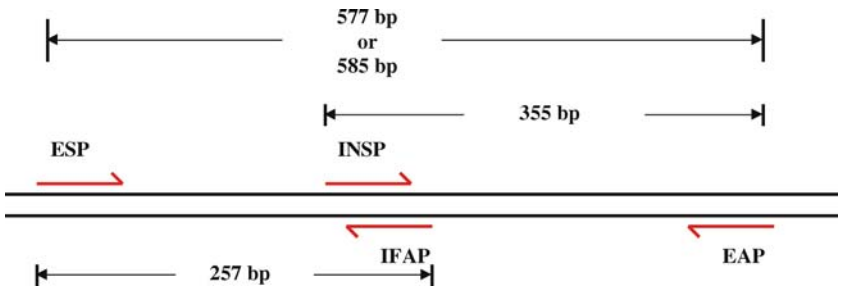

Figure 1. Relative positions of PCR primers used in fragrance PCR.

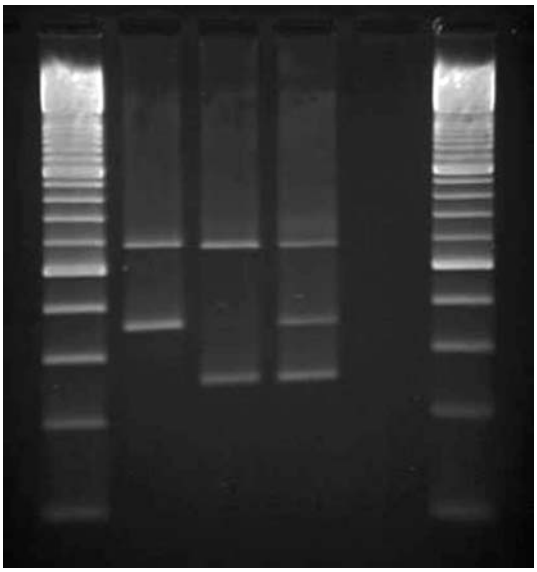

Figure 2. Agarose gel showing (lane 2–5) a non-fragrant variety (Nipponbare), a fragrant variety (Kyeema), a heterozygous individual (Kyeema/Gulfmont) and a negative control (water) flanked by Roche DNA Ladder XIV (100 bp).

sample. Fragrant individuals have a second product of 257 bp in size while non-fragrant individuals give a product of 355 bp in size, heterozygotes can also be discriminated by the presence of all three PCR products (Figure 2).

The assay predicted the phenotype of 168 F_2 progeny segregating for fragrance with 100% accuracy (46 homozygous fragrant, 80 heterozygotes, 42 homozygous non-fragrant) (Figure 3). The assay also allows discrimination between fragrant and non-fragrant grains using DNA derived from rice grains using a simple NaOH extraction protocol (Bergman et al. 2001) and leaves using a simple 10 min boiling protocol.

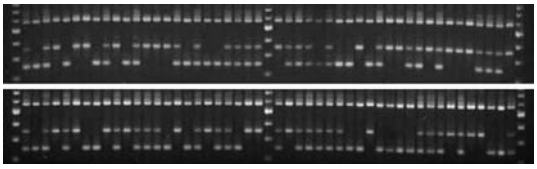

Figure 3. Agarose gel showing 96 individuals from an unselected F2 population segregating for fragrance and analysed using single tube ASA. The band of approximately 580 bp corresponds to the positive control amplified by both external primers (ESP and EAP). The 355 bp band corresponds to a PCR product amplified from the non-fragrant allele by the internal non-fragrant sense primer (INSP) and the external antisense primer (EAP). The 257 bp band corresponds to a PCR product amplified from the fragrant allele by the internal fragrant antisense primer (IFAP) and the external sense primer (ESP).

Further evaluation demonstrated the capacity of the assay to work on a broard range of fragrant varieties such as Basmati 370, Kyeema, Khao Dwak Mali 105 and Moosa Tarom (results not shown).

Discussion

Fragrance in Basmati and Jasmine style rice is a recessive trait (Lorieux et al. 1996) which results principally from the presence of elevated levels of the compound 2-acetyl-1-pyrroline (2AP) in the aerial parts of the plant. A deletion in the gene encoding BAD2 on chromosome 8 which disables the BAD2 enzyme is the most likely cause of fragrance (Bradbury et al. 2005). Functional BAD2 is either responsible for metabolising 2AP which means the presence of the non-functional enzyme results in accumulation of 2AP and hence fragrance, or functional BAD2 is active in a pathway that competes for substrate which would otherwise be used in the production of 2AP and so a non-functional BAD2 enzyme results in increased flux of substrate down the pathway of 2AP production. Knowledge of the most likely genetic cause of fragrance has allowed us to develop a perfect assay for fragrance in rice. A single tube allele specific PCR which allows determination of the genotypic status of an individual rice plant, either homozygous fragrant, homozygous non-fragrant or heterozygous non-fragrant, has practical utility for rice breeders worldwide. The assay is a simple robust method for screening rice to determine its fragrance status across a wide range of rice varieties and within segregating populations using DNA isolated from rice following simple, inexpensive and rapid extraction protocols.

The PCR products can be analysed easily and inexpensively on agarose gel or alternatively using more sophisticated high throughput equipment, making the assay a very versatile tool.

Acknowledgements

Thanks are due to Yanco Agricultural Institute, NSW Agriculture, for supplying the mapping population used in this study.

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