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Identification of Badh2 Mutation Type among Indonesian Fragrant Rice Varieties

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Abstract
The premium price as well as the high and increasing world market demand for fragrant rice has triggered the development of various fragrance markers. The application of these markers on genotyping of various popular Indonesia rice varieties is reported in this paper. PCR profiles of popular Indonesia non-fragrant (Ciherang, Fatmawati) and fragrant (Pandan wangi, Rojo Lele, Mentik Wangi, Gunung Perak, Pulu mandoti, Pare Kembang, Sintanur) rice varieties were compared using aromatic markers of Bradbury *et al*. (2005b), Lang and Buu (2008), Shi *et al*. (2008), and Sakthivel *et al*. (2009). For comparison, IR64, Nipponbare and Taipei 309 varieties were included. Rice genomic DNA was isolated from young leaves using the method as described by Doyle and Doyle (1990), PCR amplified using each of the above fragrance markers and PCR products were analysed by agarose-gel-electrophoresis. Fragrance markers of Bradbury *et al*. (2005b), Shi *et al*. (2008), and Sakthivel *et al*. (2009) were only able to discriminate fragrant Mentik Wangi and Gunung Perak from non-frangrant rice varieties, while other fragrant rice varieties (Pandan wangi, Rojo Lele, Pulu mandoti, Pare Kembang, Sintanur) showed similar band patterns as those of non-frangrant rice varieties. This suggests there are at least two groups of badh2-deletion patterns among Indonesia fragrant rice varieties. Group 1 include Mentik Wangi and Gunung perak, while group 2 includes Pandan wangi, Rojo Lele, Pulu mandoti, Pare Kembang and Sintanur. Only the RM 223 marker of Lang and Buu (2008) was able to distinguish both fragrant groups from non-fragrant Ciherang. The difference in RM233-amplicon sizes between fragrant groups 1 and 2, also supports the variation of badh2 mutation pattern among Indonesia fragrant rice.
Keywords: Bradbury, Badex7-5, FM-E7, FM-E2A, RM 223, fragrance, Pandan Wangi

1. Introduction

Various fragrance analysis have been developed to assist selection in rice breeding for aroma. Those methods include: organoleptic based on taste (Reinke *et al.* 1991, Petrove *et al.* 1996) or odor (Sood and Sidiq 1978, Paule and Powers 1989); *BADH2* enzyme-bioassay (Srivong *et al.* 2008); 2-AP detection using mass spectrophotometry/selective ion monitoring (Tanchotikul and Hsieh 1991), GC (Lorieux *et al.* 1996, Widjaja *et al.* 1996) or stable isotope (Yoshihashi 2002) and the use of RFLP (Restriction Fragment Length Polyorphisms), SNPs (Single Nucleotide Polymorphisms) or SSRs (Simple Sequence Repeats) markers (Ahn *et al.* 992, Cordeiro *et al.* 2002). However, these methods are considered difficult, labour intensive, unreliable, time consuming and require large samples, or are unable to predict the fragrace gene or allele statues (Bradbury *et al.* 2005b, Sakthivel *et al.* 2009). In addition, most of these methods are inapplicable for fragrance analysis in heterozygote progenies, since fragrance in rice is a recessive trait (Lorieux *et al.* 1996, Dong *et al.* 2000, Bradbury *et al.* 2005a, Borquis *et al.* 2008). Fragrant-marker-assisted PCR, followed by agarose-gel-electrophoresis, has been considered as the most potential and amenable approach for routine fragrance genotyping in large scale breeding materials, due to its low-cost, speed, simplicity, and the sensitivity of this method (Bradbury *et al.* 2005b, Lang and Buu 2008, Shi *et al.* 2008, Sakthivel *et al.* 2009).

The finding of an 8 bp and 3 SNPs mutation in the *badh2* gene of fragrant rice has led to the development of a specific fragrant marker-assisted PCR method (Bradbury *et al.* 2005a, Borquis *et al.* 2008) that can be used to facilitate early selection of non-fragrant and fragrant rice, as well as their cross and backcross progenies which are always heterozygous. The size difference in the *badh2* gene between non-fragrant and fragrant rice results in a different amplicon size (Bradbury *et al.* 2005b). Lang and Buu (2008) have also reported fragrant marker RM 223 that produces ~160 and ~120 bp amplicons for non-fragrant and fragrant rice, respectively. Besides the exon 7-deletion (*badh2.7*) that had been previously reported (Bradbury *et al.* 2005a, Borquis *et al.* 2008), Shi *et al.* (2008) also reported another deletion in the exon2 of *badh2* gene (*badh2.2*), and constructed fragrance markers FM-E7 and FM-E2 that flanked the 8 bp or 7 bp deletion in *badh2.7* or *badh2.2*, respectively. However, the suggestion of using PAGE method to resolve PCR products were not quite practical for routine genotyping in large scale breeding materials/germplasms.

Although various fragrance markers (Bradbury *et al.* 2005b, Lang and Buu 2008, Shi *et al.* 2008, Sakthivel *et al.* 2009) have been reported which succesfully discriminate between various fragrant rice varieties in Australia, India, Thailand, and China; none of these have been applied to Indonesia rice varieties. Fragrant genotyping was carried out on popular Indonesia non-fragrant (Ciherang, Fatmawati) and fragrant (Pandan wangi, Rojo Lele, Mentik Wangi, Gunung Perak, Pulu mandoti, Pare Kembang, Sintanur) rice varieties using various reported fragrance markers (Bradbury *et al.* 2005b, Lang and Buu 2008, Shi *et al.* 2008, Sakthivel *et al.* 2009). For comparison, IR64, Nipponbare and Taipei 309 were included.

2. Materials and Methods

2.1 Plant materials and reagents

Rice samples were supplied by the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development as well as by the Indonesian Center for Rice Research. The Bradbury marker was obtained from the Midland Certified Reagents Co. RNAse, PCR buffer, fastart Taq DNA polymerase and dNTP were supplied by Roche Diagnostics Indianapolis. One kb standard DNA size marker and agarose low EE0 were supplied by Invitrogen and Applichem Biochemical Chemica Synthesis Service, respectively. All other chemicals, of the highest purity available, were purchased from Sigma.

2.2 DNA extraction

Genomic DNA was extracted from fresh young leaves following the protocol described by Doyle and Doyle (1990).

2.3 PCR and electrophoresis

The composition of PCR mix and cycling conditions, as well as the electrophoresis conditions were as described by the previous authors (Bradbury *et al.* 2005b, Lang and Buu 2008, Shi *et al.* 2008, Sakthivel *et al.* 2009), except for the source of reagents which are described above. In addition, agarose gel was used for the FM-E7 and FM-E markers PCR products, in place of the recomended polyacrylamide gel (Shi *et al.* 2008).

3. Results and Discussion

3.1 Genotyping using Bradbury marker

Isolated DNA from non-fragrant and fragrant rice samples were PCR amplified using Bradbury *et al.*(2005b) primer, and analysed by agarose-gel-electrophoresis. The results (Figure 1) showed that only fragrant Mentik Wangi and Gunung Perak rice PCR profiles were as previously reported (Bradbury *et al.* 2005b), and these were different from those of non-fragrant samples (Ciherang, Fatmawati, IR64, Niponbare, Taipei 306). However, other frangrant (Pandan wangi, Rojo Lele, Pulu mandoti, Pare Kembang, Sintanur) were similar. Based on these results, it was deduced that there were two groups of *badh2*-deletion patterns among Indonesia fragrant rice varieties. Group 1 includes Mentik Wangi and Gunung perak, while group 2 includes Pandan wangi, Rojo Lele, Pulu mandoti, Pare Kembang, and Sintanur.

Bradbury Marker has been proved to positively amplify the *badh2* gene of various rice varieties (Bradbury *et al.* 2005b, Amarawathi *et al.* 2008). The existence of a 585 bp *badh2* band (Figure l) indicates that the *badh2* gene of all the examined Indonesia rice variety samples were identical to those rice varieties of other countries. The presence of the fragrant band (257 bp) in group l fragrant rice samples (Mentik Wangi and Gunung Perak) indicated similarity of the *badh2.7* mutation pattern with those previously reported fragrant rice samples (Bradbury *et al.* 2005b, Borquis *et al.* 2008). However, those of Group 2 (Pandan wangi, Rojo Lele, Pulu mandoti, Pare Kembang, and Sintanur) which did not possess the fragrance band seemed to have a different *badh2*-mutation pattern. Similar results have also been reported previously (Amarawathi *et al.* 2008). The *badh2* mutation in group 2 may be shorter than that of group l (8 bp) as illustrated in Figure 2. As a results, the internal fragrant primer (IFAP) becomes unsuitable, and therefore no fragrance bands were observed. On the other hand, the non-fragrance internal primer (INSP) becomes suitable, and therefore unexpected non-fragrance bands (385 bp) were observed in the samples of group 2 fragrant rice samples. Consequently, the size of group 2 *badh2* gene should be larger than that of group l fragrant rice varieties.

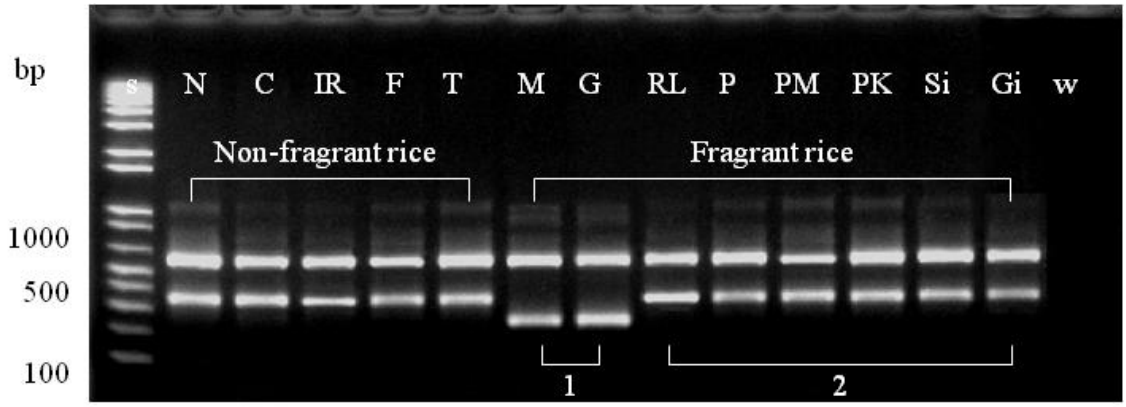

Figure 1. Bradbury-PCR profiles
s = standart size marker, N = Nippon bare, C = Ciherang, IR = IR64, F = Fatmawati, T = Taipei 306, M = Mentik Wangi, G = Gunung Perak, RL= Rojo Lele, P = Pandan Wangi, PM = Pulu Mandoti, PK = Pare Kembang, Si = Sintanur, Gi = Gilirang, and w = water (negative controll). 1 and 2 indicate members of group 1 and 2, respectively.

3.2 Genotyping using FM-E7 or Badex marker

The PCR profiles of non-fragrant and fragrant rice varieties were further compared using FM-E7 (Shi *et al.* 2008) or Badex7-5 (Sakthivel *et al.* 2009) marker. The results showed that both FM-E7 (Figure 3) and Badex 7-5 (Figure 4) markers were able to distinguish between non-frangrant and fragrant rice of group 1, but not of group 2.

The PCR profile results using FM-E7 (Shi *et al.* 2008) (Figure 3) or Badex 7-5 (Sakthivel 2009) (Figure 4) markers also supported the view that there are 2 groups of exon 7-*badh2* deletion patterns among Indonesian fragrant rice varieties. The group members are identical to those obtained previously using the Bradbury *et al.* (2005b) marker. The difference in band migration distance was relatively small (Sakthivel *et al.* 2009). The use of polyacrylamide gel has been suggested to resolve PCR amplicons resulted using the Shi *et al.* (2008) rnarker. However, no further experiments were carried out since these markers were unable to discriminate Pandan Wangi and Rojo Lele from non-fragrant rice samples.

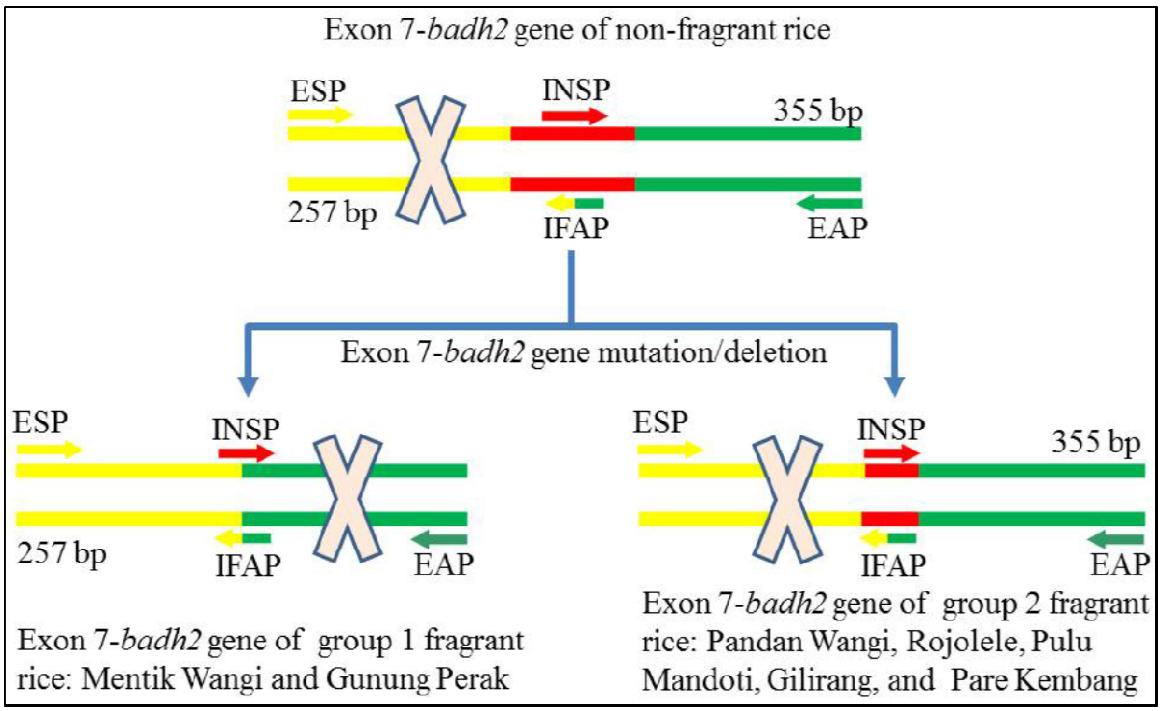

Figure 2. Suggested mutation-pattern differences between group 1 and 2-fragrant rice varieties

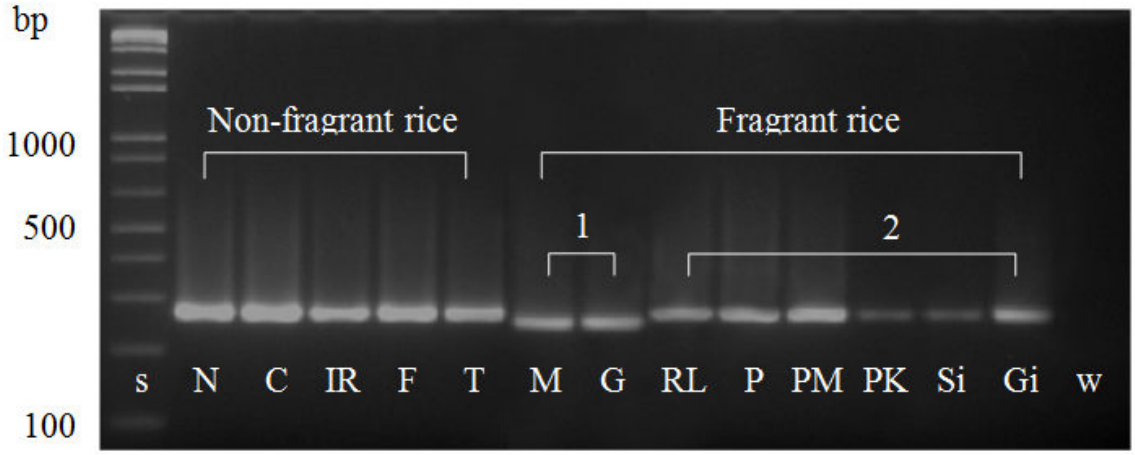

Figure 3. FM-E7-PCR profiles
s = standard marker, N = Nippon bare, C = Ciherang, IR = IR64, F = Fatmawati, T = Taipei 306, M = Mentik Wangi, G = Gunung Perak, RL= Rojo Lele, P = Pandan Wangi, PM = Pulu Mandoti, PK = Pare Kembang, Si = Sintanur, Gi = Gilirang, and w = water. 1 and 2 indicate members of group 1 and 2, respectively.

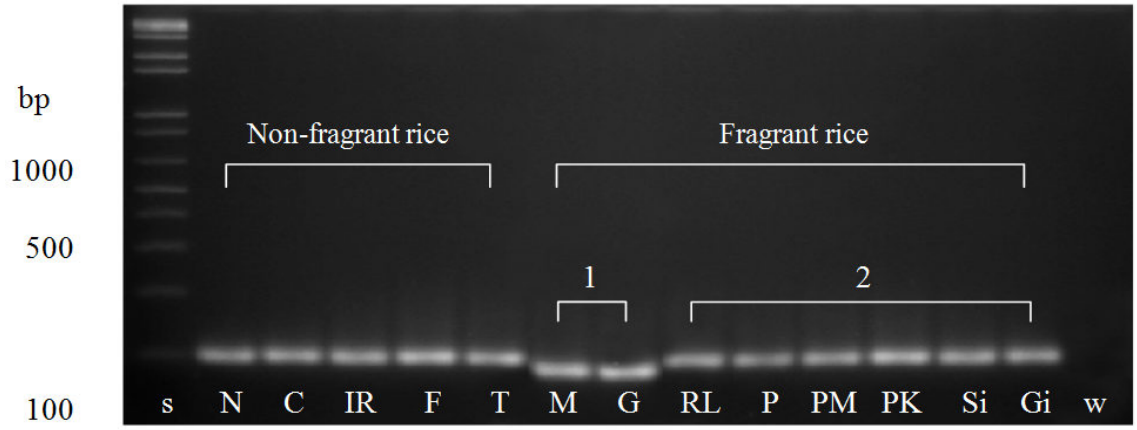

Figure 4. Badex7-5-PCR profiles.
s = standard marker, N = Nippon bare, C = Ciherang, IR = IR64, F = Fatmawati, T = Taipei 306, M = Mentik Wangi, G = Gunung Perak, RL= Rojo Lele, P = Pandan Wangi, PM = Pulu Mandoti, PK = Pare Kembang, Si = Sintanur, Gi = Gilirang, and w = water. 1 and 2 indicate members of group 1 and 2, respectively.

3.3 Genotyping using FM-E2A marker

An earlier report has found the *badh2.2* mutation in fragrant rice (Shi *et al.* 2008). Therefore, experiments were done to compare non-fragrance and fragrance PCR profiles using the exon 2 fragrance marker (FM-E2A). However, the results (Figure 5) showed no differences in the PCR profiles of all samples.

The fact that no difference between fragrant, as well as with those of non-fragrant rice samples (Figure 5) in the PCR profiles was obtained using the FM-E2A marker (Shi *et al.* 2008) suggested that the previously reported exon 2-*badh2* mutation is not applicable to most of the examined Indonesian fragrant rice samples.

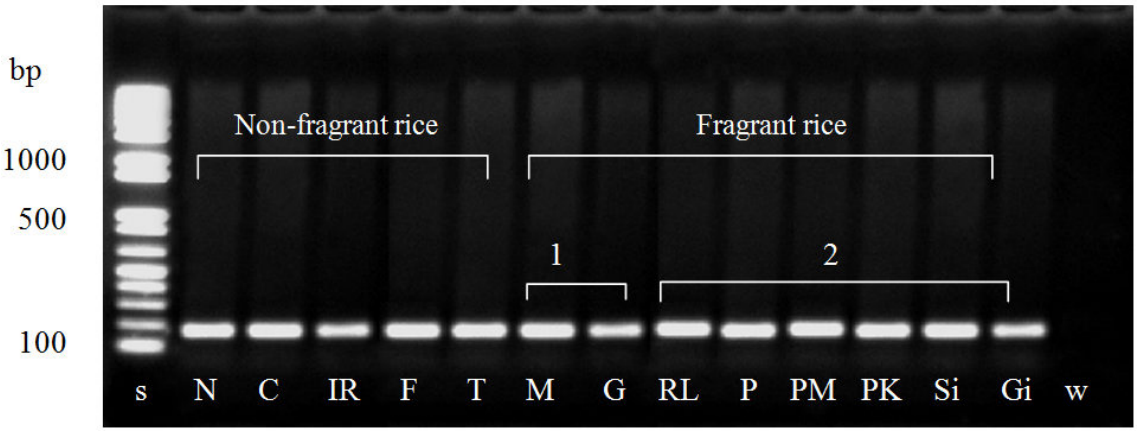

Figure 5. FM-E2A-PCR profiles.
s = standard size marker, N = Nippon bare, C = Ciherang, IR = IR64, F = Fatmawati, T = Taipei 306, M = Mentik Wangi, G = Gunung Perak, RL= Rojo Lele, P = Pandan Wangi, PM = Pulu Mandoti, PK = Pare Kembang, Si = Sintanur, Gi = Gilirang, and w = water. 1 and 2 indicate members of group 1 and 2, respectively.

3.4 Genotyping using the RM223 marker

In attempt to find suitable fragrance marker for Pandan Wangi or Rojo Lele (group 2), their PCR profiles were further compared with non-fragrant Ciherang and fragrant rice of group 1 (Mentik Wangi and Gunung Perak) using the RM223 marker of Lang and Buu (2008) as primer. The results (Figure 6) showed that RM223 was able to discriminate Pandan Wangi and Rojo Lele from non-fragrant Ciherang. The size of the group 2 amplicons was observed to be smaller than those of the group 1 fragrant rice variety samples.

Apart from the ability of the RM 223 SSR marker (Lang and Buu 2008) to discriminate fragrant from non-fragrant rice samples (Figure 6), the results also suggest that there are differences between group 1 (Mentik Wangi and Gunung Perak) and group 2 (Pandan Wangi and Rojo Lele) rice samples, and that the size of group 1amplicons were also found to be smaller than those group 2. This supports the results obtained previously using the Bradbury marker (Bradbury et al 2005b) in Figure 1.

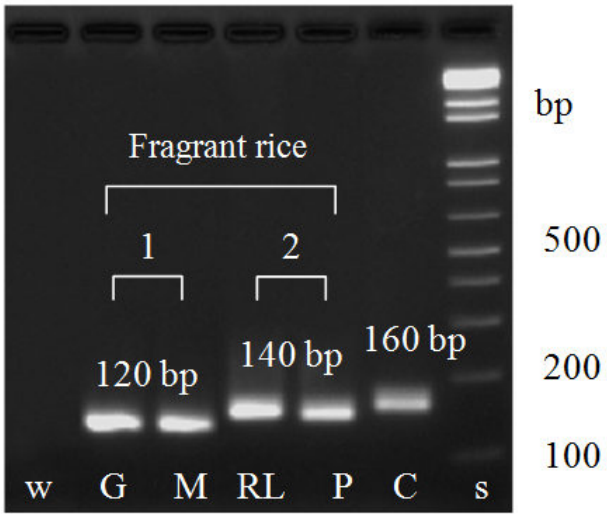

Figure 5. RM233-PCR profiles.
w = water; G and M= Gunung Perak and Mentik Wangi (~120 bp); RL and P = Rojo Lele and Pandan Wangi (~140 bp); C = non-fragrant Ciherang (~160 bp); and s = standard marker. 1 and 2 indicate members of group 1 and 2, respectively.

4. Conclusion

The results of fragrance genotyping using various fragrance (Bradbury, FM-E7, Badex 7-5, and RM 223) markers supported the view that there are at least 2 groups of exon7 *badh2*-mutation patterns among Indonesian fragrant rice varieties. Group 1 includes mentik Wangi and Gunung Perak; while group 2 includes Pandan wangi, Rojo Lele, Pulu mandoti, Pare Kembang, and Sintanur. No *badh2.2* mutation was found among Indonesian rice

varieties.

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