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SCREENING OF AROMATIC RICE LINES BY USING MOLECULAR MARKER AND SENSORY TEST

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ABSTRACT

Aromatic rice has become popular owing to its aroma. Growing demand for aromatic rice has spurred interest of breeders. In this study, twenty five BC_1F_1 rice lines of backcross hybrid combinations were used to evaluate aromatic characteristics through sensory test and using microsatellite markers. Twelve lines containing aroma and one without aroma were chosen through sensory test. Allele Specific Amplification with four primers (External Antisense Primer, External Sense Primer, Internal Non-fragrant Sense Primer, and Internal Fragrant Antisense Primer) were used for identifying fgr gene locus in 25 rice lines. Analysis of PCR results showed that BC_1F_1 population segregated into 1:1 ratio (homozygous fragrant: heterozygous non-fragrant). Thus, twelve promising lines were identified with homozygous fragrant genotype.

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1 INTRODUCTION

Grain aroma is one of the most attractive characteristic of high quality rice. Numerous chemical constituents such as different volatile compounds are the major sources of aroma in cooked rice along with environmental factors. Bergman *et al*. (2002) reported that 2-acetyl-1-pyrroline is the key aroma constituent of fragrant rice. Identification of aromatic in rice grain has been developed traditionally by selection, hybridization and back crossing. The conventional methods of plant selection for aroma are not easy because of the environment factors and the low narrow sense heritability of aroma. More recently molecular markers, such as SNPs and simple sequence repeats (SSRs), which are genetically linked to fragrance have been developed for the selection of fragrant rice (Cordeiro *et al*., 2002). Previous studies reported that chromosomes (3, 4, 8 and 12) were implicated through mapping with aromatic trait (Loriex *et al*., 1996; Amarawathi *et al*., 2008). Lorieux *et al*. (1996) confirmed close linkage between RG28 and fgr (5,8 cM) on chromosome 8 and identified two quantitative trait loci for fragrance, on chromosome 4 and chromosome 12. Then, Bradbury *et al*. (2005a) showed that a functional BADH2 enzyme inhibits 2AP biosynthesis which is a major component of aroma. Non-fragrant varieties possess a fully functional copy of the gene encoding BAD2 while fragrant varieties possess a copy of the gene containing eight base pair deletion resulting in a frame shift mutation disabling the BAD2 enzyme. Recent studies by Kovach *et al*. (2009) also confirmed that BADH2 is the major genetic determinant of fragrance in rice. Ghaffar (2011) also used BADH2 primer for monitoring the presence or absence of aroma genes in F_2 population of rice. This experiment was conducted to choose aromatic rice lines using molecular markers and sensory test.

2 METHODS

2.1 Plant material

ST20 (Soc Trang) were used as control for aroma and OM10043 (OM6677 / OM1490) for nonaroma. A segregating population was developed by crossing ST20 (a aromatic variety) with OM10043 (a non-aromatic variety with bph4 gene related to tolerance to all four biotypes of brown planthopper) for developing tolerant to brown planthopper of aromatic rice lines at the Biotechnology Research and Development Insitute. Twentyfive BC_1F_1 rice genotypes along with their parents were used to evaluate aroma detection through sensory test and genotypic analysis using microsatellite markers. BC_1 individuals were generated by crossing the F_1 plants (heterozygous non-fragrant) with parent ST20.

2.2 Sensory test using KOH method

Twenty five BC_1F_1 seeds were grown under net house condition. At 30 days after sowing, determination of presence or absence of aroma was made according to the method described by Sood and Siddiq (1978). Two grams of green leaves were taken from individual plants cut into small pieces and kept in the test tubes. Ten mL of 1.7% potassium hydroxide (KOH) solution was added to each test tube. The test tubes were covered immediately and left at 65°C temperature for about 20 minutes. The test tubes were opened one by one and was immediately evaluated by smelling. Parents are used as controls. The samples were scored on 0-2 scale with 0, 1 and 2 corresponding to non-aroma, slight aroma and aroma, respectively. According to the average of rating scale value, samples were divided into 3 groups: aromatic (> 1.0), questionable (0.5- 1.0), non - aromatic (< 0.5). The score for each sample was randomly recorded by a panel of ten people at age of 20 to 22.

2.3 Molecular marker for genotyping

DNA was isolated from 10 to 15 days old plant leaf samples (~100 mg each) using CTAB method (Roger and Bendich, 1988) from 25 lines rice and parents. In this study, we have used markers from previous study (Bradbury *et al*., 2005) for screening aroma in BC_1F_1 hybrids, namely EAP (5′-AGTGCTTTACAAAGTCCCGC-3′), ESP (5′-TTGTTTGGAGCTTGCTGATG-3′), INSP (5′-TGGTAAAAAGATTATGGCTTCA-3′) and IFAP (5′-CATAGGAGCAGCTGAAATATATACC-3′) in a multiplex PCR. The PCR reaction was conducted in a reaction volume of 25 µL containing 3.25 µL of double distilled water (DDW); 0.25 µL of Taq DNA Polymerase (~1,25 unit); 4 µL of genomic DNA (~50-100 ng); 2.5 µL of 10X buffer, 4 µL of 2 nM $MgCl_2$, 3 µL of 0.2mM dNTPs, 2 µL of each primer (~200nM). Polymerase chain reaction (PCR) amplification was performed with initial denaturation at 95°C for 3 minutes followed by 35 cycles of 95°C for 30 second, 59°C for 30 second, 72°C 30 second and final extension at 72°C for 5 min before cooling at 10°C. Amplification products were stored at 20°C till further use. PCR products were analyzed by electrophoresis in Safe View (0.5 µg/mL) 1.5% agarose gel was used. A 100 bp ladder molecular weight standard was used to estimate PCR fragment size.

3 RESULTS AND DISCUSSION

3.1 Aroma detection by KOH

In this investigation, leaf of selected parents (2 genotypes) and their BC_1F_1 hybrid individuals were used for aroma evaluation. The lines were classified into three groups including fragrant, slight fragrant and non-fragrant. There are twelve lines including line 3, line 5, line 6, line 7, line 9, line 10, line 18, line 19, line 15, line16, line 21 and line 22 were scored as fragrant, while twelve lines were ranked as questionable. Line 1 was scored as non-fragrant. This result could be due to this method is a crude method thus the ability to distinguish between mildly aromatic and non-aromatic samples is limited. The chances of error by any analyst cannot be ruled out, thus using molecular marker for detecting aroma trait were used this research.

3.2 Aroma detection by molecular markers

Aroma is one of the most important traits of aromatic rice grain quality. Molecular markers to the aroma are now available to facilitate direct selection in a rice breeding program. In this result, BADH2 primer (Bradbury *et al*., 2005b) was used to identify aroma gene. Bradbury *et al*. (2005b) reported that the relationship between this marker and fragrance trait holds in 100%.

As shown in Figure 1, external primers, ESP and EAP produced expected fragment of approximately 580 bp as a positive control in all genotypes. Internal primers, INSP and IFAP produced two bands of 355 and 257 bp when paired with external primers, ESP and EAP. IFAP primer has been designed specifically for detecting aromatic genotype and INSP primer has been designed specifically for identifying non-aromatic genotype. Accordingly, ESP and IFAP primer pair amplified a 257 bp band showing the marker for fragrant genotype. Results show that 13 lines: 4, 6, 8, 9, 10, 12,13 (Fig. 1) and 5, 6, 8, 9, 11, 12 (Fig. 2) were homozygous aromatic, 12 remaining lines were heterozygous genotype in ST20×OM10043.

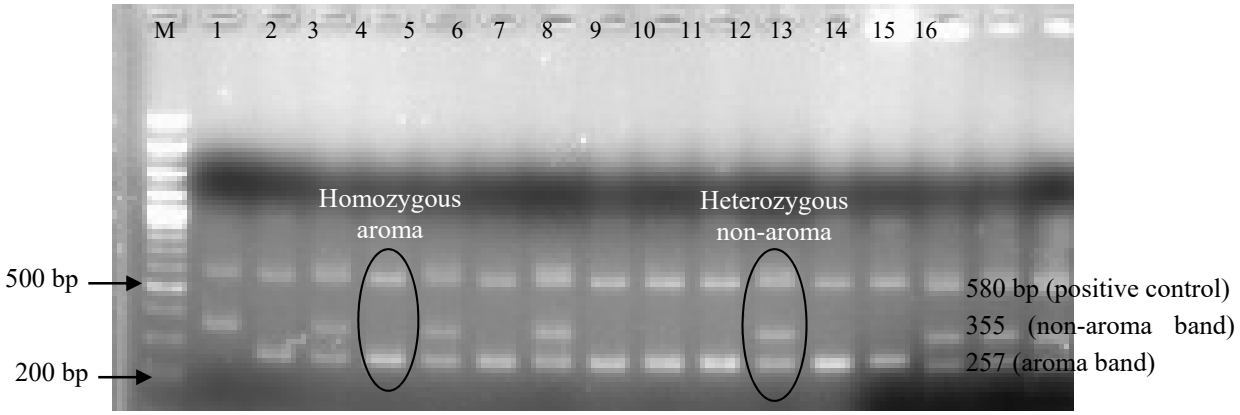

Fig. 1: PCR analysis of BC_1F_1 plants in ST20 (aromatic) x OM10043 (non-aromatic) population for presence of aroma gene. Lane 2 (ST20), Lane 4, 6, 8, 9, 10, 12 and 13 are homozygous aromatic plants; Lane 1 (OM10043) is non aromatic; Lane 3 (F_1), 5, 7, 11, 13, 14 and 16 are heterozygous genotype and M is 100 bp ladder

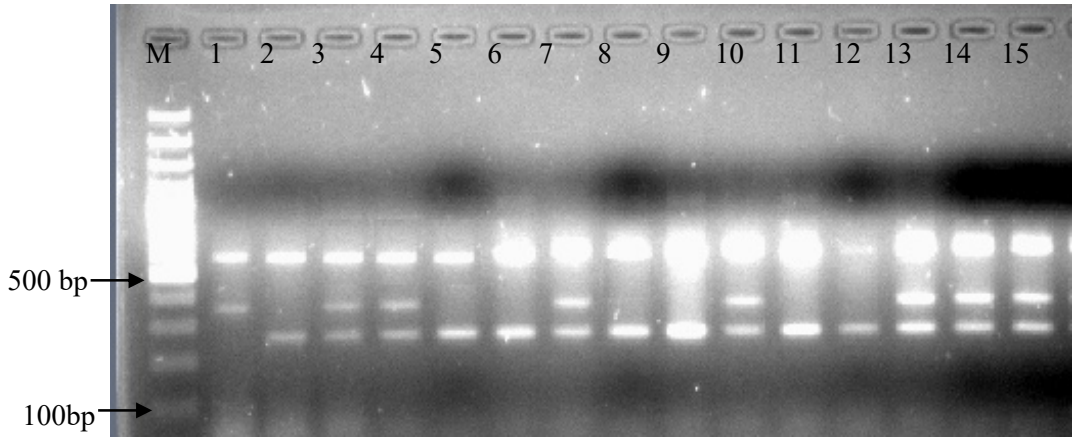

Fig. 2: PCR analysis of BC_1F_1 plants in ST20 (aromatic) x OM10043 (non-aromatic) population for presence of aroma gene. Lane 2 (ST20), Lane 5, 6, 8, 9, 11 and 12 are homozygous aromatic plants; Lane 1 (OM10043) is non aromatic; Lane 3 (F_1), 4, 7, 10, 13, 14 and 15 are heterozygous genotype and M is 100 bp ladder

ST20 rice is known for its typical aroma, whereas OM10043 was non-aromatic. Based on the Mendel's law of segreration, in case a genotype of ST20 plant is aa cross with AA (OM10043), the theoretical ratio of F_1 plant is Aa, being the backcross with aa, BC_1F_1 phenotype segreration could be expected to be 1Aa:1aa. In this result, the joint segregation analyses in BC_1F_1 showed significant chi-square (χ^2) value, indicating that homozygous fragrant and heterozygous non-fragrant segregated into 1:1 ratio.

BADH2 primer was used in aromatic rice breeding programs to produced new aromatic rice lines. Mohamad *et al*. (2008) reported that there were 28 homozygous aromatic: 2 heterozygous non-aromatic: 45 homozygous non-aromatic, indicating 28 aromatic and 47 non-aromatic rice individuals in their screening process. Meanwhile, another group of researchers, Bounphanousay *et al*. (2008), detected 36 homozygous aromatic: 3 heterozygous non-aromatic: 17 homozygous non-aromatic. While, Sarhadi *et al*. (2008) found 10 aromatic: 18 nonaromatic. These researchers confirmed that this primer would high-efficient detect this aromatic allele.

Table 1: Phenotypic and genotypic screening of BC_1F_1 plants in populations of rice

Veriety	Molecular method		Sensory method		
	Homozygous aromatic plants	Heterozygous non-aromatic plants	Aroma	Questionable	Non- aromatic
Line 1	X				X
Line 2		X		X	
Line 3	X		X		
Line 4		X		X	
Line 5	X		X		
Line 6	X		X		
Line 7	X		X		
Line 8		X		X	
Line 9	X		X		
Line 10	X		X		
Line 11		X		X	
Line 12		X		X	
Line 13		X		X	
Line 14		X		X	
Line 15	X		X		
Line 16	X		X		
Line 17		X		X	
Line 18	X		X		
Line 19	X		X		
Line 20		X		X	
Line 21	X		X		
Line 22	X		X		
Line 23		X		X	
Line 24		X		X	
Line 25		X		X	

Integration of sensory methods and molecular markers to detect the presence or absence of aroma in BC_1F_1 population (Table 1) showed that the BC_1F_1 individuals which were classified having aroma in sensory test also showed presence of aroma alleles. In other case, line 1 had aroma alleles but it still showed non-aromatic in KOH test. These results indicated that only molecular marker analysis or sensory methods could not represent the complete aromatic conditions. In 2008, Bounphanousay *et al*. (2008) showed that the results of molecular marker and chemical analysis were same in most of the rice varieties, except local aromatic rice variety, Kai Noi Leuang, which was identified as homozygous non-aromatic by molecular marker analysis but produced aroma. They suggested that different gene location might be responsible for the observed aroma or the presence of another major aromatic compound. Another research of Yi *et al*. (2009) also reported that variation in the sensory score may arise from minor genes or environmental factors and that some rice varieties may carry minor QTLs which have an influence on rice aroma.

4 CONCLUSIONS AND SUGGESTIONS

Molecular marker is a powerful tool for detecting aromatic trait of rice effectively. From this research, it can be concluded that twelve aroma lines with homozygous aromatic gene were chosen based on two methods for next research in aromatic rice breeding programme.

For further study, sensitive method such as gas chromatography/mass-spectrometry, should be used to detect aroma rice beside the two above methods.

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