J. Agric. Sci. Technol. (2004) Vol. 6: 129-137

Mapping the Gene for Aroma in Rice (*Oryza sativa* L.) by Bulk Segregant Analysis via RAPD Markers

Gh. A. Nematzadeh[1], N. Huang[2] and G. S. Khush[2]

ABSTRACT

The sensory qualities of food such as aroma, taste, and texture are the most important criteria for distinguishing Basmati type rices from non-Basmati types. To map the gene(s) controlling aroma, bulked segregant analysis (BSA) using Random Amplified Polymorphic DNA (RAPD) markers was applied in an F_2/F_3 population of Basmati 370 (aromatic) and IR36 (non-aromatic). DNA samples from homozygous aromatic and homozygous non-aromatic plants identified on the basis of progeny tests were bulked and used for BSA. A total of 550 random primers were used and the primers, AG8 and AN1, produced polymorphism between aromatic and non-aromatic types. Association of AG8-AR, AN1-AR1, and AN1-AR2 with a gene for aroma was determined by surveying F_2 individuals. The three RAPD markers AG8-AR, AN1-AR1, and AN1-AR2 were found to be linked to the gene for aroma with a distance of 6.9, 8.9 and 16.4 cM, respectively. Southern analysis with AG8-AR as a probe with 125 F_2 individuals confirmed linkage between AG8-AR and the gene for aroma. AG8-AR was mapped on chromosome 8 flanked by two tightly linked markers, RZ617 and RG978, at 2.1 and 1.7 cM distances, respectively, indicating that this gene in Basmati 370 is located on chromosome 8.

Keywords: Basmati rice, Map of aroma gene, RFLP and RAPD markers.

INTRODUCTION

Aromatic rices command a special price in national and international markets. Basmati 370, one of the donors for aroma, is being used in breeding programs worldwide. It is characterized by its tall stature, droopy leaves, intermediate amylose content and intermediate gelatinization temperature. It also boasts a soft gel consistency, slender and translucent grains, and good grain elongation ability. However, Basmati 370 has a low yield potential.

Breeding aromatic varieties with high yield potential is one of the major breeding objectives in many rice improvement programs. The lack of desirable recombinants from crosses involving modern semidwarf varieties and Basmati varieties is one of the major barriers to developing aromatic rice with a high yield potential.

Although the inheritance of aroma has been studied extensively, the results are inconsistent. Some studies reported a single recessive gene for aroma (Sood and Siddiq, 1978; Berner and Hoff, 1986; Huang and Zou, 1992; Dong *et al*., 1992; Ali *et al*., 1993). Pinson (1994) reported that aroma is controlled by a single recessive gene in Jasmine 85 and p1467917 and by two genes in Amber and Dragon Eyeball. Digenic segregation for aroma were also reported by Lin (1991). A single recessive gene for aroma in Lemont was mapped on chromosome 8 through Restriction Fragment Length Polymorphism (RFLP) analysis (Ahn *et al*., 1992).

[1] College of Agricultural, Mazandaran University, P. O. Box:578, Islamic Republic of Iran.
[2] Division of Plant Breeding, Genetics and Biochemistry, International Rice Research Institute. P. O. box: 933, 1099. Manila. Philippines.

RFLP markers are most commonly used in genetic map construction and gene mapping. In rice, several agronomical important genes have been mapped using RFLP markers (see Hittalmani *et al.*, 1995 for a review). The development of RAPD analysis (Williams *et al.,* 1990) speeds up the process of gene mapping. The analysis is based on the amplification of genomic DNA with random decamer using polymerase chain reaction (PCR). The RAPD technique has been used to construct genetic maps of plant species (Reiter *et al*., 1992).

Combining RAPD analysis with bulked segregant analysis (BSA), Michelmore *et al.* (1991) demonstrated the possibility of identifying markers linked to specific gene or genomic region. The RAPD technique has been used to map genes for resistance to bacterial blight in rice (Zhang *et al*., 1994). Here, we report the RAPD mapping of a gene for aroma via BSA in the cross Basmati 370/IR36.

MATERIALS AND METHODS

Population Development

Basmati 370, an aromatic variety (as female parent), has been crossed with IR36, an improved high-yielding non-aromatic variety, in 1995 at the International Rice Research Institute (IRRI). The F_1 was grown and selfed to produce an F_2 population. F_2 plants and their progeny, F_3 families, were used for linkage mapping. Linkage analysis was performed using an RAPD marker via BSA and the RAPD markers linked to the gene for aroma were analyzed with the MAPMAKER computer program (Lander *et al*., 1987).

Aroma Evaluation

Aroma was evaluated according to the method suggested by Sood and Siddiq (1978). Seeds from 125 F_2 plants (F_3 seeds) were dehulled using a Satake dehuller and milled with test tube miller for one hour. Rice kernels were then individually ground for 10 seconds at a medium speed using a Wig-L-Bug grinder. Rice powder from each grain was placed in a 5-x-5-cm plastic box and 500 µI of 1.70 % KOH was added. The boxes were kept at room temperature (25$^{\circ C}$). After 30 minutes, each box was opened and the degree of aroma was evaluated by sniffing and was scored using the following scale: 1= non-aromatic; 2= slightly aromatic; 3= moderately aromatic; and 4= strongly aromatic. Initially, 10 grains per F_3 family were analyzed. Genotypes of corresponding F_2 plants were determined to be homozygous for aromatic when all 10 seeds were moderately or strongly aromatic, or homozygous non-aromatic when all 10 seeds were non-aromatic. To confirm the homozygosity, an additional 30 seeds from the homozygous families were analyzed. To examine the distribution, the aromatic values of all 10 grains were summed up and plotted.

DNA Manipulation

DNA from 125 F_2 plants as well as from Basmati 370 and IR36; along with another mapping population, including 135 doubled haploid lines and their parents Azucena /IR64, were extracted from fresh leaf tissues according to Dellaporta *et al.* (1984). A portion of the genomic DNA was diluted for BSA using RAPD markers, and other portions of the genomic DNA of Basmati 370 and IR36 along with Azucena and IR64 were digested with 19 restriction enzymes (*Eco*RI, *Eco*RV, *Sca*I, *Xho*I, *Dra*I, *Apa*I, *Bgl*II, *Xba*I, *Bam*HI, *Rsa*I, *Hae*III, *Msp*I, *Bst*E, *Sma*I, *Aat*I, *Hin*dIII, *Hin*FI, *Alu*I *and Taq*I). The digested DNA was then subjected to electrophoresis on 0.9% agarose gels and transferred to Hybond N+ membrane according to the manufacturer's instructions. Further analysis was done using the Southern blotting technique (Ausubel *et al*., 1993).

Bulked Segregant Analysis

The genetic nature of any F_2 single plant was determined through the phenotyping aroma analysis of F_3 single plant (allelism test). Then the same quantity of DNA was bulked from every eight homozygous aromatic the same as from eight homozygous non-aromatic F_2 plants. The two bulk samples were used as templates for RAPD analysis along with DNA from Basmati 370 and IR36.

Genetic Markers

The genetic markers used for surveying of the parental lines were 550 RAPD primers (ten-base oligonucleotide primers purchased from Operon technologies, Alameda, California, USA). The reaction conditions, were as follows: 2.5ml dNTP 1 mM, 1.5 unit *Taq* DNA polymerase, 1.5 ml primer 10 mM, 2.5 mM PCR buffer 10X, 3.2 ml $Mgcl_2$ 15 mM and 3 ml DNA template (50 ng) including 10.8 ml pure water, as described by Williams *et al*. (1990). The PCR profile amplification was done according to the following method:

Starting with 94°C for 2 minutes, then 45 cycles run continuously for 1 minute at 92°C (denaturation), 1 minute at 34°C (annealing) and 2 minutes at 72°C (extension).

Localization of Linked Markers on the Rice Chromosome

A linkage between RAPD markers and the aroma gene was determined through bulk segregant analysis (BSA) in the F_2 and F_3 populations then the chromosomal location of these linked markers was determined on a mapping population consisting of DH lines generated from a cross between Azucena and IR64 through anther culture (Huang *et al*. 1994). A polymorphism survey of the parental lines, Azucena and IR64, was carried out with the RAPD markers isolated from agarose gel. For southern blotting the membranes were hybridized with amplified RAPD product (polymorphic band) that had been ^{32}P-labeled by the random hexamer method (Feinberg and Vogelstein, 1984).

RESULTS AND DISCUSSION

Genetic Analysis of Aroma

The result of F_3 aroma analysis indicated that the minimum aromatic value for a family is 10 which is the same as IR36 while the maximum is 40 as for Basmati 370 .The aromatic values were plotted and the result

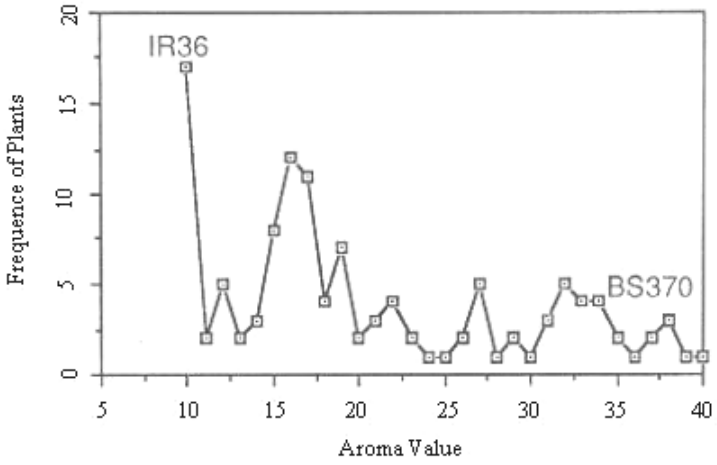

Figure 1. Distribution of aromatic values of each F_3 family in a population derived from Basmati 370 and IR36. See text for the measurement of aromatic values for each family.

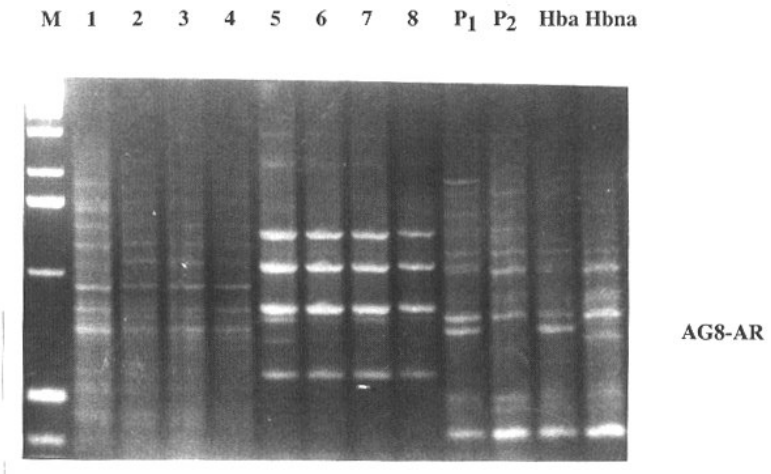

Figure 2. RAPD products generated by primers AG6, AG7, and AG8 among Basmati 370 (P1), IR36 (I), homozygous bulk aromatic (Hba), and homozygous bluk non-aromatic (Hbna). Putative linked marker (AG8 - AR) was revealed by RAPD marker, AG8. M= Molecular weight marker. The primer sequence of AG8 is GGTGGCCAAG, AG7 is CACAGACCTG, and AG8 is AAGAGCCCTC.

did not indicate any clear-cut single gene segregation (Figure 1).

Bulked Segregant Analysis

The RAPD patterns were obtained using DNA from Basmati 375(B), IR36(I), homozygous aromatic bulk (Hba) and homozygous non-aromatic bulk (Hbna). More than 90% could amplify the template DNA out of 550 RAPD primers used (Figure 2). An average of eight bands for each primer was scored, of which two were polymorphic between Basmati 370 and IR36. Most of the polymorphic RAPD bands in the parents were also reproduced in both the aromatic and non-aromatic bulk or at

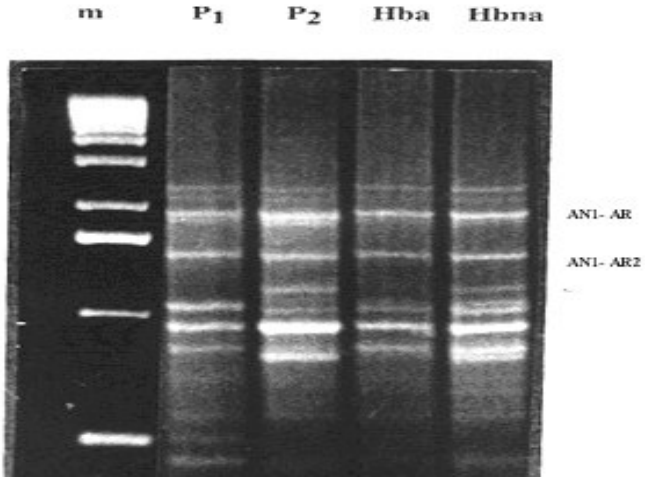

Figure 3. Polymorphism revealed by RAPD markers (AN1-AR and AN1-AR2) for IR36 (P1), Basmati370, homozygous bulk of aroma (Hba) and homozygous bulk of non aroma (Hbna). M= Molecular marker (kb ladder BRL).

least in one bulk. Only two primers, AG8 and AN1, showed polymorphism between basmati / homozygous aromatic bulk and IR36/ homozygous non-aromatic bulk. An 800-bp band from primer AG8 was amplified from DNA of Basmati 370 and homozygous aromatic bulk (Figure 2), and two 1200-and 900-bp bands from AN1 were amplified only from IR36 and homozygous nonaromatic bulk (Figure 3). Linkage analysis between AG8-AR, AN1-AR1, AN1-AR2 and the gene for aroma was done using the segregating F_2 population and a linkage map was generated (Figure 4). The product was labeled with ^{32}P and used as a probe for parental survey. The marker AG8-AR showed polymorphism between Basmati 370 and IR36 only, when digested with the *Taq*I restriction enzyme, while it showed polymorphism between the parents of the DH (double haploid) mapping population (IR64/Azucena). AN1-AR1 and AN1-AR2 RAPD markers did not show any polymorphism between parents in spite of the 19 restriction enzymes used, so the further analysis of AN1-AR1 and AN1-AR2 was not continued.

AG8-AR was mapped on a dihaploid

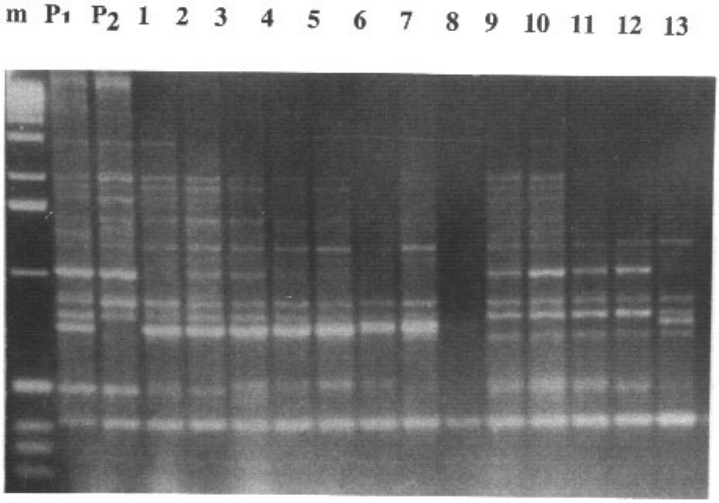

Figure 4. Linkage analysis of aroma in a cross between Basmati370 (P_1), IR36 (P_2) and (F_2) using AG8-AR via the PCR technique. M= Molecular weight marker (Kb ladder, BRL). Lanes 1-8 =F_2 homozygouse aromatic plants and 9-13 = F_2 homozygouse non-aromatic plants.

RAPD markers flanked the gene for aroma with less than 10 cM.

Locating the RAPD Marker AG8-AR on the Rice Chromosome

The marker AG8-AR (RAPD band) was isolated from agarose gel and reamplified by the AG8 RAPD primer. To identify a probe-enzyme combination revealing polymorphism between Basmati 370 and IR36, extracted DNA was digested with a set of 19 restriction enzymes. The PCR mapping population (135 lines) of IR64/Azucena, available at IRRI (Huang *et al*., 1994). The genomic DNA of dihaploid lines were digested with a *Taq*I restriction enzyme and Southern blot analysis was done using AG8-AR (RAPD reamplified band). The autoradiogram from hybridization of AG8-AR with genomic DNA of the dihaploid line showed 43 homozygous lines with Azucena allele (lower band) and 88 homozygous individuals with IR64 allele (upper band) (Figure 5).

The data of AG8-AR were integrated with the existing RFLP data set of DH population

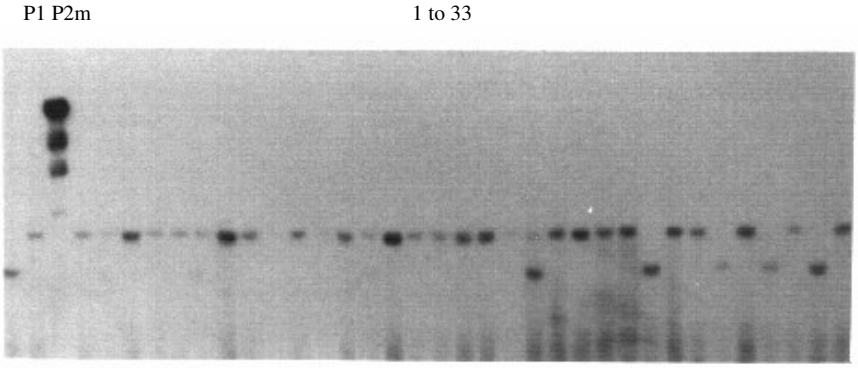

Figure 5. Segregation pattern of double haploid lines (DHlS) in IR64 (I) /Azucena (A) cross (Huang *et al*., 1994), using RAPD marker (AG8-AR) via Southern analysis when digested with *Taq*I. M= molecular weight marker (λ/*Hind*lll). Lower band indicates Azucena allele and upper band indicates IR64 allele.

and analyzed using MAPMAKER software. The location of AG8-AR was determined on the basis of its linkage with RFLP markers on the linkage map.

Results indicate that the marker (AG8-AR) is located on chromosome 8. RFLP markers RZ617 and RG978 on chromosome 8 were found to be 2.1 and 1.7 cM away from AG8-AR (Figure 6A and B). The gene for aroma is therefore located on chromosome 8 near RZ617 and RG978.

There is a wide range of rice varieties in the world. The lifestyles of rice consumers and rice cooking methods are also different. Aroma, taste, and texture are the three principal sensory characteristics that determine the quality of cooked rice. Of these, aroma is the most important.

Various techniques have been used to evaluate rice aroma to study its inheritance. However, there is no consensus about the mode of inheritance of aroma. A large

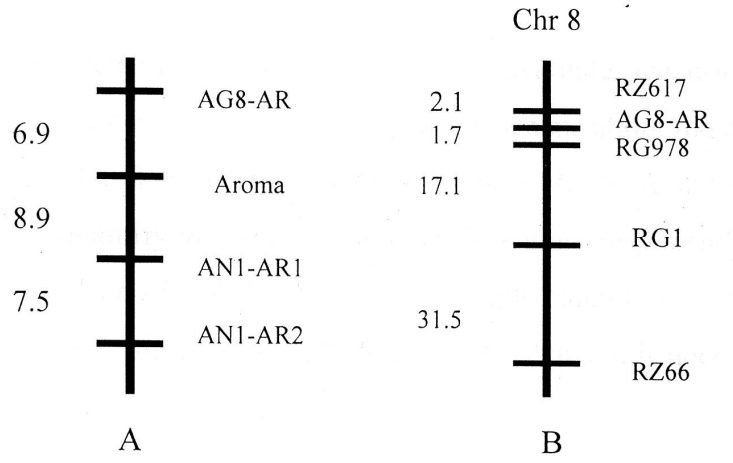

Figure 6. A. Genetic map of the aroma gene and its linked RAPD markers. Numbers are genetic distances between loci in cM. B. Chromosomal localization of the RAPD marker, AG8-AR, linked to the aroma gene. The localization is based on the doubled haploid mapping population (Huang *et al*. 1994). Genetic distances between marker loci in cM are given on the left.

amount of samples (at least up to 3 g) is required to quantify the volatile compounds that are responsible for aroma (Uraiwan *et al*. 1991). Such a large sample cannot be obtained from single grains of F_2, F_3 or BC populations. Thus, it is necessary to study recombinant inbred lines or DH lines.

This study was carried out to map the gene for aroma in an aromatic rice variety, Basmati 370, using RAPD markers via BSA. Among the various molecular markers, RAPD markers are among the suitable DNA markers that can be applied for various purposes in biological research. BSA as a rapid and alternative technique to RFLP can easily be utilized for identifying markers linked to a specific gene. We report here an amplified RAPD product, 800 bp, which was generated by AG8 RAPD primer, closely linked to the gene for aroma (Figure 6A). This RAPD marker was mapped on chromosome 8 between two tightly flanking RFLP markers, RZ617 and RG978 (Figure 6B). Another gene for aroma, *fgr*, was mapped on chromosome 8 using a different population involving the aromatic variety, Lemont, via RFLP analysis (Ahn *et al*., 1992). It has been linked to RG28. The aromatic allele of Lemont was derived from Della. An allelic test is being carried out to see if the gene for aroma from Basmati is different from the gene from Della.

Loriex *et al.* (1996) confirmed the close linkage between RG28 and aroma (5.8 cM), and concluded that a single recessive gene was responsible for the production of aromatic plants. The same result (single recessive gene controlling aroma) was obtained in this research with different aromatic varieties in the same chromosome number, but at different locations. Given that RZ617 and RG978 were linked with a 2.1 and 1.7 cM distance to the AG8-Ar RAPD marker and also this marker is linked with 6.9 cM distance to the aroma gene, thereby these markers are suitable for different genotypes of aroma and the large numbers of aromatic and non aromatic varieties identified. These markers will be highly useful in the Iranian rice breeding program as well as rice breeding programs in general.

ACKNOWLEDGEMENT

The authors would like to thank Arnold Parco and Gerard Magpantay for their technical assistance. The technical support of GML and the quality lab of IRRI, and especially of Mrs. N. D. Cruz is also greatly appreciated.

REFERENCES

1. Ahn, S. W., Bollich, C. N. and Tanksley, S. D. 1992. RFLP Tagging of a Gene for Aroma in Rice. *Theor. Appl. Genet*., **84**: 825-828.
2. Ali, S. S., Jafri, S. J. H. Khan, M. J. and Butt, M. A. 1993. Inheritance Studies for Aroma in Two Aromatic Varieties of Pakistan. *Int. Rice Res. Newsl*., **18**: 2-6.
3. Ausubel, F. M., Brent, R. Kingston, R. E. Moore, D. D. Seidman, J. G. Smith, J. A. and Struhl. K. 1993. *Current protocols in Molecular Biology*. Greene Publishing Associations inc. New York, USA.
4. Berner, D. R. and Hoff, B. J. 1986. Inheritance of Scent in America Long Grain Rice. *Crop Sci*., **26**: 876-878.
5. Dellaporta, S. L., wood, J. and Hicks, J. B. 1993. A Plant DNA Minipreparation: Version ll. *Plant Mol. Biol. Rep*., **1**: 19-21.
6. Dong, Y., Zhang, H. and Shi, S. 1992. Genetic Studies of Aroma in the Elite Cytoplasmic Male Sterile (CMS) Aromatic Japonica Line Shanghai A. *Int. Rice Res. Newsl*., **17**: 2-5.
7. Feinberg, A. P. and Vogelistein, B. 1984. A Technique for Radiolabeling DNA Restriction Fragment to a High Specific Activity. *Anal. Biochem*., **132**: 6-13.
8. Hittalmani, S., Mew, T. Khush, G. S. and Huang, N. 1995. DNA Marker-assisted Selection for Disease Resistance in Rice. *Proceedings of the International Rice Research Conference*. International Rice Research Institute, Manila, Philippines.
9. Huang, N., McCouch, S. R. Mew, T. and Parco, A. 1994. Development of an RFLP Map from a Doubled Haploid Population in Rice. *Rice Genet. Newsl*., **11**: 134-137.

10. Huang, Q. H. and Zou, X. Y. 1992. Inheritance of Aroma in Two Aromatic Rice Varieties. *Int. Rice Res. Newsl*. **17**: 5-6.
11. Lander, E. S., Green, P. Abrahamson, J. Barlow, A. Daly, M. J. Lin-Coin, S. E. and Newburg, L. 1987. MAPMAKER: An Interactive Computer Package for Constructing Primary Genetic Linkage Maps of Experimental and Natural Population. *Genomics* **1**: 174-181.
12. Loriex, M., Petrov, M. Huang, N. Guiderdoni, E. and Ghesquiere, A. 1996. Aroma in Rice: Genetic Analysis of Quantitative Traits. *Theor. Appl. Genet*., **93**: 1145-1151.
13. Lin, S. 1991. Rice Aroma: Methods of Evaluation and Genetics. In: *Rice Genetics*ll, (Eds.) International Rice Research Institute, Manila, Philippines.
14. Michelmore, R. W., Paran I. and Kesseli, R.V. 1991. Identification of Markers Linked to Disease Resistance Genes by Bulked Segregant Analysis. A Rapid Method to Detect Marker in Specific Genomic Regions by Using Segregating Population. *Proc. Natl. Acad. Sci. USA*, **88**: 9828-9832.
15. Pinson, S. R. M. 1994. Inheritance of Aroma in Six Rice Cultivars. *Crop Sci*., **34**: 1151-1157.
16. Reiter, R. S., Williams, J. G. K. Feldman, R. A. Rafalski, J. A. Tingey, S. V. and Scolnik. P. A. 1992. Global and Local Genome Mapping *Arabidopsis thaliana* by Using Recombinant Inbred Lines and Random Amplified Polymorphic DNA. Proc. *Natl. Acad. Sci. USA*., **89**: 1477-1481.
17. Sood, B. C. and Siddiq, E. A. 1978. A Rapid Technique for Scent Determination in Rice. *Indian J. Genet. Plant Breed*., **38**: 268-271.
18. Uraiwan, T., Thomas, C. and Hsieh, Y. 1991. An Improved Method for Quantification of 2-acetyl-1 Pyroline, a "Popcorn"-like Aroma in Aromatic Rice by High-resolution Gas Chromatography/Mass Spectrometry/Selection Ion Monitoring. *J. Agric. Food Chem*., **39**: 944-947.
19. Williams, J. G. K., Kubelik, A. R. Rafalski, J. A. and Tingey, S. V. 1990. DNA Polymorphism Amplified by Arbitrary Primers is Useful as Genetic Markers. *Nucleic Acid Res*., **18**: 6531-6535.
20. Zhang, G., Angeles, E. R. Angeles, M. L. P. Khush, G. S. and Huang, N. 1994. Molecular Mapping of a Bacterial Blight Resistance Gene on Chromosome 8 in Rice. *Rice Genet. Newsl*., **11**: 142-144.

تعیین نقشه ژن عطر و طعم در برنج (*Oryza sativa* L.) از طریق تجزیه توده در حال تفکیک (BSA) با استفاده از مارکرهای RAPD

ق. ع. نعمت‌زاده، ن. هانگ و ج . ص. خوش

چکیده

برنج های معطر در مقایسه با برنج های غیر معطر از اهمیت بیشتری برخوردار هستند. کیفیت خوراک غذا از جمله عطر و طعم، مزه و بافت آن از معیارهای مهم در تشخیص ارقام باسماتی و غیرباسماتی می باشند. برای تعیین نقشه ژن (های) کنترل کننده عطر و طعم در برنج از طریق تجزیه توده در حال تفکیک (BSA) F_2 و F_3 از طریق مارکرهای RAPD استفاده گردید. به کمک آزمون آللی، گیاهان خالص معطر و گیاهان خالص غیرمعطر از یکدیگر شناسائی شده و از توده یا بالک آنها برای تعیین مارکرهای پلی مورفیک استفاده گردید. از مجموع ۵۵۰ پرایمر RAPD آزمون شده فقط پرایمرهای AG8 , AN1 و AN2 تولید

باندهای پلی مورفیک بین انواع معطر و غیرمعطر نمودند. با تجزیه و تحلیل گروه همبستگی (لینکاژ) در جامعهٔ F_2، فاصله ژنتیکی آنها با ژن کنترل کنندهٔ عطر و طعم برنج به ترتیب ٦/٨، ٨/٩ و ١٦/٤سانتی مورگان برآورد شدند. AG8 – AR تکثیر شده مجدداً از روی ژل استخراج و از آن به عنوان کاوشگر (پروب) استفاده گردید و از طریق سادرن بلات با ۱۲۵ تک بوته F_2 مورد بررسی قرار گرفت. نتیجهٔ بدست آمده، تجزیه و تحلیل همبستگی ژن مورد نظر را با پرایمر RAPD مورد تأیید قرار داد. سپس با استفاده از لاین دابل‌هاپلوئید جایگاه نشانگر AG8 – AR بر روی کروموزوم شماره ۸ مشخص گردید. بطوریکه این نشانگر با دو نشانگر جانبی RFLP (RG978 و RZ617) همبستگی خوبی نشان داد و فاصله آنها نیز به ترتیب ۲/۱و ۱/۷ سانتی مورگان برآورد گردید.