

STUDY ON APPLICATION OF MOLECULAR TECHNIQUES (RAPD-PCR AND RAMP-PCR) TO DETECT MUTATION IN RICE BREEDING

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ABSTRACT: Project: “Study on application of molecular techniques (RAPD-PCR and RAMP-PCR) to detect mutation in rice breeding was carried out in 2007 with the purpose of consideration for using the two simple and inexpensive molecular techniques to estimate changes in DNA of rice mutant after gamma irradiation.

Three rice cultivars: Basmati370, Tam Thom (TT1), IR64 and three gamma irradiated mutants BDS, TDS and VND 95-20 respectively, were used. Suitable DNA extraction procedure was obtained. PCR optimization was conducted on three important factors including: amount of MgCl₂, DNA concentration and annealing temperature. 2.5mM of MgCl₂ for RAPD-PCR and 3.75mM for RAMP-PCR were found the best. 40 ng DNA provided a good amplification for RAMP-PCR; this figure was 50ng for RAPD-PCR. Annealing temperatures were determined at 36⁰C for RAPD primer and at 55⁰C for Microsatellite primer.

Final results showed that, both RAPD-PCR and RAMP-PCR could detect changes in DNA of rice mutants after gamma irradiation compared to their parents. Percentage of DNA changes determined by RAPD-PCR and RAMP-PCR on Basmati370 and its mutant BDS were 11.49% and 21.2% respectively; These on TT1 and TDS were 8.98% and 15.4%; and on IR64 and VND 95-20 were 3.45% and 4.95%.

1. INTRODUCTION

Spontaneous and induced mutations are the ultimate source of all existing genetic variation in plants, and are commonly used in plant breeding. However the occurrence of spontaneous mutations in nature is relatively rare and difficult to identify because they are mainly recessive, or are deleterious and quickly eliminated [1]. Therefore, increasing the rate of mutation (ie induced mutations) can provide additional sources of variant genotypes important in plant breeding.

The objectives of this study were to consider whether or not the two simple and inexpensive molecular techniques RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction) and RAMP-PCR (Random Amplified Microsatellite Polymorphism-Polymerase Chain Reaction) can be used to estimate changes in DNA of rice mutant after gamma irradiation in rice breeding.

RAPD-PCR amplifies random genomic DNA sequences using single, short arbitrary primers, and these can be effectively used as genetic markers. The RAPD technique therefore surveys (scans) numerous loci in the genome, which makes this method particularly attractive for analysis of genetic distance and similarity between closely related species [3,4]. A new method to compensate for some weakness in RAPD-PCR has been developed by using microsatellite primers, called RAMP (random amplified microsatellite polymorphisms)-PCR [5]. The method utilizes the ubiquitous and highly polymorphic nature of microsatellites. RAMP-PCR has been reported to

potentially detect and map co-dominant polymorphisms in DNA without cloning and sequencing [6].

2. MATERIALS AND METHODS

2.1. Plant material

Tissue culture leaves of varieties Tam Thom (TT1) and its mutants TDS, Basmati 370 - its mutants BDS, and IR 64 - its mutants VND 95-20 (induced by gamma irradiation) were used.

2.2. Extraction of genomic DNA

Genomic DNA from leaf of rice mutants, varieties Tam Thom (TT1) TDS, Basmati 370 BDS IR 64 and VND 95-20 were extracted using CTAB method [7,8].

2.3. PCR optimization

To obtain reproducible results from a PCR reaction, it was necessary to determine the optimum reaction conditions for each primer. In this project, different amounts of genomic DNA (10, 20, 25, 30, 40 and 50 ng) and three different concentrations of Mg^{2+} (2.5 mM, 3.75 mM and 4.0 mM) were tested, along with the three different PCR program temperature profiles described below.

2.4. RAPD and RAMP amplification

RAPD-PCR was carried out on DNA samples using 50 RAPD primers from UBC set 1 (University of British Columbia) in an Eppendorf Master Cycler Gradient. Each reaction was performed in a final volume of 25 μ l containing 2.5 μ l of 10 X *Taq* Polymerase Reaction Buffer [67 mmol/L Tris-HCl - pH 8.8, 16.6 mmol/L $[NH_4]_2SO_4$, 0.45% (w/v) Triton X-100, 0.2 mg/ml gelatin] (Biotech), 0.6 mM betaine, 200 M of each of dATP, dTTP, dCTP, dGTP (mix dNTP- Biotech), varying amounts of genomic DNA (10, 25, 40, 50, and 70 ng), $MgCl_2$ (2.5 mM, 3.0 mM and 4.0 mM), and *Taq* DNA polymerase (1.0 and 1.5 units) (Biotech), with 12 nmol/mL of each primer. Three annealing temperatures 40 $^{\circ}$ C; 35 $^{\circ}$ C and 32 $^{\circ}$ C were tested with following profile: Denaturation at 94 $^{\circ}$ C for 5 minutes, followed by 40 cycles of denaturation at 94 $^{\circ}$ C for 1 minute, annealing at (40 $^{\circ}$ C or 36 $^{\circ}$ C and 32 $^{\circ}$ C) for 1 minute and extension at 72 $^{\circ}$ C for 2 minutes, with a final round of extension at 72 $^{\circ}$ C for 2 minutes.

2.5. RAMP-PCR

Each PCR reaction was performed in a final volume of 25 μ l containing 2.0 μ l of 10 X *Taq* Polymerase Reaction Buffer including $MgCl_2$; 1.0 μ l of mix 10mM dNTPs; 20ng DNA; 1.25 Unit *Taq* DNA polymerase; 0.8 μ l of 10nmol/ μ l of each forward and reverse primers and varying amounts of sterile distilled water to make the final volume up to 25 μ l. Reactions were carried out in a PCR cycler (Eppendorf Master Cycler Gradient). Three annealing temperatures 52 $^{\circ}$ C \pm 3 $^{\circ}$ C, 55 $^{\circ}$ C \pm 3 $^{\circ}$ C and 57 $^{\circ}$ C \pm 3 $^{\circ}$ C were tested with following profile: Denaturation at 94 $^{\circ}$ C for 5 minutes, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 1 minute, annealing at (52 $^{\circ}$ C \pm 3 $^{\circ}$ C or 55 $^{\circ}$ C \pm 3 $^{\circ}$ C or 57 $^{\circ}$ C \pm 3 $^{\circ}$ C) for 1 minute and extension at 72 $^{\circ}$ C for 2 minutes, with a final round of extension at 72 $^{\circ}$ C for 5 minutes.

PCR products were separated on 1.5% agarose gel containing 25% of fine agarose and 75% of routine agarose.

2.6. Data analysis

Data was analysed with PopGen and Phylip Tree software.

3. RESULTS AND DISCUSSION

3.1 PCR optimization

The optimum amount of MgCl₂ required in each PCR reaction varied according to the particular primer combinations. Optimum concentration of MgCl₂ was determined based on the brightness, sharp and the consistency of bands [9]. In this experiment, 2.5mM of MgCl₂ was found suitable and produced strong and consistent banding patterns for the RAPD primers and 3.75mM of MgCl₂ was the best for RAMP-PCR. At a concentration of MgCl₂ lower than these amounts (about 1.5 mM for RAPD and 2.5 mM for RAMP) bands were faint and difficult to record. Higher concentrations of MgCl₂ (approximately 4.0 mM) showed no further improvement on the pattern and strength of the bands obtained, or on their reproducibility.

Of the six genomic DNA concentrations tested (10 ng, 20 ng, 25 ng, 30 ng, 40 ng and 50 ng.), 50 ng of genomic DNA produced the strongest and most consistent bands in RAPD and 40 ng genomic DNA was found the best for RAMP-PCR.

The most suitable thermal profile for RAPD-PCR in this study was: denaturation at 94⁰C for 5 minutes, followed by 40 cycles of denaturation at 94⁰C for 1 minute, annealing at 36⁰C for 1 minute and extension at 72⁰C for 2 minutes, with a final round of extension at 72⁰C for 5 minutes. Two other thermal profiles, with different annealing temperatures, were tested. At a higher annealing temperature (i.e. 40⁰C), low molecular weight bands were absent from the profile, while at a lower annealing temperature (i.e. 32⁰C), the bands were faint and inconsistent. Optimum thermal profile for RAMP-PCR in this study was: denaturation at 94⁰C for 5 minutes, followed by 35 cycles of denaturation at 94⁰C for 1 minute, annealing at 55⁰C ±3⁰C for 1 minute and extension at 72⁰C for 2 minutes, with a final round of extension at 72⁰C for 5 minutes.

RAPD-PCR products of IR64 - VND95-20 and Basmati 370 BDS with 2.5 mM MgCl₂, 50 ng genomic DNA, annealing temperature at 36⁰C and primer UBC9 separated on 1.5% agarose gel, is shown in figure 1a. Figure 1b shows RAMP-PCR products of IR64 - VND95-20 and Basmati 370 BDS with 3.75 mM MgCl₂, 40 ng genomic DNA, annealing temperature at 55⁰C ±3⁰C and primers RM238, RM243, RM256, RM259, RM265, RM272.

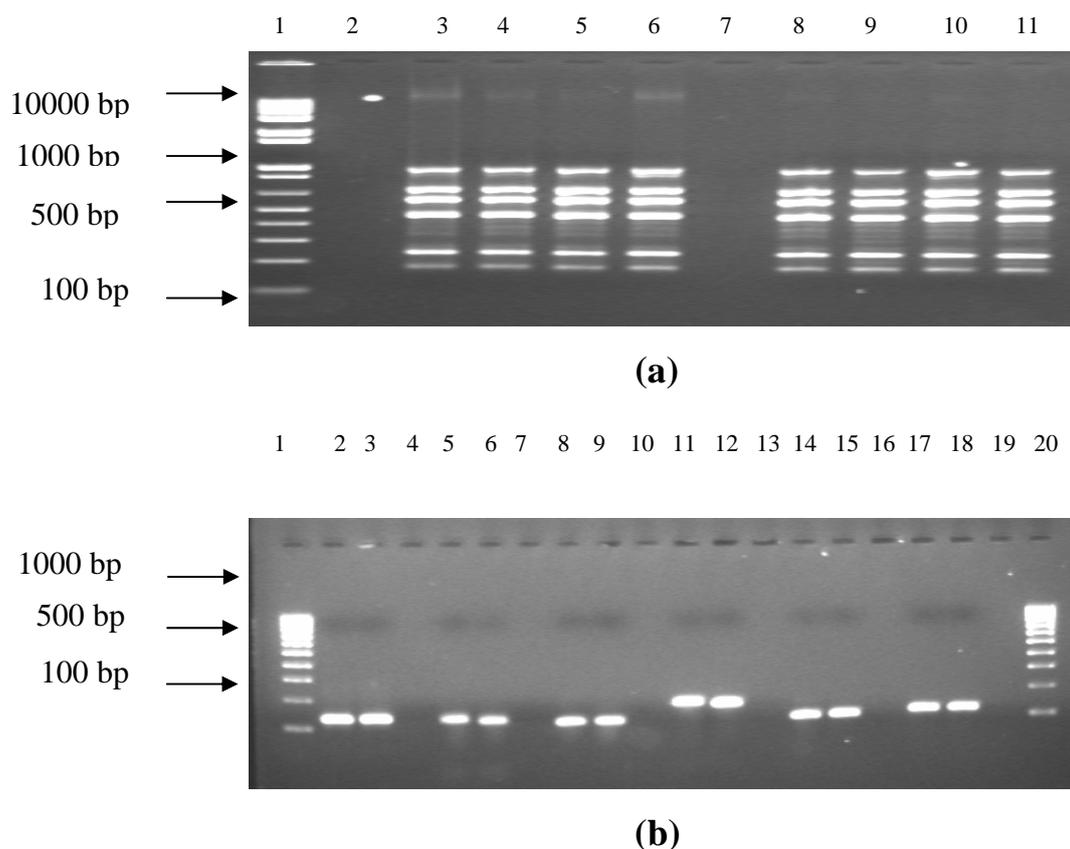


Fig. 1: PCR performance with optimized amounts of $MgCl_2$, genomic DNA and temperature profile in IR64 - VND95-20 and Basmati 370 - BDS. Gel (a): PCR with RAPD primer UBC9. Lane 1 contained a DNA ladder (10Kb plus, Biofly); lanes 3-6 contained PCR products of IR64 and VND95-20; Lane 7 contained the products of a negative control (all components of the PCR reaction except genomic DNA). Lanes 8-11 contained PCR products of Basmati 370 and BDS; Gel (b): PCR with Microsatellite primers. Lanes 1, 20 contained a DNA ladder (1Kb plus, Promega); lanes 2,3,5,6,8,9,11,12,14,15,17 and 18 contained PCR products of TT1 and TDS with RM238, RM243, RM256, RM259, RM265, and RM272 respectively; Lane 19 contained the products of a negative control (all components of the PCR reaction except genomic DNA).

3.2. RAPD-PCR

The total number and number of polymorphic bands generated by RAPD primers in Basmati 370 BDS; Tam Thom (TT1) -TDS; and IR 64 - VND 95-20 is given in table 1.

Table 1: DNA Polymorphism between Basmati 370 BDS; Tam Thom (TT1) – TDS; and IR 64 - VND 95-20 as detected by RAPD-PCR using 50 RAPD primers. Results are for duplicate PCR and only consistent bands were recorded.

RAPD-PCR	Basmati 370 BDS	Tam Thom (TT1) –TDS	IR 64 - VND 95-20
Total bands scored	174	178	174
Number of polymorphic bands	20	16	6
% Polymorphism	11.49	8.98	3.45

Of the 50 RAPD primers tested, 27 primers could produce shape, bright and consistent bands in all cultivars and mutants examined. Amongst these, 13 primers generated different bands in Basmati 370 and BDS, these figures were 8 primers in Tam Thom (TT1) –TDS and 5 primers for IR 64 - VND 95-20. Percentage of polymorphism between parent and its mutant varies from 3.45% in IR 64 - VND 95-20 to 8.98% in Tam Thom (TT1)–TDS and 11.49% in Basmati 370 and BDS. An example of polymorphic bands between parents and mutants detected by UBC11 is shown in figure 2.

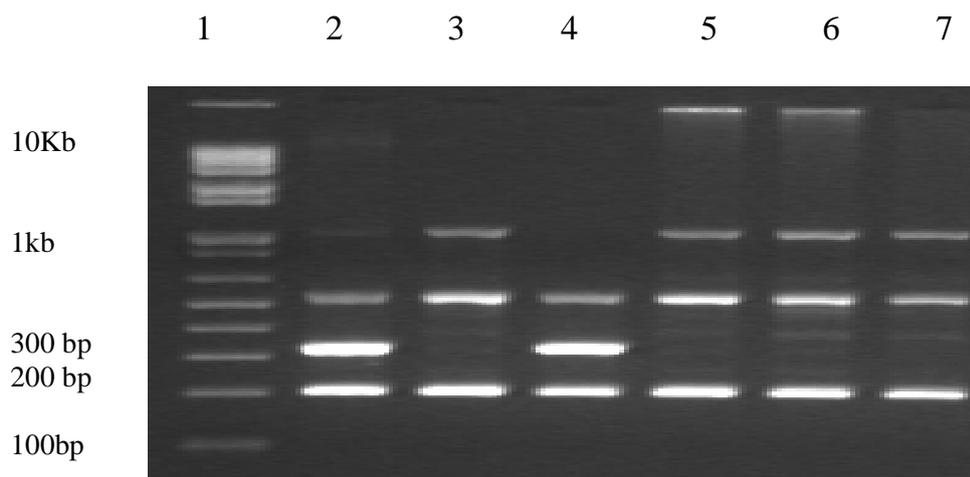


Fig. 2: Polymorphic bands between Basmati370 - BDS and TT1 TDS detected by RAPD primer UBC11. Lane 1 DNA ladder (10Kb, Biofly); lane 2-3 contained PCR of Basmati370 and BDS; lanes 4-5 contained PCR of TT1 and TDS; lanes 6-7 contained PCR of IR64-VND 95-20.

The dendrogram of similarity index (Figure 3) resulting from analysis of the presence and absence of bands generated by 27 RAPD primers in 6 cultivars and mutants not only shows significant differences in the relationship between each parent and mutant but also separates 6 cultivars and mutants into groups. There are two main groups in Figure 3. The first was Basmati 370 and TT1, the second was the rest. This result agrees with the fact that Basmati 370 and TT1 are quite close to each others in terms of aroma, height, life cycle, photoperiod sensitive,... As the same trend, IR 64, VND 95-20, TDS, BDS were grouped together. They are quite similar in the duration of growth, height, high yield,...Our results support the suggestion of Williams *et.al*, 1990

[4]; Chen and DeFillipis, 1996 [10] and Linh, 2002 [11] that RAPD primers are good in distinguish between individuals within species.

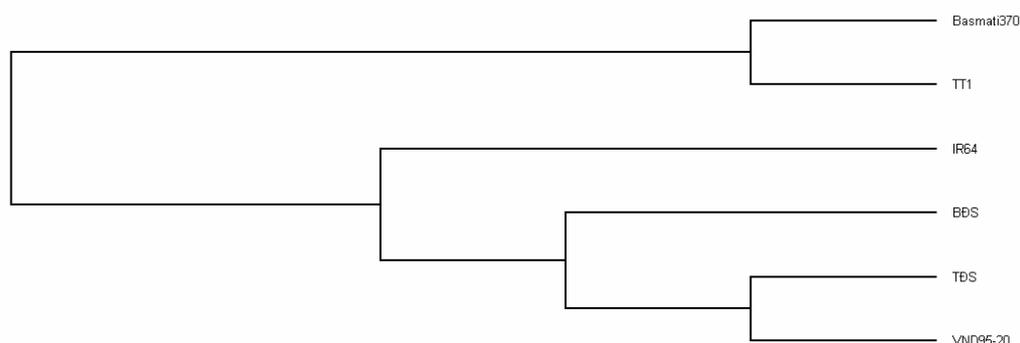


Fig. 3: Genetic relationship (i.e. similarity index) between Basmati 370, BDS, TT1, TDS, IR64 and VND 95-20 determined by RAPD-PCR. Analysis was based on the presence and absence of bands produced by 27 well-working RAPD primers using the Population Genetic program. Results are for triplicate PCR, and only consistent bands were recorded.

3.3. RAMP-PCR

The total number and number of polymorphic bands generated by Microsatellite primers in Basmati 370 BDS; Tam Thom (TT1) –TDS; and IR 64 - VND 95-20 is given in table 2.

Table 2: DNA Polymorphism between Basmati 370 BDS; Tam Thom (TT1) –TDS; and IR 64 - VND 95-20 as detected by RAMP-PCR using 60 Microsatellite primers. Results are for triplicate PCR and only consistent bands were recorded.

RAMP-PCR	Basmati 370 BDS	Tam Thom (TT1) –TDS	IR 64 - VND 95-20
Total bands scored	101	103	99
Number of polymorphic bands	21	15	5
% Polymorphism	21.2	15.4	4.95

Of the 60 Microsatellite primers tested, 50 primers could produce shape, bright and consistent bands in all cultivars and mutants examined. Amongst these, 11 primers generated different bands in Basmati 370 and BDS, these figures were 8 primers in Tam Thom (TT1) –TDS and 3 primers for IR 64 - VND 95-20. Percentage of polymorphism between parent and its mutant varies from 4.95% in IR 64 - VND 95-20 to 15.4% in Tam Thom (TT1) –TDS and 21.2% in Basmati 370 and BDS.

3.4. RAPD versus RAMP

Though there was a difference in percentage of DNA polymorphism between parents and mutants examined by RAPD and RAMP, both could detect changes in DNA between cultivars and mutants tested. Percentage of DNA Polymorphism detected by Microsatellite markers is higher than that of RAPD markers. The differences might be

resulted from the natural Dominant and Co-dominant characteristics of RAPD and Microsatellite markers. The two techniques supplement each other and can be used to detect changes in DNA of rice mutants after gamma irradiation.

4. CONCLUSIONS

The success or failure of a PCR reaction depends upon the optimization of conditions for the PCR. Different primers in PCR have different optimal conditions. Optimum amounts of MgCl₂ in this study were 2.5mM for RAPD-PCR and 3.75mM for RAMP-PCR. Suitable concentrations of genomic DNA for RAPD and RAMP were 50ng and 40ng respectively. Annealing temperatures were determined at 36⁰C for RAPD primer and at 55⁰C for Microsatellite primer.

The most suitable thermal profile for RAPD-PCR in this study was: denaturation at 94⁰C for 5 minutes, followed by 40 cycles of denaturation at 94⁰C for 1 minute, annealing at 36⁰C for 1 minute and extension at 72⁰C for 2 minutes, with a final round of extension at 72⁰C for 5 minutes.

The most suitable thermal profile for RAMP-PCR in this study was Denaturation at 94⁰C for 5 minutes, followed by 35 cycles of denaturation at 94⁰C for 1 minute, annealing at 55⁰C 3⁰C for 1 minute and extension at 72⁰C for 2 minutes, with a final round of extension at 72⁰C for 5 minutes.

Percentage of DNA polymorphism between Basmati370 and BDS, TT1 and TDS, IR64 and VND 95 -20 determined by RAPD were 11.49%; 8.98% and 3.45% respectively. Those determined by RAMP were 21,2%; 15,4% and 4,95%.

Both RAPD and RAMP can detect changes in DNA of parents and mutants. The two techniques should be used to assist rice breeding.

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