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Genetic variation in gamma ray induced mutants in blackgram as revealed by RAPD and ISSR markers

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Abstract

Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers were used to study the DNA polymorphism in 12 gamma ray induced morphological mutants of blackgram [*Vigna mungo* (L.) Hepper]. Of the 35 random and eight ISSR primers used, 15 random and five ISSR primers detected polymorphism among the mutants. Total number of bands varied from 2 to 9 for RAPD and from 6 to 10 for ISSR. The number of polymorphic bands varied from 1 to 3 for RAPD and from 1 to 6 for ISSR. Percentage of polymorphism ranged from 12.5 to 50.0 for RAPD and 12.5 to 44.4 for ISSR. Significant DNA polymorphism among the mutants were observed using RAPD (25.8%) and ISSR (33.3%) markers. An young leaf chlorina mutant and a smooth pod mutant showed more DNA polymorphism as compared to the parent. DNA polymorphism data revealed by RAPD and ISSR could facilitate selection of mutants to be involved in cross breeding and genome mapping.

Key words : Blackgram, gamma rays, mutants, RAPD, ISSR, genetic variation

Introduction

Induced mutation has been proved to be a valuable approach in generating genetic variability for breeding improved varieties employing conventional and/or molecular means. Application of gamma rays and mutagens for crop improvement in the past 70 years has increased crop biodiversity and productivity in several crops in different parts of the world. To date world wide, 2252 mutant varieties have been officially registered. Of which 1585 were released as direct mutants, and 667 were mutant derivatives. Gamma ray treatment was employed for the development of 64% of the mutant varieties. Of the 311 legume mutants, four were in blackgram [1].

Mutants and mutant derivatives when used in cross breeding were found to be more productive in the development of improved varieties of blackgram [2, 3]. Moreover, induced mutations have recently become the subject of molecular investigations leading to descriptions of the structure and function of related genes. Mutated genes have therefore, become valuable material to plant breeders and molecular biologists for understanding not only the function but also in isolating and shuffling the genes between varieties.

The identification and analysis of mutants using molecular techniques of DNA fingerprinting and mapping with PCR based markers such as RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and STMS (sequence-tagged microsatellite sites) and mutant tagging could bring a new dimension in gene technology [4].

The evaluation of genetic diversity and construction of linkage maps may promote the efficient use of the vast array of genetic variation in a breeding programme [5]. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers [6]. RAPD markers have been used for the identification of cultivars and the genetic relationships among cultivars of several crops including *Phaseolus vulgaris* [7], cowpea [8], mungbean [9, 10] and *Vigna angularis* [11]. RAPD markers were also used for the study of induced mutants in groundnut [12], chickpea [13] and for mapping mutation in *Arabdopsis thaliana* [14].

Inter simple sequence repeat (ISSR) analysis involves the PCR amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat (SSR) containing primer anchored at the 3' or 5' end by 2 to 4 arbitrary, often degenerate nucleotides [15]. ISSR markers have been successfully utilized for analysis of repeat motifs in mungbean [16] and genetic relationships in the genus *Vigna* [17]. In the present study RAPD and ISSR markers were used to analyze the DNA polymorphism in 12 gamma ray induced blackgram mutants.

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Materials and methods

Plant materials

A blackgram variety TU 94-2, determinate with 2 to 3 branches, stem colour green with light purple tinge, photoinsensitive and resistant to yellow mosaic virus developed at the Bhabha Atomic Research Centre was used for mutation studies. Seeds of TU 94-2 were irradiated with 400 Gy gamma rays. Treated seeds were sown in a single unreplicated plot with 3 meter rows. The row to row and plant to plant spacing were kept as 30 cm and 10 cm, respectively. About 1500 M_1 plants were harvested individually and grown to develop M_2 population following plant to progeny row method. The M_2 population was raised in 3 m rows at 30 cm × 10 cm spacing. Various morphological and chlorophyll mutants were isolated. Along with the parent variety (A) TU 94-2, twelve true breeding mutants (B) Young leaf chlorina (C) narrow curly leaf with late maturity, (D) small leaf, (E) late and narrow leaves, (F) virescent, (G) white pods, (H) smooth pods, (I) 2/3 seeds per pod, (J) large seed, (K) shiny seed coat, (L) extreme dwarf and (M) erect plant were selected for the present study.

DNA isolation

DNA was extracted from young leaf tissue [18] diluted to 25 ng μl^{-1} for PCR amplification.

DNA amplification

PCR amplification [19] was performed with random decamer primers obtained from Operon Technologies Inc., Almeda, CA, USA. Amplification was performed in a 25 µl reaction volume containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5mM $Mgcl_2$, 0.01% gelatin. 0.2mM each of dNTPs, 0.2 µmoles of RAPD primer, 50 ng of total DNA and 0.5 units of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.) Amplifications were performed in an Eppendorf Master cycler gradient (Eppendorf Netheler-Hinz GMBH, Hamburg). Amplification conditions were 1 cycle of 94°C for 4 min, and 45 cycles at 94°C for 1min, 37°C for 1 min, 72°C for 2 min followed by 1 cycle of 5 min at 72°C. Amplified products were separated by electrophoresis in 1.5% agarose gel using TBE buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1mM EDTA) at 50 V. The gels were stained in 0.5µg/ml ethidium bromide solution and visualized under UV light.

ISSR amplification

ISSR amplification reactions were performed in 25 µl volume containing 50 ng template DNA, 0.5 units of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.), 0.2 mM each of dNTPs, 1.0 µmoles primer in a reaction buffer containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 0.01% gelatin. Amplification was performed in an Eppendorf Master cycler gradient (Eppendorf Netheler-Hinz GMBH, Hamburg). Amplification conditions were 5 cycle at 94°C for 8min, and 1 cycle at 94°C for 30 sec, 55°C-50°C for 45 sec, 72°C for 2 min and 35 cycles at 94°C for 30 sec, 50°C for 45 sec, 72°C for 2 min. followed by 1 cycle of 7 min at 72°C. Amplified products were separated by electrophoresis in 2% agarose gel using TBE at 75 V. RAPD and ISSR bands were scored as presnet (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Data were used for similarity based analysis using the programme of NTSYS-PC (version 2.02) [20]. The SIMQUAL programme was used to calculate Jaccard's coefficient, a common estimator of genetic identity. Similarity coefficients were used to construct the UPGMA (unweighted pair group method with arithmetic average) dendrogram. Reproducibility of the bands was tested by repeating twice the amplification.

Results and discussion

RAPD

A total of 235 DNA bands were amplified across all the mutants with 35 RAPD primers revealing an average of 6.4 bands per primer in each mutant. Twenty random primers (OPA 1, OPA 2, OPA 4, OPA 5, OPA 6, OPA 7, OPA 8, OPA 10, OPA 11, OPA 12, OPA 13, OPI 4, OPI 12, OPK 1, OPK 2, OPK 5, OPK 10, OPL 1, OPL 2, OPN 20) showed monomorphic banding pattern over the mutants. Fifteen primers showed polymorphism

Table 1. List of RAPD and ISSR primers which showed polymorphism among the mutants studied

	Primer	Total no. of bands	No. of polymorphic band	Percentage of polymorphism
RAPD	OPA 04	9	1	11.1
	OPA 05	8	2	25.0
	OPA 09	6	3	50.0
	OPA 14	2	1	50.0
	OPE 20	5	1	20.0
	OPI 04	7	3	42.9
	OPI 14	6	2	33.3
	OPK 04	8	1	12.5
	OPK 06	8	3	37.5
	OPK 19	5	1	20.0
	OPN 07	5	1	20.0
	OPN 08	8	1	12.5
	OPN 09	6	1	16.6
	OPN 14	6	2	33.3
	OPN 18	4	1	25.0
ISSR	807 $(AG)_8$ T	8	2	25.0
	808 $(AG)_8$ C	8	1	12.5
	810 $(GA)_8$ T	10	6	44.4
	834 $(AG)_8$ YT	7	1	14.3
	841 $(GA)_8$ YC	9	4	44.4

Y-Pyrimidine

among the mutants (Table 1). The size of the amplified DNA bands ranged from 200 bp (OPL 1) to a maximum of 2500 bp (OPA 1). A total of 24 (25.8%) polymorphic DNA bands were scored among all the mutants. The number of RAPD marker loci detected in the mutants ranged from 2 (OPL 1, OPI 12 and OPA 4) to 9 (OPA 4). Number of polymorphic bands ranged from one to three. Percentage of polymorphism ranged from 12.5 (OPK 4) to 50.0% (OPA 9 and OPA 14). Primer OPK

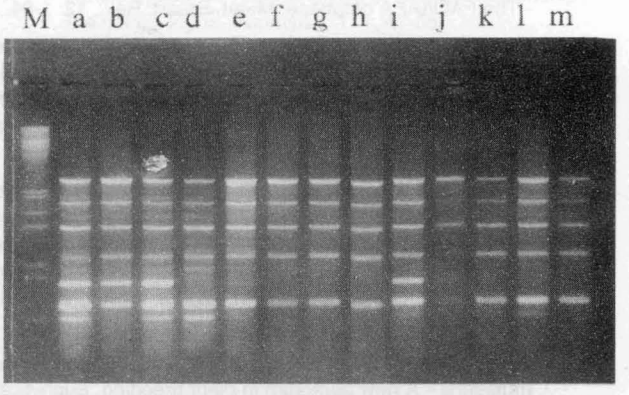

Fig. 1. RAPD profile of blackgram variety TU 94-2 and its mutants using the primer OPK 6. Lane M: Lambda DNA *Eco* RI and *Hind* III double digest marker.

6 showed more deletion mutation (Fig. 1). OPN 9 primer showed more bands in 2/3 seeds per pod mutant than parent. Similar results of mutants showing more bands than parents were observed in chickpea mutants [13]. This could be attributed to large chromosomal rearrangements due to radiation [12].

ISSR

A total of 63 bands were produced by eight ISSR primers with an average frequency of 7.8 bands per primer. Five UBC primers exhibited polymorphism among different mutants (Table 1). Amplified DNA fragments varied from 300 bp, with primer UBC 807 to 2,027 bp, with primer UBC 841. Fourteen (33.3%) of the 42 fragments were observed to be polymorphic among the 12 mutants. Number of polymorphic bands produced

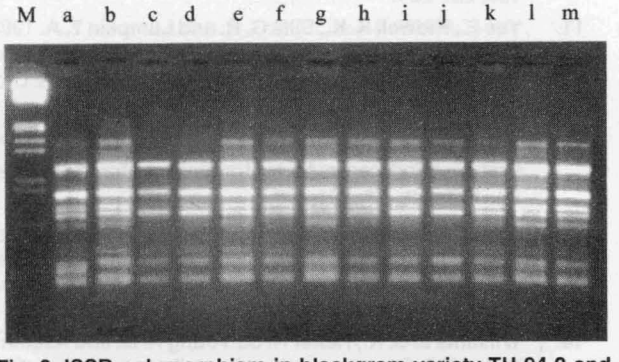

Fig. 2. ISSR polymorphism in blackgram variety TU 94-2 and its mutants using the primer UBC807. Lane M: Lambda DNA *Eco* RI and *Hind* III double digest marker.

by a single primer varied from one, with UBC 808 and 834 to 6 with UBC 10. Percentage of polymorphism ranged from 12.5 (UBC 808) to 44.4 (UBC 810 and 841). A representative amplification profile using the primer UBC 807 is shown in the Fig. 2.

Dendrogram using the RAPD and ISSR data was constructed based on similarities using UPGMA

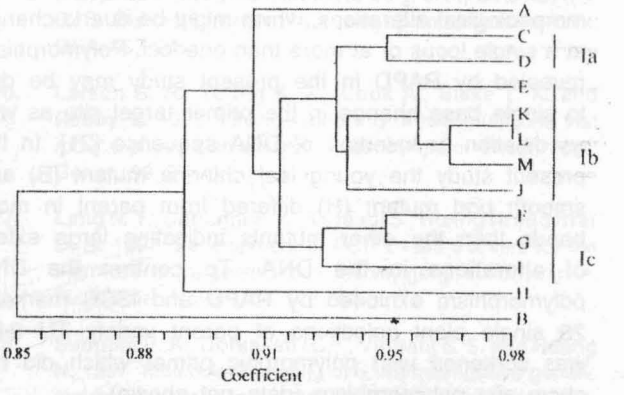

Fig. 3. Dendrogram generated using UPGMA analysis showing relationships between cultivar TU 94-2 and its 12 mutants using RAPD and ISSR markers

(NTSYS-PC 2.0) analysis (Fig. 3). Mutants were grouped in three clusters. Cluster 1 contains 3 sub clusters 1a, 1b and 1c. Subcluster 1a includes narrow curly leaf (C) and small leaf (D) mutants. Cluster 1b consists of late narrow leaves (E), shiny seeds (K), extreme dwarf (L), erect plant (M) and bold seed (J) mutants. Shiny seed mutant (K) and extreme dwarf mutant (L) showed more similarity among them (98%).

Cluster 1c consists of virescent (F), white pod (G) and 2/3 seeds per pod (I) mutants, which also exhibited maximum variation compared with other mutants and parent TU 94-2(A) in cluster 1. Smooth pod mutant (H) and young leaf chlorina mutant (B) which formed as separate cluster II and III respectively showed maximum polymorphism (11% and 15%) compared to the parent TU 94-2. Similar results of DNA polymorphism revealed by RAPD among different mutants were observed in groundnut [12]. Large amount of variation at the DNA level was observed among different mutants though phenotypically they look similar to parent TU 94-2 in many characters. The level of polymorphism detection was more with ISSR primers compared to random primers. Polymorphism observed with ISSR primers could occur whenever one genome is missing the repeated sequence or has a delection or insertion that modifies the distance between the repeats. For 5' anchored primers, polymorphisms also occur due to differences in the length of the microsatellite. The sequence of repeats and anchored nucleotides is randomly selected and has the advantage

of analyzing multiple loci in a single reaction. Hence significant levels of polymorphism among the 12 mutants using RAPD (25.5%) and ISSR (33.3%) markers were observed.

RAPD and ISSR fingerprinting has been successful in detecting variation in gamma ray induced blackgram mutants in the present investigation. The mutants examined in the present study represent gross morphological alterations, which might be due to change at a single locus or at more than one loci. Polymorphism revealed by RAPD in the present study may be due to single base change in the primer target site as well as deletion or insertion of DNA sequence [21]. In the present study the young leaf chlorina mutant (B) and smooth pod mutant (H) differed from parent in more bands than the other mutants indicating large extent of alterations in the DNA. To confirm the DNA polymorphism exhibited by RAPD and ISSR markers, 20 single plant selections of parent variety TU 94-2 was screened with polymorphic primer which did not show any polymorphism (data not shown).

Analysing induced mutants at molecular level have been carried out in *Arabdopsis*. A chalcone flavanone isomerase (CHI) mutation obtained following fast neutron irradiation revealed a 1.4 Kb inversion within the CHI gene and a 272 bp insertion adjacent to another inversion from the same chromosome [22]. A dihydroflanol-4-reductase (DFR) allele, induced by X-ray irradiation contained two 72 bp and 7.4 Kb deletions flanking a 2.8 CM inversion [22].

Li *et al.* [23] demonstrated that deletion mutants can be identified for targeted plant genes by screening fast neutron- mutagenized populations via an efficient PCR screening procedure. Mutations that reduce seed phytic acid have been isolated, characterized and genetically mapped in rice [24], maize [25] and barley [26]. The two gamma-ray induced mutations for TGMS (thermogenic male sterility), *tms*2, and *tms*3, and the spontaneous *tms*1 have already been mapped with molecular markers [27, 28]. Most of the radiation induced mutations analyzed have mainly revealed deletions, and others inversions and deletions [12]. The nature of alteration was pinpointed in the studies mentioned earlier because mutations in known and well characterized genes were available for comparison in those crops along with the classical phenotypic maps.

Molecular analysis of induced mutants with markers could facilitate selection of suitable mutants for crossing to exploit heterosis. Heterosis in the F_1 generation resulting from crosses between independently selected maize mutants was reported by D. F. Jones [29]. Since then, mutant heterosis has been described in many plant species [30]. Genetic variation among blackgram mutants based on RAPD and ISSR analysis could be useful to select parents to be crossed for generating appropriate populations intended for genome mapping and breeding purposes. RAPD and ISSR markers could provide useful tools for mutants characterization, conservation and utilization, as well as for genetic and breeding studies in blackgram.

References

1. **Maluszynski M., Nichterlein K., Vanzanten L. and Ahloowalia B. S.** 2000. Officially released mutant varieties - The FAO/IAEA database. Mutat. Breed. Rev., **12**: 1-84.
2. **Pawar S. E., Thakare R. G. and Bhatia C. R.** 1988. Genetic improvement of pulse crops: induced mutations and their use in cross breeding. *In*: Proc. FAO/IAEA workshop on improvement of grain legume production using induced mutations. Vienna: 235-249.
3. **Pawar S. E., Manjaya J. G., Souframanien J. and Bhatkar S. M.** 2000. Genetic improvement of blackgram using induced mutations. *In*: DAE-BRNS symposium on the use of nuclear and molecular techniques in crop improvement, 6-8, December, 2000, BARC, Mumbai 170-174.
4. **Ahloowalia B. S. and Maluszynski M.** 2001. Induced mutations - A new paradigm in plant breeding. Euphytica, **118**: 167-173.
5. **Paterson A. H., Tanksley S. D. and Sorrells M. E.** 1991. DNA markers in plant improvement. Adv. Agron. **46**: 39-90.
6. **Soller M. and Beckmann J. S.** 1983. Genetic polymorphism in varietal identification and genetic improvement. Theor. Appl. Genet., **67**: 25-33.
7. **Skroch P. W. dos Santos J. B. and Nienhuis J.** 1992. Genetic relationships among *Phaseolus vulgaris* genotypes based on RAPD marker data. Annu. Rep. Bean Improv. Coop., **35**: 23-24.
8. **Mignouna H. D., Ng N. Q., Ikea J. and Thottapilly G.** 1998. Genetic diversity in cowpea as revealed by random amplified polymorphic DNA. J. Genet and Breed., **53**: 151-159.
9. **Santalla M., Power J. B. and Davey M. R.** 1998. Genetic diversity in mungbean germplasm revealed by RAPD markers. Plant Breed., **117**: 473-478.
10. **Lakhanpaul S., Chadha S. and Bhat K. V.** 2000. Random amplified polymorphic DNA (RAPD) analysis in Indian mungbean (*Vigna radiata* (L.) Wilczek) cultivars. Genetica, **109**: 227-234.
11. **Yee E., Kidwell K. K., Sills G. R. and Lumpkin T. A.** 1999. Diversity among selected *Vigna angularis* (Azuki) accessions on the basis of RAPD and AFLP markers. Crop Sci., **39**: 268-275.
12. **Anjali B., Krishna T. G. and Bhatia C. R.** 1997. RAPD analysis of induced mutants of groundnut (*Arachis hypogea* L.). J. Genet., **76**: 201-208.
13. **Banerjee H., Pal R. A. and Sharma R. P.**. 1999. Restriction fragment length polymorphism and random amplified polymorphic DNA analysis of chickpea accessions. Biologia Plantarum, **42**: 197-208.
14. **Williams J. G. K., Reiter R. S., Young R. M. and Ascolnik P. A.** 1993. Genetic mapping of mutations using phenotypic pools and mapped RAPD markers. Nucl. Acids Res., **21**: 2697-2702.

15. **Zietkiewicz E., Rafalski A. and Labuda D.** 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, **20**: 176-183.

16. **Singh S., Reddy K. S. and Jawali N.** 2000. PCR analysis of mungbean genotypes using anchored simple sequence repeat primers. *In*: DAE-BRNS symposium on the use of nuclear and molecular techniques in crop improvement, 6-8 December, 2000, BARC, Mumbai, 359-369.

17. **Ajibade S. R., Weeden N. F. and Chite S. M.** 2000. Inter simple sequence repat analysis of genetic relationships in the genus *Vigna*. Euphytica, **111**: 47-55.

18. **Dellaporta S. L., Wood J. and Hicks J. B.** 1983. A plant DNA mini-preparation: version II. Plant Mol. Biol. Rep., **1**: 19-21.

19. **Williams J. G. K., Kubelik A. R., Livak K. J., Rafalaski J. A. and Tingey S. V.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., **18**: 6531-6535.

20. **Rohlf F. J.** 1990. NTSYS. Pc Numerical taxonomy and multivariate analysis system, version 2.02. Applied Biostatistics, New York.

21. **Caetano-Anolles G., Bassam B. J. and Gresshoff P. M.** 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology, **9**: 553-557.

22. **Shirley B. W., Hanley S. and Goodman H. M.** 1992. Effect of ionizing radiation on a plant genome : Analysis of two *Arabdopsis* transparent testa mutations. Plant Cell, **4**: 333-347.

23. **Li X., Song Y., Century K., Straight S., Ronald P., Dong X., Lassner M. and Zhang Y.** 2001. A fast neutron deletion mutagenesis - based reverse genetics system for plants. Plant J., **27**: 235-242.

24. **Larson S. R., Neil Rutger J., Young K. A. and Raboy V.** 2000. Isolation and genetic mapping of a non-lethal rice (*Oryza sativa* L.) low phytic acid 1 mutation. Crop Sci., **40**: 1397-1405.

25. **Raboy V. and Gerbasi P.** 1996. Genetics of *myo*-inositol phosphate synthesis and accumulation. *In*: *Myo*-inositol phosphates, phosphonositides and signal transduction (ed. B. B. Biswas). Plenum Publishing Company, New York, USA: 257-285.

26. **Larson S. R., Young K. E., Cook A., Blake T. K. and Raboy V.** 1998. Linkage mapping of two mutations that reduce phytic acid content of barley grain. Theor. Appl. Genet., **99**: 27-36.

27. **Lang N. T., Subudhi P. K., Virmani S., Huang N. and Brar D. S.** 1997. Development of PCR based markers for the thermosensitive genetic male sterility gene, tms3(t) in rice. Rice Genet. Newsl., **14**: 102-103.

28. **Subudhi P. K., Borkakati R. P., Virmani S. S. and Huang N.** 1997. Molecular mapping of a thermosensitive genetic male sterility gene in rice using bulked segregant analysis. Genome, **40**: 188-194.

29. **Jones D. F.** 1945. Heterosis resulting from degenerative changes. Genetics, **30**: 527-542.

30. **Maluszynski M. Fuglewicz A., Szarejko I. and Micke A.** 1989. Barley mutant heterosis. *In*: Current options for cereal improvement: Doubled hapoids, mutants and heterosis. Kluwer Academic Publisher. Dodrecht, Germany, 129-146.