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Evaluation of genetic variation in mutants of Black gram (*Vigna mungo* (L.) Hepper) as revealed by RAPD markers

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Abstract: One of the most possibilities is the genetic marker system to find out the genetic variation. The advent of large-scale DNA sequencing technology has generated a tremendous amount of sequence information for many important organisms. The genetic variation was evaluated in four mutants (high seed protein, tall, bushy and dwarf mutants) along with parent cultivar (control) by 20 random primers which generated 202 fragments scored with 58 polymorphic DNA bands. The average DNA bands were 10.1 per locus ranged from 1 to 9. The average polymorphic rates were 38.37 % among mutants and parent cultivar (control) through the 20 primers. Primer OPK–06 and OPK-11 revealed 62.5 % DNA polymorphism. Five genotypes were used to constructed dendrogram based on the similarity matrix, suggested that genetic distance from 0.621 to 0.785. The DNA variation might have been caused by mutation due to gamma rays and ethyl methane sulphonate. Hence, research is needed to analyse function of mutated genes for their mutants characters for future prospects because, such mutants and their genes when using in cross breeding/transgenic technologies will found more production in the development of improved crop varieties like high seed protein, lodging resistances, semi dwarf with high yield.

Key words: Gamma rays, Ethyl methane sulphonate, DNA polymorphism.

تقييم التباين الوراثي في طفرات العدس الصيفي (*Vigna mungo* (L.) Hepper) باستخدام تقنية التضاعف العشوائي المتعدد للمادة الوراثية (RAPD)

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الملخص: يعتبر استخدام نظام المعلمات الوراثية من أهم الطرق في دراسة الاختلاف الوراثي. ظهور تكنولوجيا التسلسل للحمض النووي أدت إلى معرفة معلومات هائلة لتسلل المادة الوراثية لكائنات كثيرة وهامة. تم تقيم الاختلاف الوراثي لأربعة طفرات (بذور عالية البروتين، الارتفاع، كثافة النمو والتقزم) جنبا إلى جنب مع صنف الأب (الشاهد) باستخدام 20 بادئ تضاعف عشوائي ولدت 202 جزء مسجل مع 58 حزمة متعددة الأشكال من المادة الوراثية. معدل حزمة المادة الوراثية 10.1 لكل موقع تراوح من 1 إلى 9. معدل التنوع في أشكال المادة الوراثية 38.37% بين الطفرات والصنف إلاب (الشاهد) خلال 20 بادئ. البادئ OPK–06 والبادئ OPK-11 كشف 62.5% من التنوع في اشكال المادة الوراثية. خمس أصناف تم استخدامهم لبناء مخطط التحليل التجميعي (dendrogram) بناءا على علاقة التشابه بينهما، حيث كانت المسافة الجينية من 0.621 إلى 0.785. الاختلاف في المادة الوراثية قد يكون ناجما عن الطفرة بأشعة جاما وإيثيل ميثان سلفونات. وبالتالي، يجب إجراء البحوث اللازمة لتحليل وظيفة الجينات التي تعرضت للطفرة وصفاتها في المستقبل، لأن مثل هذه الطفرات وجيناتها عند استخدام في تربية النبات وتكنولوجيات التعديل الوراثي ستعمل على تحسين وتطوير إنتاج أنواع محسنة من المحاصيل تمتاز بصفات مثل نسبة عالية من البروتين في البذور، مقاومة الانبطاح وشبه التقزم مع إنتاج عالي.

الكلمات المفتاحية: اشعة جاما، إيثيل ميثان سلفونات، تعدد أشكال المادة الوراثية.

Introduction

One of the most possibilities is the genetic marker system to find out the genetic variation. The advent of large-scale DNA sequencing technology has generated a tremendous amount of sequence information for many important organisms (Jinguo and Brady, 2003). The genetic diversity studies in various species of the genus *Vigna* using DNA base marker system are limited to a large extent (Amadou et al., 2001). Till today, no significant study of genetic diversity of *V. mungo* using DNA base marker has been reported (Sivaprakash et al., 2004). The systematic collection of black gram is displayed inadequate variability for biotic and abiotic desirable genes (Deepalakshmi and Anandakumar, 2004). It is possible that genes for high productivity could have been lost due to overriding role of natural selection (Roopalakshmi et al., 2003) and the creation of variability is difficult through hybridization due to its high self pollination (Deepalakshmi and Anandakumar, 2004). This has resulted low seed productivity and its protein content with other polygenic traits.

Among the various genus of *Vigna, V. mungo* has been the least studied crop within the pulse. No international system for black gram according to the Consultative Group of International Agricultural Resources (CGIAR) as a mandate crop (Ghafoor et al. 2002). The mutation assisted breeding can play an important role in crop improvement either directly or by supplementing the conventional breeding. The direct use of mutation by gamma rays and ethyl methane sulphonate (EMS) is a valuable approach to plant breeding, particularly when it is desired to improve one or a few characters in well-adapted variety. The principle objective of the present study was to find genetic variation among mutant along with parent cultivar of black gram by RAPD profile.

Materials and Methods

Four mutants and parent cultivar of black gram were subjected to RAPD assay in the study (Table 1). Mutants were derived from gamma rays and ethyl methane sulphonate (EMS) on M_4 generation.

Five sets of hundred well-matured seeds of black gram for each dose were subjected to gamma irradiation with following doses 0, 20, 40, 60, 80,100 and 120 kR of gamma rays. Irradiation was accomplished in Sugarcane Breeding Institute (ICAR) at Coimbatore, TN, India. The labeled Cobalt (^{60}Co) was used for source of gamma rays.

Five hundred well-matured healthy seeds were subjected to the mutagenic treatment for each concentration. The seeds were pre-soaked in distilled water for five hours at room temperature (28 ± 2°C) prior to treatment. After pre-soaking the excess of moisture in the seeds was removed by filter paper. Then, the seeds soaked in the freshly prepared aqueous solution which about three times than that of volume of seeds with corresponding concentrations of EMS *viz,* 0.02, 0.04, 0.06, 0.08, 0.1 and 0.12 % for 6 hours at room temperature (28 ± 2 °C) with an hour of intermittent shaking. The pH of aqueous solution was adjusted to 8.5 by using 0.2 M solution of sodium tetra borate (Borax). After the treatment, seeds were washed thoroughly with distilled water. The untreated seeds (pre-soaked in water for 3 hours) were used as parent cultivar (control). Both treated and parent cultivar (control) seeds were sown in the field in a randomized block design (RBD) with three replications to rise the M_1 generation. M_1 plants were harvested and grown in successive seasons and developed M_2, M_3 and M_4 generations.

The mutants scored in M3 generation were bulked and raised in petriplates to obtain M4 generation and field. The four mutants selected by morphological and biochemical (protein profile) criteria,

namely, tall and high seed protein mutants derived from gamma rays and dwarf and bushy mutants derived from EMS (Table1).

Table 1. Effect of mutagen and mutants of black gram (*Vigna mungo* L. Hepper).

No.	Mutants	Mutagen induction
1.	Parent	-
2.	High seed protein	Gamma rays
3.	Tall	Gamma rays
4.	Bushy habit	EMS
5.	Dwarf mutant	EMS

Five gram (5 gm) of young, fresh leaf tissue from each genotype was ground to fine powder in a mortar containing liquid nitrogen. The DNA was extracted according to modified method Dellaporta et al. (1983). Twenty random arbitrary decamer primers were selected and screened to produce polymorphic bands for this investigation obtained from *Operon Technologies Inc.* (Alameda, CA, USA) (Table 3). The polymerase chain reaction (PCR) amplification was performed in each 25 µl reaction mixture (Eppendorf PVT Ltd, Germany) containing 10 mM of Tris HCl (pH-8.3), 50mM KCl, 0.5U of Taq DNA polymerase, 0.01% gelatin, 0.2mM each dNTPs, 2.5mM $MgCl_2$, 50pmol µl-^{1} of random primer and 20ng/µl of genomic DNA. The amplifications were performed in TECHNE Master Cycler System (TECHNE Ltd, Cambridge, Duxford, U.K) with the following sequential thermal cycling as, initial denaturation step at 94° C for 4 min, followed by 40 cycles at 94° C for 1 min, an annealing step at 37° C for 1 min and an extension at 72° C for 1 min, with a final extension step at 72° C for 5 min. The final products were held at 3° C. The amplified products were run o 1.5% agarose gel (Bangalore Genie Pvt Ltd, India) using with 1X TAE buffer at 6.5V/cm for 1 h. The gel was stained with (5mg/ml) ethidium bromide for 15 min and visualizedunder UV light at 290 nm. The size of amplified fragments was determined by co-electrophoresed low range marker (Bangalore Genie, PVT Ltd, India).

The fragments obtained from amplification were scored the presence or absence of each single fragment for a binary data matrix. The binary data were used to compute a pair wise similarity/distance matrix using the Jaccard's distance index. The similarity matrix was subjected to cluster analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic Average) algorithm on NTSYS-pc version 2.10 software (Rohlf, 2000). The genetic distance was analyzed between mutants and parent, as per Nei and Li (1979).

Results

Quantitative traits of mutants and parent cultivar

The quantitative traits were observed among parent cultivar of black gram with four mutants**.** The quantitative traits of mutants were recorded and compared with parent cultivar (Table 2). The seed protein mutant had high protein content (256.96±5.78mg g^{-1}) than parent and other mutants. Bushy mutant showed high vegetative growth with more fruit clusters (16.58±1.36), pods plant-^{1} (31.25±1.82) and seed yield plant^{-1} (7.96±0.22g plant^{-1}) compared to other mutants and parent. Tall mutant showed highest plant height (75.69±1.14cm) and more seed yield plant^{-1} (7.28±0.07g). The dwarf mutant had more seed protein (244.96±5.98mg g^{-1} seed meal) with lodging resistance compared to parent cultivar (Table 2).

Table 2. Quantitative traits of parent cultivar and different mutants of black gram.

Quantitative traits	Parent cultivar	High seed protein mutant	Bushy type mutant	Tall mutant	Dwarf mutant
Plant height plant^{-1}	50.68±1.63	29.88±1.25	22.31±1.41	75.69±1.14	12.36±1.14
No. of branches plant^{-1}	3.21±0.11	2.16±0.36	6.96±0.45	4.69±0.42	1.01±0.04
No. of leaves Plant^{-1}	25.22±1.12	10.48±1.31	40.14±1.10	32.62±1.25	7.26±0.03
No. of clusters plant^{-1}	15.25±0.96	8.63±0.23	16.58±1.36	15.87±0.89	6.23±0.09
No. of pods plant^{-1}	29.26±1.52	19.47±1.12	31.25±1.82	30.36±1.12	5.36±0.96
Seed yield plant^{-1} (g)	6.86±0.16	5.23±0.26	7.96±0.22	7.28±0.07	4.26±0.16
Seed protein (mg g^{-1} seed meal)	230.36±6.24	256.96±5.78	232.52±3.63	220.56±7.45	244.96±5.98

SE 0.9843 SED 1.6121 (P= 0.05) 0.3722 (P=0.01) 0.4898

Polymorphism of RAPD markers

Twenty random decamer primers were used to evaluate the genetic variation of the five genotypes of black gram. The total amplified fragments, polymorphic bands, polymorphic rate (%) and genetic distance are presented in Table 3.

Twenty random primers were yielded 202 alleles, all of them showed polymorphic fragments with an average of 7.0 alleles per locus (range of 1-9 alleles). The average polymorphic rate was 767.49% within the 5 genotypes and they were ranged from 256 bp to 2896 bp. (Table 3). The RAPD profiles generated by the primers OPK-06 and OPK-11 were shown in figure 1 and 2 respectively. The genetic distance ($GD_{mut\ -\ par}$) mutants and parent was 0.19 according to Nei and Li (1979).

Clustering distance analysis of RAPD markers (UPGMA)

A dendrogram was constructed using data from UPGMA cluster analysis. Based on the genetic distance (GD), coefficient matrix of five genotypes was analyzed using the primers OPK-06 and OPK-11 (Figure 3, 4). The dendrogram grouped into three clusters a, b and c and showed genetic distance among the mutants and parent plant was from 0.621 to 0.785. Cluster-a indicated parent and tall mutant grouped in to a single cluster. Dwarf and bushy mutants were grouped into an individual group as c and the genetic distance between cluster c and parent was 0.621 to 0.722. Whereas, high seed protein mutant was grouped a single cluster–b and showed highest genetic diversity (0.686 to 0.725) than parent (Table 4; Figure 3).

Table 3. The total bands, polymorphic bands, a polymorphic rate and genetic distance (GD) of the RAPD primers using 5 genotypes (4 mutants & parent) of black gram.

Sl. No.	Primer name	Total bands	Polymorphic bands	Polymorphic rate (%)	G.D.
1.	OPA04	2	2	100.00	0.02
2.	OPA07	33	12	33.36	0.31
3.	OPA09	3	1	33.33	0.06
4.	OPA11	3	1	33.33	0.21
5.	OPA13	4	1	25.00	0.00
6.	OPA1	36	9	25.00	0.71
7.	OPA16	4	2	50.00	0.00
8.	OPA17	2	1	50.00	0.19
9.	OPA19	5	2	40.00	0.16
10.	OPA20	2	1	50.00	0.28
11.	OPA04	4	1	25.00	0.11
12.	OPI04	40	4	10.00	0.36
13.	OPI08	28	8	28.57	0.31
14.	OPI11	6	2	33.33	0.16
15.	OPK04	7	2	28.57	0.08
16.	OPK06	4	1	25.00	0.10
17.	OPK11	8	3	37.50	0.22
18.	OPK13	4	2	50.00	0.11
19.	OPK17	5	2	40.00	0.20
20.	OPK20	2	1	50.00	0.15
	Minimum	1	1	10.00	0.00
	Maximum	40	9	100.00	0.71
	Total	202	58	767.49	3.74
	Mean	10.1	2.9	38.37	0.19

G.D. = Genetic Distance

Genetic variation in mutants

There were 202 alleles derived from 20 primers, which were used to construct the genetic variation among mutants with parent. The average of DNA polymorphic rate of this investigation is 38.37% revealed by 20 primers. The number of RAPD loci detected in the mutants with parent ranged from 1 to 9 across 20 primers. For the present study OPK-06 and OPK-11 were considered ad shown in Figs. 1and 2. Comparatively, all the mutants revealed addition and deletion of DNA bands than parent. RAPD loci revealed a tremendous variation in DNA profile and it was 62.5 per cent of total polymorphism across two primers; primer OPK-06 and OPK-11 (Figure 1 and 2) revealed 25 and 10 per cent of polymorphism respectively among the mutants when compared to parent (Table 3).

Discussion

Quantitative parameters of mutants and parent cultivar

The quantitative traits of mutants and parent cultivar (control) were recorded (Table 2) showed that high plant height (tall mutant), seed yield (bushy mutant), high seed protein content (seed protein mutant), lodging resistance (dwarf). Seed protein mutant showed 25.6 per cent of protein when compared to the parent (23.3%) cultivar (control). The dwarf mutant showed more seed protein (24.4%) content than parent with lodging resistance. Bushy mutant was showed more productivity rather than parent cultivar. Juliet Hepziba and Subramanian (2002) observed various macromutants such as tall, bushy, erect type mutants with quantitative modifications in black gram in M_4 generation induced by gamma rays and EMS treatments.

Odeigah et al. (1998) recorded quantitative traits of cowpea such as plant height, peduncles plant^{-1}, pods plant^{-1}, 1000 seed weight, seeds pod^{-1} were increased than control with effect of EMS and gamma rays in M_2 generation with improved seed protein mutant.

RAPD profile

DNA polymorphism by RAPD of this investigation was 38.37% revealed by 20 decamer primers. Mutants revealed in this study addition and deletion of DNA bands compared to parent cultivar (control). Across two primers OPK-06 and OPK-11 (Figure 1 & 2) 62.5% of DNA polymorphism was recorded for four mutants and parent cultivar (Table 3).

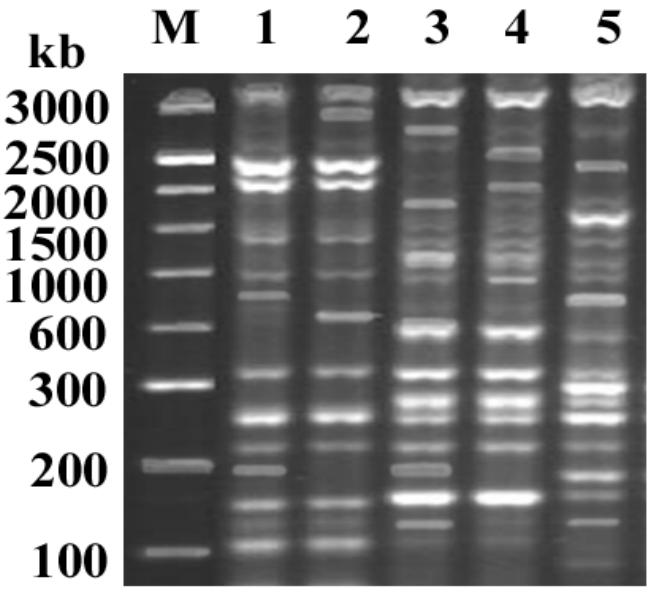

Figure 1. RAPD profile generated by primar OPK-6 from genomic DNA of mutant and parent cultivar (control). M- DNA molecular marker; 1- Parent cultivar (Control); 2- High seed protein mutant; 3- Bushy type mutant; 4- Tall mutant; 5- Dwarf mutant

DNA polymorphisms in mutants of black gram were 25.5% by RAPD analysis (Souframanien et al., 2002). The RAPD technique has been applied to assess molecular polymorphism in Vigna (Akito et al., 1996), chickpea (Sonnante and Beckman, 1997), *Vigna ungularis* (Yee et al., 1999), mung bean (Santalla et al., 1998; Lakshanpaul et al., 2000) and black gram (Souframanien et al., 2002).

Distance of Genetic variation among mutants and parent cultivar (control)

UPGMA dendrogram showed three cluster a, b and c and range of genetic distances was from 0.621 to 0.785 (Table 4). Tall and parent was clustered (a) in single group which deviated from other mutants dwarf and bushy mutants (Cluster-c). Mutant high seed protein mutant was too isolated from parent and grouped in single cluster b (Figure 3). It is due to mutations accumulate at constant rate throughout the living systems (molecular clock hypothesis), that the same is true for rapidly evolving markers (deletions and duplications in genome) detected by a RAPD technique (Abid Hussain et al., 2008). Changes in DNA are caused by mutagens result in genetic variation detected by RAPD analysis. It has to be noted that direct acting alkylating agents such as EMS primarily cause point mutations, which are the result of a single base pair deletion, addition or substitution (Schy and Plewa, 1989).

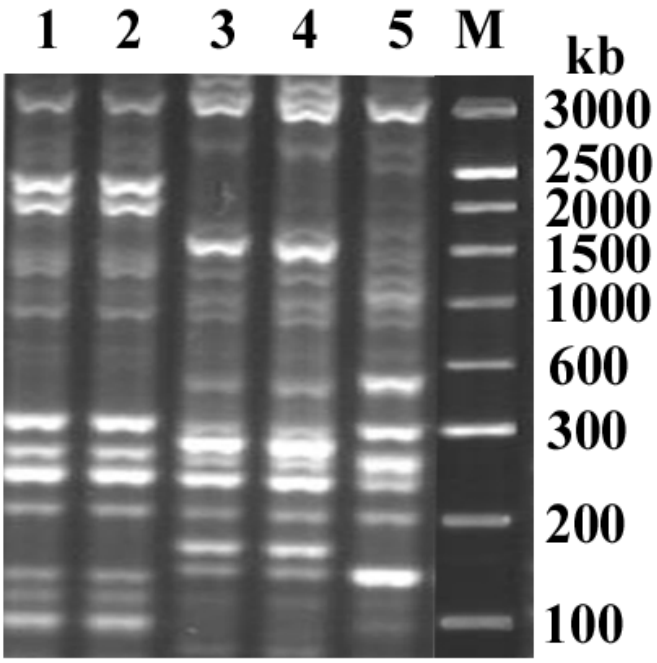

Figure 2. RAPD profile generated by primar OPK-11 from genomic DNA of mutant and parent cultivar (control). M- DNA molecular marker; 1- Parent cultivar (Control); 2- High seed protein mutant; 3- Bushy type mutant; 4- Tall mutant; 5- Dwarf mutant

Most of the radiation induced mutations analysis has revealed deletions and others inversions (Anjali et al., 1997). It is the prerequisite for map-based cloning is the development of a highly saturated map in the region contains the gene of interest. The markers close to this gene can be used as starting point for chromosomal walking and eventually finding and cloning the target gene (Hu et al., 2008). The polymorphism revealed by RAPD may be due to single base changes in the primer target sites as well as deletion or insertion of DNA sequence (Caetano Anolles et al., 1991).

Table 4. Distance matrix of the black gram mutants with parent analyzed based on Jaccard's index (Primer-OPA-14 and OPI-04).

Genetic Distance Matrix	Parent cultivar (Control)	High seed protein	Bushy mutant	Tall mutant	Dwarf mutant
Parent cultivar (Control)	0.621	-	-	-	-
High seed protein	0.686	0.621	-	-	-
Bushy mutant	0.769	0.648	0.621	-	-
Tall Mutant	0.785	0.625	0.728	0.621	-
Dwarf mutant	0.722	0.618	0.617	0.629	0.621

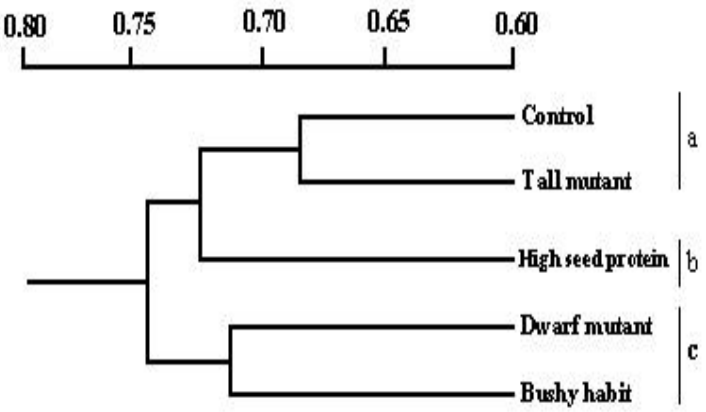

Figure 3. RAPD dendrogram based on algorithm of unweighted pair group method with arithmetic average among mutants and parent cultivar (control) of black gram by primers (OPK-6 and OPK-11).

Conclusion

The genetic variation was significant in the primers OPK-06 and OPK-11 which was 62.5 percent of DNA polymorphism. This variation due to deletion, addition may be caused for variation in DNA banding pattern by gamma rays and EMS. The polymorphic DNA by RAPD assay suggested that the mutated gene can be isolated, sequenced and analyse the function of them for development of black gram

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