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RAPD based DNA fingerprinting and analysis of genetic diversity in radiation induced mutants of cowpea [*Vigna unguiculata* (L.) Walp.]

R. N. Pandey, P. Dhanasekar and J. Souframanien

Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai 400 085

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Abstract

Thirteen radiation-induced mutants of cowpea [*Vigna unguiculata* (L.) Walp.] cv. V-130 showing distinct morphological differences, besides the parental line, were screened for random amplified polymorphic DNA (RAPD) variation. Sixty-four random decamer primers used for amplification generated a total of 495 bands, of which 230 (46.5%) were polymorphic. Mutant-specific polymorphic markers either alone or in combination, were detected. Eight mutants and the parent can be identified by using specific markers, while combination of two markers could identify three other mutants. The Jaccard's similarity coefficient revealed considerable genetic diversity among the mutants. The dissimilarity between the mutants was as high as 61 per cent. The UPGMA based dendrogram showed two clusters, as supported by bootstrapping, with seven sub-clusters. The high range of genetic diversity observed among the mutants affirms the potentiality of radiation in inducing variability in cowpea. DNA fingerprinting of the mutants will facilitate their identification, registration and determination of seed purity.

Key words: Cowpea, DNA fingerprinting, genetic diversity, mutants, RAPD markers

Introduction

Genetic markers specific to cultivars are desirable for identification, germplasm protection and seed purity determination. Morphological traits have been traditionally used to identify a cultivar. However, during last three decades, molecular markers have been developed for unambiguous identification of the cultivars. DNA markers like RAPD give high degree of polymorphism and thus aid in differentiating even closely associated cultivar [1, 2]. RAPD technique has also been used to investigate genetic diversity in germplasm of several crops including cowpea [3], mungbean [4,5], *Phaseolus vulgaris* [6] and *Vigna angularis* [7].

Cowpea, one of the most important food legume crops of the tropical and subtropical regions of the world, has a great potential in India, as it can be grown during *Kharif* and summer seasons in northern India and throughout the year in the peninsular India [8]. With an objective of developing suitable varieties suited to specific requirements of the regions and also to create additional variability by inducing mutations, seeds of cowpea cultivar V-130 were irradiated with 200 Gy gamma rays, and a number of morphological mutants were obtained [9, 10]. The breeding behaviour of the mutants was studied for several generations and stable mutants were identified. In the present investigation, RAPD based DNA fingerprinting of some of the mutants was carried out for precise identification, and the polymorphism observed was analyzed to assess the genetic variability induced by radiation treatment.

Materials and methods

The cowpea mutants included in this investigation, along with their plant characteristics are listed in Table 1.

DNA extraction and quantification: DNA was extracted from the seed tissues following the procedure described by Krishna and Jawali [11] with minor modification. About 1 g seed tissue of each sample obtained after removing seed coat of the soaked seeds was crushed and ground with 10 ml extraction buffer (0.1 M Tris-HCl, pH 8, 0.05 M EDTA, pH 8, 0.5 M NACl) and transferred to centrifuge tubes. To this 1 ml of 20% sodium dodecyl sulphate was added. The tubes were maintained at 65°C for 15 min. and then cooled. Five ml of 5 M potassium acetate was added to each tube and vortexed. The tubes were maintained at 4°C for 20 min. The slurry was centrifuged at 4°C for 20 min at 14000 g (subsequent centrifugations were also at 14000 g). The supernatants obtained were precipitated (1:1, v/v) with isopropanol and incubated at room temperature for 15 min. The contents of the tube were centrifuged at 4°C for 10 min. The pellets obtained were dissolved in 700 µl of TE buffer (0.01 M Tris-HCl, pH 8, 0.001 M EDTA, pH 8). To this 500 µl of a mixture of chloroform : isopropanol (24:1) was

Table 1. Radiation induced cowpea mutants and their plant characteristics

Mutant/variety	Height (cm)	No. of pods	Pod length (cm)	Yield (g)	100 seed wt. (g)
V-130 (Parent)	117.0±7.53	12.2±3.20	13.00±0.42	8.59±2.86	7.91±0.36
TCM 121-8	117.4±10.57	40.2±8.29*	10.40±0.26	33.64±7.61*	10.46±0.15*
TCM 13-3	164.4±14.71*	27.0±8.29*	12.10±0.33	26.80±10.11*	13.20±0.23*
TCM 77-4	59.2±2.53*	28.2±3.04	13.25±0.70	25.55±4.11	11.05±0.30*
TCM 121-15 (Stippled black seed)	134.0±12.59	24.2±5.81*	10.32±0.39	12.83±4.09	7.15±0.26
TCM 121-16 (Turkey grey seed)	161.0±14.2*	19.0±4.00	12.17±0.71	16.10±5.13	8.77±0.43
TCM 10-1	149.3±7.52	20.3±3.53	13.38±0.34*	13.30±2.10	11.40±0.19*
TCM 10-2	111.5±9.90	12.2±1.76	11.80±0.53	7.58±0.79	9.55±0.19*
TCM 134-2	82.3±2.74*	15.1±1.97	12.93±0.39	9.60±1.72	9.06±0.28*
TCM 173-22 (Bi-coloured seed: dark brown and white)	143.7±18.00	8.0±2.65	12.75±0.29	7.87±1.96	11.73±0.67*
TCM 42-1	96.0±22.27	9.3±3.38	12.67±0.30	8.20±0.20	8.61±0.71
TCM 55-5 (Bi-coloured seed: brown and white)	130.4±6.80	29.9±4.03*	10.90±0.35	22.72±2.92*	10.90±0.16*
TCM 119-9	133.2±4.73*	31.0±4.51*	11.41±0.23	21.84±3.25*	11.88±0.27*
TCM 148-1	113.9±3.78	14.4±4.45	11.29±0.55	10.64±2.41	10.41±0.32*

*Indicates major difference from the parent

added. The contents of the tubes were centrifuged at 4°C for 10 min. and supernatant collected. 1 ml of ethanol (100%) was added to each supernatant for DNA precipitation and centrifuged for 2 min. Supernatants were discarded and the pellets (DNA) were dissolved in 500 µl of TE buffer. Five µl of RNase was added to each tube and incubated at 37° C for 1 h followed by addition of 500 µl of chloroform. The contents of the tubes were centrifuged at 4° C for 10 min. 500 µl of ethanol (100%) was added to the supernatants and centrifuged for 5 min. Supernatants were discarded and pellets washed with 70% ethanol and kept for drying for 30 min. DNA thus obtained was dissolved in 100 µl of TE buffer, and was quantified using Hoefer DyNA Quant 200-Fluorometer with the reagents (10X TNE buffer, calf thymus DNA as standard and Hoechst dye). DNA was diluted with sterile water to get 50 ng/µl for PCR amplification.

DNA amplification: The random primer kits (C, G, H, I, K, L and N) used for amplification were obtained from Operon Technologies (Alameda, CA, USA). The DNA amplification mixture (25 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dNTPs, 20 pmoles of RAPD primer, 150 ng of DNA and 0.5 unit of Taq DNA polymerase (Bangalore Genie Pvt. Ltd.). Amplifications were performed in an Eppendorf Master Cycler gradient (Eppendorf Netheler - Hinz GMBH, Hamburg). DNA was amplified using the following programme: 1 cycle of 94° C for 4 min., 45 cycles of "94° C for 1 min., 37° C for 1 min., 72° C for 2 min.", followed by 72° C for 5 min.

Amplified DNA was analyzed on a 1.5% agarose gel in 1X TBE buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA) at 75 V. The bands were visualized by ethidium bromide staining (0.5 µg/ml) by inclusion in the gel. The gels were photographed under UV with Polaroid Gel Cam or by Gel Documentation System. Bands were scored as '1' (present) or '0' (absent). Reproducibility of the bands was confirmed by repeating the experiment twice (extraction and amplification).

Data analysis: Data were analyzed for similarity by using the NTSYS-pc Version 2.02 [12]. Pairwise genetic similarity values were estimated using Jaccard's similarity coefficient [13]. Unweighted pair group method using arithmetic averages (UPGMA) was employed for cluster analysis. Bootstrap support for the branches of the dendrogram was generated with 1000 bootstrapped samples in the WinBoot programme [14].

Results and discussion

DNA fingerprinting: A total of about 495 amplification products or bands were generated by 64 decamer primers (Table 2), of which 230 (46.5%) were polymorphic. The number of amplified bands for various primer, varied from 3 (OPG 13, OPI 05 and OPH 1 1) to 14 (OPL 08) with an average of 7.7 bands per primer, while the number of polymorphic bands ranged from I to 9 with an average of 3.6 per primer. The size of the amplified bands ranged from 125 bp (OPG 16) to 7345 bp (OPL 03). while several polymorphic bands were observed in the mutants (Fig. 1a), some bands were observed to be present or absent only in a particular mutant (Fig. 1b). Such bands were taken as mutant- specific markers that can identify the mutants, either alone or in combination. Markers specific to mutants/cultivar are listed in Table 3. Eight mutants and the parent can be identified using specific marker. Combination of two markers is required to identify three

Table 2. List of the decamer primers and the polymorphism observed in the study

Primer	Primer sequence (5' to 3')	Total No. of bands	No. of polymorphic bands	Primer	Primer sequence (5' to 3')	Total No. of bands	No. of polymorphic bands
OPC 11	AAAGCTGCGG	7	4	OPN 20	GGTGCTCCGT	9	8
OPG 02	GGCACTGAGG	9	7	OPL 01	GGCATGACCT	4	3
OPG 08	TCACGTCCAC	4	3	OPL 02	TGGGCGTCAA	5	3
OPG 13	CTCTCCGCCA	3	0	OPL 03	CCAGCAGCTT	4	0
OPG 16	AGCGTCCTCC	11	5	OPL 05	ACGCAGGCAC	8	4
OPI 01	ACCTGGACAC	9	7	OPL 06	CIAGGGAAGAG	8	8
OPI 04	CCGCCTAGTC	5	1	OPL 07	AGGCGGGAAC	11	3
OPI 05	TGTTCCACGG	3	1	OPL 08	AGCAGGTGGA	14	9
OPI 06	AAGGCGGCAG	11	4	OPL 12	GGGCGGTACT	11	1
OPI 08	TTTGCCCGGT	11	9	OPL 13	ACCGCCTGCT	12	2
OPI 09	TGGAGAGCAG	6	3	OPL 14	GTGACAGGCT	6	1
OPI 12	AGAGGGCACA	8	1	OPL 15	AAGAGAGGGG	10	4
OPI 13	CTGGGGCTGA	6	4	OPL 16	AGGTTGCAGG	11	4
OPI 14	TGACGGCGGT	4	3	OPL 17	AGCCTGAGCC	7	1
OPI 16	TCTCCGCCCT	8	5	OPH 01	GGTCGGAGAA	6	5
OPI 17	GGTGGTGATG	4	2	OPH 02	TCGGACCITGA	8	7
OPI 18	TGCCCAGCCT	9	5	OPH 03	AGACGTCCAC	8	1
OPI 19	AATGCGGGAG	4	1	OPH 04	GGAAGTCGCC	8	2
OPI 20	AAAGTGCGGG	6	3	OPH 05	AGTCGTCCCC	12	8
OPK 02	GTCTCCGCAA	4	2	OPH 07	CTGCATCGTG	6	2
OPK 03	CCAGCTTAGG	4	3	OPH 08	GAAACACCCC	7	2
OPK 07	AGCGAGCAAG	13	5	OPH 09	TGTAGCTGGG	5	3
OPN 01	CTCACGTTGG	5	5	OPH 11	CTTCCGCAGT	3	1
OPN 02	ACCAGGGGCA	7	3	OPH 12	ACGCGCATGT	10	6
OPN 04	GACCGACCCA	4	2	OPH 13	GACGCCACAC	9	2
OPN 05	ACTGAACGCC	7	1	OPH 14	ACCAGGTTGG	10	3
OPN 11	TCGCCGCAAA	11	7	OPH 15	AATGGCGCAG	10	4
OPN 12	CACAGACACC	10	2	OPH 16	TCTCAGCTGG	8	4
OPN 15	CAGCGACTGT	10	8	OPH 17	CACTCTCCTC	12	1
OPN 16	AAGCGACCTG	7	3	OPH 18	GAATCGGCCA	11	4
OPN 17	CATTGGGGAG	5	5	OPH 19	CTGACCAGCC	13	4
OPN 18	GGTGAGGTCA	5	4	OPH 20	GGGAGACATC	9	2

other mutants namely TCM 121-8, TCM 77-4 and TCM 42-1. These specific markers will aid in unambiguous identification of the mutants and to maintain their seed purity.

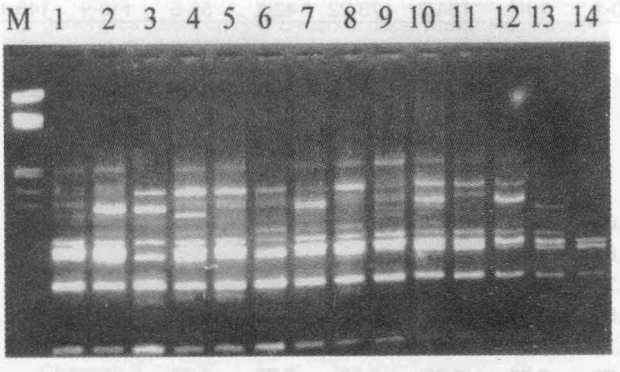

Fig. 1a. RAPD polymorphism in cowpea cultivar V-130 (No. 1) and its mutants (Nos. 2-14) using the primer OPL 16. M is the Lamda DNA *EcoR* I and *Hind* III double digest marker

Genetic diversity. The genetic similarity between the parent and the mutants based on Jaccard's similarity coefficient ranged from 0.54 (TCM 173-22) to 0.83 (TCM 42-1) (Table 4). The similarity coefficient among the mutants varied from 0.39 (TCM 173-22 and TCM

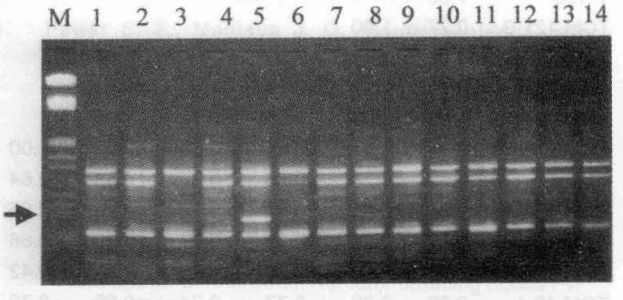

Fig. 1b. RAPD fingerprint of cultivar V-130 and its mutants using the primer OPH 20. Arrow indicates the mutant specific marker

Table 3. Specific RAPD markers identified in the cowpea mutants

Mutant/cultivar	Specific marker	Present (+) or absent (-)
V-130 (Parent)	OPL 15 (1493 bp)	-
TCM 121-15	OPH 20 (542 bp)	+
TCM 121-16	OPG 02 (2027 bp)	+
TCM 10-1	OPI 01 (1206 bp)	-
TCM 134-2	OPI 18 (735 bp)	+
TCM 173-22	OPH 05 (3228 bp)	-
TCM 119-9	OPH 14 (3364 bp)	+
TCM 148-1	OPL 11 (2845 bp)	-
TCM 10-2	OPI 08 (1176 bp)	+
TCM 77-4, TCM 10-2	OPH 12 (348 bp)	-
TCM 10-1, TCM 119-9, TCM 148-1	OPI 14 (1167 bp)	-
TCM 121-8, TCM 10-1, TCM 119-9, TCM 148-1	OPH 01 (628 bp)	+
TCM 121-15 and TCM 121-16	OPH 16 (487 bp)	+
TCM 42-1,TCM 121-15, TCM 121-16	OPH 05 (682 bp)	-

121-15) to 0.89 (TCM 42-1 and TCM 134-2). The UPGMA based dendrogram (Fig. 2) shows only two major clusters, though there are seven sub-clusters. But for the mutant TCM 173-22, all the mutants and the parent V-130 fall in one large cluster. Among the seven sub-clusters, five with high bootstrap values have two or more individuals, while two sub-clusters have one individual each.

Genetic similarity analysis thus showed considerable variability in the cowpea mutants at DNA level as a result of radiation treatment. The range of variability was considerably high as observed between the mutants TCM 173-22 and TCM 121-15 with 61% dissimilarity. This indicates the potentiality of radiation treatment in inducing genetic variability in cowpea.

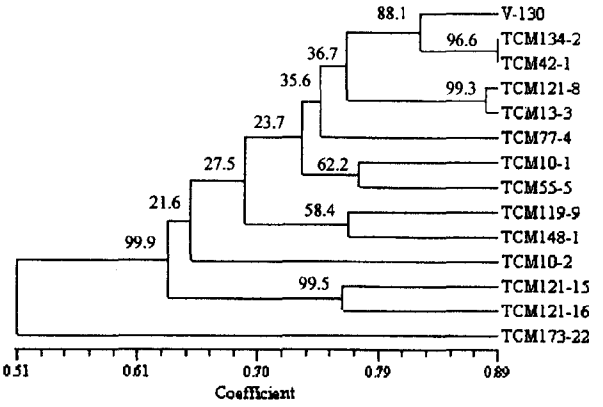

Fig. 2. UPGMA based dendrogram with bootstrap values depicting relationships among the cultivar V-130 and the 13 mutants

Radiation induced variability at DNA level has been reported in a few crops like groundnut [15] and blackgram [16]. The high range of variability can be attributed to the presence of transposable elements in cowpea [17], which possibly gets activated with radiation treatment exhibiting wide range of mutations. High range of induced variability owing to activation of mutator system was reported in cowpea variety Pusa Phalguni [18], from which the cultivar V-130 has been derived. Reactivation of the mutator transposable element system following gamma irradiation of seed has been reported in some crops [19].

DNA markers have been used to quantify the genetic diversity and determine phenetic relationships in several plant species [20, 21]. Information on the genetic diversity in a crop species is important for selection of parental strains and in the prediction of hybrid performance [22]. The genetic diversity observed in the mutants in the present investigation shall aid in selecting parents for crossing programme. For example,

Table 4. Genetic similarity coefficients based on the RAPD dataset among the cultivar V-130 and its 13 mutants

	V-130	TCM 121-8	TCM 13-3	TCM 77-4	TCM 121-15	TCM 121-16	TCM 10-1	TCM 10-2	TCM 134-2	TCM 173-22	TCM 42-1	TCM 55-5	TCM 119-9	TCM 148-1
V-130	1.00													
TCM 121-8	0.77	1.00												
TCM 13-3	0.81	0.88	1.00											
TCM 77-4	0.74	0.77	0.76	1.00										
TCM 121-15	0.65	0.63	0.65	0.70	1.00									
TCM 121-16	0.64	0.64	0.64	0.66	0.77	1.00								
TCM 10-1	0.72	0.78	0.75	0.67	0.58	0.64	1.00							
TCM 10-2	0.61	0.65	0.63	0.72	0.60	0.65	0.68	1.00						
TCM 134-2	0.82	0.73	0.79	0.74	0.65	0.66	0.73	0.67	1.00					
TCM 173-22	0.54	0.58	0.59	0.56	0.39	0.42	0.53	0.49	0.51	1.00				
TCM 42-1	0.83	0.73	0.77	0.74	0.65	0.70	0.73	0.69	0.89	0.52	1.00			
TCM 55-5	0.71	0.75	0.77	0.70	0.61	0.61	0.78	0.69	0.76	0.56	0.75	1.00		
TCM 119-9	0.68	0.75	0.72	0.63	0.61	0.62	0.75	0.60	0.76	0.44	0.75	0.73	1.00	
TCM 148-1	0.62	0.70	0.67	0.61	0.56	0.55	0.65	0.54	0.66	0.55	0.67	0.69	0.77	1.00

a cross between TCM 173-22 and V-130 or TCM 134-2 is likely to generate promising recombinants. In blackgram, mutants and mutant derivatives when used in cross breeding have been reported to be more productive in the development of improved varieties [23,24].

Some mutants falling in the same cluster are also observed to be similar in morphological characteristics. For example, TCM 134-2 and TCM 42-1, the mutants with dmeterminate growth habit were grouped in a single sub-cluster. TCM 121-8 and TCM 13-3, the members of the same sub-cluster, are high yielding with large number of pods and bold seeds. Similarly, TCM 121-15 and TCM 121-16 are seed colour mutants and similar in other agronomic attributes. Thus, understanding of the genetic relationships among these mutants, together with analysis of their agronomic performance, may help in their further utilization in breeding programmes.

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