Seed coat color, weight and eye pattern inheritance in gamma-rays induced cowpea M2-mutant line

Reda M. Gaafar a,*, Marwa Hamouda a, Abdelfattah Badr b

a Botany Department, Faculty of Science, Tanta University, 31527 Tanta, Egypt
b Botany and Microbiology Department, Faculty of Science, Helwan University, 11790 Cairo, Egypt

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Abstract Gamma radiation is a very effective tool for inducing genetic variation in characters of many plants. Black seeds of M2 mutant were obtained after exposure of an Egyptian cowpea cultivar (Kaha 1) to a low dose of gamma rays. Segregation of seed coat color, weight of 100 seeds and seed eye pattern of the black seeds of this mutant line were further examined in this study. Four colors were observed for seed coat in the M3 plants ranging from cream to reddish brown and three eye patterns were distinguished from each other. SDS–PAGE of the seed storage proteins showed 18 protein bands; five of these bands disappeared in the seeds of M3 plants compared to M2 and M0 controls while other 5 protein bands were specifically observed in seeds of M3 plants. PCR analysis using twelve ISSR primers showed 47 polymorphic and 8 unique amplicons. The eight unique amplicons were characteristic of the cream coat color and brown wide eye pattern (M03-G10) while the polymorphic bands were shared by 6 coat-color groups. A PCR fragment of 850 bp was amplified, using primer HB-12, in M3-G04 which showed high-100 seed weight. These results demonstrated the mutagenic effects of gamma rays on seed coat color, weight of 100 seeds and eye pattern of cowpea M3 mutant plants.

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1. Introduction

Cowpea (Vigna unguiculata L.) is one of the most essential crop species in the developing countries of the tropics and subtropics, especially in sub-Saharan Africa [1]. It is one of diploid species with $2n = 22$ [2]. According to FAOSTAT [3], worldwide green pod production in 2010 was 4.5 million tons, intended for human consumption. Cowpea seeds are considered as a major source of carbohydrates (64%), proteins (23–28%), vitamins and minerals [4–6]. Compared to other crop plants, their storage proteins are rich in lysine and tryptophan. Therefore, cowpea represents a remarkable part of the dietary protein of the people worldwide and particularly for the people inhabiting the tropics and subtropics areas [7].

Gamma radiation is a very effective tool that induces genetic variation in characters of many plants and may cause changes in different plant characters depending on the level of irradiation [8–10]. Furthermore, irradiations have been successfully employed for mutation in breeding of different ornamentals and crop plants [11].

* Corresponding author.
E-mail address: redagaafar@science.tanta.edu.eg (R.M. Gaafar).
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can be generated by few dosages of gamma rays within a short time span in order to study the modifications in any morphological characteristics in cowpea [12]. Since the 1970s, mutation breeding has been utilized for creating genetic changes in grain legumes in order to improve crop productivity, quality of protein content, disease resistance and other useful agricultural traits. Moreover, an increase in useful genetic variability has been reported [13].

A number of mutations breeding projects in various countries have involved several important grain legume species, high yield cultivars have been developed [14]. Irradiation mutations encourage the expression of recessive genes and produce new genetic variation [15,16]. In cowpea, it has been reported that irradiation mutations have resulted in changes in morphological and reproductive traits [17,18]. At the biochemical level, two cowpea cultivars have shown variation in number as well as intensity of protein bands after exposure to gamma rays [19,20].

Molecular markers are of great value to plant breeders in: isolating, identifying and evaluating markers-linked genes affecting specific traits [21]. One type of these molecular markers is the inter-simple sequence repeats (ISSRs) that uncover the regions that lie within the micro-satellite repeats [22]. ISSRs have been implemented in evaluating the intragenomic and inter-genomic diversity and also have been frequently utilized in breeding and legume diversity analysis [7,18,23]. A dose of 50 Gy of gamma radiation resulted in an increase of growth parameters and enhanced yield components in the three varieties Dokki 331, Amzerly and Cream 7; while the dose of 100 Gy resulted in higher growth rate and yield in var. Kaha 1 and var. Giza 6. Analysis of seed protein and RAPD and ISSR markers induced more genetic variation in the genotypes of cv. Kaha 1 and cv. Dokki 331 compared to other three cultivars [20,23].

Several reports on the inheritance of morphological criteria and disease resistance characteristics of cowpea have been described as early as 1985 [24]. Monogenic segregation pattern was outlined for hastate leaf shape [25], flower color [26], dehiscent pod [27], narrow eye pattern [28], dry pod color [29], brown seed coat color [28] and smooth seed coat [30]. According to the above reports, hastate leaf shape was dominant over subglobose leaf shape, purple flower was dominant over white flower, shattering of dry pod was dominant to non-shattering, and narrow seed eye pattern was recessive to the solid eye pattern. Moreover, the brown color of seed coat was dominant to the white seed coat color, while the smooth seed coat was dominant to the rough seed coat texture. Previous studies at Tanta University showed that cv. Kaha 1 produced black seed coat following exposure to 50 Gy of gamma rays as a new trait in the M2 generation, while in other cultivars, different helium colors were scored compared to control. It was concluded that these two observations require further attention [20,23]. It is well known that M1 plants are produced directly from seeds treated with a mutagen, while the next generation is termed the M2, followed by M3, etc. The main objective of the present study was to determine the inheritance pattern of seed coat color, seed weight (weight of 100 seeds), seeds eye pattern and seed coat texture of M3 cowpea plants, which were the descendants of an M2 mutant that has black seeds. In addition, seed protein profiling as a biochemical markers and ISSR as DNA markers were examined to find out relationship between these markers and seed characteristics particularly the seed coat color, seed weight (weight of 100 seeds) and seeds eye pattern.

2. Material and methods

2.1. Plant materials

Egyptian cowpea cultivar Kaha 1 was originally obtained from Agriculture Research Center, Giza, Egypt. M1 plants were produced as follows: the dry seeds of the Egyptian cowpea cultivar Kaha 1 were exposed to 50 Gy of gamma rays using cobalt 60 as a source of gamma radiations. The irradiation was done at the Atomic Energy Center, Nasr City, Cairo, Egypt. The control seeds (M0) were not exposed to gamma irradiation. The exposed and control seeds of the cowpea Kaha 1 were grown to maturity in 50 cm wide pots (4 plants per pot). The seeds of the M1 plants were then sown in the soil under the recommended conditions for growing cowpea until maturity. It was observed that the seed color of some M1 plants (M2 seeds) was changed from the normal white color to dark black color [31]. In this study, the dark black seeds of M2 (provided by Mr. Mohamed Halawa) and the control seeds of the cowpea cultivar Kaha 1 (M0) were grown to maturity in 50 cm wide pots (4 plants per pot). Seeds were collected from all M3 plants. Weight of 100 seeds/genotype was determined. Seed coat-color, seed texture (smooth or rough) and eye patterns of M0, M2 and the 10 M3 genotypes were visually recorded. A one-way analysis of variance (ANOVA) was used to compare the productivity (weight of 100 seeds) among M0, M2 and the 10 M3 genotypes. SigmaStat ver. 3.5 was used for an analysis of variance (DUNDAS SOFTWARE Ltd., Germany).

2.2. SDS–PAGE analysis of seeds protein

Total seed proteins were extracted from 100 mg of seeds powder with 0.5 ml of 0.03 M Tris–HCl buffer (pH 8.0), extraction buffer containing 5 μl of 2-mercaptoethanol. Seed protein components were separated with slab type SDS–PAGE in 12% polyacrylamide gel as described by Laemmli [32]. The gel was then photographed and the presence or absence of bands was scored as 1 or 0; whereby 1 = band present and 0 = band absent. Molecular weight marker (5 μl) composed of 10–200 KDa proteins (Fermentas, Germany) were used as standard markers. The molecular weight of the protein molecules was determined by comparison to the standard markers.

2.3. ISSR analysis

ISSR fingerprinting was performed based on the procedure described by [23,33] with some modifications. In brief, twelve ISSR primers (Operon Nippon EGT Co. Ltd.) were used for the amplification of PCR products from the M0 plants of the cowpea cultivar Kaha 1 (control) and the plants of the M3 generation. Sequences of the primers and their properties are listed in Table 1. PCR amplification was conducted using Bio-Rad thermo-cycler according to the following cycle profile: initial denaturation at 94 °C for 4 min, followed by 44 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C, and 8 min at 72 °C for final product extension. The amplified PCR products were resolved by electrophoresis in a 2%
agarose gel containing ethidium bromide (0.5 μg/ml) in 1× TAE buffer at 100 V. The ISSR fingerprinting was visualized using a Gel Works 1D advanced gel documentation system (UVP, UK) and photographed under UV light. The size of each band was estimated using 100 bp DNA ladder (Fermentas, Germany) as a standard marker. Molecular weights of protein bands and the size of ISSR bands were determined using Lab Image software version 2.7 (Kapelan GmbH, Germany).

3. Results

3.1. Seed coat color, texture, eye pattern and seed weight

The results showed that black seed of M2 generation was segregated in M3 generation into various distinct categories depending on coat color (Fig. 1 and Table 2). Moreover, distinct seed-coat textures and eye patterns were observed in the seeds of M3 plants (Table 2). The coat color ranged from cream, dark cream, pale brown to reddish brown (Fig. 1). Seed texture fell into two forms rough or smooth, while eye-pattern was either black or brown and narrow or wide hilum ring. The cream seed-coat color was the dominant color which represented 79.9% of the total obtained seeds. While the brown color designated 15.9% of the total seeds (Table 2) and the reddish brown seed coat was 4.2%. The low dose (50 Gy) of gamma rays induced a significant increase in the weight of 100 seeds in M2 mutant plants compared to cv. Kaha 1 (M0 control) see Table 2. The M2 line showed a 20.65 g/100 seeds, while cv. Kaha 1 exhibited 15.76 g/100 seeds (Table 2). The M3 plants were highly variable, where the weight of 100 seeds ranged from the lowest 100 seeds weight (14.76; M3-G09 mutant plants) to the highest 100 seeds weight (22.05; M3-G04 mutant plants). The statistical analysis of the productivity (weight of 100 seeds) results showed that there is a significant difference between M3-G04 and M0 (Table 2).

| Table 1 | Sequences of 12 ISSR primers with annealing temperatures (Tm) that produced polymorphism in cowpea M3 segregating plants, number of amplified bands/ primer, number of polymorphic bands/ primer and the percentage of polymorphism. |
| Primer code | Primer sequence | Tm | No. of amplified bands/ primer | No. of polymorphic bands/ primer | % of polymorphism/ primer (%) | Markers size range (bp)/primer |
|------------|-----------------|-----|-----------------------------|-----------------------------|-----------------------------|-------------------------------|
| 809        | 5'AGAGAGAGAGAGAGAGG3' | 34.3 | 9 | 6 | 66.67 | 699–168 |
| 825        | 5'ACACACACACACACACT3' | 35.3 | 6 | 5 | 83.33 | 781–203 |
| 841        | 5'GAGAGAGAGAGAGAGAYC3' | 35.8 | 8 | 6 | 75.00 | 845–282 |
| 844        | 5'CTCTCTCTCTCTCTCTAC3' | 31.4 | 5 | 1 | 20.00 | 629–265 |
| 17898-A    | 5'CACACACACACACACAC3' | 42 | 8 | 4 | 50.00 | 913–237 |
| 17899-A    | 5'CACACACACACAAG3' | 42 | 8 | 7 | 87.50 | 703–119 |
| 17899-B    | 5'CACACACACACAG3' | 44 | 7 | 3 | 42.86 | 206–657 |
| HB-8       | 5'GAGAGAGAGAGAGAG3' | 44 | 9 | 4 | 44.44 | 1087–360 |
| HB-9       | 5'GTGTGTGTGTGTGG3' | 44 | 7 | 1 | 14.29 | 779–333 |
| HB-10      | 5'GAGAGAGAGAGACC3' | 44 | 8 | 4 | 50.00 | 627–201 |
| HB-12      | 5'CACACACACACGG3' | 38 | 8 | 3 | 37.50 | 918–418 |
| UBC-827    | 5'ACACACACACACACACG3' | 41 | 4 | 2 | 50.00 | 649–305 |

Figure 1  Photographs illustrating ten segregates of seed shape, color and seed eye pattern of M3 cowpea mutant genotypes (1–10), M2 plants and of the parent cultivar Kaha 1 (M0).
3.2. SDS-proteins analysis of M3 plants

Twenty-one protein bands of molecular weights ranging between 10 and 175 KDa were observed in cowpea SDS–PAGE protein profiles (Fig. 2). Two bands with molecular weights of 26 and 27 KDa appeared only in control plant (Kaha 1: M0); while they were absent in M3 plants (progenies of the M2 black seeds, which were derived from Kaha 1 after exposing its seeds to a low dose of gamma-rays). On the other hand, the two protein bands of molecular weights 125 and 115 KDa were de novo synthesized in protein profiles of M2 mutant plants (lane 11, Fig. 2). Interestingly, two other protein bands (15 KDa and 17 KDa) were found specifically in M3 mutant groups M3-G02, M3-G03, M3-G04 and M3-G09. Moreover, 29 KDa protein band was absent in M3 plants M3-G01 (17.81 g/100 seeds), which were derived from Kaha 1 after exposing its seeds to a low dose of gamma-rays). On the other hand, the two protein bands of molecular weights 125 and 115 KDa were de novo synthesized in protein profiles of M2 mutant plants (lane 11, Fig. 2). In contrast, two protein bands (20 KDa and 150 KDa) were more intense in M0 control compared to M2 and M3 plants. Interestingly, two high molecular weight protein bands (110 KDa and 135 KDa) were only observed in M2 mutant line (lane 11, Fig. 2), while two low molecular weight protein bands (26 KDa and 28 KDa) were only shown in M0 control (lane 12, Fig. 2). 96 KDa protein band was characteristics of M3-G04 mutant plants (Fig. 2), where it showed highest weight (22.05 g) of 100 seeds (Table 2).

3.3. ISSR fingerprinting of M3 plants

Using 12 primers, the total number of ISSR markers observed among the M3 cowpea mutant plants was 87 bands (loci), which included 34 monomorphic, 46 polymorphic (Table 1) and 8 unique loci (Table 3). The ISSR primers 809 and HB-9 amplified 9 bands, while ISSR primer UBC-827 amplified only 4 loci (Table 1). ISSR primer 17899-A exhibited the highest polymorphic percentage (7 loci; 87.5%), while the primers 844 and HB-9 showed the lowest percentage (1 band; 20%) and (1 band; 14.29%), respectively (Table 1). The percentage of polymorphism in the ISSR profiles in the genomes of M0, M2 and M3 mutant plants is shown in Table 3. The highest polymorphism

| Genotype | Seed coat-color | Seed eye-pattern | Seed texture | Weight of 100 seeds (g) | Seed number obtained |
|----------|----------------|------------------|--------------|-------------------------|----------------------|
| M0       | White          | Black/narrow     | Smooth       | 15.76*                  | –                    |
| M2       | Black          | Black/solid      | Smooth       | 20.65*                  | –                    |
| M3-G01   | Dark Cream (black dots) | Black/wide       | Rough        | 17.81                   | 9                    |
| M3-G02   | Cream          | Black/wide       | Rough        | 18.49                   | 24                   |
| M3-G03   | Cream          | Black/narrow     | Smooth       | 18.43                   | 46                   |
| M3-G04   | Light brown    | Brown/narrow     | Smooth       | 22.05*                  | 13                   |
| M3-G05   | Cream          | Black/narrow     | Rough        | 18.43                   | 48                   |
| M3-G06   | Pale brown     | Brown/narrow     | Smooth       | 18.71                   | 14                   |
| M3-G07   | Light brown (white patches) | Brown/narrow     | Smooth       | 19.20                   | 3                    |
| M3-G08   | Reddish-brown  | Black/wide       | Smooth       | 14.76*                  | 8                    |
| M3-G09   | Cream          | Brown/wide       | Rough        | 18.31                   | 13                   |
| M3-G10   | Cream          | Brown/narrow     | Rough        | 18.30                   | 11                   |
| Total    |                |                  |              |                         | 189                  |

* There is a statistically significant difference (P ≤ 0.001).
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Table 3 Number of polymorphic bands and percentage of polymorphism in the ISSR profiles in the genome of M0, M2 and M3 mutant cowpea plants.

| Name of M3 mutant group | Total no. of amplified bands | No. of monomorphic bands | No. of polymorphic bands | % of polymorphism | No. of unique bands |
|------------------------|-------------------------------|--------------------------|--------------------------|------------------|-------------------|
| M3-G01                 | 60                            | 34                       | 26                       | 43.33            | 0                 |
| M3-G02                 | 61                            | 34                       | 27                       | 44.26            | 0                 |
| M3-G03                 | 58                            | 34                       | 24                       | 41.37            | 0                 |
| M3-G04                 | 57                            | 34                       | 23                       | 40.35            | 0                 |
| M3-G05                 | 62                            | 34                       | 28                       | 45.16            | 0                 |
| M3-G06                 | 61                            | 34                       | 27                       | 44.26            | 0                 |
| M3-G07                 | 63                            | 34                       | 29                       | 46.03            | 0                 |
| M3-G08                 | 63                            | 34                       | 21                       | 38.18            | 0                 |
| M3-G09                 | 55                            | 34                       | 21                       | 46.87            | 0                 |
| M3-G10                 | 64                            | 34                       | 30                       | 37.03            | 0                 |
| M2                     | 54                            | 34                       | 20                       | 46.87            | 0                 |
| M0                     | 64                            | 34                       | 30                       | 46.87            | 0                 |

(46.87%) was observed in the genome of M3-G10 mutant plants compared to M3 mutant plants while the lowest genome variability (37.03% polymorphism) was found in the genome of the M2 mutant line (Table 3). Although M0 genome showed high genome variability (46.87% polymorphism) compared to M2 and M3 mutant plants, it showed null unique bands (loci). Interestingly, ISSR profiles of M3-G10 mutant plants displayed 8 unique loci (Table 3). Meanwhile, the ISSR profiling produced by the 12 primers in the examined genotypes is illustrated in Fig. 3. Three primers (HB-8, HB-9 and HB-12) showed polymorphism among the M3 mutant plants M2 and M0 in 3 loci of 500 bp, 700 bp and 900 bp, respectively (Fig. 3). The M3-G10 mutant plants (cream, brown-narrow eye, rough seeds and 18.30 g/100 seeds) showed a unique 8 amplicons of 800 bp and 850 bp (ISSR HB-8), 750 bp (ISSR HB-9), 900 bp (ISSR HB-12), 950 bp (ISSR 17898-A), 850 (ISSR 841), 500 bp and 600 bp (ISSR 809). The primer 17899-B showed only one polymorphic locus (450 bp). Primer UBC-827 showed two polymorphic two amplicons (550 bp and 600 bp), which were produced in the genotype 5 of M3 (cream coat, black-narrow eye, rough seed texture and 18.43 g/100 seeds) and that were also found in the M2 generation (black seed, black-solid eye, smooth texture and 20.65 g/100 seeds). Meanwhile, only the 550 bp amplicon was amplified in the M3-G07 mutant (light brown coat, black-narrow eye, smooth seed texture and 18.43 g/100 seeds) and M3-G10 (cream coat, brown-narrow, rough seed texture and 18.30 g/100 seeds). Primers 809, 825 and 17899-A showed polymorphism among M3 mutant plants, M2 and M0 control plants, while primers 841, 844, 179898-A and HB-10 exhibited variation only among M3 mutant plants (Fig. 3). Interestingly, primer HB-12 amplified 850 bp PCR fragment in M3-G04 (light brown coat, brown-narrow eye, smooth seed texture and 22.05 g/100 seeds).

4. Discussion

The low dose of gamma rays (50 Gy) resulted in a remarkable change in coat color of Kaha 1 (M0 control) from white to black in M2 generation as well as an increase in the weight of 100 seeds in M2 and M3 mutant plants. However, black seed-coat color, seed-eye pattern, seed texture and high weight of 100 seeds characters of M2 were segregated in M3 generation. Seed color trait was segregated into various categories depending on seed color. Moreover, distinct textures and eye patterns were shown in the seeds of M3 plants. These results are in agreement with those obtained by previous studies reporting seed mutations as different seed color in legume crops (e.g. buff and black in arhar [34] and golden yellow in chickpea [35]). It is well known that exposure to gamma radiations produces morphological, physiological and biochemical mutants [36].

Gamma rays are a class of ionizing radiation, which interact with atoms or molecules to produce free radicals in cells. These radicals can modify important components of plant cells and consequently affect different morphological (e.g. seed-coat color, eye pattern etc.), anatomical, biochemical and physiological characters of plants. These changes are predominately depending on the dose of irradiation. Furthermore, it was proposed that these mutational effects could have induced changes in the cellular structure and metabolism, like dilation of thylakoid membranes, alteration in photosynthesis and pigment accumulation which later change the color and texture of seed [37,38]. Although M2 showed high yield (20.65 g/100 seeds) compared to M0 (15.76 g/100 seeds), the weight of 100 seeds character was highly variable among the M3 plants, where M3-G04 showed high productivity (22.05 g/seeds) and M3-G08 exhibited less yield (14.76 g/100 seeds). This result is in agreement with what has been obtained by Halawa and Badr et al. [20,31].

Additionally, gamma rays were also found to cause alteration in protein electrophoretic patterns by inducing appearance and/or disappearance of some protein bands [39]. In the present work (Fig. 2), three types of modifications are observed in the protein patterns of cowpea seeds, some protein bands (130, 110, 28 and 26 KDa) disappeared in all M3 plants, other proteins (15, 17 KDa in M3-G09; 20 KDa in Kaha 1 control (M0) and 120 KDa in all M3 mutant plants) were selectively increased in intensity and synthesis of a new set of proteins was induced (Fig. 2). Two protein bands of molecular weight (125 and 115 KDa) were de novo synthesized in M2 mutant seeds (lane 11, Fig. 2).

This may be due to effect of gamma radiations that led to formation of disulfide bridge between polypeptide chains which may be resulted in aggregation of the low molecular weight proteins [40,41]. Since irradiation dose used is considered very important, it has been shown that significant
change in protein constituents occurred after seed treatment with low dose of gamma irradiation, which was due to deamination of the proteins [42] or aggregation of proteins during disintegration through the decrease of the sulfhydryl group and increase the disulfide bond [43]. In addition, gamma rays caused rearrangement of the small molecular weight proteins to a high molecular weight and causes decrease in protein solubility [43].
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5. Conclusion

Gamma irradiation is very important tool in mutation breeding which has been used to create genetic variation that has been used to develop new varieties with required characteristics including disease resistant, cold and salt tolerant and high quality crops. In this study, it was obvious that lower dose (50 Gy) of gamma irradiation caused several variation in the seed-coat color, eye pattern, yield (weight of 100 seeds) and texture in M3 mutant plants. Because mutations induced by γ-radiation are mainly recessive and can only be selected in advanced generations, several seed-coat colors were observed among M3 plants, which might be caused by structural rearrangements in DNA. The observed morphological and yield characteristics can be utilized for identification and characterization of cowpea gene(s) that stand beyond them and study their functions at the molecular level. From the M3 analysis, irradiation dose at 50 Gy might have induced several mutations in the genes controlling seed-coat color, eye pattern and seed-coat texture. The found unique ISSR markers can be used in marker assisted selection (MAS) of the brown-narrow eye-pattern phenotype and high yield genotypes and in identification of gene responsible of that phenotypes.

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