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Genetic variability of mutant rice (*Oryza sativa*) genotype induced by gamma rays

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Abstract. *Ishak I. 2023. Genetic variability of mutant rice (*Oryza sativa*) genotype induced by gamma rays. Biodiversitas 24: 3300-3306.* Rice seeds of Sidenuk cultivars were irradiated by gamma rays creating a broad spectrum of genetic variability among individual plants at M3 generation progeny. Gamma Irradiated seeds created Mutation among the somatic cells population in mature embryos at M1 generations. Somatic cell mutation influenced the genetic structures of gametes cells formation on gametogenesis at M1 plants. Observation of M2 plants showed that black and brown colors of rice's pericarp were found with rice's lemma, and palea color was different compared to the wild type (Sidenuk cultivars). Seven plants with black rice pericarp colors out of 330 were obtained at M2 plants. Chlorophyll mutation was observed at M4 generation; the results showed that chimera, green, and albino leaf blade was obtained in several individual plants. There were also the variability of Palea and lemma colors among individual plant such as green, white, and purple. The cellular biology approach will explain these mutations before molecular biology identification is made.

Keywords: Genetic variability, gamma rays, rice mutants

INTRODUCTION

Induced plant mutation using physical mutagens such as gamma rays creates genetic variability in plant cells. The biological effects of ionizing radiation, such as gamma rays, and subsequent risks associated with such exposure are that only cells "hit" by the radiation are likely to carry the legacy of radiation damage. When a cell is hit, the deposition of energy can directly damage the genetic material or indirectly damage critical nuclear targets through the radiolysis of water (Morgan and Sowa 2005). Mutation of the gene creates genetic variability among individual plants in offspring because the gene controls characteristic appearance in the living organism. Hahn et al. (2021) mentioned that The exposure of dry DNA to X-rays leads to strand breaks at the sugar-phosphate backbone, while deoxyribose and nucleobases are less affected. In contrast, a substantial increase in DNA damage is observed in water, where OH-radicals are produced. Therefore, the water content in a cell influences water radiolysis and free radicals formations.

Gene mutation also can create genetic variability within species. Leaf blade chimera in the rice leaves due to gamma irradiation caused changes in genetic structure compared to the wild type. Mutations cause changes in the genetic structure that occur in the chloroplast genome in the DNA coding frame. The stability of leaf-color chimera mutants was higher than that of leaf-shape chimeras. Stability depends on the chimera type and location of a mutation in the shoot apical meristem cell layers (Kim et al. 2020). The word chimera comes from the Greek chimaira, a mythological animal composed of parts of three different animals. It is traditionally applied in biology to define a plant (or an animal, an organ or a tissue) consisting of somatic sectors having different genetic constitutions (Prina et al. 2012). Short-term γ-irradiation was more effective in inducing mutations in *Cymbidium* (Kim et al. 2020).

A rice mutant with a purple color in the leaf blade, pl6, was developed from wild type (WT), Zhenong 41, with gamma-ray treatment (Khan et al. 2020). Besides that, Nguyen et al. (2021) reported the photosynthetic properties and transcriptomic profiles of chlorophyll (Chl) b-deficient mutant (ch11) and wildtype rice (*Oryza sativa* L.). Chl b-deficient rice showed irregular chloroplast development, affecting the photosynthesis pathway in absorbing light energy. Rice mutants of st1-2 and st1-3 were temperature sensitive and Chl b deficient, increasing temperature and chlorophyll contents (Chen et al. 2015).

Somatic cell mutation was caused by physical mutagens such as gamma rays creating genetic variability among somatic cell populations in the mature embryos. Somatic cells mutation in plant cells can be transmitted to offspring. According to Watson et al. (2016), mutations acquired during vegetative growth can be passed on to offspring; it is generally believed that older plants will acquire more such mutations over their lifetimes due to replication during cell division. Somatic embryogenesis (SE) is a model system for understanding the physiological, biochemical, and molecular biological events to form bipolar structure during plant embryo development (Méndez-hernández et al. 2019). Each germ cell line in the flower become undergo meiosis, and the resulting spores subsequently develop into highly polarized and differentiated haploid gametophytes that harbor the gametes. The research aimed to study genetic changes in the plant caused by gene mutation caused by gamma rays irradiation.

MATERIALS AND METHODS

Two rice panicle seeds containing 160 to 180 seeds of the Sidenuk cultivar were gamma irradiated at a dose of 250 Gy. These irradiated seeds were germinated and planted in a greenhouse for 21 days. These seedlings were transferred to the field with a spacing of 20 cm, and only one seedling was planted in each hole. In total, they were 330 plants in the field. The basal fertilizer (NPK) with a dosage of 15 g/m^2 was applied one week after seedlings were transferred to the field, and the second and third fertilizer with a dose of 30 g/m^2 was carried out four and eight weeks after planting. Plants' growth and development were observed during the vegetative and generative phases. The research scheme from seeds irradiation to mutants selection is presented as follows: (i) Seeds along with panicles were irradiated by gamma ray with a dose of 250 Gy. (ii) Irradiated seeds were germinated to obtain M1 plants produced M2 seeds at the generative phase. M1 Plants segregation has not occurred because gametogenesis in the generative phase results in M2 seeds. (iii) M2 seeds from selected plants were grown into M2 plants. Plants' growth during vegetative and generative phases was observed. The first selection of mutants was carried out at the M2 plants generation. At this time, the genetic variability of plants was observed. Seeds (M3) were selected from the best plant performance at M2. (iv) Observations were continued at M3 plants to M6 plants. The plant genetics variabilities included plant height, rice pericarp colors, lemma and palea colors, leaf blade chimera, and albino plants. All these characteristics were observed from M2 to M6 plants generation.

Vegetative and generative phases

M2 Seeds from Four rice panicles were chosen for germination on the trays (30 cm x 40 cm). In one panicle containing approximately about 130 seeds, there were 520 seeds germinated on these trays. Observations were carried out at ten days of the seedling stage in the nursery; the plant's chimeras were recorded at the M2 plant. At 21 days, the rice seedlings were transferred to the field, one seedling in a hole. The flag leaves started to emerge 60-70 days after transplanting. Harvesting was done 100 days after transplanting. The selection was made by choosing the best plant performance, namely one clump of plants originating from one individual seedling for each mutant, and the selection was continued at M3 to M5 plant's generations.

M3 to M6 plants

Selected M3 seeds germinated in the greenhouse until growing into seedlings. After the rice seedlings were 17 days old, they were transferred to the field, with only one seed per hole. After one week of planting, fertilization was done with NPK at a dose of 15 g/m^2; the second was done four weeks after planting at 30 g/m^2. Genetic variability was observed among individual plants, starting one week after planting. The characters observed included lemma and palea color, rice's pericarp, chimeras of the leaf blade, semi-albino plants, and plant height. Similar methods were carried out for selecting M4 to M6 plant generation. Selected plants were given an ID number.

RESULTS AND DISCUSSIONS

Irradiation seeds of the wild type (WT) variety are presented in Figure 1. The variability of a somatic cell population by gamma-ray effect creates heterogeneity among somatic cell populations. The heterogeneity of somatic cells influenced male or female gametes formation in gametogenesis. The mutation of cell gametes creates genetic variability in leaf blade, rice's pericarp colors, lemma and palea color, and plant height. Seeds were irradiated using ionizing radiation of gamma rays with a dose of 250 Gy. Genetic variability in M1 plants could not be seen phenotypically in the vegetative or generative phase. Genetic alterations such as plant height, rice pericarp colors, lemma and palea colors, leaf blade chimera, and albino plants in the rice genome were observed. Some of the seeds in a panicle in the M2 plant generation were empty due to the influence of gamma radiation, which affects the genetic structure in the formation of gametes.

Observation of plants at M2 generation

The results showed seven plants with black pericarp color and one with red pericarp color. The plants' black rice pericarp color had a panicle length of 20 to 24 cm, while that of the wildtype was 28 to 30 cm. Presumably, the black or red rice pericarp color is linked to panicle length because the panicle length of black rice or red rice in M4 generation remained at the same size from 20 to 24 cm. During the harvest, no panicle length of black rice pericarp was found to be equal to or longer than that of the wild type or mutant white rice pericarp. There were three types of the black color of rice's pericarp; (i) Dark black pericarp included scutellum area, (ii) Scutellum area with white color, and (iii) Less intensity of black pericarp color, with the scutellum area, is whitish. The palea and lemma color of the seeds during maturation also differed among black rice's pericarp. The dark black pericarp mutant had a dark brown lemma, and the palea color and the color of palea and lemma of other white pericarp mutants were similar to the wild type. Each selected M2 black rice pericarp mutant was given an ID number such as N250.1.Bp, N250.2.Bp., N250.3.Bp., and so on. According to Mackon et al. (2021) anthocyanin-enriched rice or pigmented rice cultivars are a possible alternative to reduce malnutrition around the globe. Kim et al. (2021) reported that OsKala4 was a regulator for the black rice pericarp, and their inactivation results in rice with a white pericarp. Switch on of this regulator gene affected mutation, which may change the white rice pericarp to black rice pericarp in the rice.

Genetic variability caused by gene mutation can be explained using cellular biology theory about cell growth and development. The cell biology theory explains that all living things are composed of one or more cells; the cell is the basic unit of life, and new cells arise from existing cells. Zygote formation from self-pollination of rice was a single cell, then grew and developed into mature embryos. Mature embryo on the seed consisting of somatic cells was not differentiated yet, but they were groups of cells having the capability for the formation of adult plants through the somatic embryogenesis pathway. The single plant cell can differentiate into a whole plant, called the cell totipotency. Mutagenic treatments caused heterogeneity among somatic cells population in an embryo because gene mutation occurred in the genome. The heterogeneity of somatic cells influenced the formation of the genetic constituent of pollen grains and ovum at gametogenesis in plants. According to Wilson and Yang (2004), the late transition of the vegetative phase of a diploid cell to the generative phase of haploid cells depends on the genetic structures of each diploid cell to be inherited. The transition from the vegetative into the generative phase occurs, followed by gametogenesis in meiosis I and II forming gamete cells. Therefore, gametogenesis occurs late in development, and somatic mutations can be transmitted to the next generation. Longer periods of growth are believed to result in an increase in the number of cell divisions before gametogenesis, with a concomitant increase in mutations arising due to replication errors (Watson et al. 2016).

Gamma rays hit mature rice embryos, causing genetic variability among the somatic cell population and leading to heterogeneity. Somatic cells in mature rice embryos have not had polarization yet for organ development, such as a leaf, gamete cells, and flower. Irradiated rice seeds caused a mutation in the nuclear or chloroplast genome because photon energy from gamma rays hits cells randomly among the somatic cell population in an embryo. So somatic cells that grow and develop from cells that have undergone mutations will multiply until the end of the vegetative phase. Mutations that occur in cells-groups will form gametes cells, resulting in heterogeneity at gametogenesis in meiosis I and II. Cells growth and development and cell gamete formation can be illustrated as follows: (i) irradiated mature embryos on the seeds, (ii) somatic cells Growth and development, (iii) adult plants grown to the generative stage, (iv) mature embryos at the seed, (v) selfing resulted in zygotes, (vi) gametogenesis results in pollen grain or ovum variability, (vii) mutant plants with variances in phenotype appearance. Self-pollination developed in embryos gave rise to different characteristics in M2 plants originating from single seeds (M1). Because somatic mutation produces genetic variability in the germ cell line, it can be transmitted to the next generation.

Black and red rice at the M2 plant was caused by anthocyanin accumulation in the rice pericarp due to a gene mutation responsible for anthocyanin biosynthesis. White rice pericarp turned to black rice pericarp was interesting to study at the gene level (Figure 1). According to Meng et al. (2021), anthocyanin caused purple, brown, or red colors in various tissues of rice plants, but the specific determinant factors and regulatory systems for anthocyanin biosynthesis in almost all tissues remain largely unknown. Genetically, the black rice's pericarp traits were controlled by a dominant gene, and a recessive gene controlled the white rice's pericarp. Tong et al. (2021) confirmed that only one dominant gene, temporarily designated Rp (Red pericarp), controlled the segregation of the red pericarp in the F2 population. Wang et al. (2020) stated that the gene resources of red rice containing anthocyanin are of great value for developing nutritious, high-anthocyanin-content rice. According to Wang et al. (2020), weedy rice pericarps had much higher anthocyanin content than cultivated rice, beneficial trace elements, free amino acids, and unsaturated fatty acids for food nutrition value. The gene resources and novel genetic systems of rice anthocyanin biosynthesis explored in this study are of great value for developing nutritious, high anthocyanin-content rice. Anthocyanins are the primary metabolites that give rice various colors. Three types of genes control anthocyanin accumulation in rice tissues, MYB TFs, bHLH TFs, and a functional enzyme DFR (Xia et al. 2021).

Chimeras of leaf blade color from different ID numbers were observed; ID N250.6Br and N250.6B exhibited stripe appearance with different morphology (Figure 2). These results showed that chlorophyll mutation occurred in the chloroplast genome. According to Hasib (2022), the frequency of chlorophyll mutations was high in higher dosages. Among the chlorophyll mutants studied, albina was the most frequent, followed by alboxantha, alboviridis, xantha, viridis and striata.

Seedlings aged about 17-20 days old at the nursery were transferred to the field; after 15 to 30 days at the field, the leaves gradually turned green, and on the 40th day, hundreds percent of the leaves turned green (Figure 5). Sunlight activated chlorophyll formation after transplanting to the field; the possibility chlorophyll activation for photosynthesis was highly dependent on light strength.

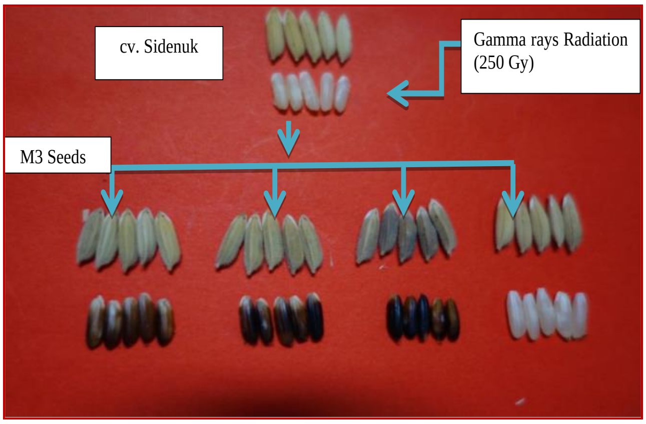

Figure 1. Mutant of rice's pericarp color variation was caused by mutation from white pericarp color (WPC) to black pericarp color (BPC) gene

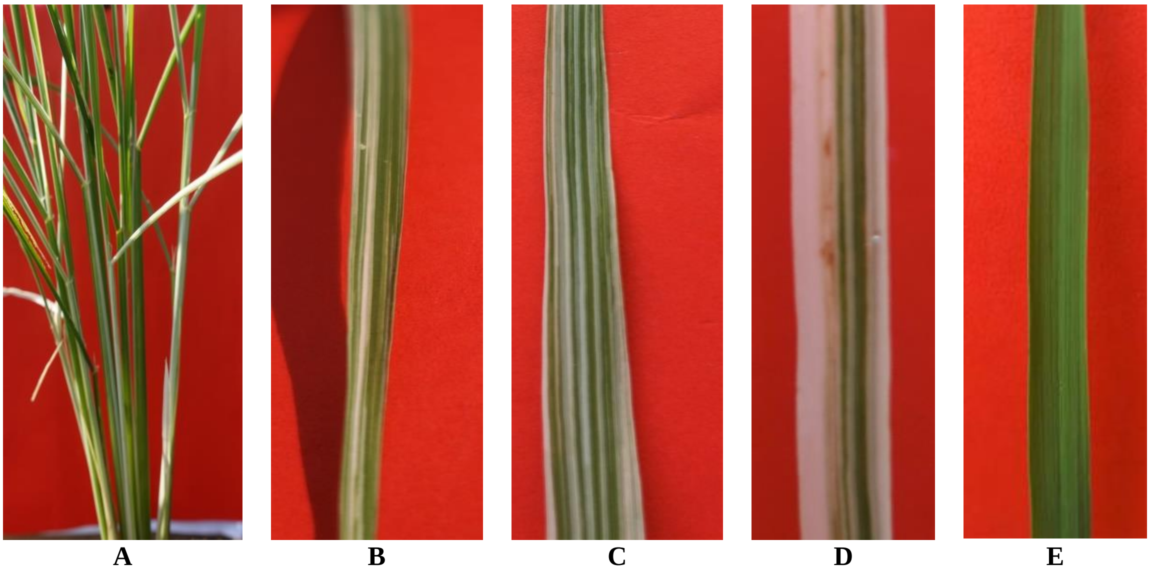

Figure 2. Genetic variability of rice mutant's leaf blade chimera. A. Rice seedling at 30 days plants old. B. Leaf blade with two stripes. C. Leaf blade with four stripes. D. Albino leaf margin. E. Leaf blade's green color of the wild type

Observation of M3 to M6 plants

The accession number of N250.5 with white rice pericarp was found in one plant with black rice pericarp. This plant's black rice pericarp was given ID number N250.5B, and the seeds of this plant were M4 generation. Theoretically, the emergence of black rice from white rice deviates from the previous theory about genetic segregation in monogenic character because a recessive gene controls the pericarp of white rice. There is likely a nucleotide deletion or insertion in the coding frame of an exon in the gene that controls anthocyanin biosynthesis in the rice's pericarp. When a gene suppressor loses its function for anthocyanin biosynthesis, loss of function affects gene mutation. According to Rahman et al. (2013), the dominant Pb allele was a primary factor for anthocyanin synthesis in rice pericarps. When the Pp allele in rice is incompletely dominant to the recessive pp allele; thus, the number of dominant Pp alleles determines the concentration of cyanidin-3-O-glucoside in black rice.

Observation of N250.5B in M4 plant generation showed segregation between white pericarp and black pericarp with a ratio of 1:1. This segregation has not followed Mendelian law in monogenic characters segregation pattern. Because a dominant gene controlled black rice pericarp, the segregation ratio should be 3:1 in heterozygous conditions. The segregation did not follow Mendel's law, which caused DNA methylation. According to Moore et al. (2013), DNA methylation regulates gene expression or inhibits the binding of transcription factor(s) to DNA. During development, the pattern of DNA methylation in the genome changes due to a dynamic process involving *de novo* DNA methylation and demethylation.

According to Tong et al. (2021), the segregation ratio of the red pericarp individuals to the white pericarp individuals was 3:1. Single dominant gene is responsible for the red pericarp of GER-3, and the red pericarp expression of the mutant gene Rp is not affected by the cytoplasm. Lemma and palea color in black rice pericarp during anthesis from green turned to purple, while in white rice pericarp, there were two colors of lemma and palea, i.e., green and white. Observation of black rice pericarp in M5 plant generation revealed three types of black rice pericarp, i.e., (i) dark pericarp color, (ii) black rice pericarp with whitish scutellum area, and (iii) brown color of rice pericarp (Figure 3).

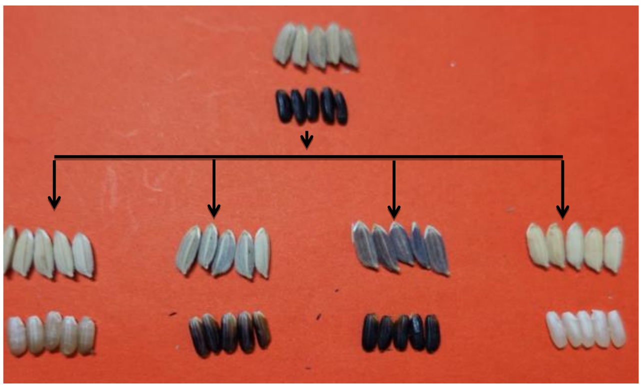

Figure 3. Rice mutant line with number of N250.5B at M 4 generation was segregated into: A. brown pericarp, B. black pericap, C. heavy black pericarp, D. white pericarp

Leaf blade chimeras caused by chlorophyll mutation were generally expressed at the seedling stage and can be divided into eight types: albino, greenish-white, white emerald, light green, greenish-yellow, etiolation, yellow-green, and striped (Zhao et al. 2020). Our observation showed that chimeras on the leaf blade at the M5 generation plants were obtained on the accession number N250.6B, N250.6w, and the N250.6Br mutant lines with black, white, and brown pericarp colors, respectively. These mutant lines were the results of segregation from a mutant with the accession number N250.5B in the M4 generation. The observation showed that leaf blade chimeras gradually disappeared during plant growth in the field (Figures 4 and 5). According to Khan et al. (2020), multiple mutations such as single nucleotide polymorphism (SNP) could upregulate the expression of OsPL6 to accumulate anthocyanin. Gene signaling between nuclear gene and chloroplast biosynthesis in a chloroplast genome depends on nuclear gene expression. These results indicate that the expression of nuclear genes encoding various chloroplast proteins might be feedback regulated by the level of Chl or Chl precursors (Wu et al. 2007). Rice plants with color mutations typically have less efficient photosynthesis, which can result in poor growth and yield reduction (Li et al. 2022).

White rice pericarp of the mutant line at the M3 plant generation with the accession number of N250.5 produced a single plant with black rice pericarp, and the other plants remained with white rice pericarp. This plant with black rice pericarp was given ID number N250.5B, and the seeds of this plant were M4 generation. Theoretically, the emergence of black rice from white rice deviates from the previous theory about genetic segregation in monogenic character because a recessive gene controls the pericarp of white rice. Is there a nucleotide deletion or insertion in the coding frame of an exon in the gene that controls anthocyanin biosynthesis in the rice pericarp? The possibility of gene suppressor for anthocyanin biosynthesis loss of function affected gene mutation. According to Rahman et al. (2013), the dominant Pb allele was a primary factor for anthocyanin synthesis in rice pericarps. When the Pp allele in rice is incompletely dominant to the recessive pp allele; thus, the number of dominant Pp alleles determines the concentration of cyanidin-3-O-glucoside in black.

Figure 4. Semi albino rice seedling mutant, leaf blade was 60% white with strip in color gradually turned into green leaf at 15 to 30 days plant old after planting in the field

Figure 5. White leaf blade gradually turned to green from 15 days to 30 days after planting, while Sidenuk cultivar as WT had green leaf from seedling stage in nursery

Figure 6. Genetic variability of rice mutant's lemma and palea color at M3 generation: A. rice mutants with lemma and palea whites color, B. rice mutants with lemma and palea's purple color, C. rice mutants with lemma and palea's green color, D. wild type with lemma and palea's green color

At the seedlings stage at M5 generation, the accession numbers of N250,6B, N250.6w, and N250.6Br showed leaf blade chimeras with mixed green and albino colors (Figure 4). At this time, the photosynthesis in the leaves was not fully occurred because there was very little chlorophyll content in the leaf blade. The leaves gradually turned green, and on the 40th day, all the leaves turned green (Figure 5). Sunlight activated chlorophyll formation after transplanting to the field, the possibility of chlorophyll activation for photosynthesis was highly dependent on light strength.

According to Liu et al. (2018), chlorophyll-deficient mutant plants showed abnormal grana stacking during the seedling stage, and there was the deletion of 33-bp in the 3'-untranslated region (UTR) of the cpSRP54 gene. Prina et al. (2012) stated that the M2 generation is not chimerical since mitosis derives every plant from a zygote. The uni-cellular constitution of gametes and zygotes does not allow the transmission of chimeras based on nuclear genes from one generation to another. This report was not valid because our results showed that the chimeras still exist on the leaf blade of the M3 and M4 generations. Another interesting finding of this study is that the tiller had seeds with black or white pericarp in one clump of rice. This clump of rice came from one seedling. Rice plant tillers result from somatic cell division in a vegetative stage, developing into adult plants. Each tiller produces one panicle with the number of seeds per panicle ranging from 70-130. Observation of M6 generation found different lemma and palea color during anthesis from accession numbers N250.6w and N250.Br, and 250.6B (Figure 6).

In conclusion, induced mutation using gamma rays on seed embryos of rice created genetic variabilities in rice, such as pericarp color, plant height, palea and lemma colors, and leaf blade chimeras. Mutation in somatic embryos has been transmitted to progeny, giving rise to different phenotypic performances from M2 to M6 plant generation.

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