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Chronic Gamma Irradiation Changes Phenotype and Gene Expression Partially Transmitted to Next-Generation Tomato Seedlings

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Abstract: Compared to the studies on acute irradiation of seeds, fewer studies have reported on the chronic irradiation of seedlings, especially in fruit-bearing vegetables. We examined the effects of chronic gamma irradiation on tomato (*Solanum lycopersicum* 'Micro-Tom') seedlings exposed to gamma rays (50, 100, 150, and 200 Gy) for 4 weeks. As the total dose of gamma rays increased, leaf length, trichome density, and seed number were reduced in the irradiated seedlings (M_1). Additionally, a change in fruit shape was observed. Chronic gamma irradiation reduced the expression of two trichome-related genes and affected the expression levels of 11 reactive oxygen species (ROS)-related genes. We examined the transmittance of these effects using M_2 plants. The trichome density and fruit shape were similar between M_2 and control plants; however, a reduction in leaf length and seed number was detected in M_2 plants. Interestingly, changes in the expression of four ROS-related genes (*ZAT10*, *Mn-SOD*, *POD3*, and *RBOH1*) found in M_1 were detected in M_2 plants. Thus, the changes in phenotype and gene expression induced by chronic gamma irradiation were transmitted to the next generation. Additionally, we found novel mutants from M_2 plants, suggesting that chronic gamma irradiation may be considered in tomato mutation breeding.

Keywords: chronic gamma irradiation; fruit shape; mutation breeding; reactive oxygen species-related gene; tomato; trichome

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1. Introduction

Irradiation of plant tissues causes various biological effects at both morphological and physiological levels of plant organisms and cellular levels [1]. At the morphological and physiological levels of plant organisms, the irradiation of rice with carbon beams or gamma rays results in morphological and functional defects, including reduction in fertility [2] and plant height [3]. Gamma irradiation also causes other developmental changes, such as increased trichome density in *Arabidopsis* [4] and elongation of internode diameter in *Brachypodium* [5]. At the cellular level, gamma rays alter the amount of sucrose in potato [6] and anthocyanin in wheat [7]. Furthermore, gamma irradiation causes damage to cell walls in *Brachypodium* [5] and alters the structure of mitochondria and chloroplasts in *Arabidopsis* [8]. Notably, irradiation significantly modifies genetic properties by causing DNA mutations [9], by introducing epigenetic modifications [10], and by altering gene expression [11]. Among these genetic properties, heritable changes can be considered

for radiation mutation breeding, which is the best-known and the most widely applied radiation technology in plants [12].

A mutant population can be developed and established by irradiating seeds or plants to harbor random mutations. Individual lines containing useful and inheritable characteristics are selected in later generations and are used as breeding materials for the development of new cultivars. However, most phenotypic changes observed in irradiated plants (M_1 plants) are caused by physiological responses rather than heritable genetic changes [13]. Therefore, it is generally accepted that changes in certain characteristics found in M_1 plants are mostly not detected in their progeny (M_2 plants) when mutagenesis is performed in seed-propagated plants with homozygous genomes [1]. In contrast, phenotypes due to inherited DNA mutations are rarely detected in M_1 plants because recessive mutations account for most mutations [1]. For the transmission of DNA mutations, through the analysis of two successive generations of gamma-irradiated *Arabidopsis*, it was found that most DNA structural variations induced by irradiation were not transferred to the next generation. This was presumably because considerable DNA structural variations hamper the viability of gametes in the haploid state [14]. Regarding mutation efficiency, many factors such as radiation dose, linear energy transfer (LET) of radiation, irradiation conditions, and irradiated tissues affect the frequency and spectrum of mutations [15]. Yamaguchi et al. [16] have suggested that the dose corresponds to the shoulder (the dose at which the survival rate of plants begins to rapidly decrease) in the dose-survival rate curve, as the optimal dose to obtain the highest number of mutants per sown M_1 seeds. Recent genomics studies have shown that high-LET radiation, pertaining to the application of heavy ion beams (e.g., argon and carbon ion beams) and fast neutrons, induces more marked and increasingly complex DNA structural variations, thus resulting in a higher frequency of gene mutations than those observed with low-LET radiation, such as gamma rays in *Arabidopsis* and rice [17–19]. The irradiation dose rate also affects the genomic characteristics. Acute irradiation, which is conducted at a relatively high dose rate in a short period (usually ranging from several hours to 1 day), has been widely used for conducting mutation breeding [17,20,21]. Recently, the impact of acute irradiation on DNA mutations was investigated through whole-genome sequencing analyses by subjecting *Arabidopsis* and rice to treatment with several radiation sources (e.g., fast neutrons, heavy ion beams, and gamma rays) [15]. However, chronic irradiation, characterized by a low dose rate over a relatively long period (ranging from several days to months) during plant development, has been less studied than its counterpart.

Treatment of plants with chronic irradiation requires special facilities for the growth of plants subjected to irradiation. For example, the gamma field in Japan [22], gamma phytotron in the Republic of Korea [23], and the gamma greenhouse in Malaysia [24] have demonstrated successful results pertaining to the treatment of plants with chronic irradiation. Several mutants with novel traits have been obtained through chronic irradiation, including a black spot disease-resistant pear variety ('Gold Nijisseiki') [25] and 10 chrysanthemum cultivars with variation in flower color and shape [26]. A few studies have shown that chronic irradiation subsequently induces physiological responses or inflicts damage, such as reduction in the fertility in rice [3], alterations in the expression patterns of diverse genes in *Arabidopsis* and wheat [7,11], and degradation of lignocellulose in *Brachypodium* [5]. Whole-genome sequencing of *Arabidopsis* lines that were chronically or acutely subjected to irradiation with gamma rays for five generations showed that chronic irradiation could generate a higher frequency of heritable DNA mutations than those observed with acute irradiation [17]. However, the heritability of various biological effects of chronic irradiation has not been examined in successive generations. When we consider the differences in dose rate and duration of irradiation between acute and chronic irradiation, the biological effects and their inheritance following subjection to chronic irradiation may differ from those following subjection to acute irradiation. Therefore, research is warranted to elucidate the specific effects of chronic irradiation exerted on plants and their progeny.

Tomato (*Solanum lycopersicum*) is a vegetable crop that is cultivated globally with considerable production [27]. Additionally, as tomato is considered a model plant, assembly of a high-quality genome has been realized (Tomato Genome Consortium, 2012), and diverse plant materials, including mutant lines, have been developed for functional genomics studies and breeding [28–30]. In the development of mutant populations, the application of acute gamma irradiation or ethyl methanesulfonate (EMS) treatment has mainly been considered for mutagenesis. The frequency and spectrum of DNA mutations induced by gamma irradiation and EMS treatment were determined in mutant lines of dwarf tomato ('Micro-Tom') via whole-genome sequencing [31]. For chronic irradiation, the radiosensitivity of tomato has been reported in several studies [32,33]. However, research on the physiological and genetic effects of chronic irradiation in tomatoes remains limited.

In the present study, changes in the physiological characteristics and expression levels of genes related to radiation responses were investigated following the use of chronic gamma irradiation in tomatoes. Additionally, the inheritance of these characteristics was examined in the next generation. Our results provide essential information on the biological effects and inheritance patterns necessary for consideration of the application of chronic irradiation technology for the development of tomato genetic resources.

2. Materials and Methods

2.1. Plant Growth and Gamma Irradiation Conditions

Tomato 'Micro-Tom' (LA3911) seeds were sown in 72-hole seedling trays (Bumnong Co., Jeong-eup, Korea) with a soil mixture (Super-baroker, Seoul Bio Co., Eumseong, Korea) and subjected to growth in a greenhouse under natural light conditions for 16/8 h (light/dark) with daily and nightly temperature 26 °C and 20 °C, respectively, and 60% relative humidity. After a period of two weeks, plants were individually transferred into 11 cm-diameter pots. Three-week-old plants were moved to a gamma-phytotron facility and maintained under conditions of photoperiod 16/8 h (light of intensity 250 μmol m^{-2} s^{-1}/darkness) with daily and nightly temperature 26 °C and 20 °C, respectively, and relative humidity of 60% (Figure S1). Tomato seedlings were placed at different distances and irradiated with gamma-rays for 4 weeks at 50 Gy (74.4 mGray/h), 100 Gy (148.81 mGray/h), 150 Gy (223.21 mGray/h), and 200 Gy (297.62 mGray/h) by using a ^{60}Co irradiator (20 TBq of capacity, Ottawa, ON, Canada) at the Korea Atomic Energy Research Institute (KAERI). For consideration of 0 Gy as a control, tomato seedlings were subjected to protection by using a lead wall to prevent gamma-ray exposure. The gamma-irradiated M_1 seedlings were transferred to the greenhouse and cultivated under the same conditions as described above to harvest M_2 seeds. M_2 seeds were germinated in 72-hole seedling trays with a soil mixture and cultivated in the greenhouse under the same conditions mentioned above. After a period of two weeks, each M_2 plant was transferred into 11 cm-diameter pots and cultivated to harvest M_3 seeds.

2.2. Microscopic Observations and Trichome Counting

A dissecting microscope (Leica CH-M205A, Wetzlar, Germany) equipped with LED5000 RL light sources (Leica) and a Leica MC170 HD camera was used to observe trichome morphology and to measure trichome density, as per methods previously described [34]. Seven-week-old M_1 (4 weeks after gamma irradiation) and M_2 plants were used. The primary leaflet of the second compound leaf underneath the shoot apical meristem was selected, and the adaxial leaf surfaces of the middle region of the leaflet were used for trichome counting (Figure S2).

2.3. Total RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The leaves of the control and gamma-irradiated plants were collected from the same region used for trichome observation. Total RNA isolation and cDNA synthesis were conducted using the TRIzol reagent (#15596018, Invitrogen) and a cDNA synthesis kit (#K1622, Thermo Fisher Scientific, Waltham, MA, USA). Genes involved in trichome development

(*Wo*, *CycB2*, *MYC1*, and *TPS5*) or those related to reactive oxygen species (ROS) signaling and scavenging (*HsfA4a*, *WRKY39*, *CML51*, *ZAT10*, *Mn-SOD*, *cAPX*, *GPX*, *ph-GPX*, *POD3*, *CAT1*, *CAT3*, and *RBOHI*) were selected for quantitative real-time polymerase chain reaction (qRT-PCR). *ACT7* was selected as an internal standard. Primer sets for each gene are listed in Table S1. qRT-PCR was performed in 20-μL reaction mixtures containing the cDNA template equivalent to 20 ng of total RNA, 10 μM of each primer, 10 μL of Solg^{TM} 2 × real-time PCR smart mix, and 1 μL of EvaGreen (#SRH71-m40h, Solgent, Daejeon, Korea) using the AriaMx Real-time PCR system (G8830A, Agilent Technologies, Santa Clara, CA, USA). The PCR conditions included the following: 95 °C for 15 min, followed by 40 cycles at 95 °C for 20 s and at 60 °C for 40 s.

3. Results

3.1. Chronic Gamma Irradiation Reduces Plant Growth, Trichome Density and Seed Number, and Induces Abnormal Fruit Shape in Tomato

To investigate the effect of chronic gamma irradiation exerted on tomato plants, 3-week-old tomato seedlings were irradiated with 50, 100, 150, and 200 Gy of gamma rays for a period of 4 weeks (Figure S1). We then compared several phenotypes between the gamma-irradiated M_1 plants and the control plants (0 Gy). First, the gamma-irradiated M_1 plants showed different growth rates. The leaf length of plants irradiated with 50 and 100 Gy was similar to that of the control plants. However, the leaf length of plants irradiated with 150 and 200 Gy was reduced by 19.7% and 20.0%, respectively, compared to that of the control plants (Figure 1A). In *Arabidopsis* and soybean, trichome density is altered by gamma irradiation [4,35,36]. Therefore, we measured the number of type I and VI trichomes (Figure S2), which are representative of tomato trichomes, to determine whether gamma irradiation also induced trichomes in tomato. As the total dose of gamma rays increased, the number of type I trichomes on the leaves gradually decreased. However, the number of trichomes between gamma-irradiated plants and control plants did not significantly differ (Figure 1B,C). The density of type VI trichomes on leaves also decreased when the intensity of gamma rays increased. The number of type VI trichomes on 50 Gy-irradiated leaves slightly decreased by 12% compared to that on the control leaves. However, the number of type VI trichomes on 100 Gy-, 150 Gy-, and 200 Gy-irradiated leaves was significantly reduced by 25%, 33%, and 59%, respectively, compared to that on the control leaves (Figure 1B,D). These results indicated that chronic gamma irradiation negatively affected trichome density in tomatoes.

In addition to the trichome density changes, chronic gamma irradiation also affected the shape of the tomato fruit. As the total dose of gamma irradiation increased, gamma-irradiated plants exhibited the development of a peanut-shaped fruit with a higher length-to-width ratio than that observed on control plants, which demonstrated a typical round shape (Figure 1E). In particular, the length-to-width ratio of 100 Gy- and 150 Gy-irradiated fruits significantly increased to 21% and 31%, respectively, compared with that of control fruits. In 200 Gy-irradiated plants, fruit size was markedly smaller than that of control plants, which resulted in the achievement of a similar length-to-width ratio between 200 Gy-irradiated fruit and control fruit (Figure 1E,F). Chronic gamma irradiation also affected the seed set. The number of M_2 seeds obtained from 50 Gy- and 100 Gy-irradiated M_1 plants slightly decreased to 7–19%, but was not significantly different from that obtained from control plants. The number of M_2 seeds obtained from 150 Gy- and 200 Gy-irradiated M_1 plants significantly decreased to 35% and 92%, respectively, compared to that obtained from control plants. (Figure 1G). These results indicated that chronic gamma irradiation affected several aspects of growth and development in tomatoes.

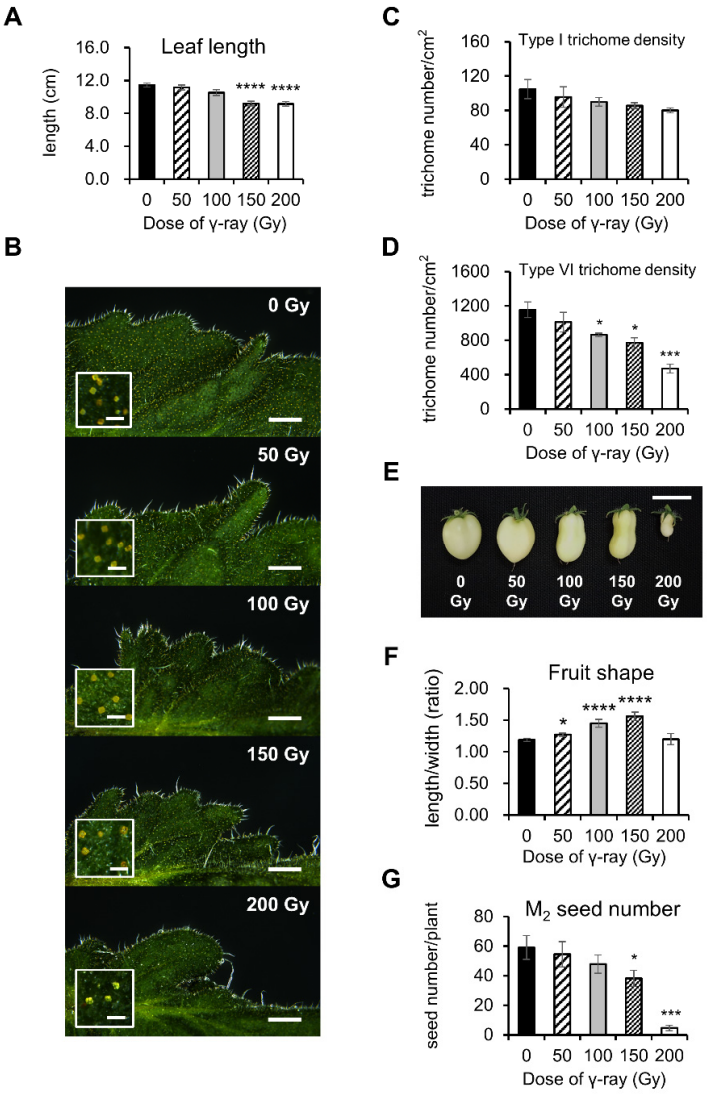

Figure 1. Effect of chronic gamma irradiation on growth and development of tomato M_1 plants. Plants irradiated with 50, 100, 150, or 200 Gy of gamma rays for 4 weeks were compared with control (0 Gy) plants. (**A**) Leaf length of control and gamma-irradiated plants. The length from the center of the plant to the edge of the largest terminal leaflet was measured. Data are presented as mean ± SE of 16 biological replicates for each dose. Asterisks represent significant differences between gamma-irradiated and control plants (unpaired *t*-test: **** $p < 0.0001$). (**B**) Dissecting micrographs of the adaxial leaf surfaces of the control and gamma-irradiated plants. Scale bars represent 2 mm. Insets show representative type VI trichomes, and scale bars in the insets indicate 0.2 mm. (**C**,**D**) Density of type I (**C**) and VI (**D**) trichomes on the adaxial leaves of control and gamma-irradiated plants. Data are presented as mean ± SE of four biological replicates for each dose. Asterisks represent significant differences between control and gamma-irradiated plants (unpaired *t*-test: * $p < 0.05$; *** $p < 0.001$). (**E**) Shape of mature green fruit in the control and gamma-irradiated plants. Scale bar indicates 2 cm. (**F**) Length-to-width ratio of mature-green fruit in control and gamma-irradiated plants. Data are shown as mean ± SE of 12 biological replicates. Asterisks represent significant differences between control and gamma-irradiated plants (unpaired *t*-test: * $p < 0.05$; **** $p < 0.001$). (**G**) M_2 seed number in control and gamma-irradiated M1 plants. Following gamma irradiation, M_1 plants were transferred to a greenhouse and cultivated until 28 weeks from sowing to collect M_2 seeds. Data are presented as mean ± SE of 12 biological replicates for each dose. Asterisks represent significant differences between gamma-irradiated and control plants (unpaired *t*-test: * $p < 0.05$; *** $p < 0.001$).

3.2. Chronic Gamma Irradiation Affects the Expression of Genes Involved in Trichome Development and the ROS Signaling Pathway

To examine which genes triggered the changes in trichome density in the gamma-irradiated plants, the expression levels of genes involved in trichome development were analyzed by qRT-PCR. Since 100 Gy- and 200 Gy-irradiated plants presented with mild and severe phenotypes, the leaves of these plants were used for gene expression analysis. The expression levels of the *Wo* and *CycB2* genes, which regulate the initiation of type I trichomes in tomato [37,38], were similar between gamma-irradiated and control leaves (Figure 2A). However, the expression level of the *MYC1* gene, which is essential for the initiation of type VI trichomes [39], was significantly reduced by 48–56% in 100 Gy- and 200 Gy-irradiated leaves, compared to that in control leaves. The expression level of the *TPS5* gene, which is highly expressed in type VI trichomes and whose expression regulates the synthesis of monoterpenes [40], also decreased by 49–54% in 100 Gy- and 200 Gy-irradiated leaves compared to that in control leaves (Figure 2A).

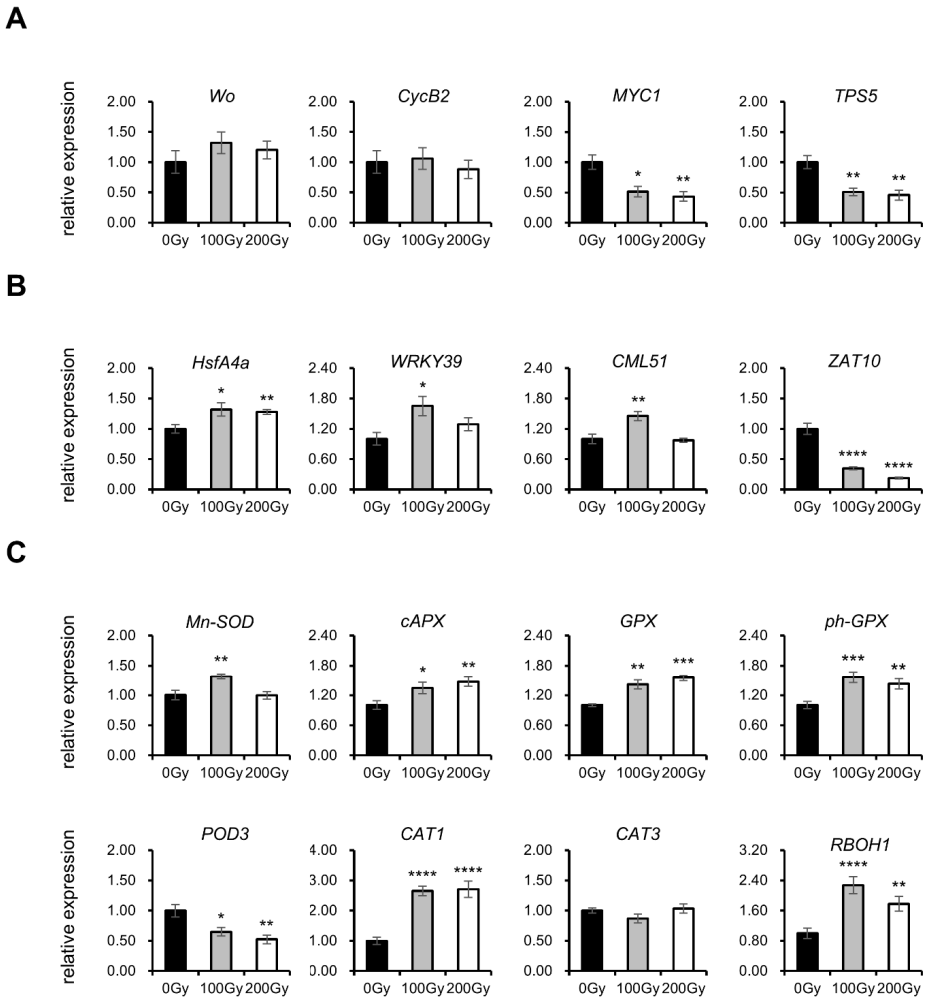

Figure 2. Expression levels of genes involved in trichome development, reactive oxygen species (ROS) signaling, and scavenging in leaves of gamma-irradiated M_1 plants. Control (0 Gy) plants and gamma-irradiated plants (100 and 200 Gy) were used. Gene expression levels were analyzed via quantitative real-time polymerase chain reaction (qRT-PCR) and the values were normalized to the levels in control plants. Data are presented as mean ± SE of four biological replicates. Asterisks represent significant differences between control and gamma-irradiated plants (unpaired *t*-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). (**A**) Genes involved in trichome development. (**B**) Genes related to reactive oxygen species (ROS) signaling. (**C**) Genes involved in ROS scavenging.

Acute and chronic gamma irradiation is known to alter the expression of several ROS signaling-related genes, such as heat shock factors (*HSFs*), *WRKYs*, calcium binding proteins, and zinc transporters (*ZATs*) in *Arabidopsis* [36]. Therefore, we analyzed the expression levels of genes related to the ROS signaling pathway. The expression level of *HsfA4a* increased to 33% and 29% in leaves irradiated with 100 Gy and 200 Gy, respectively, compared with that in the control leaves. *WRKY39* and *CML51* showed a 65% and 45% increase in 100 Gy-irradiated leaves, respectively, compared to the findings observed in control leaves. However, the expression level of *ZAT10* decreased to 65–81% in 100 Gy- and 200 Gy-irradiated leaves compared to that in control leaves (Figure 2B). ROS signaling promotes the expression of genes encoding antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), phospholipid hydroperoxide GPX (phGPX), peroxidase (POD), and catalase (CAT) [41–43]. To investigate antioxidant-related responses to chronic gamma irradiation, the expression of the corresponding genes was analyzed. The transcript level of *Mn-SOD* increased to 32% in 100 Gy-irradiated leaves compared to that in control leaves. The expression levels of *cAPX*, *GPX*, *ph-GPX*, and *CAT1* increased to 34–165% in 100 Gy-irradiated leaves and 42–171% in 200 Gy-irradiated leaves compared with those in control leaves. However, the expression levels of *POD3* decreased to 35% and 48% in leaves irradiated with 100 and 200 Gy, respectively. The expression levels of *CAT3* were similar between the gamma-irradiated and control leaves. In the case of respiratory burst oxidase homologue1 (*RBOH1*), which encodes NADPH oxidase [44], the transcript level was upregulated to 125% and 73% in 100 Gy- and 200 Gy-irradiated leaves compared with that in control leaves (Figure 2C).

3.3. M_2 Plants Obtained from Gamma-Irradiated M_1 Plants Exhibit Normal Trichome Density and Fruit Shape but Demonstrate Reduced Leaf Size and Seed Number

To investigate whether the phenotypes observed in the gamma-irradiated M_1 plants were maintained in the next generation, M_2 seeds were germinated and cultivated in a greenhouse. Then, the phenotypes of 7-week-old tomato plants were examined. First, the leaf length of M_2 plants obtained from 50 Gy- and 100 Gy-irradiated M_1 (hereafter referred to as 50-Gy M_2 plants and 100-Gy M_2 plants) was similar to that of M_2 plants obtained from control (0 Gy) M_1 plants (hereafter referred to as control M_2 plants). The leaf length of 150- and 200-Gy M_2 plants was reduced by 9–18% compared with that of control M_2 plants (Figure 3A). The density of type I and type VI trichomes on the leaves of the gamma-irradiated M_2 plants was similar to that of the control M_2 plants (Figure 3B–D). The fruit of the gamma-irradiated M_2 plants exhibited a normal shape, similar to the fruit of control M_2 plants (Figure 3E). However, the seed production of gamma-irradiated M_2 plants was similar to that of gamma-irradiated M_1 plants. The number of M_3 seeds obtained from 50-Gy M_2 plants decreased to 19%; however, this was not significantly different from that of control M_2 plants. The number of M_3 seeds obtained from 100-, 150-, and 200-Gy M_2 plants was significantly reduced to 42–51% compared with that of control M_2 plants (Figure 3F). These results indicated that the changes in phenotype (leaf length and seed number) were maintained in M_2 plants obtained from gamma-irradiated M_1 plants.

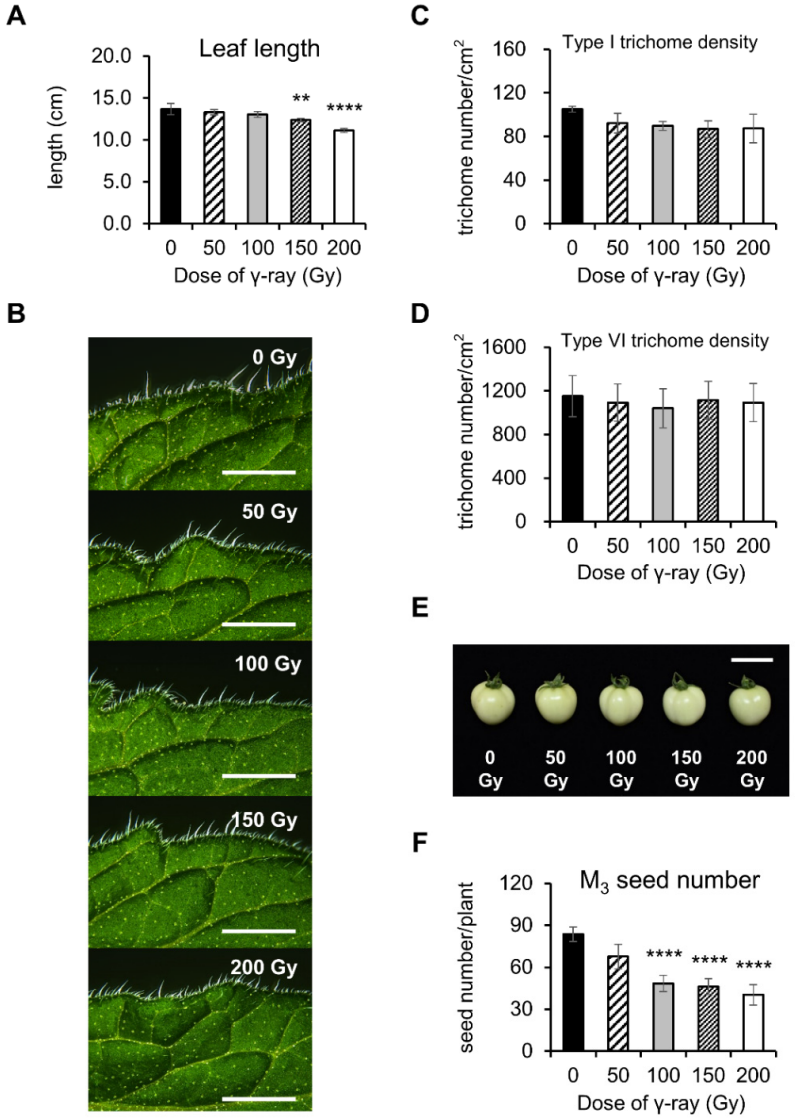

Figure 3. Effect of chronic gamma irradiation on growth and development of tomato M_2 plants. M_2 seeds harvested from the control (0 Gy) M_1 and M_1 plants irradiated with 50, 100, 150, or 200 Gy of gamma rays were germinated, grown for 7 weeks, and used. (**A**) Leaf length of control and gamma-irradiated plants. The length from the center of the plant to the edge of the largest terminal leaflet was measured. Data are presented as mean ± SE of 12 biological replicates for each dose. Asterisks represent significant differences between control and gamma-irradiated plants (unpaired *t*-test: ** $p < 0.01$; **** $p < 0.0001$). (**B**) Dissecting micrographs of the adaxial leaf surfaces of the control and gamma-irradiated plants. Scale bars represent 2 mm. (**C**,**D**) Density of type I (**C**) and VI (**D**) trichomes on the adaxial leaves of control and gamma-irradiated plants. Data are presented as mean ± SE of six biological replicates for each dose. (**E**) Shape of mature green fruit in the control and gamma-irradiated plants. Scale bar indicates 2 cm. (**F**) M_3 seed numbers of the control and gamma-irradiated M_2 plants. M_2 plants were grown for 28 weeks in a greenhouse to harvest the M_3 seeds. Data are presented as mean ± SE of 12 biological replicates for each dose. Asterisks represent significant differences between the control and gamma-irradiated plants (unpaired *t*-test: **** $p < 0.0001$).

3.4. Expression Levels of ZAT10, Mn-SOD, POD3, and RBOH1 Are Altered in M_2 Plants

The expression levels of the *Wo* and *CycB2* genes in 100- and 200-Gy M_2 plants were similar to those observed in control M_2 plants (Figure 4A). The expression levels of *MYC1* and *TPS5* genes were also similar between 100- and 200-Gy M_2 plants and control M_2 plants

(Figure 4A). These results were consistent with the similar density of type I and VI trichomes between the gamma-irradiated M_2 plants and control M_2 plants (Figure 3B). In general, the expression of genes related to ROS signaling and scavenging was not different between the gamma-irradiated M_2 and control M_2 plants (Figure 4B,C). However, the transcript level of *ZAT10* was downregulated to 28% in 100- and 200-Gy M_2 plants compared with that in control M_2 plants. In contrast, *Mn-SOD* expression was upregulated to 76% in 100-Gy M_2 plants. The expression level of *POD3* was reduced to 33–31% in 100- and 200-Gy M_2 plants, respectively, compared with that in control M_2 plants. Moreover, the expression level of *RBOH1* was highly upregulated to 102–59% in 100- and 200-Gy M_2 plants compared with that in control M_2 plants (Figure 4B,C). The changes in the expression of *ZAT10*, *Mn-SOD*, *POD3*, and *RBOH1* were examined at least twice. These results indicated that the effects of chronic gamma irradiation on M_1 plants were transmitted to the next generation.

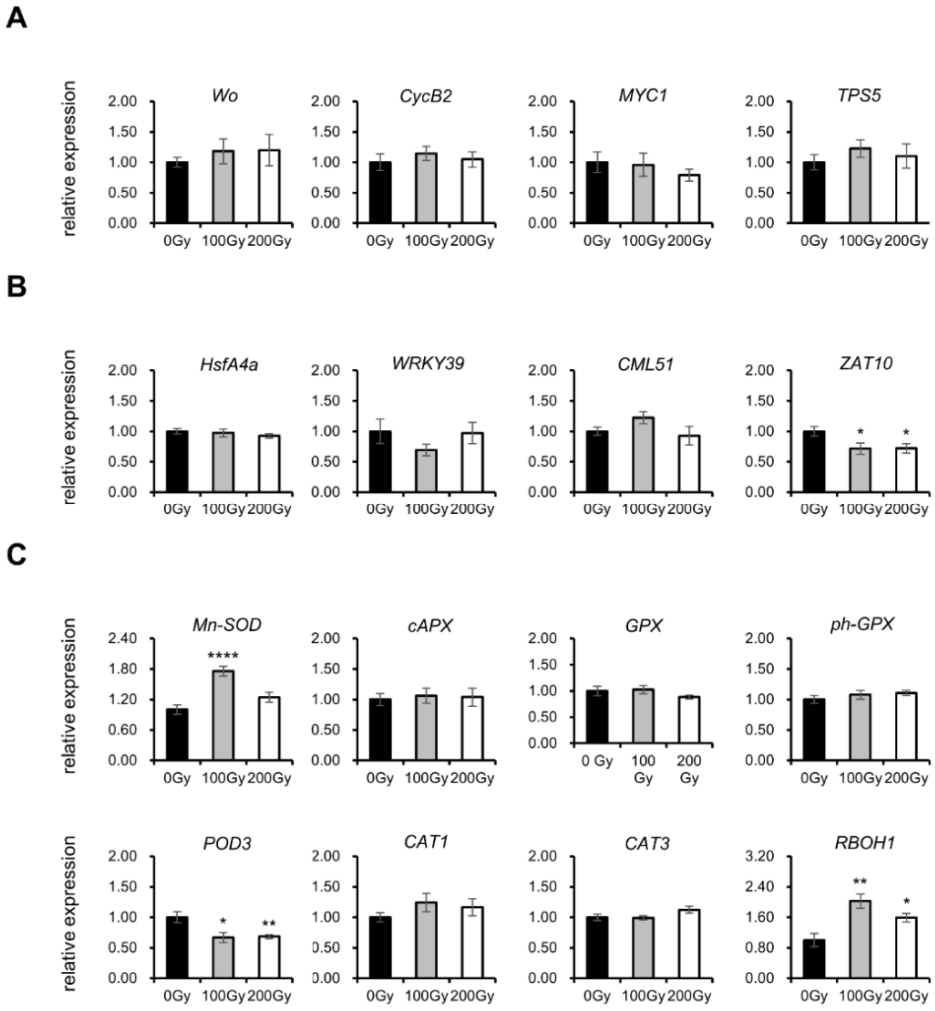

Figure 4. Expression levels of genes involved in trichome development, reactive oxygen species (ROS) signaling, and scavenging in leaves of gamma-irradiated M_2 plants. M_2 seeds harvested from the control (0 Gy) M_1 plants and the M_1 plants irradiated with 100 or 200 Gy of gamma rays were germinated, cultivated for 7 weeks, and assessed. Gene expression levels were analyzed via qRT-PCR, and the values were normalized to the levels in control plants. Data are presented as mean ± SE of six biological replicates. Asterisks represent significant differences between gamma-irradiated and control plants (unpaired *t*-test: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$). (**A**) Genes involved in trichome development. (**B**) Genes related to ROS signaling. (**C**) Genes involved in ROS scavenging.

3.5. M_2 Mutant Generation from Chronically Gamma-Irradiated M_1 Plants

In general, seeds are subjected to irradiation with acute gamma rays to facilitate the establishment of a mutant population [45,46]. To examine the feasibility of using chronically gamma-irradiated tomato plants in the establishment of a mutant population, 184 M_2 seeds (40 seeds from 0 Gy- 50 Gy-, 100 Gy-, and 150 Gy-irradiated M_1 lines, and 24 seeds from 200 Gy-irradiated M_1 lines) were sown. Phenotypes of M_2 lines were screened to identify mutants. Three mutants with different phenotypes were observed compared to the control plants. The mutant-1 (m-1) plants presented with chlorotic leaves compared to control plants that presented with green leaves (Figure 5A). Compared with the control fruit, which exhibited a round shape, the mutant-2 (m-2) plants presented with elongated fruit with abnormal tip regions developed from the stigma of the flowers (Figure 5B). The mutant-3 (m-3) plants presented with bush leaves and floral buds, which did not undergo development into flowers (Figure 5C). The findings in the M_2 plants implied that chronic gamma irradiation induced mutations that were transmitted to the next generation; thus, chronic gamma irradiation could be considered for the establishment of mutant populations in tomato.

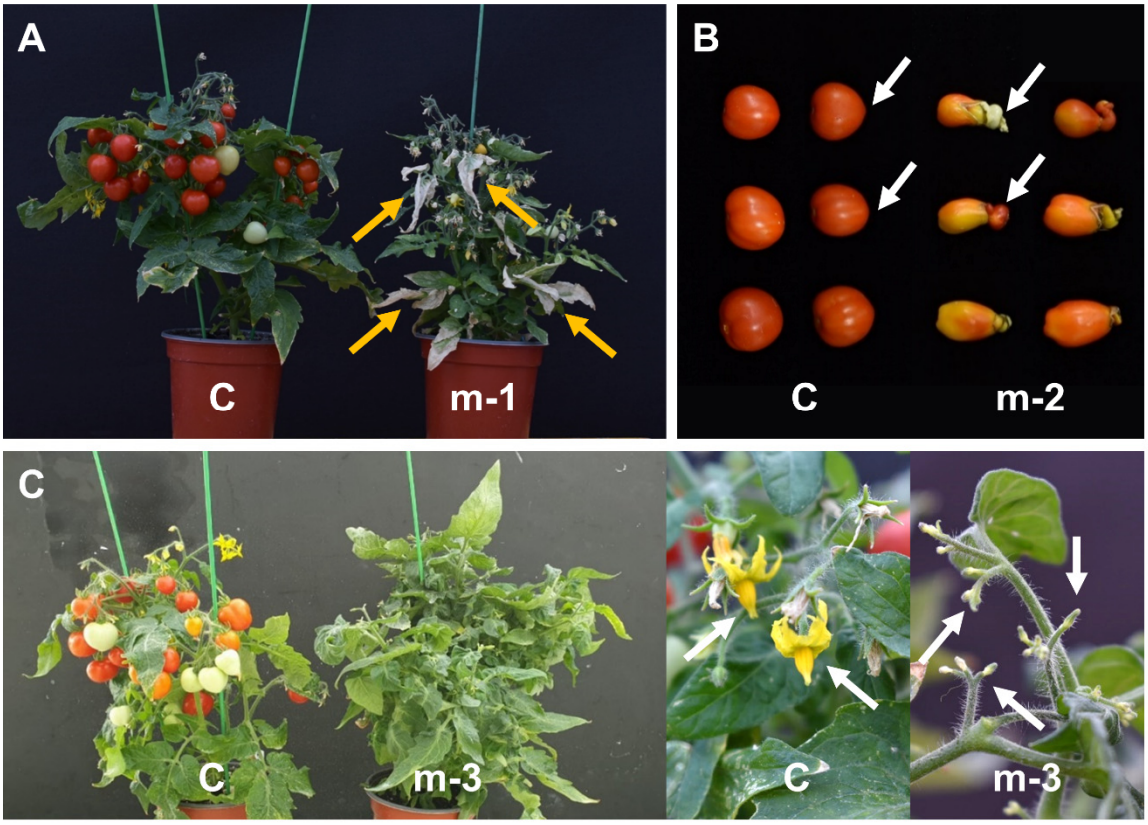

Figure 5. Various phenotypes of mutant M_2 plants. Mutants (m-1, m-2, and m-3) with different phenotypes compared with control (C) plants are shown. (**A**) An m-1 mutant with chlorotic leaves (indicated by arrows). (**B**) An m-2 mutant with abnormal fruit shape. Arrows indicate the tip regions developed from the stigma of the flowers. (**C**) m-3 mutant with bushy leaves and floral buds, which did not develop into flowers. Arrows indicate flowers and floral buds in WT and m-3 plants, respectively.

4. Discussion

The physiological and mutagenic effects of acute radiation on plants have been widely studied, but relatively limited data are available on the effects of chronic irradiation on plants [20]. As the biological effects of radiation can be continuously monitored during various developmental stages, investigation of the effects of chronic irradiation will facilitate a more multifaceted understanding of plant response to radiation. This understanding

is necessary for predicting plant development and inheritance in radiation-exposed terrestrial areas or non-terrestrial space environments where cosmic radiation exists, and for the application of chronic irradiation to mutation breeding [47]. Although physiological characteristics or gene expression patterns in response to chronic irradiation have been analyzed [3,7,11], integrative studies combining both research areas are limited. Additionally, there has been limited research on the analysis of inheritance of characteristics under uncontrolled irradiation conditions [48,49]. Therefore, we investigated the changes in biological characteristics induced in tomato via subjection to chronic irradiation, and the inheritance of such characteristics, to facilitate a comprehensive understanding of radiation biology in plants.

We used the dwarf tomato cultivar 'Micro-Tom', which exhibits a short life cycle (70–90 days) [30], therefore, conducting irradiation for a period of 4 weeks could cover multiple developmental stages, including vegetative growth, flowering, and fruit set, and helped elucidate the changes occurring at each stage. The biological effects investigated during and after chronic irradiation were categorized into the following three groups: deleterious effects on plant growth and reproduction (reduced leaf length and seed set); developmental and morphological effects (changes in trichome density and fruit shape); and alteration of the expression patterns of genes related to antioxidant activity and trichome development (Table 1). The reduction in leaf length and seed set might be attributable to both the physiological and genetic effects of radiation. Irradiation at low doses (1–5 Gy irradiation) during vegetative stages stimulates growth in several plant species such as pepper, pea, and wheat [50–52]. However, irradiation at high doses (>50 Gy irradiation) during vegetative stages usually leads to deleterious physiological effects, including infliction of damage to cellular components, by inducing ROS production [47], by diminishing the ATP/ADP ratio presumably by impairing the photosynthesis system [53], and by lowering the content of indoleacetic acid (IAA) via reduction of IAA synthesis [54] and acceleration of its degradation [55]. These effects may be related to reduced leaf length and seed set observed in our study, considering that IAA positively regulates plant growth [56] and a substantial amount of energy is required for gametophyte development [57,58]. Regarding genetic effects, radiation often induces chromosomal aberrations such as chromosome breaks, deletions, chromatid exchanges, and chromatin bridges [59]. Chromosomal abnormalities can hamper the normal segregation of chromosomes during mitosis and meiosis [60–62], which may cause retarded growth and reduced fertility, respectively. Additionally, the accumulation of various DNA mutations in the haploid genome of gametophytes may affect the function of gametophyte development-related genes. In our analysis, a reduction in seed set was detected following subjection to irradiation at high doses. Hase et al. [17,63] showed that the frequency of DNA mutations, including single-substitutions, small InDels, and DNA structural variation, increased when irradiation was applied at higher doses.

Table 1. Summary of biological changes caused by chronic irradiation of the tomato cultivar 'Micro-Tom'.

Categories of Biological Change	Changes in M_1 Plants	Transmission to the Next Generation (M_2)
Impaired growth and reproduction	Reduced leaf length	Partially transmitted
	Reduced seed set	Partially transmitted
Developmental and morphological changes	Reduced trichome density	Not transmitted
	Increased length-to-width ratio of fruits	Not transmitted
Alteration of gene expression pattern	Downregulation of expression of trichome development genes	Not transmitted
	Upregulation of expression of ROS response-related genes	Partially transmitted (four out of the twelve genes tested)

Trichomes are specialized cells that undergo differentiation from the epidermis of plants and play an essential role in conferring protection to plants against various biotic and abiotic stresses [64–66]. We detected a decrease in the density of type VI trichomes on leaves when gamma rays were used for chronic irradiation at a dose of 100–200 Gy for 4 weeks. This decrease might be attributable to a decrease in the expression of the *MYC1* gene, which is involved in the initiation of type VI trichomes through the jasmonic acid (JA) signaling pathway [39]. Since salicylic acid (SA) signaling is triggered by ROS accumulation [67], and as there exists an antagonistic crosstalk between SA and JA [68], the reduction in type VI trichomes could be explained by the continuous SA signaling induced by ROS. Goh et al. [36] showed that trichome formation was promoted in *Arabidopsis* seedlings irradiated with a gamma-ray dose of 200 Gy for both 1 h and 1 week. The reason for the inconsistent trichome phenotypes between *Arabidopsis* and tomato is unknown. However, the difference may be attributed to the sensitivity of plant species to radiation, duration of radiation, or dose rate. For example, different dose rates may affect gene expression to a variable extent.

Comparative analysis between groups of *Arabidopsis* plants subjected to irradiation with ^{137}Cs at 1 Gy for a period of 14 days (chronic irradiation) and a single pulse (acute irradiation) showed that the two groups shared <10% differentially expressed genes [69]. The dose rate of chronic irradiation reported by Goh et al. [36] was 1.2 Gy/h, while it was markedly lower in our study [0.15 Gy/h (total 100 Gy) to 0.30 Gy/h (total 200 Gy)]. We also showed that gamma-irradiated (50, 100, and 150 Gy) tomato lines could develop fruits with a higher length-to-width ratio. Rodríguez et al. [70] classified tomato fruit shape into eight groups and found that the allele distribution of four major genes was strongly related to fruit morphology. The expression of two genes, *SUN* and *OVATE*, regulated the elongated shape, and the expression of other genes, namely *FASCIATED* and *LOCULE NUMBER*, modulated fruit locule number and the flat shape [70]. The unique peanut-shaped morphology of fruit derived from irradiated tomato plants did not match any of the eight groups classified by Rodríguez et al. [70]. This suggests that changes in the expression level of unidentified fruit shape-related genes may be responsible for the abnormal fruit shape observed in our study. The tomato fruit with unique characteristics found in our study could be used as a novel research material for investigating the relationship between environmental stress, gene expression, and fruit morphology.

Regarding gene expression, the expression levels of *HsfA4a*, *WRKY39*, and *CML51*, which are upstream transcription factors that exhibit responses to ROS [71–73], increased in M_1 plants. This was similar to the findings reported in *Arabidopsis* [74]. However, the expression of *ZAT10* decreased after subjection to chronic gamma irradiation. In *Arabidopsis*, the overexpression of *ZAT10* conferred resistance to osmotic stress and salinity; however, resistance to the same stresses was also detected when *ZAT10* was subjected to knockout [75]. The expression of *ZAT10* increased in *Arabidopsis* following subjection to acute gamma irradiation [74], however, in tomato, the expression decreased following subjection to chronic gamma irradiation in this study. This result implies that the method of controlling gene expression to counter stress attributable to irradiation may differ according to the plant species or dose rate. The expression of the antioxidant gene *RBOH1*, which is induced by photo-oxidative stress [44], increased at doses of 100 and 200 Gy. Interestingly, among the genes exhibiting an upregulated expression, expression levels were higher in the 100-Gy group than those in the 200-Gy group. It was presumed that a dose of 200 Gy exceeded the limit below which the defense mechanism could effectively mount responses against ROS in tomato. In contrast, the transcription of *POD3* decreased at doses of 100 and 200 Gy. Similarly, Goh et al. [36] showed that POD activity decreased during 3 weeks of subjection to chronic gamma irradiation in *Arabidopsis* seedlings. When tomato leaves were subjected to drought stress for a period of 4 weeks, the formation of a new isoform of *POD3* was observed [76]. Further studies are warranted to determine the effect of chronic gamma irradiation on *POD3*. Expression levels of a considerable proportion of the tested tomato genes (*Mn-SOD*, *cAPX*, *GPX*, *phGPX*, and *CAT1*) encoding ROS-scavenging

enzymes were also upregulated after subjection to chronic gamma irradiation, as observed for their *Arabidopsis* orthologs [74].

Our results showed that the transmissibility of biological changes caused by chronic irradiation depended on the type of effect (Table 1). Changes in trichome density and fruit shape were not inherited to the next generation, suggesting that they were caused by changes in gene expression or protein function in response to irradiation, without the occurrence of inheritable genetic modifications. This conclusion was supported by the absence of a significant difference in the expression levels of trichome development-related genes between progenies from gamma-irradiated and wild-type tomatoes. In contrast, there was partial inheritance of reductions in leaf length and seed set and changes in the expression of several antioxidant genes in the progenies, indicating the involvement of inheritable genetic modifications. The inheritance of large structural variation in DNA can be a potential mechanism because chromosomal aberrations may result in the retardation of growth and reduction in fertility in the irradiated plants. However, this was unlikely to be the main mechanism because the majority of DNA structural variation in M_1 plants was lost, presumably during gametophyte development [14]. Hase et al. [17] demonstrated that the progeny of *Arabidopsis* plants obtained following treatment with chronic gamma irradiation for five successive generations harbored a high ratio of small DNA mutations (e.g., single-substitutions and small InDels) compared to a considerable structural variation. They also found that the frequency of DNA mutations was higher following subjection to chronic gamma irradiation (168 homozygous mutation events per plant on average with 916 Gy irradiation) than acute gamma irradiation (16.3 homozygous mutation events per plant on average with 1000 Gy irradiation). Therefore, the additive effects of the accumulated small DNA mutations might partly contribute to the deleterious phenotypes observed in the M_1 and M_2 generations.

In addition to DNA mutations, irradiation has been shown to affect epigenetic modifications by increasing the expression of epigenetic regulators (*MET1*, *CMT3*, and *SUVH5*) in *Arabidopsis* subjected to irradiation with X-rays [77]; furthermore, modifications pertaining to hypermethylated DNA sequences in *Pinus sylvestris* trees exposed to radiation after the Chernobyl accident [78] and hypomethylated DNA sequences in rice subjection to irradiation with gamma rays [3] have also been observed. Sidler et al. [75] hypothesized that epigenetic changes (hypermethylation in this case) following radiation exposure might constitute an adaptive response of plants to prevent genome instability and reshuffling. The inheritance of epigenetic changes in plants has been reported in various biological areas [79–81]. As changes in the expression pattern of a few ROS-related genes (*ZAT10*, *Mn-SOD*, *POD3*, and *RBOH1*) were found to be inherited to the next generation, it might be inferred that the epigenetic effects of irradiation could affect the expression of radiation-responsive genes and, in turn, might cause the inheritance of certain phenotypic changes and gene expression patterns. Boratyński et al. [48] showed that the progenies of carrots subjected to chronic irradiation around Chernobyl showed higher resistance to irradiation. The upregulation of expression of ROS-related genes in our M_2 lines may be related to this type of adaptation to radiation. Further genome-wide investigation into the changes in gene expression patterns and epigenetic modifications in M_1 and M_2 generations is necessary to clarify the mechanism underlying the inheritance of phenotypic and genetic changes.

Large mutant populations with mutant phenotype databases have been developed via subjection to acute gamma irradiation and EMS treatment of 'Micro-Tom' [30,82,83]. Since Hase et al. [17] have shown that chronic gamma irradiation is more effective for the accumulation of DNA mutations with less radiation-associated damage than acute irradiation, and have suggested that further development of mutant populations can be attempted by applying chronic irradiation. The present study showed that phenotypic mutants could be obtained in the M_2 generation, as expected. The optimal irradiation dose for mutagenesis should be determined for the development and establishment of large mutant populations. For 'Micro-Tom', Matsukura et al. [80] established a mutant population following subjection to acute gamma irradiation at a dose of 300 Gy, which

reduced the germination rate by 30%. In our previous study conducted on acutely gamma-irradiated 'Micro-Tom', the survival rate significantly decrease from 300 Gy [46]. Thus, a dose of 300 Gy was considered as the shoulder dose that could help generate the highest number of mutants per sown M_1 seed [16]. The number of seeds per fruit in plants subjected to irradiation with a dose of 300 Gy was 84% of that in the original plants (data not shown). At instances where the seed set rate in the present study was considered, the significance of the biological effects exerted by acute gamma irradiation at a dose of 300 Gy seemed similar to that observed with chronic gamma irradiation at a dose ranging from 50–100 Gy. This result was consistent with that reported by Hase et al. [63], who showed that *Arabidopsis* seeds were six times more tolerant to carbon beams than the tolerance exhibited by seedlings. However, when the transgenerational effect on the seed set is considered, chronic irradiation at a dose of 100 Gy may hamper the maintenance of mutant populations as a significant decrease in the seed set could be detected in the M_2 generation. Therefore, we suggest the application of a dose of 50 Gy as an optimal dose for mutant population establishment using chronic gamma irradiation in 'Micro-Tom'.

5. Conclusions

We investigated the biological changes and their inheritance in 'Micro-Tom' plants following subjection to chronic gamma irradiation. The biological effects detected in the M_1 generation included reduction in leaf length and seed set, changes in trichome density and fruit shape, and alteration of the expression pattern of genes involved in trichome development and antioxidant response. Few changes in phenotype and gene expression patterns were also detected in the M_2 generation, suggesting the existence of transgenerational inheritance of biological changes caused by irradiation. Meanwhile, the optimal dose for chronic gamma irradiation for the development of a 'Micro-Tom' mutant population was determined to be 50 Gy, considering the biological effects in both the M_1 and M_2 generations. Based on our findings, we suggest that this study will provide valuable information for understanding plant response to radiation and will provide a resource for executing the application of chronic irradiation for conducting functional genomics studies and breeding in tomatoes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11081638/s1, Table S1. Primers used for qRT-PCR. Figure S1. Schematic diagram of tomato seedling treatment with chronic gamma irradiation. Figure S2. Dissecting micrograph of the adaxial leaf surface derived from 7-week-old 'Micro-Tom' plants.

Author Contributions: J.-H.K. and J.-B.K. designed the experiments. S.-M.K., Y.D.J. and J.-I.C. performed the experiments. S.-M.K., Y.D.J., J.-B.K. and J.-H.K. analyzed the data. S.-M.K., Y.D.J. and J.-H.K. wrote the first draft of the manuscript. J.-H.K. prepared the final draft and was responsible for the correspondence. All authors have read and agreed to the published version of the manuscript.

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