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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Chronic Sr-90 and Cs-137 radionuclide exposure in Lemna minor: Effects on phenotype, redox balance, and methylation

Yelltrich Reymen

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Ann CUYPERS

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Chronic Sr-90 and Cs-137 radionuclide exposure in *Lemna minor*: Effects on phenotype, redox balance and methylation.

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*Running title: *Chronic radionuclide exposure in Lemna minor.*

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ABSTRACT

Ionizing radiation is known to induce DNA damage and oxidative stress in plants. However, this has mainly been studied for acute high-dose exposures. As sessile organisms, plants cannot avoid long-lasting exposure to ionizing radiation in case of a nuclear accident. Therefore, it is important to study the effects of low-dose chronic ionizing radiation exposure on multiple generations of plants, together with the mechanisms that regulate these responses. In this research, strontium-90 (Sr-90) and cesium-137 (Cs-137) were used to investigate the effect of chronic ionizing radiation in *Lemna minor*. Two experiments were set up that lasted 6 weeks and applied the following activity concentrations: 40 Bq/L, 400 Bq/L, and 4,000 Bq/L for Sr-90, and 40 Bq/L, 400 Bq/L, and 40,000 Bq/L for Cs-137. Phenotypic traits, pigment concentration, and photosynthetic activity were determined. Moreover, an expression analysis with genes involved in epigenetic regulation and oxidative stress was performed using qPCR. Finally, glutathione content was analyzed. Growth was significantly affected after 6 weeks of exposure to Cs-137. Pigment concentration and photosynthetic activity were, however, not altered. Sr-90 induced changes in the expression of epigenetically regulating genes, while Cs-137 altered the expression of genes involved in glutathione synthesis. Glutathione levels remained stable after Sr-90 exposure, while an increasing trend was observed after Cs-137 exposure. These findings highlight a potential regulating role for S-adenosylmethionine in response to ionizing radiation stress, which needs further investigation. Moreover, additional research is needed on the effect of ionizing radiation on plants to complete the overview of response mechanisms.

INTRODUCTION

All organisms on the planet are continuously exposed to cosmic and natural background radiation, which includes ionizing radiation (1, 2). However, the exposure rates increase significantly in case of a nuclear accident that leads to the release of radionuclides into the atmosphere. The best-known examples of such an event are the Chornobyl disaster from 1986 and the incident at the Daiichi power plant in Fukushima in 2011 (3). Both events involved the release of numerous radionuclides with unstable nuclei, of which the decay resulted in unseen quantities of ionizing radiation (4). As a consequence, both animals and plants were exposed to unusual doses of radioactivity, including gamma radiation. This

high-energy type of ionizing radiation has the highest penetration capability compared to alpha and beta particles that were also emitted (5). The direct effect of ionizing radiation on plants is energy deposition into DNA, inducing single and double-strand breaks (6, 7). In addition, high-energy radiation interacts with multiple molecules, such as proteins and salts, and leads to the formation of reactive oxygen species (ROS). For instance, H_2O can be ionized to $H_2O^{\bullet+}$ + SE(e^-). The resulting electron causes DNA damage and the $H_2O^{\bullet+}$ reacts with H_2O again, which forms a $OH^{\bullet}$ radical, leading to additional damage (7). As a response to an increase in oxidative stress, the plant's antioxidant system intervenes. The activity of antioxidant enzymes increases, while the redox status of antioxidant molecules, such as glutathione (GSH) and ascorbic acid (AA) is simultaneously changed to act as a buffer (8). Over an extended period, exposure can eventually lead to an increased radiation sensitivity in offspring, up to numerous generations (9). Due to their sessile nature, plants suffer more severely from the consequences of ionizing radiation in case of a nuclear accident (10). Moreover, they have been forced to evolve particularly refined and specific methods to cope with stressors. Therefore, it is important to investigate the effects of ionizing radiation exposure on these organisms together with the mechanisms that are responsible for handling this stress.

Previous research has already identified epigenetic modifications as a key regulator during various stress responses, including radiation stress (11-14). The three main types of epigenetic modifications that alter chromatin structure and thus gene expression in plants include DNA methylation, histone modifications, and RNA interference (RNAi) (15). In this paper, DNA methylation refers to the addition of a methyl group to the 5'-carbon of a cytosine. Adenine and guanine are also targets for methylation in plants, but this type of methylation is significantly less abundant (16). In vertebrates, DNA methylation occurs mostly at CpG sites. However, in plants, CHG and CHH (where H = T, A, or C) sites can be methylated as well (17, 18). De novo methylation in plants is executed by the Domains Rearranged Methyltransferase 1&2 (DRM1, DRM2), while methylation maintenance is secured by DNA Methyltransferase 1 (MET1), Chromomethylase 2 (CMT2), and CMT3 (19, 20). MET1 maintains CG methylation and CMT2 and CMT3 preserve CHH and CHG methylation, respectively (21). In contrast, repressor of silencing 1 (ROS1) plays a role in demethylation, thereby ensuring a stable methylation balance (22-24). In most cases, DNA methylation reduces gene expression by repressing the transcription process (25). However, there are exceptions: For instance, DNA methylation within intronic regions facilitates the recruitment of histone modifiers, leading to increased transcription (26). Histone modifications include the post-translational addition of functional groups on the N-terminal tail, mostly acetyl or methyl groups (25, 27). Depending on the functional group that is added, its position inside the nucleosome, and its molecular environment, the modification will result in either gene expression or repression (28, 29). Besides DNA methylation and histone modifications, RNAi is considered a significant epigenetic regulator as well. It usually involves non-coding RNA sequences that interact with transcribed RNA from functional genes to repress gene expression (30). These non-coding RNA sequences can be divided into long and short non-coding RNAs (lncRNAs and sncRNAs). lncRNAs are subdivided depending on their relationship to protein-coding genes (e.g. long intergenic ncRNA or lincRNA) and sncRNAs can also be further divided into numerous subgroups: snRNA, snoRNA, siRNA, miRNA, piRNA, among others, each fulfilling their function (31-33).

Along with these 3 key epigenetic modifications, chromatin remodeling in plants is also achieved through the activity of a group of intracellular proteins, called sirtuins. Sirtuins, such as sirtuin 1 (SRT1), catalyze the deacetylation of histones and non-histone protein substrates, which is β-nicotinamide adenine dinucleotide (β-NAD^+) dependent. This can result in significant changes in the chromatin structure (34, 35). In addition, telomere maintenance is another layer of epigenetic regulation in plants. Telomeres act as epigenetic regulatory elements through the so-called telomere positioning effect (TPE). This refers to the ability of telomeres to repress or activate gene transcription depending on their position towards neighboring genes (36). Telomere length and thus structure in plants is

maintained by telomerase. This protein consists of the telomere reverse transcriptase (TERT) and telomerase RNA (TR) subunits, which are responsible for the catalytic activity and for providing a reverse transcription template respectively. These subunits interact with other proteins to preserve telomeres (37).

As mentioned, the most important epigenetic mechanisms have already been investigated in the context of plants and their reaction to different biotic and abiotic stresses in the environment (25, 38). Firstly, cold influences epigenetic regulation: It was found that a cold environment induces genome-wide DNA methylation in maize seedlings (39). Methylation might also be responsible for conserving a cold-stress memory in plants (40). Furthermore, histone acetylation was identified to play an important role in cold stress responses, leading to increased expression of cold-responsive genes (41). On the other hand, several epigenetic regulators have been known to be involved in heat stress responses to protect plants from damage (42). In *Arabidopsis thaliana*, increased methylation was reported by Naydenov et al. as a result of heat conditions (43). In contrast, Folsom et al. showed that DNA and histone methylation decreased in rice seeds, leading to reduced seed size (44). Histone acetylation is also found to be involved in heat stress, changing the transcription of responsive genes (45). Finally, miRNA expression is reported to be changed in multiple poplar species under heat conditions which affected gene expression (46, 47). Besides cold and heat stress, drought can severely affect plants. Again, epigenetics is involved in handling this distress. Both DNA methylation changes and histone modifications are observed when plants are subjected to drought (48). More specifically, histones undergo methylation, acetylation, sumoylation, and phosphorylation, altering nucleosome structure (45, 49). The affected plants are thought to develop a drought stress memory through the deposition of these modifications (50). Another abiotic stress factor that changes the epigenetic framework of plants, is salinity. It induces differences in whole genome methylation patterns combined with histone modifications (51). One study found a correlation between salt stress and the expression of DNA methyltransferases (52). The histone modifications are achieved mainly through an altered activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (50). Aside from the abiotic stresses, plants are also affected by biotic interactions. The review from Alonso et al. provides a clear overview of the relationship between plant epigenetics and biotic stresses (53). For example, it is mentioned that methylation patterns are correlated with the expression of genes that are essential in the defense against pathogens. Furthermore, it highlights the importance of non-coding RNAs in plant immunity.

When considering ionizing radiation as a stress factor, adaptations have been found for plant DNA methylation, as well as for histone modifications and non-coding RNA interference (54). In a study by Volkova et al., ionizing radiation exposure was associated with DNA hypermethylation in pine trees collected from Chornobyl (55). In addition, lab-exposed *A. thaliana* showed a dose-rate-determined change in total methylation levels (56). Similarly, Kovalchuk et al. reported differences in DNA methylation for *A. thaliana* (57). These studies suggest that epigenetic-mediated regulatory mechanisms might play a pivotal role in plants that experience ionizing radiation stress.

However, more research is needed to establish a clear relationship between ionizing radiation exposure and changes in the molecular organization of plants. Moreover, the effects of ionizing radiation and the epigenetic regulation of stress responses in plants over multiple generations have not yet been documented properly. Therefore, we investigated the chronic effect of strontium-90 (Sr-90) and cesium-137 (Cs-137) exposure on *Lemna minor* in two separate 6-week experiments. These radioactive isotopes are relevant because of their high, long-lasting impact on the environment. In addition, they are commonly released during nuclear accidents, including the Chornobyl and Fukushima disasters (58-60).

L. minor is an aquatic plant that mainly duplicates by division and has a rapid reproduction time of approximately 2.5 days (61). Since its genome has been fully sequenced recently, this organism also allows for the analysis of environmental effects on molecular levels such as the genetic and epigenetic level (4, 62). In

combination with its easy use and relatively small size, it is commonly used in laboratory experiments that involve toxicity testing and ecotoxicological research (63, 64).

It was hypothesized that exposure to the Sr-90 and Cs-137 sources leads to notable phenotypic changes in *L. minor,* together with a disturbance of the redox balance. In addition, it was expected that photosynthetic activity will be altered and that methylation patterns will be affected. Eventually, this research will improve overall environmental risk assessment regarding ionizing radiation exposure by implementing the epigenetic layer.

EXPERIMENTAL PROCEDURES

Experimental setup – Lemna minor (serial number 1007 and ID number 5500) collected from a pond in Blarney, Co. Cork, Ireland, (University College Cork, Ireland) were utilized in this experiment (65). They were pre-cultured in blacked-out containers filled with 1/10 strength Hoagland solution. Square transparent Petri dishes were placed on top of the containers to limit evaporation and the deposition of particulates. These containers were stored in a controlled climate chamber (Microclima MC1000E, Snijders Scientific B.V., Tilburg, The Netherlands) with a 14 h photoperiod (photosynthetically active radiation (PAR) of 116 µmol m-2 s-1 at the leaf level)(supplied by three Sylvania BriteGro F36WT8/2084 lamps and one Philips MASTER TL-D 90 De Luxe 36 W/950 1SL/10 lamp) with 65% humidity and a day/night temperature of 22°C/18°C.

At the start of the irradiation experiments, 60 sterile 250 mL transparent Nalgene® pots (15 per condition), each containing 100 ml of fresh medium, were seeded with 7 mature plants that had 3-4 fronds. In addition, a sterilized red square of $1cm^2$ was added onto the medium as a reference to determine the growth. The pots were covered with a transparent and sterile Petri dish to minimize evaporation of the medium, while still allowing essential light transmission and exchange of atmosphere. During 6 weeks, the *L. minor* plants were chronically exposed to gamma radiation by a Sr-90 or Cs-137 source that was added to the growth medium. Different activity concentrations were applied: 40 Bq/L, 400 Bq/L, and 4,000 Bq/L for Sr-90 and 40 Bq/L, 400 Bq/L, and 40,000 Bq/L for Cs-137. All plants were continuously grown at 24°C and light was supplied by LED lights for a 14 h photoperiodic period with a PAR of 200 µmol m^{-2} s^{-1} at the leaf level. The pots were placed randomly in the climate chamber to ensure proper homogeneity for all conditions. Each week, seven mature plants were transferred from each pot to a new one with fresh medium.

Phenotypic trait analysis – Phenotypic changes were monitored for six weeks by taking top-view pictures on days 0, 2, 4, and 7. The pots were covered with a circular, sterile Petri dish to ensure an equal distance between the solution and the camera. The total surface frond area was determined using EasyLeafArea software and ImageJ. The red square method was applied to serve as a reference measurement. Afterwards, the mean frond area and average specific growth rate (ASGR) were calculated according to the OECD guidelines.

Pigment analysis – Pigments were first extracted by adding dimethylformamide (DMF) in a ratio of 0.5ml/20mg fresh weight and incubating in the dark for 24h at 4°C. Afterwards, the absorbance was measured in triplicate (1:1, 1:2, and 1:4 dilution series) at wavelengths of 664, 647, and 480nm. The absorbance values were corrected for the light path using factor 0.294. To determine the amount of chlorophyll A, chlorophyll B, and carotenoids, the following formulas were utilized respectively: $C_a = 11.65A_{664} - 2.69A_{647}$; $C_b = 20.81A_{647} - 4.53A_{664}$; $C_{x+c} = (1000A_{480} - 0.89C_a - 52.02C_b)/245$.

Photosynthetic activity – The photosynthetic activity was determined with the DUAL-PAM-100 (Walz) and its accessory software. Prior to analysis, plants were left in the dark for 1h in a sterile 6-well plate filled with demineralized water. Then, the DUAL-PAM-100 was calibrated using a fluorescence standard. For each measurement, the cuvette was filled with demineralized water, and the *Lemna* were placed on top. Afterwards, the position of the LED array was adjusted to start the measurement. All measurements were performed in the dark using a green desk light.

Gene expression analysis – First, specific primers were designed for every gene of interest. A detailed explanation of the primer design procedure and calculations is added in the supplementary material (method S1).

RNA was extracted to analyze gene expression levels. Initially, frozen plant samples of approximately 50 mg were shredded using a Mixer Mill MM 400 (Retsch) and 2.0 mm Zirconia (zirconium(IV)oxide) beads (BioSpec Products) for 3 min at 30 Hz. The RNeasy® Plant Mini Kit (Qiagen) was utilized to extract the RNA, applying the manufacturer's instructions. Afterwards, RNA quality and quantity were determined spectrophotometrically at 230, 260, and 280 nm using the NanoDrop ND1000. The final RNA was then treated with the Invitrogen™ TURBO DNA-free™ kit (Thermo Fisher Scientific) to remove DNA contamination before converting it to cDNA using the PrimeScript 1st strand cDNA synthesis kit (TaKaRa Bio). The cDNA was then used to examine gene expression levels. The qPCR runs were performed with 96-well plates using the QuantStudio3 machine (Applied Biosystems) and the corresponding QuantStudio™ software. Each run contained 40 cycles with the following program: 95°C for 5 seconds, 60°C for 12 seconds.

Glutathione measurement – For each condition in week 1, week 2, and week 6, 4 biological replicates of approximately 100 mg were shredded similarly as for the gene expression analysis. 200 mM HCl was added to the samples to extract the glutathione, before centrifuging for 15 min. at maximum speed at 4°C. Afterwards, 1/10 200 mM NaH_2PO_4 was added to the supernatant, and pH was adjusted to 4-5 using a 200 mM NaOH solution. For measuring oxidized glutathione levels, 2-Vinylpyridine (2-VP) was subsequently added to the samples and incubated at room temperature for 30 min. Next, the 2-VP was precipitated by centrifuging for 15 min. at maximum speed at 4°C. To determine total and oxidized glutathione levels, a reduced L-glutathione (GSH) and oxidized L-glutathione (GSSG) standard were prepared respectively. A master mix consisting of 200 mM NaH_2PO_4 – 10mM EDTA, 10 mM NADPH, 12 mM DTNB in DMSO, and glutathione reductase was added to each well containing standard or sample to initiate the reaction. Samples were analyzed using the BioTek® PowerWave XS plate reader that measured the absorbance at 412 nm during 5 min 16s with 39s intervals. Standards were analyzed in duplicate and samples is triplicate. Afterwards, the concentration of reduced glutathione was calculated by subtracting GSSG from total glutathione, taking into account that 1 GSSG is equivalent to 2 GSH.

Statistical analysis – Statistical analysis was performed with RStudio 2022.02.2 build 485 (version 4.1.0) and 2023.03.1 build 446 (version 4.3.0). Normality was checked with a Shapiro-Wilk test in combination with a Q-Q plot, and homoscedasticity was examined with a Bartlett test and Levene's test. If assumptions were fulfilled, a two-way analysis of variance (ANOVA) was executed together with a posthoc Tukey HSD test. Otherwise, the non-parametric Kruskal-Wallis test was preferred in combination with a Wilcoxon pairwise comparison.

RESULTS

Phenotypic trait analysis – For the plants exposed to Cs-137, the mean frond area was calculated for each condition on days 0, 2, 4, and 7 of each week (Fig. S1). Afterwards, the average specific growth rate (ASGR) was determined according to the OECD guidelines (Table 1). A comparison between week 1 and week 6 is shown in figure 1. The control plants showed no difference in growth when comparing week 1 and week 6. In week 1 and week 6, the plants exposed to 40 Bq/l showed a significantly higher ASGR compared to the control plants. A concentration of 400 Bq/l did not affect ASGR in week 1. However, the plants exposed to 400 Bq/l showed a significantly higher ASGR compared to the unexposed plants in week 6. In contrast, exposure to the highest activity concentration of 40,000 Bq/l led to a significantly low ASGR in both weeks. The ASGR for all other weeks is presented in figure S2.

Table 1: Effect of Cs-137 exposure on average specific growth rate (ASGR) in *L. minor.* P-values are shown for each week. Data are analyzed using a non-parametric Kruskal-Wallis test ($\alpha = 0.05$).

Week	P-value
1	$P < 0.001$
2	$P = 0.001$
3	$P < 0.001$
4	$P = 0.496$
5	$P = 0.171$
6	$P < 0.001$

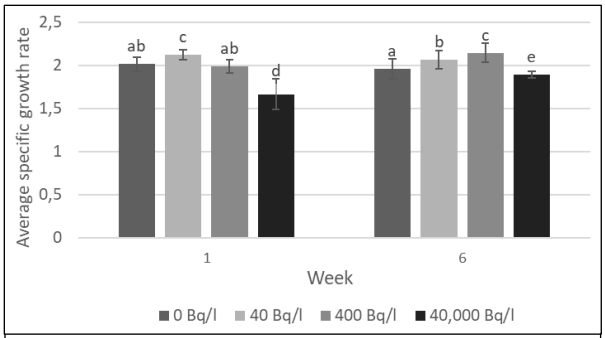

Fig. 1: Comparison of the *L. minor* ASGR for week 1 and week 6 during chronic Cs-137 exposure. Data are presented as mean ±SD (n=15). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Cs-137 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 40,000 Bq/l. Different letters indicate groups that are significantly different (p-value < 0.05; Kruskal-Wallis test). *Bq = Becquerel, l = liter*

Pigment analysis – The pigments chlorophyll A, chlorophyll B, and carotenoids were measured (data not shown). In the Sr-90 experiment, ionizing radiation had no significant effect on pigment concentration. Similarly, no significant differences were observed for either of the pigments in the Cs-137 experiment. However, a similar increase in pigment concentration was observed for chlorophyll B and carotenoids in week 2, both for Cs-137 exposed and control plants.

Photosynthetic activity – The Cs-137 experiment showed no significant effect of ionizing radiation on photosynthetic activity in *L. minor* (data not shown).

Gene expression analysis – Primers were successfully designed for 9 genes of interest that are involved in either epigenetic regulation or maintaining the redox balance. The function of each gene, primer sequences, and associated efficiencies are shown in table 2.

The qPCR results from the Sr-90 experiment are shown in figure 2. Expression of SRT1 was not significantly affected by Sr-90 exposure. However, the increase in expression observed in week 6 was borderline significant ($p = 0.068$). GR expression showed a clear decreasing trend in weeks 1-4. This trend is significant for week 1 ($p = 0.017$) and week 2 ($p = 0.042$), and borderline significant for week 3 ($p = 0.096$) and week 4 ($p = 0.06$). CMT3 expression also showed a decreasing trend, with a significant difference in week 2 ($p = 0.043$) and week 3 ($p = 0.025$). In contrast to the other weeks, in weeks 4 and 6, the decreasing trend is not visible. ROS1 expression was not affected by Sr-90 ionizing radiation exposure. The expression of MET1 showed a decreasing trend during the first 3 weeks. In week 2 and week 3, the expression was significantly lowered with p-values of 0.033 and 0.029, respectively. For TERT expression, no trend appeared as a result of Sr-90 exposure. However, a significant effect was observed in week 3 (p =

Table 2: Genes of interest and their developed primer pairs. Primers were developed for 9 genes of interest. The gene's name, function, primer sequences and primer efficiency are shown. *DNA = deoxyribonucleic acid0*

Name	Function	Primer sequences	Primer efficiency
DNA methyltransferase 1 (MET1)	Methylation maintenance	ACGGCGAAGAAGCATAACCA GGAAGCACATGCCGACTTTG	103.27%
Chromomethylase 3 (CMT3)	Methylation maintenance	AACCATTCGCTAGGCTCTGG TCGTCAGAACCCTGTCTTGC	100.06%
Repressor of silencing 1 (ROS1)	Demethylation	ACCAGGCAACCTGTTTCTCC CTCTCATCGCCGTCCTACAA	102.24%
Sirtuin 1 (SRT1)	Histone deacetylation	GCGGTGGAGGGAAGATTGTA CACTAACCCGTGGATCACCAA	89.8%
Glutamate-cysteine ligase (GSH1)	Glutathione biosynthesis	AGTGGGCTCTCTTGGAGTTG TAATGCAAGACCGGCACGAA	106.95%
Glutathione synthetase 2 variant 1 (GSH2.1)	Glutathione biosynthesis	GAAATGCTTTGCTGGGCTGTG TTGTTCCCTCCTCCTTCCCTT	98.68%
Glutathione synthetase 2 variant 2 (GSH2.2)	Glutathione biosynthesis	TGCTTTGCTCCCTGTTTCGT TCCAAACTGACGCGATCCAC	97.07%
Glutathione reductase (GR)	Glutathione metabolism	GATCGAAGGTCGCGGAAAGA GCCGTCCACCAACAGAAATG	105.2%
Telomere reverse transcriptase (TERT)	Telomere maintenance	TCTCTGAAATGGTGGTCCCC TTTGGTGCTTCCCGAACTCA	126.06%

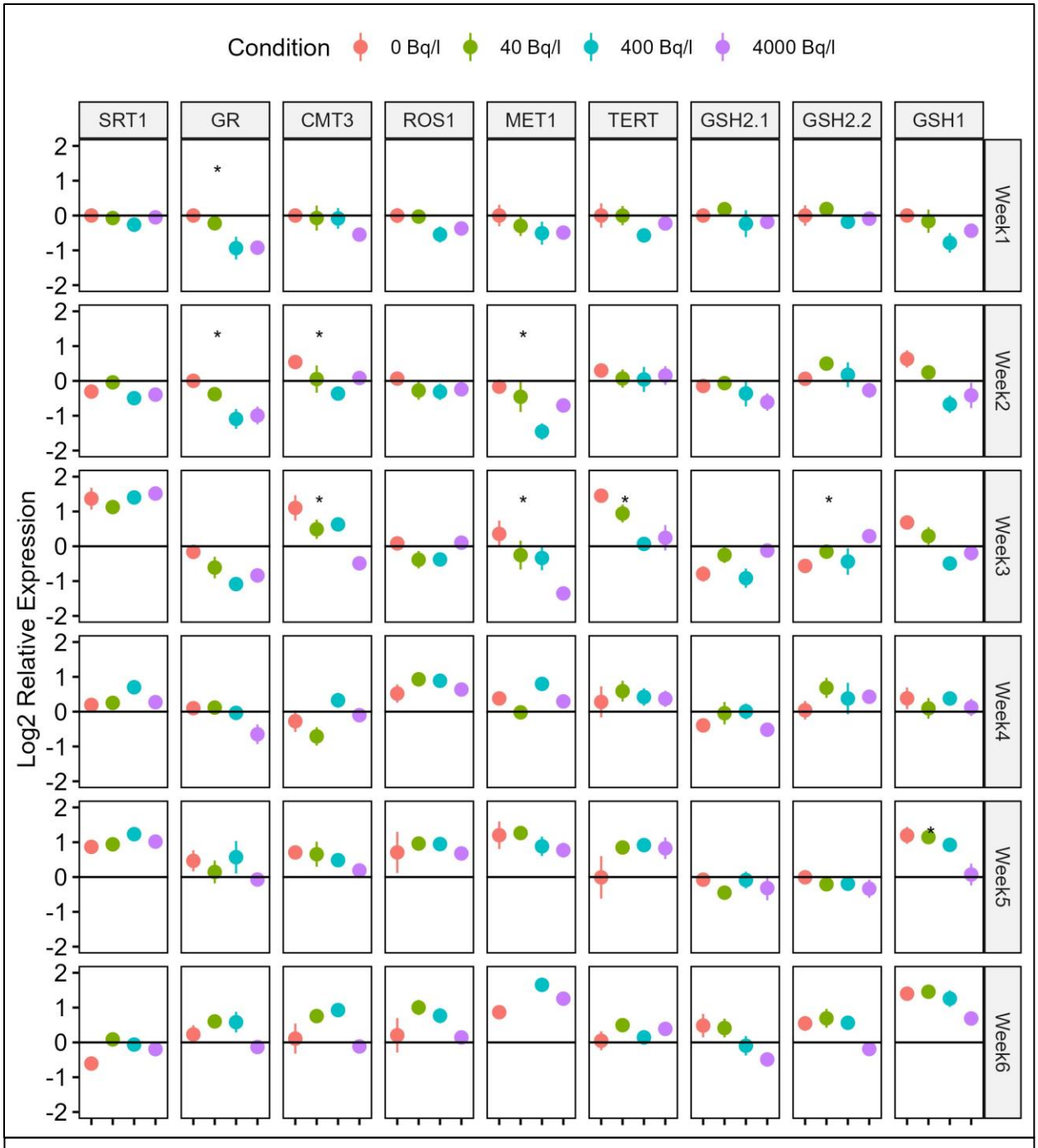

Fig. 2: qPCR data representing changes in gene expression during chronic Sr-90 exposure. Data are presented as mean ±SE (n=4). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Sr-90 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 4,000 Bq/l. Weeks with a significant effect of Sr-90 exposure on gene expression are indicated with an asterisk ($p < 0.05$, Kruskal-Wallis test). *Bq = Becquerel, l = liter, SRT1 = sirtuin 1, GR = glutathione reductase, CMT3 = chromomethylase 3, ROS1 = repressor of silencing 1, MET1 = DNA methyltransferase 1, TERT = telomere reverse transcriptase, GSH2.1 = glutathione synthetase 2 variant 1, GSH2.2 = glutathione synthetase 2 variant 2, GSH1 = glutamate-cysteine ligase*

0.021). 2 genes involved in the glutathione metabolism, GSH2.1, and GSH2.2, showed no increasing or decreasing trend in their expression after exposure to Sr-90. Nevertheless, the expression of GSH2.2 was significantly affected in week 3 ($p = 0.041$). Finally, GSH1 expression showed an overall decreasing trend with a statistically significant effect in week 5 ($p =$

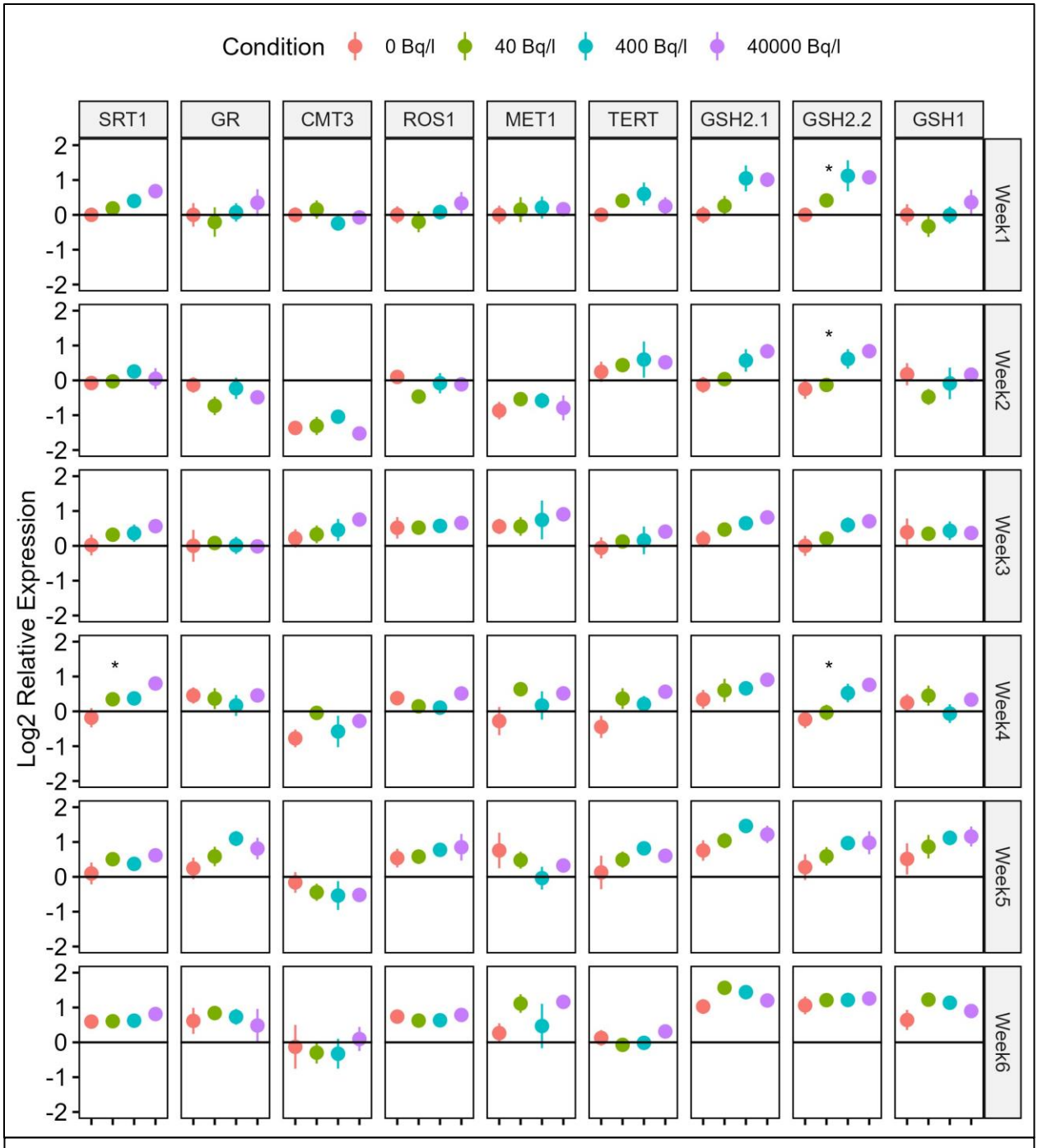

Fig. 3: qPCR data representing changes in gene expression during chronic Cs-137 exposure. Data are presented as mean ±SE (n=4). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Cs-137 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 40,000 Bq/l. Weeks with a significant effect of Cs-137 exposure on gene expression are indicated with an asterisk ($p < 0.05$, Kruskal-Wallis test). *Bq = Becquerel, l = liter, SRT1 = sirtuin 1, GR = glutathione reductase, CMT3 = chromomethylase 3, ROS1 = repressor of silencing 1, MET1 = DNA methyltransferase 1, TERT = telomere reverse transcriptase, GSH2.1 = glutathione synthetase 2 variant 1, GSH2.2 = glutathione synthetase 2 variant 2, GSH1 = glutamate-cysteine ligase*

0.045). In addition, weeks 2, 3 and 6 showed a borderline significant effect with p-values of 0.051, 0.051, and 0.082, respectively. The outcome of the Wilcoxon pairwise comparison of the significant and borderline significant results reported above is shown in figure S3.

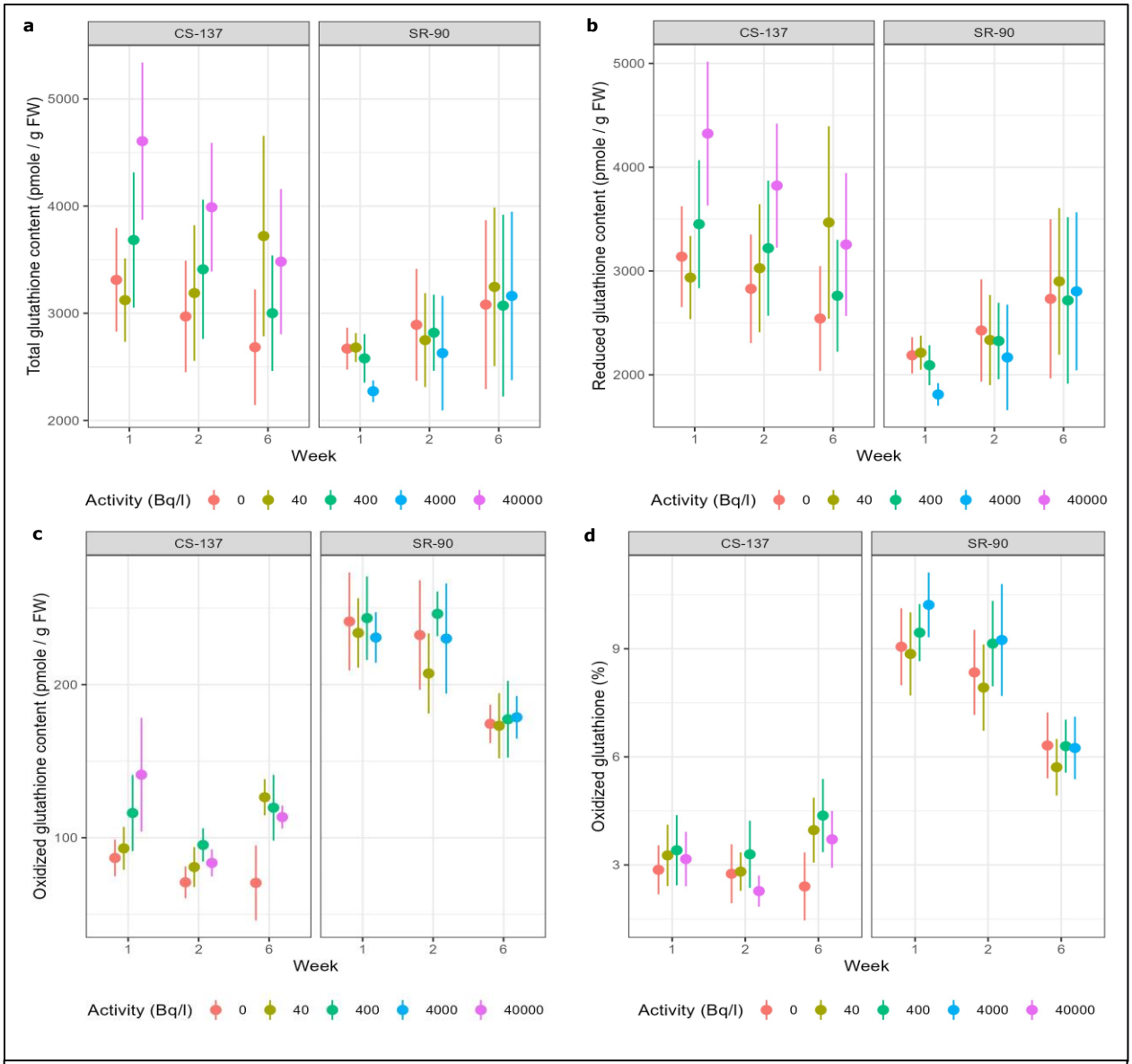

Fig. 4: Glutathione measurements for week 1, 2, and 6 during chronic Cs-137 or Sr-90 exposure in *L. minor*. Data are presented as mean ±SE (n=4). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Cs-137 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 40,000 Bq/l. (A) Total glutathione content; (B) Reduced glutathione content; (C) Oxidized glutathione content; (D) Percentage oxidized glutathione. Pmole = picomole, g = gram, FW = fresh weight, *Bq = Becquerel, l = liter*

The qPCR results of the Cs-137 experiment are presented in figure 3. SRT1 expression showed an increasing trend with raising concentrations throughout the 6 weeks, of which week 4 presented a significant effect ($p = 0.039$). No effect was observed for GR expression. Similarly, the expression of the genes involved in DNA methylation, CMT3, ROS1, and MET1, were not affected by Cs-137 exposure. In addition, TERT expression showed no change in response to Cs-137 irradiation, although a slightly increasing trend was noticed with increasing activity concentrations. For GSH2.1 expression, a clear increasing trend was observed with increasing concentrations. No significant values were reported, but week 1 and week 2 showed a borderline significant effect with p-values of 0.087 and 0.053, respectively. In parallel, an increasing trend was observed for GSH2.2 expression. Here, a significant difference was found for week 1 ($p = 0.02$), week 2 ($p = 0.029$), and week 4 ($p = 0.023$). For both GSH2.1 and GSH2.2, the observed trend is less clear in week 6 compared to the other weeks. Lastly, no clear trend can be

identified for GSH1 expression. The outcome of the relevant Wilcoxon pairwise comparisons from the results mentioned above are presented in figure S4.

Glutathione measurement – The levels of reduced, oxidized, and total glutathione were determined to reflect the effect of ionizing radiation exposure on the redox balance (Fig. 4). Total glutathione content was not significantly affected in the Sr-90 experiment. However, an attenuating, decreasing trend was observed during week 1 and week 2 when comparing exposed and control plant samples. A similar outcome was observed for the level of reduced glutathione. Oxidized glutathione levels remained relatively stable. Therefore, the percentage of oxidized glutathione showed an increasing tendency when the activity concentration raised in week 1 and week 2, but nowhere significant. For the Cs-137 experiment, total glutathione content showed a clear increasing trend. Reduced glutathione levels presented the same steady rise. In addition, oxidized glutathione content followed the same pattern. However, no significant results were noted for these measurements. The percentage of oxidized glutathione was constant during week 1 and week 2 while showing a non-significant increase in week 6 after Cs-137 exposure.

DISCUSSION

This study investigated the effect of chronic Sr-90 and Cs-137 ionizing radiation exposure on phenotypical and molecular parameters in *L. minor*. As mentioned before, these radionuclides were relevant due to their high, long-lasting impact and presence in nuclear accident releases (58-60). In addition, *L. minor* was an appropriate species for investigating chronic exposure consequences because of its asexual generation time of approximately 2.5 days (61). The length of 6 weeks for each experiment allowed us to investigate the effect of ionizing radiation over approximately 17 generations. For the Sr-90 experiment, the lowest activity concentration was 40 Bq/l. This concentration is currently present in Chornobyl and lies above the detection threshold (59). The activity concentration of 400 Bq/l and 4,000 Bq/l each represented a ten-fold increase in exposure. Similarly, the Cs-137 experiment used 40 Bq/l and 400 Bq/l as the lowest activity concentrations. The highest concentration of 4,000 Bq/l was replaced by 40,000 Bq/l. Control plants were only exposed to background radiation levels in both experiments. Taken together, the experiment duration and applied activity concentrations of Sr-90 and Cs-137 have the potential to fill research gaps since few controlled experiments have investigated chronic low-dose ionizing radiation exposure (66).

The mean frond area was calculated for each day of each week and each activity concentration of Cs-137 radiation. Afterwards, the average specific growth rate (ASGR) was calculated according to the OECD guidelines. A comparison of control plants between week 1 and week 6 showed that the ASGR remained constant for weeks. However, taking into account the weeks in between highlighted some deviations in ASGR. Although significant according to statistical analysis, these changes might be attributable to biological variation. In addition, these variations might be caused by minimal changes in environmental parameters, such as temperature and light intensity, as described by Van Dyck et al. (67). As expected, the highest concentration of 40,000 Bq/l caused a significant decrease in ASGR for most weeks, which was already clearly visible in week 1. This is consistent with previous research of the research group that showed similar effects. In contrast, the activity concentrations of 40 Bq/l and 400 Bq/l elevated ASGR, although this was not yet visible in week 1 for 400 Bq/l. Growth of *L. minor* was stimulated by these low-dose exposures compared to the control plants in most weeks. This phenomenon where low levels of a harmful substance can improve the plant's performance is called hormesis (68). Hormesis has already been regularly described in literature in relation to ionizing radiation exposure and plant growth improvement (69). Gudkov et al. summarized several studies that reported beneficial effects on plant growth in response to acute low-dose radiation exposure (70). In addition, Hong et al. showed that chronic low-dose exposure also positively affects growth in *T. aestivum* (71). Collectively, growth can be stimulated by both acute and chronic exposure to low-dose radiation sources. Due to time restrictions, the growth analysis for the Sr-90 experiment could not be included in this paper.

The three main pigments in plants, chlorophyll A, chlorophyll B, and carotenoids, were measured as an indicator of overall plant health and photosynthetic capacity (72). No significant differences were found for the Sr-90 experiment. Besides, the reliability of these results is questioned since the measurements were not performed in 100% dark conditions. For the Cs-137 experiment, the workflow was completely according to the protocol, which makes those results reliable. Similar to the Sr-90 experiment, no significant effect was observed in the Cs-137 experiment comparing exposed and control plants. However, chlorophyll B and carotenoid presence showed a slight increase in week 2. This is not due to the Cs-137 radiation since the control shows the same elevation. Another unknown factor may have led to this deviation. All other small differences, including those for chlorophyll A content, can probably be assigned to biological variation. Other studies found that relatively high concentrations of ionizing gamma radiation significantly decrease pigment concentrations (73, 74). For example, Marcu et al. reported a significant decrease in chlorophyll A, chlorophyll B, and carotenoid levels after gamma radiation exposure (75). For lower concentrations similar to the ones applied in this study, no significant effects have been reported in literature.

No significant differences were observed between all conditions in the Cs-137 experiment. In contrast, low-dose ionizing radiation exposure has been repeatedly described in literature for its beneficial effects on photosynthetic capacity. Grinberg et al. found that photosynthesis was stimulated in tobacco plants after irradiation with a Sr-90 gamma source (76). 2 other studies found an increase in photosynthetic activity in *A. thaliana* when irradiated with a Cs-137 and Americium-241 (Am-241) source (77, 78).

9 genes of interest were included in the gene expression analysis. 5 of these genes are active on different levels of epigenetic regulation. For example, SRT1 codes for one of the sirtuin proteins that change the structure of chromatin. Together with SRT2, this gene is the most important regulator of chromatin remodeling within the sirtuin family (35). It was aimed to include SRT2 in the gene expression analysis along with SRT1 to get a more complete understanding of the role of sirtuins in response to ionizing radiation in *L. minor*. However, primers could not be developed successfully for this gene. Besides the SRT1 gene, 3 genes involved in DNA methylation were included. MET1 and CMT3 are both responsible for methylation maintenance, while ROS1 demethylates DNA (20, 24). This combination allowed to observe disturbances in the methylation balance by including both sides of the methylation spectrum. In addition to these genes, DRM2 was also targeted since its function is de novo methylation (19). However, similar to SRT2, no functional primers were found for DRM2 in *L. minor*. The next gene responsible for epigenetic regulation that was investigated in relationship to ionizing radiation exposure is TERT. As the catalytic subunit of telomerase, the protein that is derived from this gene plays a major role in its activity and functionality (37). Therefore, its expression can represent the ability of telomerase to perform epigenetic regulation of gene expression through the telomere positioning effect (36). The primer efficiency of TERT was 126.06%, which is higher than the predetermined range. However, it was decided to utilize these primers since the other parameters were acceptable and TERT was expected to be expressed in low quantities (79). The last genes that were included in the gene expression analysis all play an important role in maintaining the redox balance. GSH1 catalyzes the following reaction: cysteine + glutamate → γ-glutamyl cysteine (Fig. S5). This reaction is highly dependent on cysteine availability and additionally expression of GSH1. Therefore, this step is considered the rate-limiting step in glutathione synthesis (80, 81). GSH2 expression is also regulated strictly and upregulation can further enhance glutathione synthesis through the following reaction: γ-glutamyl cysteine + glycine → reduced glutathione. Both the GSH1 and GSH2 catalyzed reactions require ATP as substrate (80). Reduced glutathione is used to neutralize radicals that cause oxidative stress and disturb the redox balance (82, 83). Afterwards, GR can reduce the oxidized glutathione again using NADPH (84). The combination of these genes was included in the gene expression analysis to provide a clear overview of the effect of ionizing radiation on the glutathione metabolism in plants. Both variants of GSH2 that are found in *A. thaliana* were included.

GSH2.1 synthesizes glutathione primarily in chloroplasts, while GSH2.2 performs the synthesis in the cytosol (85, 86).

The results of the gene expression analysis showed several clear trends and significant differences between exposed and control plants. Sr-90 exposure caused no notable effect on SRT1 expression, while a clear increasing trend was visible with increasing activity concentrations of Cs-137 exposure. This trend was even significant in week 4, indicating that Cs-137 ionizing radiation exposure stimulates the expression of SRT1, leading to changes in chromatin structure. Members of the plant sirtuin protein family have not yet been described in literature in relationship with ionizing radiation, making these results the first of its kind. TERT expression showed no consistent increase or decrease in expression after Sr-90 exposure, although a significant effect was reported in week 3. Similarly for the Cs-137 experiment, no statistical effects were observed, but a minimal, consistent incline was noticed. Taken together, TERT expression seems not affected by ionizing radiation. No literature was found about the effects of environmental stress on telomere length in plants. Sr-90 and Cs-137 exposure had different outcomes on the expression of genes involved in DNA methylation. No effect was found on MET1, CMT3, and ROS1 expression after exposure to Cs-137 radiation. In contrast, MET1 and CMT3 expression demonstrated a decreasing trend with higher activity concentrations after Sr-90 exposure. For both genes, this effect was significant in week 2 and week 3. However, this trend became less clear towards the end of the experiment, which might be the result of adaptation from *L. minor* to radiation exposure. ROS1 expression was not affected by Sr-90 exposure and showed differences that are most likely attributable to biological variation, comparable with Cs-137. One other study measured CMT3 and ROS1 expression levels while investigating the effect of gamma radiation on DNA methylation in plants. It observed an increase in ROS1 expression and a decrease in CMT3 expression through applying doses of 5-200 Gray (87). In addition, Naydenov et al. and Yang et al. both reported that ROS1 is upregulated in response to several abiotic stresses (88, 89). The genes involved in maintaining the redox balance also showed different responses depending on the ionizing radiation source. Sr-90 exposure caused no increasing or decreasing trend in GSH2.1 and GSH2.2 expression. For the Cs-137 experiment, both GSH2.1 and GSH2.2 showed an increasing trend in expression with increasing activity concentrations, of which some differences were significant and some additional ones borderline significant. GSH1 expression was lower in plants that were exposed to Sr-90 compared with control plants, while its expression remained constant after Cs-137 exposure. Finally, GR expression showed a clear decreasing trend with some significant differences during the Sr-90 experiment, while its expression was not altered by Cs-137 exposure. One other study exposed *L. minor* to a Sr-90 and Cs-137 source and executed an RNA sequencing analysis, including GSH2.1, GSH2.2, GSH1, and GR (4). These authors found no significant change in expression for those genes while applying higher doses of ionizing radiation. However, Cs-137 was applied externally in contrast with the internal contamination in the medium from this study.

There is a clear difference between the expression of genes involved in DNA methylation and genes involved in maintaining the redox balance when comparing Sr-90 and Cs-137 as exposure. Analysis of the glutathione metabolism pathway may provide a plausible explanation for this observation. As mentioned, cysteine is needed in the synthesis process of glutathione. Its availability determines the amount of glutathione that is produced. Cysteine is synthesized from homocysteine, a molecule that is part of the methionine cycle together with S-Adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) (Fig. S6) (90). However, SAM also acts as an important methyl donor for DNA methylation genes, such as MET1 (91). Under normal conditions, SAM can provide methyl groups and function as a precursor for cysteine. When plants are exposed to abiotic stress, the availability of SAM is hampered. In this study, it is suggested that Sr-90 exposure has shifted the SAM balance towards DNA methylation, while it was mainly used for the synthesis of glutathione during the Cs-137 experiment.

Reduced, oxidized and total glutathione levels were measured to analyze the effect of

ionizing radiation on the redox balance. Afterwards, the percentage of oxidized glutathione was calculated. No significant differences were found between exposed and non-exposed plant samples for any of the measured parameters. This is attributable to the high variation caused by the low amount of replicates (n = 4). The results seemed to differ between Sr-90 and Cs-137 exposure. Glutathione measurements showed no differences for Sr-90, while they reported a steady rise for the Cs-137 experiment. In addition, oxidized glutathione content was elevated in the Cs-137 experiment, while its levels remained consistent during the Sr-90 experiment. Finally, the percentage of oxidized glutathione was higher during week 1 and week 2 for the Sr-90 experiment, while this effect was only observed during week 6 in the Cs-137 experiment. Increases in the GSH:GSSG ratio have already been observed in previous studies as a response to environmental stressors (92-95). In addition, increased glutathione accumulation in response to Cs-137 stress has been reported before (86). Moreover, Adams et al. showed that an increase in glutathione concentrations alleviates stress induced by ionizing radiation from a cesium source in *A. thaliana* (96). An elevated glutathione content caused by Cs-137 was also observed in *L. minor* in the study from Van Hoeck et al. (97). Taken together, glutathione concentrations in plants are frequently affected by ionizing radiation stress.

The results of the glutathione measurement support the findings of the gene expression analysis. In the Sr-90 experiment, the biggest effect was noticed for the expression levels of genes involved in DNA methylation, while the genes involved in the glutathione metabolism were not affected. The measured glutathione levels complemented these data by remaining relatively constant or showing a minor decreasing trend. The Cs-137 experiment demonstrated the highest differences in gene expression for the genes involved in glutathione metabolism. This matches with the increasing trend that was found for the levels of total, reduced, and oxidized glutathione.

CONCLUSION

Ionizing radiation is known for its damaging effects on plants. In this study, it was investigated how chronic, multigenerational exposure to a Sr-90 and Cs-137 source affected epigenetic regulatory mechanisms by analyzing expression levels of relevant genes. In addition, these findings were coupled with measurements of glutathione levels that reflected the redox status. Phenotypic trait analysis showed clear consequences of exposure to Cs-137 for 6 weeks. Moreover, this study showed beneficial growth effects after low-dose exposure, which supports the hypothesis of hormesis. Sr-90 exposure mainly affected the expression of genes involved in DNA methylation, while Cs-137 exposure mostly altered the expression of genes that are essential in glutathione synthesis. Comparable with these results, glutathione levels remained relatively stable after Sr-90 exposure, while a clear increasing trend in glutathione content was observed after Cs-137 exposure. Taken together, these results suggest that defense mechanisms against chronic, multigenerational ionizing radiation exposure focus on 2 key modes of action, depending on the exposure source. On the one hand, the upregulation of epigenetic mechanisms, such as DNA methylation. On the other hand, the increase of the antioxidant capacity through glutathione synthesis to fight oxidative stress. This supports the hypothesis that SAM plays a role in the response to chronic ionizing radiation stress over numerous generations. The importance of SAM as a regulating and deciding molecule still needs to be further investigated. Besides these key mechanisms, multiple other ways of coping with radiation stress are probably active. Further research is needed to complete the overview of processes that are activated in plants in response to ionizing radiation exposure.

REFERENCES

1. Sanlorenzo M, Wehner MR, Linos E, Kornak J, Kainz W, Posch C, et al. The risk of melanoma in airline pilots and cabin crew: a meta-analysis. JAMA Dermatol. 2015;151(1):51-8.
2. Shahbazi-Gahrouei D, Gholami M, Setayandeh S. A review on natural background radiation. Adv Biomed Res. 2013;2:65.
3. Horemans N, Nauts R, Vives IBJ, Van Hees M, Jacobs G, Voorspoels S, et al. Genome-wide DNA methylation changes in two Brassicaceae species sampled alongside a radiation gradient in Chernobyl and Fukushima. J Environ Radioact. 2018;192:405-16.
4. Van Hoeck A, Horemans N, Nauts R, Van Hees M, Vandenhove H, Blust R. Lemna minor plants chronically exposed to ionising radiation: RNA-seq analysis indicates a dose rate dependent shift from acclimation to survival strategies. Plant Sci. 2017;257:84-95.
5. Alsharef S, Alanazi M, Alharthi F, Qandil D, Qushawy M. REVIEW ABOUT RADIOPHARMACEUTICALS: PREPARATION, RADIOACTIVITY, AND APPLICATIONS. International Journal of Applied Pharmaceutics. 2020;12:8-15.
6. Peng Y, Allen S, Millwood RJ, Stewart CN, Jr. 'Fukusensor:' a genetically engineered plant for reporting DNA damage in response to gamma radiation. Plant Biotechnol J. 2014;12(9):1329-32.
7. Alizadeh E, Sanz AG, García G, Sanche L. Radiation Damage to DNA: The Indirect Effect of Low Energy Electrons. J Phys Chem Lett. 2013;4(5):820-5.
8. Kerchev PI, Van Breusegem F. Improving oxidative stress resilience in plants. Plant J. 2022;109(2):359-72.
9. Dutilleul M, Bonzom J-M, Lecomte C, Goussen B, Daian F, Galas S, et al. Rapid evolutionary responses of life history traits to different experimentally-induced pollutions in Caenorhabditis elegans. BMC Evolutionary Biology. 2014;14(1):252.
10. Laanen P, Cuypers A, Saenen E, Horemans N. Flowering under enhanced ionising radiation conditions and its regulation through epigenetic mechanisms. Plant Physiol Biochem. 2023;196:246-59.
11. Bossdorf O, Richards CL, Pigliucci M. Epigenetics for ecologists. Ecology Letters. 2008;11(2):106-15.
12. Grativol C, Hemerly AS, Ferreira PCG. Genetic and epigenetic regulation of stress responses in natural plant populations. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 2012;1819(2):176-85.
13. Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, et al. Herbivory in the Previous Generation Primes Plants for Enhanced Insect Resistance Plant Physiology. 2012;158(2):854-63.
14. Whittle CA, Otto SP, Johnston MO, Krochko JE. Adaptive epigenetic memory of ancestral temperature regime in Arabidopsis thalianaThis paper is one of a selection of papers published in a Special Issue from the National Research Council of Canada – Plant Biotechnology Institute. Botany. 2009;87(6):650-7.
15. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nature Reviews Genetics. 2016;17(8):487-500.
16. Jiménez-Ramírez IA, Pijeira-Fernández G, Moreno-Cálix DM, De-la-Peña C. Same modification, different location: the mythical role of N(6)-adenine methylation in plant genomes. Planta. 2022;256(1):9.
17. Cingolani P, Cao X, Khetani RS, Chen C-C, Coon M, Sammak Aa, et al. Intronic Non-CG DNA hydroxymethylation and alternative mRNA splicing in honey bees. BMC Genomics. 2013;14(1):666.

18. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nature Reviews Genetics. 2012;13(2):97-109.
19. Chan SWL, Henderson IR, Jacobsen SE. Gardening the genome: DNA methylation in Arabidopsis thaliana. Nature Reviews Genetics. 2005;6(5):351-60.
20. Law JA, Jacobsen SE. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nature Reviews Genetics. 2010;11(3):204-20.
21. Erdmann RM, Picard CL. RNA-directed DNA Methylation. PLoS Genet. 2020;16(10):e1009034.
22. Coulter JB, O'Driscoll CM, Bressler JP. Hydroquinone Increases 5-Hydroxymethylcytosine Formation through Ten Eleven Translocation 1 (TET1) 5-Methylcytosine Dioxygenase*. Journal of Biological Chemistry. 2013;288(40):28792-800.
23. Scourzic L, Mouly E, Bernard OA. TET proteins and the control of cytosine demethylation in cancer. Genome Medicine. 2015;7(1):9.
24. Halter T, Wang J, Amesefe D, Lastrucci E, Charvin M, Singla Rastogi M, et al. The Arabidopsis active demethylase ROS1 cis-regulates defence genes by erasing DNA methylation at promoter-regulatory regions. eLife. 2021;10:e62994.
25. Garima S, Pradeep S. Epigenetic Modifications in Plants under Abiotic Stress. In: Rosaria M, editor. Epigenetics. Rijeka: IntechOpen; 2019. p. Ch. 2.
26. Dhar GA, Saha S, Mitra P, Nag Chaudhuri R. DNA methylation and regulation of gene expression: Guardian of our health. Nucleus (Calcutta). 2021;64(3):259-70.
27. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. Genes Dev. 2009;23(7):781-3.
28. He G, Elling AA, Deng XW. The epigenome and plant development. Annu Rev Plant Biol. 2011;62:411-35.
29. Roudier F, Ahmed I, Bérard C, Sarazin A, Mary-Huard T, Cortijo S, et al. Integrative epigenomic mapping defines four main chromatin states in Arabidopsis. Embo j. 2011;30(10):1928-38.
30. Castel SE, Martienssen RA. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nature Reviews Genetics. 2013;14(2):100-12.
31. Jivan R. Epigenetic Regulation of Gene Expression2020.
32. Brant EJ, Budak H. Plant Small Non-coding RNAs and Their Roles in Biotic Stresses. Frontiers in Plant Science. 2018;9.
33. Wang HV, Chekanova JA. Long Noncoding RNAs in Plants. Adv Exp Med Biol. 2017;1008:133-54.
34. Soccio M, Laus MN, Alfarano M, Pastore D. Measuring Activity of Native Plant Sirtuins - The Wheat Mitochondrial Model. Front Plant Sci. 2018;9:961.
35. Zheng W. Review: The plant sirtuins. Plant Sci. 2020;293:110434.
36. Fojtová M, Fajkus J. Epigenetic regulation of telomere maintenance. Cytogenet Genome Res. 2014;143(1-3):125-35.
37. Procházková Schrumpfová P, Fojtová M, Fajkus J. Telomeres in Plants and Humans: Not So Different, Not So Similar. Cells. 2019;8(1).
38. Miryeganeh M. Plants' Epigenetic Mechanisms and Abiotic Stress. Genes (Basel). 2021;12(8).
39. Hu Y, Zhang L, Zhao L, Li J, He S, Zhou K, et al. Trichostatin A selectively suppresses the cold-induced transcription of the ZmDREB1 gene in maize. PLoS One. 2011;6(7):e22132.

40. Carter B, Bishop B, Ho KK, Huang R, Jia W, Zhang H, et al. The Chromatin Remodelers PKL and PIE1 Act in an Epigenetic Pathway That Determines H3K27me3 Homeostasis in Arabidopsis. Plant Cell. 2018;30(6):1337-52.
41. Park J, Lim CJ, Shen M, Park HJ, Cha JY, Iniesto E, et al. Epigenetic switch from repressive to permissive chromatin in response to cold stress. Proc Natl Acad Sci U S A. 2018;115(23):E5400-e9.
42. Liu J, Feng L, Li J, He Z. Genetic and epigenetic control of plant heat responses. Front Plant Sci. 2015;6:267.
43. Naydenov M, Baev V, Apostolova E, Gospodinova N, Sablok G, Gozmanova M, et al. High-temperature effect on genes engaged in DNA methylation and affected by DNA methylation in Arabidopsis. Plant Physiol Biochem. 2015;87:102-8.
44. Folsom JJ, Begcy K, Hao X, Wang D, Walia H. Rice fertilization-Independent Endosperm1 regulates seed size under heat stress by controlling early endosperm development. Plant Physiol. 2014;165(1):238-48.
45. Kim JM, Sasaki T, Ueda M, Sako K, Seki M. Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. Front Plant Sci. 2015;6:114.
46. Chen L, Ren Y, Zhang Y, Xu J, Sun F, Zhang Z, et al. Genome-wide identification and expression analysis of heat-responsive and novel microRNAs in Populus tomentosa. Gene. 2012;504(2):160-5.
47. Li B, Qin Y, Duan H, Yin W, Xia X. Genome-wide characterization of new and drought stress responsive microRNAs in Populus euphratica. J Exp Bot. 2011;62(11):3765-79.
48. Suji KK, Joel AJ. An epigenetic change in rice cultivars under water stress conditions. Electronic Journal of Plant Breeding. 2010;1:1142-3.
49. Kim JM, To TK, Ishida J, Morosawa T, Kawashima M, Matsui A, et al. Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in Arabidopsis thaliana. Plant Cell Physiol. 2008;49(10):1580-8.
50. Chen LT, Luo M, Wang YY, Wu K. Involvement of Arabidopsis histone deacetylase HDA6 in ABA and salt stress response. J Exp Bot. 2010;61(12):3345-53.
51. Pandey G, Sharma N, Sahu PP, Prasad M. Chromatin-Based Epigenetic Regulation of Plant Abiotic Stress Response. Curr Genomics. 2016;17(6):490-8.
52. Ferreira LJ, Azevedo V, Maroco J, Oliveira MM, Santos AP. Salt Tolerant and Sensitive Rice Varieties Display Differential Methylome Flexibility under Salt Stress. PLoS One. 2015;10(5):e0124060.
53. Alonso C, Ramos-Cruz D, Becker C. The role of plant epigenetics in biotic interactions. New Phytologist. 2019;221(2):731-7.
54. Horemans N, Spurgeon DJ, Lecomte-Pradines C, Saenen E, Bradshaw C, Oughton D, et al. Current evidence for a role of epigenetic mechanisms in response to ionizing radiation in an ecotoxicological context. Environ Pollut. 2019;251:469-83.
55. Volkova PY, Geras'kin SA, Horemans N, Makarenko ES, Saenen E, Duarte GT, et al. Chronic radiation exposure as an ecological factor: Hypermethylation and genetic differentiation in irradiated Scots pine populations. Environmental Pollution. 2018;232:105-12.
56. Saenen E, Lecomte C, Bradshaw C. DELIVERABLE (D-4.3) COMET Initial Research Activity on Transgenerational Effects and Role of Epigenetics: Results and Impact. COMET European Program. Contract Number: Fission-2012-34. 2017:1-604794.
57. Kovalchuk I, Abramov V, Pogribny I, Kovalchuk O. Molecular Aspects of Plant Adaptation to Life in the Chernobyl Zone. Plant Physiology. 2004;135(1):357-63.

58. Exeter Uo. Crops near Chernobyl still contaminated. ScienceDaily. 2020.
59. Labunska I, Levchuk S, Kashparov V, Holiaka D, Yoschenko L, Santillo D, et al. Current radiological situation in areas of Ukraine contaminated by the Chornobyl accident: Part 2. Strontium-90 transfer to culinary grains and forest woods from soils of Ivankiv district. Environment International. 2021;146:106282.
60. Sulaiman S, Mohamed F, Ab Rahim AN. Radioactive release during nuclear accidents in Chernobyl and Fukushima. IOP Conference Series: Materials Science and Engineering. 2018;298:012011.
61. Ziegler P, Adelmann K, Zimmer S, Schmidt C, Appenroth KJ. Relative in vitro growth rates of duckweeds (Lemnaceae) - the most rapidly growing higher plants. Plant Biol (Stuttg). 2015;17 Suppl 1:33-41.
62. Hoeck A, Horemans N, Monsieurs P, Cao H, Blust R. The first draft genome of the aquatic model plant Lemna minor opens the route for future stress physiology research and biotechnological applications. Biotechnology for Biofuels. 2015;8:1-13.
63. Aliferis KA, Materzok S, Paziotou GN, Chrysayi-Tokousbalides M. Lemna minor L. as a model organism for ecotoxicological studies performing 1H NMR fingerprinting. Chemosphere. 2009;76(7):967-73.
64. Lee H, Depuydt S, Shin K, Choi S, Kim G, Lee YH, et al. Assessment of Various Toxicity Endpoints in Duckweed (Lemna minor) at the Physiological, Biochemical, and Molecular Levels as a Measure of Diuron Stress. Biology (Basel). 2021;10(7).
65. Van Hoeck A, Horemans N, Monsieurs P, Cao HX, Vandenhove H, Blust R. The first draft genome of the aquatic model plant Lemna minor opens the route for future stress physiology research and biotechnological applications. Biotechnol Biofuels. 2015;8:188.
66. Caplin N, Willey N. Ionizing Radiation, Higher Plants, and Radioprotection: From Acute High Doses to Chronic Low Doses. Front Plant Sci. 2018;9:847.
67. Van Dyck I, Vanhoudt N, Vives i Batlle J, Horemans N, Nauts R, Van Gompel A, et al. Effects of environmental parameters on Lemna minor growth: An integrated experimental and modelling approach. Journal of Environmental Management. 2021;300:113705.
68. Sakai K. [Biological responses to low dose radiation--hormesis and adaptive responses]. Yakugaku Zasshi. 2006;126(10):827-31.
69. Volkova PY, Bondarenko EV, Kazakova EA. Radiation hormesis in plants. Current Opinion in Toxicology. 2022;30:100334.
70. Gudkov SV, Grinberg MA, Sukhov V, Vodeneev V. Effect of ionizing radiation on physiological and molecular processes in plants. Journal of Environmental Radioactivity. 2019;202:8-24.
71. Hong MJ, Yoon YH, Kim DS, Kim SH, Kang SY, Kim D-Y, et al. Phenotypic and molecular responses of wheat (Triticum aestivum L.) to chronic gamma irradiation. Journal of Agricultural Science and Technology. 2018;20:167-78.
72. Li Y, He N, Hou J, Xu L, Liu C, Zhang J, et al. Factors Influencing Leaf Chlorophyll Content in Natural Forests at the Biome Scale. Frontiers in Ecology and Evolution. 2018;6.
73. Dale MFB, Griffiths DW, Bain H, Goodman BA. The effect of gamma irradiation on glycoalkaloid and chlorophyll synthesis in seven potato cultivars. Journal of the Science of Food and Agriculture. 1997;75(2):141-7.
74. Pick A, Ling A, Jing Yi C, Hussein S, Harun AR. Physiological Responses of Citrus sinensis to Gamma Irradiation. World Appl Sci J. 2008;5.

75. Marcu D, Damian G, Cosma C, Cristea V. Gamma radiation effects on seed germination, growth and pigment content, and ESR study of induced free radicals in maize (Zea mays). J Biol Phys. 2013;39(4):625-34.
76. Grinberg M, Nemtsova Y, Ageyeva M, Brilkina A, Vodeneev V. Effect of low-dose ionizing radiation on spatiotemporal parameters of functional responses induced by electrical signals in tobacco plants. Photosynth Res. 2023.
77. Biermans G, Horemans N, Vanhoudt N, Vandenhove H, Saenen E, Van Hees M, et al. Biological effects of α-radiation exposure by (241)Am in Arabidopsis thaliana seedlings are determined both by dose rate and (241)Am distribution. J Environ Radioact. 2015;149:51-63.
78. Vanhoudt N, Horemans N, Wannijn J, Nauts R, Van Hees M, Vandenhove H. Primary stress responses in Arabidopsis thaliana exposed to gamma radiation. J Environ Radioact. 2014;129:1-6.
79. Fajkus P, Peška V, Fajkus J, Sýkorová E. Origin and Fates of TERT Gene Copies in Polyploid Plants. Int J Mol Sci. 2021;22(4).
80. Lu SC. Regulation of glutathione synthesis. Mol Aspects Med. 2009;30(1-2):42-59.
81. Hothorn M, Wachter A, Gromes R, Stuwe T, Rausch T, Scheffzek K. Structural basis for the redox control of plant glutamate cysteine ligase. J Biol Chem. 2006;281(37):27557-65.
82. Garcia-Ruiz C, Fernandez-Checa JC. Mitochondrial glutathione: hepatocellular survival-death switch. J Gastroenterol Hepatol. 2006;21 Suppl 3:S3-6.
83. Meister A, Anderson ME. Glutathione. Annu Rev Biochem. 1983;52:711-60.
84. Tanwir K, Amna, Javed MT, Shahid M, Akram MS, Ali Q. Chapter 32 - Antioxidant defense systems in bioremediation of organic pollutants. In: Hasanuzzaman M, Prasad MNV, editors. Handbook of Bioremediation: Academic Press; 2021. p. 505-21.
85. Rawlins MR, Leaver CJ, May MJ. Characterisation of an Arabidopsis thaliana cDNA encoding glutathione synthetase. FEBS Lett. 1995;376(1-2):81-6.
86. Locus: AT5G27380 2022 [Available from: https://www.arabidopsis.org/servlets/TairObject?id=130902&type=locus.
87. Kim JE, Lee MH, Cho EJ, Kim JH, Chung BY, Kim JH. Characterization of non-CG genomic hypomethylation associated with gamma-ray-induced suppression of CMT3 transcription in Arabidopsis thaliana. Radiat Res. 2013;180(6):638-48.
88. Naydenov M, Baev V, Apostolova E, Gospodinova N, Sablok G, Gozmanova M, et al. High-temperature effect on genes engaged in DNA methylation and affected by DNA methylation in Arabidopsis. Plant Physiology and Biochemistry. 2015;87:102-8.
89. Yang L, Lang C, Wu Y, Meng D, Yang T, Li D, et al. ROS1-mediated decrease in DNA methylation and increase in expression of defense genes and stress response genes in Arabidopsis thaliana due to abiotic stresses. BMC Plant Biol. 2022;22(1):104.
90. Sanderson SM, Gao X, Dai Z, Locasale JW. Methionine metabolism in health and cancer: a nexus of diet and precision medicine. Nature Reviews Cancer. 2019;19(11):625-37.
91. Detich N, Hamm S, Just G, Knox JD, Szyf M. The methyl donor S-Adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-Adenosylmethionine. J Biol Chem. 2003;278(23):20812-20.
92. El-Shabrawi H, Kumar B, Kaul T, Reddy MK, Singla-Pareek SL, Sopory SK. Redox homeostasis, antioxidant defense, and methylglyoxal detoxification as markers for salt tolerance in Pokkali rice. Protoplasma. 2010;245(1-4):85-96.

93. Hasanuzzaman M, Fujita M. Selenium pretreatment upregulates the antioxidant defense and methylglyoxal detoxification system and confers enhanced tolerance to drought stress in rapeseed seedlings. Biol Trace Elem Res. 2011;143(3):1758-76.
94. Hasanuzzaman M, Nahar K, Anee TI, Fujita M. Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. Physiol Mol Biol Plants. 2017;23(2):249-68.
95. Kocsy G, Szalai G, Galiba G. Induction of glutathione synthesis and glutathione reductase activity by abiotic stresses in maize and wheat. ScientificWorldJournal. 2002;2:1699-705.
96. Adams E, Miyazaki T, Watanabe S, Ohkama-Ohtsu N, Seo M, Shin R. Glutathione and Its Biosynthetic Intermediates Alleviate Cesium Stress in Arabidopsis. Front Plant Sci. 2019;10:1711.
97. Van Hoeck A, Horemans N, Van Hees M, Nauts R, Knapen D, Vandenhove H, et al. Characterizing dose response relationships: Chronic gamma radiation in Lemna minor induces oxidative stress and altered polyploidy level. Journal of Environmental Radioactivity. 2015;150:195-202.

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Author contributions – NH and LB conceived and designed the research. YR, LB, MvH, RN and JW performed experiments in the lab. Data analysis was executed by YR, LB and BDR. YR wrote the paper with assistance of NH and LB. All authors carefully edited the manuscript.

SUPPLEMENTARY MATERIAL

Method S1: Primer design

The qPCR primers for the selected genes of interest were developed using the following procedure: The protein structure of each gene of interest was identified in The Arabidopsis Information Resource (TAIR), a public database for *Arabidopsis thaliana.* Afterwards, this protein structure was blasted against a *L. minor* protein database that was previously constructed by the research group. This blast was performed with the National Center for Biotechnology Information (NCBI) Genome Workbench software. The best match in the *L. minor* database was identified and linked to the corresponding gene sequence. This sequence was then used together with the *L. minor* database to develop primer pairs with the NCBI Primer-BLAST tool. Before blasting, primer parameters were adjusted for the qPCR design: Melting temperature was set at 56°C - 64°C for optimal specificity, product length was adjusted to 80 – 160 base pairs. Finally, the generated primer pairs were judged on their characteristics. Primer length should be between 18 and 24 base pairs, guanine/cytosine (G/C) content should be between 40% and 60%, a 3' G/C clamp of 1-3 base pairs was preferred, and primer location should be as close to the 3' end as possible. The 2 best primer pairs were selected and tested with a 1/3 serial dilution of stocked cDNA. The resulting Ct-values were divided by the logarithm of the concentration. The slope of the associated trendline was then used to calculate the primer efficiency using the following formula: $\left(10^{-\frac{1}{SLOPE}}\right)$-1. Primers with values between 80% and 120% were considered efficient.

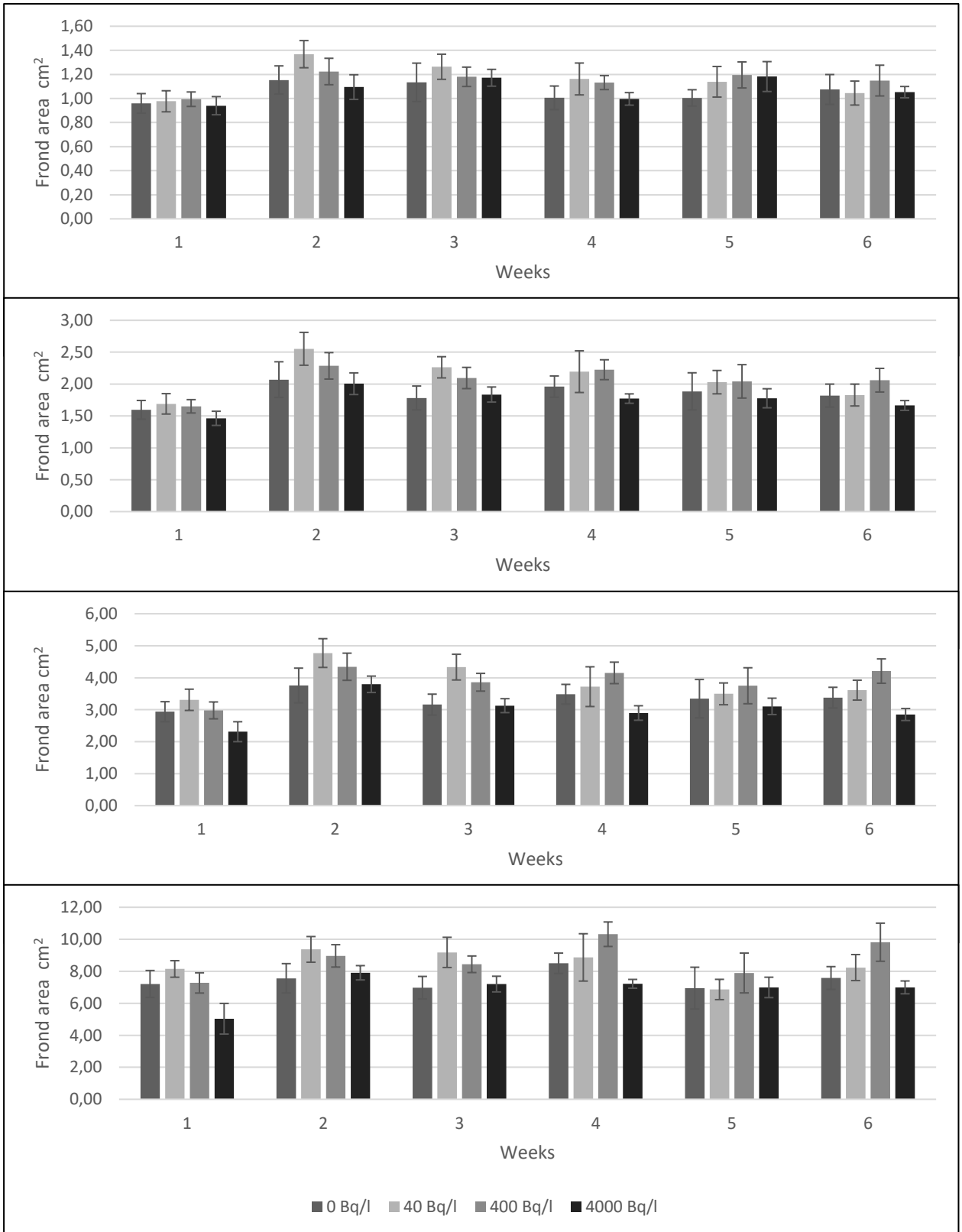

Figure S1: Frond area of *L. minor* on day 0, 2, 4, and 7 for all 6 weeks during chronic Cs-137 exposure. Data are presented as mean ±SD (n=15). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Cs-137 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 40,000 Bq/l. (A) Day 0; (B) Day 2; (C) Day 4; (D) Day 7. *Cm = centimeter, Bq = Becquerel, l = liter*

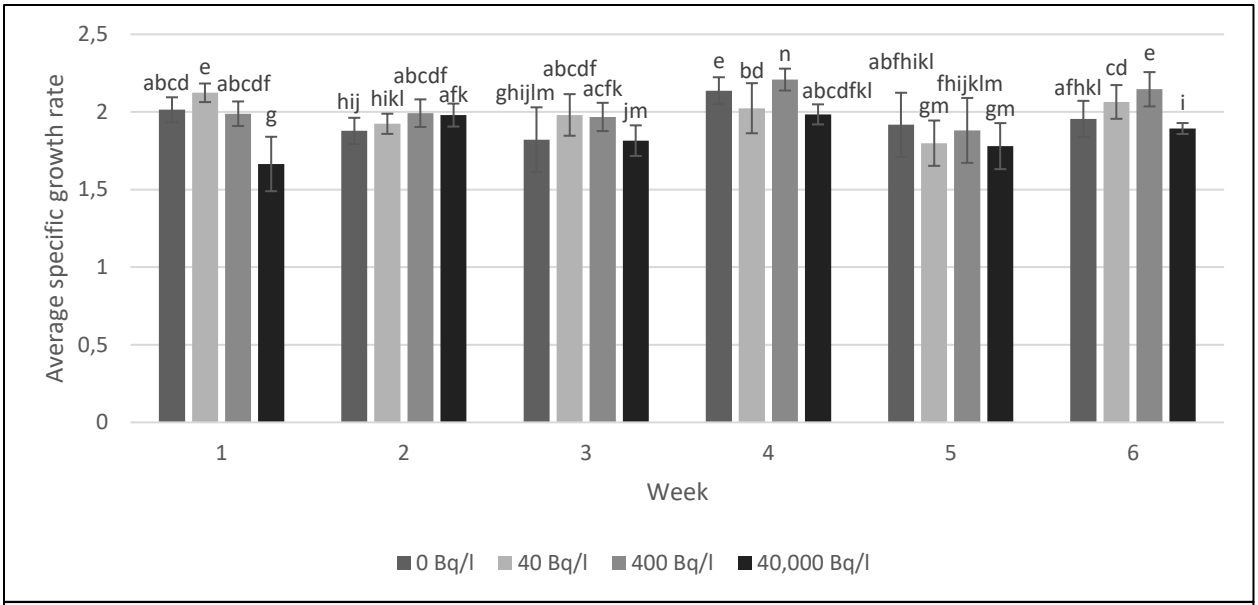

Figure S2: Comparison of the *L. minor* ASGR for all 6 weeks after Cs-137 exposure. Data are presented as mean ±SD (n=15). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Cs-137 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 40,000 Bq/l. Different letters indicate groups that are significantly different (p-value < 0.05; Kruskal-Wallis test). *Bq = Becquerel, l = liter*

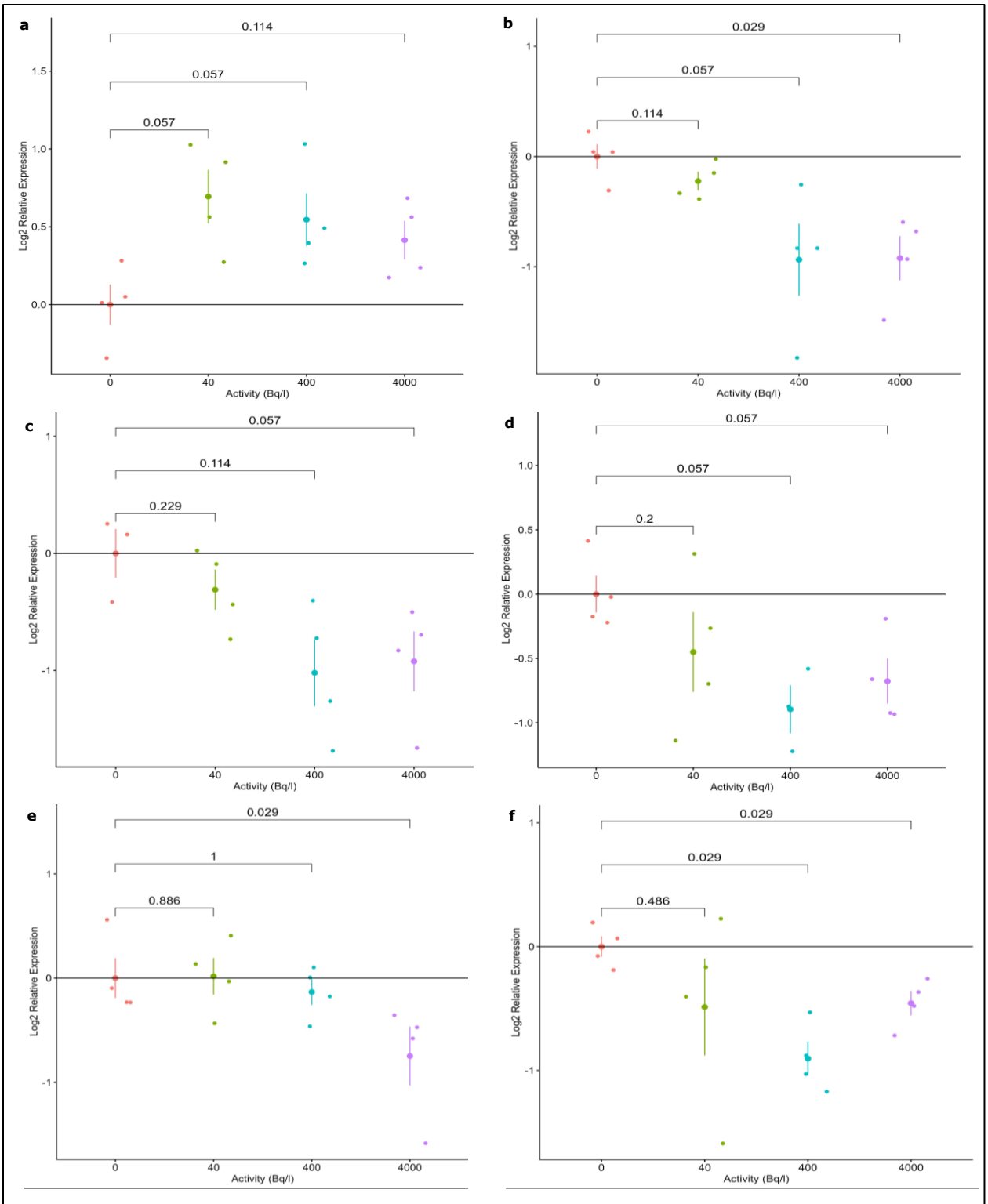

Figure S3 A-F: Comparison of the effect from the different concentrations of chronic Sr-90 exposure on changes in gene expression. Data are presented as mean ±SE (n=4). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Sr-90 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 4,000 Bq/l. (A) SRT1, week 1; (B) GR, week 1;(C) GR, week 2; (D) GR, week 3; (E) GR, week 4; (F) CMT3, week 2. P-values are shown above the brackets and considered significant if smaller than 0.05 (Wilcoxon pairwise comparison) *Bq = Becquerel, l = liter*

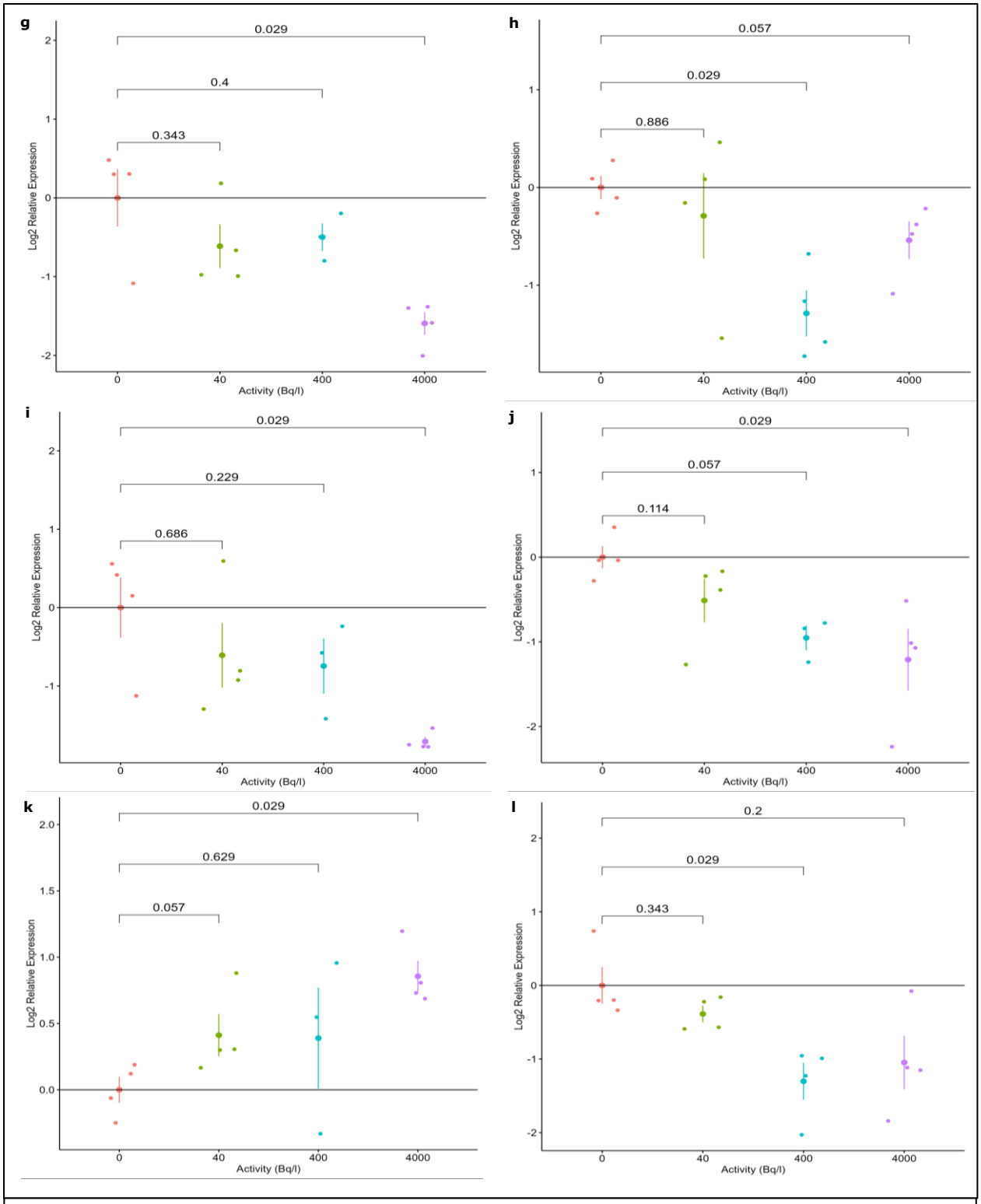

Figure S3 G-L: Comparison of the effect from the different concentrations of chronic Sr-90 exposure on changes in gene expression. Data are presented as mean ±SE (n=4). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Sr-90 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 4,000 Bq/l. (G) CMT3, week 3; (H) MET1, week 2; (I) MET1, week 3; (J) TERT, week 3; (K) GSH2.2, week 3; (L) GSH1, week 2. P-values are shown above the brackets and considered significant if smaller than 0.05 (Wilcoxon pairwise comparison) *Bq = Becquerel, l = liter*

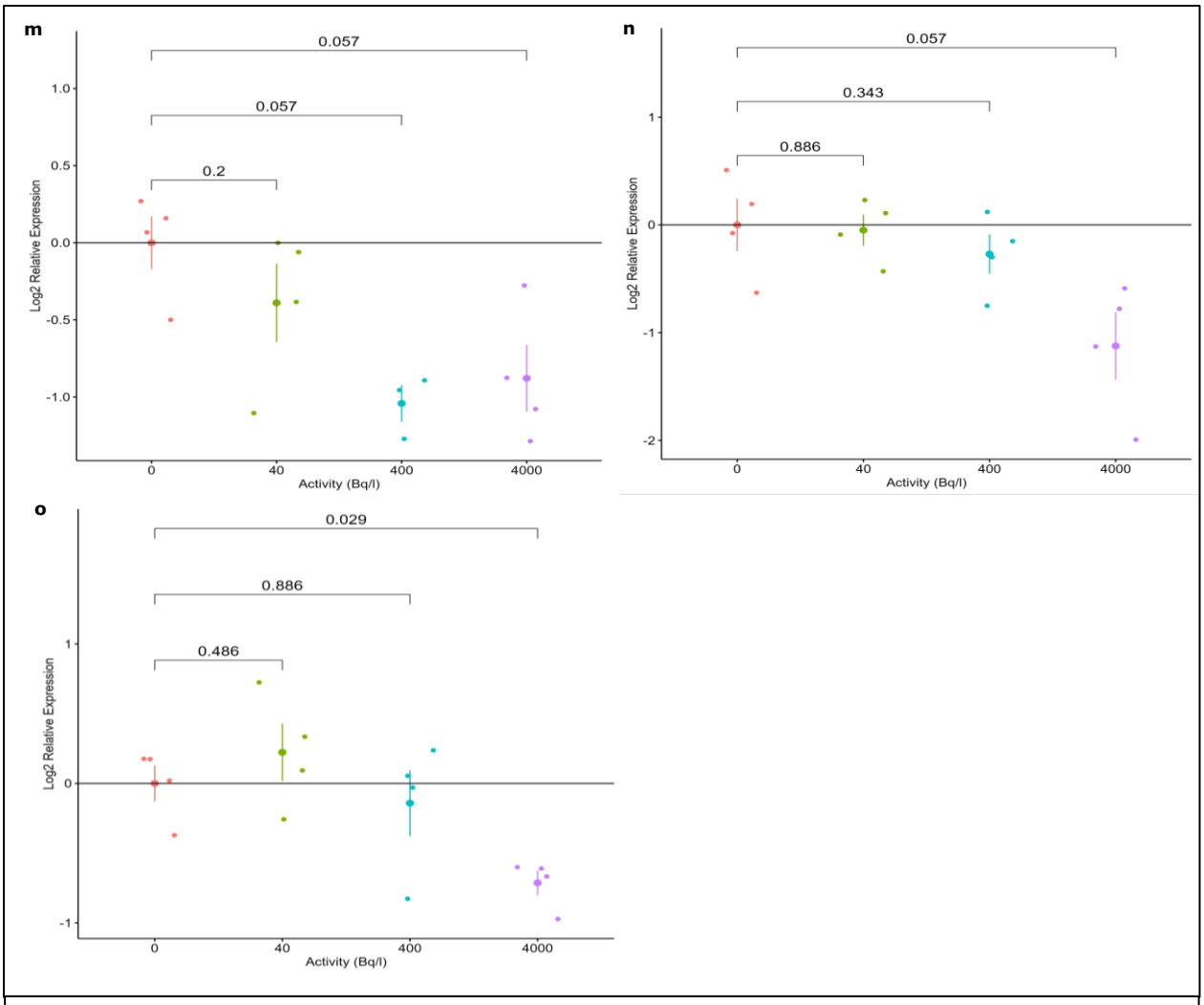

Figure S3 M-O: Comparison of the effect from the different concentrations of chronic Sr-90 exposure on changes in gene expression. Data are presented as mean ±SE (n=4). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Sr-90 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 4,000 Bq/l. (M) GSH1, week 3; (N) GSH1, week 5; (O) GSH1, week 6. P-values are shown above the brackets and considered significant if smaller than 0.05 (Wilcoxon pairwise comparison) *Bq = Becquerel, l = liter*

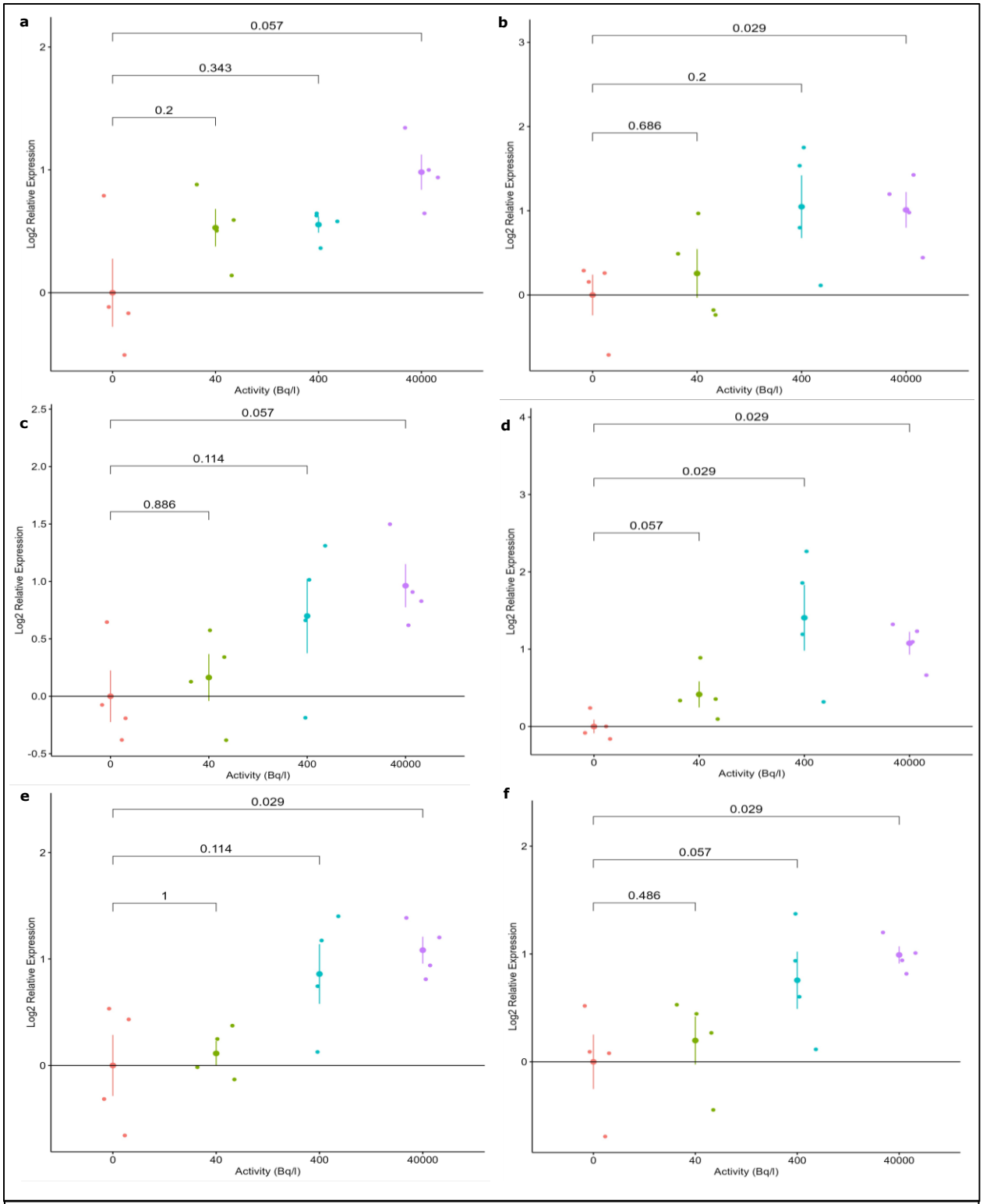

Figure S4: Comparison of the effect from the different concentrations of chronic Cs-137 exposure on changes in gene expression. Data are presented as mean ±SE (n=4). Plants were cultured in growth medium and exposed to different activity concentrations of ionizing radiation from a Cs-137 source: 0 Bq/l, 40 Bq/l, 400 Bq/l, and 40,000 Bq/l. (A) SRT1, week 4; (B) GSH2.1, week 1;(C) GSH2.1, week 2; (D) GSH2.2, week 1; (E) GSH2.2, week 2; (F) GSH2.2, week 4. P-values are shown above the brackets and considered significant if smaller than 0.05 (Wilcoxon pairwise comparison) *Bq = Becquerel, l = liter*

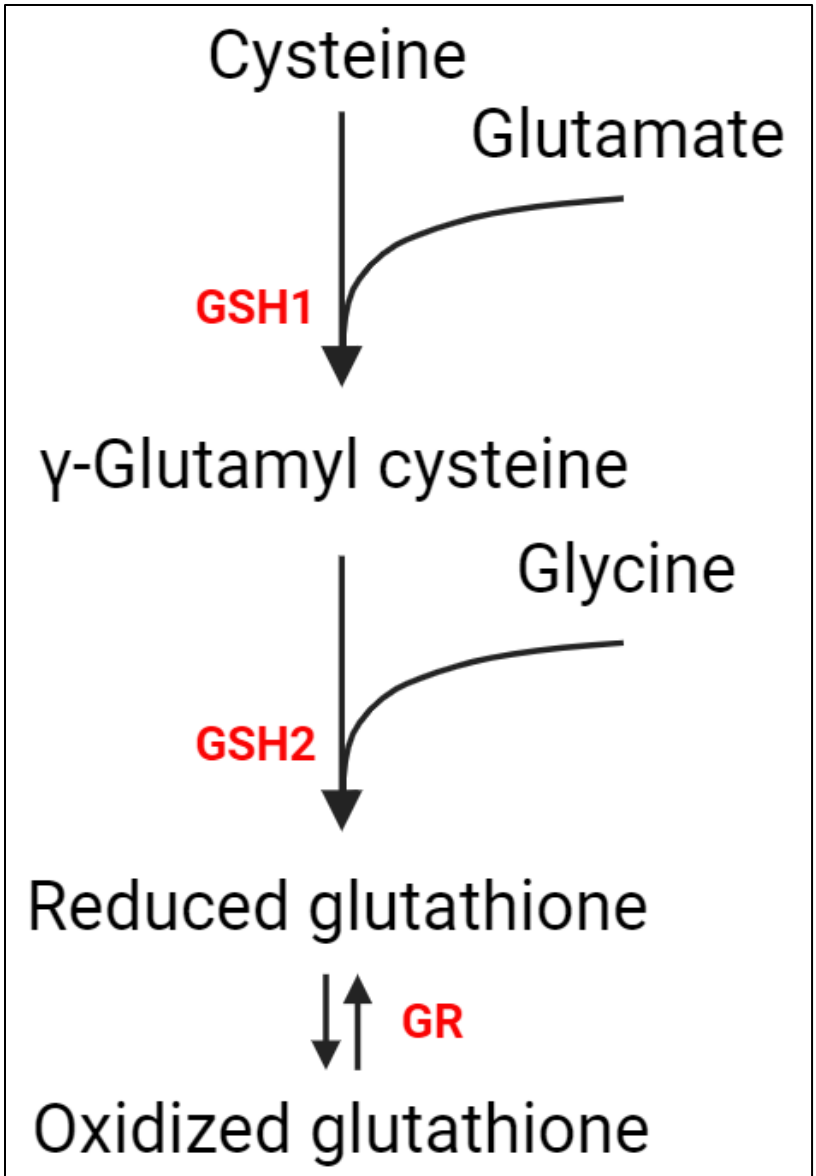

Figure S5: Overview of the glutathione synthesis pathway. Cysteine, glutamate, and glycine are required to synthesize glutathione. The first, rate-limiting step is catalyzed by GSH1. The second step is catalyzed by GSH2. GR converts oxidized glutathione back to reduced glutathione. *GSH1 = glutamate-cysteine ligase, GSH2 = glutathione synthetase 2, GR = glutathione reductase*

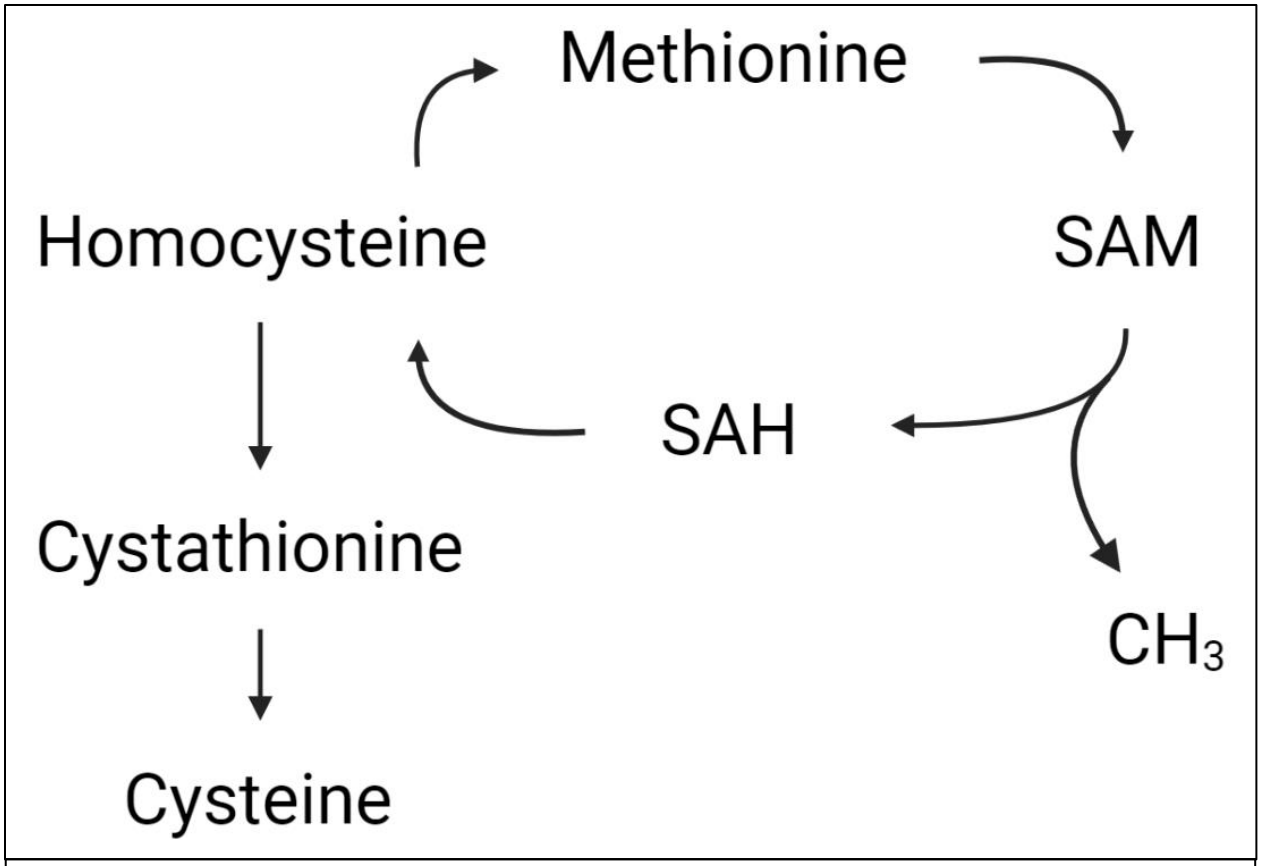

Figure S6: Link between glutathione synthesis and DNA methylation. SAM functions as a precursor of cysteine and is an important methyl group donor required for DNA methylation. *SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine, CH_3 = methyl group*