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Arabidopsis plants exposed to gamma radiation in two successive generations show a different oxidative stress response

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Abstract

When the environment gets contaminated with gamma emitting substances, plants that grow in these contaminated areas are almost always exposed to gamma radiation during consecutive generations. Therefore it is important to evaluate the gamma induced stress response in plants in and between generations. The objective of this research is to reveal differences at the level of the antioxidative stress response between generations with a different radiation history. An experiment was conducted in which 7-days old *Arabidopsis thaliana* plants were exposed for 14 days to four different gamma dose rates: 22 mGy/h, 38 mGy/h, 86 mGy/h and 457 mGy/h. Two different plant groups were used: plants that were not exposed to gamma radiation before (P0) and plants that received the aforementioned gamma treatment during their previous generation (S1). Growth, the concentration of the antioxidants ascorbate and glutathione, a number of antioxidative enzyme activities and their gene transcript levels were analysed. A dose-rate dependent induction was seen for catalase (CAT) and guaiacol peroxidase (GPX) in the roots and for syringaldazine peroxidase (SPX) in the shoots. Differences between the two generations were observed for CAT and GPX in the roots, where a significantly higher activity of these ROS detoxifying enzymes was observed in the S1 generation. For SPX in the shoots, a dose dependent upregulation was observed in the P0 generation. However, high SPX activities were present for all doses in the S1 generation. These differences in enzyme activity between generations for SPX and GPX and the involvement of these enzymes in cell wall biosynthesis, suggest an important role for cell wall strengthening in the response to gamma irradiation.

Keywords

Gamma radiation

Arabidopsis thaliana

Oxidative stress

Generations

Cell wall strengthening

1. Introduction

Gamma radiation is an important type of ionizing radiation (IR) due to its high penetrating capability and high energy, potentially causing damage in organic tissue upon interaction. A background of IR is present in the environment coming from cosmic sources or naturally occurring radioactive materials. However, due to anthropogenic activities such as medical applications, nuclear power production and nuclear accidents, there is a potential elevation of the radiation level in the environment. Although, recently considerable progress was made in the ecological risk assessment of exposure to radioactive substances, major challenges still remain (Garnier-Laplace et al., 2015; Jackson et al., 2004). To enhance the uncertainty present in risk assessment there is among other things a need for more data on responses of organisms exposed to environmental relevant dose rates for a long-term and even across generations (Hinton et al., 2013).

When organisms encounter gamma radiation, damage to DNA and tissues can be inflicted in a direct or indirect manner. Firstly, gamma radiation can cause damage by interacting directly with macromolecules, possibly leading to lipid oxidations, enzyme inactivation, DNA lesions and DNA double strand breaks (Britt, 1996; Esnault et al., 2010; Szumiel, 1998). In addition, gamma radiation, for example, through the radiolysis of water can result in the formation of reactive oxygen species (ROS) (VTJr et al., 1993) which on their turn potentially lead to cellular damage (Esnault et al., 2010; Ward, 1988). In the radiolysis of water the primary reactions of IR are excitation and ionization, producing ionized water molecules ($\text{H}_2\text{O}^{\bullet+}$) and radicals of hydrogen (H^{\bullet}) and hydroxyl ($\bullet\text{OH}$). The hydroxyl radical is a reactive and short living molecule that can cause numerous forms of cell damage as well as cell death (Koyama et al., 1998; Lee et al., 2009). In addition to hydroxyl radicals also other ROS can be induced, such as the more stable hydrogen peroxide (H_2O_2) (Esnault et al., 2010; Neill et al., 2002).

Reactive oxygen species can damage cells via the direct oxidation of proteins and macromolecules, but they are also produced in low quantities as a result of natural metabolism and some species (e.g. H_2O_2) even function as signaling molecules that regulate growth, development and stress responses (for a review see e.g. Torres et al. (2010); Niu and Liao (2016)). Therefore, controlling the concentration of ROS is a priority in plant cells (Mhamdi et al., 2012). In order to do so plants possess an antioxidative defense system that is comprised of enzymatic and non-enzymatic pathways (Mittler et al., 2004). Oxidative stress arises in plants when there is an imbalance between oxidative compounds and these antioxidative plant defense mechanisms. (Karuppanapandian et al., 2011).

In plants glutathione (GSH) and ascorbate (AsA) are the two main hydrophilic antioxidants. GSH is important in the reaction to oxidative stress, as it can directly scavenge $\bullet\text{OH}$ and $^1\text{O}_2$ (Foyer & Noctor, 2005). It is one of the principal low-molecular-weight antioxidants in plant cells and, similar to other thiols, GSH can undergo oxidation, forming GSSG. Glutathione reductase (GR) recycles GSH by reducing GSSG. The main enzymes in the GSH biosynthesis pathway are glutamate-cysteine ligase (GCL) and glutathione synthetase (GSH-S). *GSH1* encodes for GCL, which transforms cysteine into gamma-glutamylcysteine. This is further transformed to GSH, a reaction catalyzed by GSH-S, the gene product from *GSH2* (Noctor et al., 2012). Ascorbate, commonly known as vitamin C, is an important antioxidative metabolite. Ascorbate-deficient *A. thaliana vtc* mutants are hypersensitive to a number of oxidative stresses including ozone and ultraviolet B radiation (Conklin et al., 1996). Ascorbate can react directly with ROS (Smirnoff, 2000). It is also a substrate of ascorbate peroxidase (APX), which is highly specific to H_2O_2 and functions in the 'ascorbate-glutathione' pathway where reduction of H_2O_2 to water is linked to NAD(P)H oxidation via AsA and GSH pools (Noctor et al., 2012).

In addition to the aforementioned ones in the AsA-GSH cycle, there are several other enzymes, that can function in controlling the level of ROS in plants. Important enzymes in the detoxification of ROS are catalase (CAT), peroxidases (PXs) and superoxide dismutase (SOD). Catalase is an enzyme that is located in the peroxisomes catalysing the conversion of H_2O_2 to H_2O and O_2 (Zamocky et al., 2008). It is known that CAT reacts to a lot of different stressors (for a review see Gill and Tuteja (2010)) and

this is also the case for gamma stress. But the activity of CAT under different types of stressors is also known to be rather variable; Zaka et al. (2002) reported an increase in CAT activity in gamma irradiated *Stipa capillata* originating from a nuclear test site, Vandenhove et al. (2010) observed no effects of gamma stress on CAT activity and a decrease was observed by Vanhoudt et al. (2014). This variance in response might be attributed to the fact that different species and experimental setups were used. For the latter, examples are plants irradiated at different life stages (Biermans et al., 2015) or to different dose rates (Vandenhove et al., 2010; Vanhoudt et al., 2014).

Several different peroxidases are important in the antioxidative defence pathways, e.g. APX, syringaldazine peroxidases (SPX) and guaiacol peroxidases (GPX). SPX and GPX belong to a large family of class III peroxidases. Class III peroxidases have functions in the antioxidative response via the reduction of H_2O_2 through a catalytic cycle of electron scavenging from donor molecules, but they are mainly involved in other physiological processes such as plant defense reactions and cell wall metabolism via the cross-linking of lignin (Cosio & Dunand, 2009; Fry, 1986; Passardi et al., 2004; Tognolli et al., 2002). Lignin is a polymeric constituent of the plant cell wall. Lignification involves the biosynthesis of monolignols in the cytosol, in which the phenylalanine ammonia lyase (PAL) genes play a crucial role, transport of these monolignols to the cell wall and subsequent oxidation, performed by peroxidases (GPX, SPX), to form the lignin polymer (Boerjan et al., 2003).

Lignin is involved in the regulation of cell wall permeability. While information on the effect of gamma stress on lignin content is scarce, it has supportive functions and serves as a defensive agent upon biotic and abiotic stress (Christensen et al., 2001; Cuypers et al., 2002). For example, lignin content is known to increase in *Vicia sativa* after treatment with cadmium (Cd) (Rui et al., 2016) and GPX and SPX showed increased activity upon treatment with copper (Cu) and zinc (Zn) (Cuypers et al., 2002). Irradiation with UV-B is also known to stimulate lignin production (Yamasaki et al., 2007), and result in a thicker cuticle (Fukuda et al., 2008) and in the formation of thicker cell walls with an increased lignin content in *Kalanchoe pinnata*, a species considered to already be protective against UV-B radiation (Nascimento et al., 2015).

The importance of research on the transgenerational effects of gamma radiation in plants is supported by a few field studies (Kovalchuk et al., 2004; Zaka et al., 2002). Kovalchuk et al. (2004) demonstrated that *A. thaliana* originating from a contaminated area around the Chernobyl site had a higher expression of oxidative response genes (*CAT1* and *FSD3*) and DNA repair genes (*Rad1* and *Rad54*-like) after exposure to X-rays. They were also able to resist higher concentrations of DNA-damaging or free radical-producing agents. In *Stipa capillata*, originating from a nuclear test site in Kazakhstan, an increased radioresistance to acute exposure was observed due to an increased activity of antioxidative enzymes (CAT, PXs, SOD) (Zaka et al., 2002).

The hypothesis of this study states that exposure to gamma radiation leads to adaptive responses that can persist in the next generation in which the antioxidative pathways play a central role. Therefore, in this study, two generations of *A. thaliana* plants were externally exposed to gamma radiation at different dose rates for 14 days after which growth and different antioxidative endpoints were analysed.

2. Material and methods

a. Plant culture and gamma irradiation

A. thaliana (*Columbia* ecotype) seeds were vernalized on moist filter paper during three days at 4°C in order to synchronize germination. The seeds of two different generations with a different radiation history were used; P0 seeds originated from our standard seed stock and had never been irradiated while S1 seeds were harvested from plants that were irradiated in their previous generation. Subsequently, the seeds were sown on plugs from 1.5 mL polyethylene centrifuge tubes filled with a Hoagland solution that was solidified with 0.6 % agar. The plugs were placed in a PVC

cover that was put on a black container filled with a Hoagland solution (1 mM KNO₃, 0.3 mM Ca(NO₃)₂, 0.2 mM MgSO₄, 0.1 mM NH₄H₂PO₄, 1.62 μM FeSO₄, 0.78 μM Na₂-EDTA, 4.6 μM H₃BO₃, 0.9 μM MnCl₂, 32 nM CuSO₄, 55.6 nM H₂MoO₄ and 76.5 nM ZnSO₄). Every PVC cover contained 36 plugs. It was made sure that the solution touched the base of the agar plugs, preventing it from drying out. Plants were grown in a growth chamber (Snijders Scientific, Microclima 1000E) under a 14 h photoperiod (photosynthetic photon flux density of 200 μmol m⁻² s⁻¹ at the leaf level) with 65% humidity and a day/night temperature of 22°C / 18°C. When plants were 7 days old, they were transferred to the irradiation unit of SCK•CEN where they were exposed to gamma radiation during 14 days by a panoramical ¹³⁷Cs-source. The irradiated plants were divided in 4 groups, all receiving different dose rates. The containers were placed at 0.5m, 1m, 1.5m and 2m from the source, resulting in a dose rate of, respectively, 457 mGy/h, 86 mGy/h, 38 mGy/h and 22 mGy/h. After 14 days they received a total dose of, respectively, 156 Gy, 29 Gy, 13 Gy and 7 Gy. During this irradiation period of 14 days, plants were grown at 26°C and a photosynthetic photon flux density of 160 to 170 μmol m⁻² s⁻¹ at the leaf level. Control plants were grown in a separate chamber at the same temperature and light conditions. After 14 days of exposure to gamma radiation, plants were harvested. Fresh weight was determined for all samples. Pictures were taken just before harvest and analysed in ImageJ to determine the leaf surface area. The samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

b. Antioxidative enzyme capacities

To determine the capacities of some important antioxidative enzymes, two stainless steel beads and a spatula tip of polyvinylpyrrolidone (PVP) were added to plant samples of approximately 100mg. Samples were grounded under frozen conditions using a MM400 (Retsch) for 3.5 minutes at 30 Hz. After the addition of 1.5 mL extraction buffer (0.1 M TRIS, 1mM Na₂-EDTA, 1mM DTT, pH 7.8), the samples were centrifuged (13000 x g, 10 min, 4°C) and kept on ice. The supernatant was diluted ½ with the extraction buffer, after which the capacities of several antioxidative enzymes were determined by spectrophotometry. All enzymes were analysed in 96-well plates using the the

PowerWave XS plate reader (BioTek). For the measurement of CAT activity, 10 μL of sample extract was added to 190 μL of a 49 mM H_2O_2 solution. The decrease in absorbance at 240 nm was monitored during 5 minutes. For GPX, 10 μL extract was added to 150 μL 0.1M phosphate buffer and 40 μL guaiacol- H_2O_2 master mix (equal parts 90mM guaiacol and 8mM H_2O_2 solutions). Subsequently, the appearance of tetraguaiacol was monitored kinetically at 436 nm. The capacity of SPX was measured by adding 20 μL extract to a plate well containing 155 μL 0.1 TRIS (pH 7.5), 20 μL 98 mM H_2O_2 and 5 μL syringaldazine (SAZ). Oxidized SAZ was measured at 530nm. For APX, 18 μL plant extract was added to 155 μL HEPES-EDTA (0.1 M HEPES; 1 mM EDTA) buffer, 20 μL 30 mM ascorbate and 7 μL of a 20 mM H_2O_2 solution. The emergence of dehydroascorbate was measured at 298 nm. For the determination of the GR capacity, 165 μL TRIS-EDTA buffer (0.1 M TRIS; 1 mM $\text{Na}_2\text{-EDTA}$) (pH 8), 7 μL GR mastermix (1:1 mix of 82 mM GSSG and 6 mM NADPH) and 28 μL plant extract were added in each well of a 96-well UV-plate. The decrease of NADPH, used for the reduction of GSSG, is followed kinetically at 340 nm.

C. Metabolite analysis

The concentrations of reduced and oxidized glutathione (GSH) from root and leaf samples were determined spectrophotometrically using a plate reader assay as described in Queval and Noctor (2007). Samples of ca. 50 mg plant tissue in 2 mL tubes (...) were grounded under frozen conditions with two zirconium beads during 3.5 min at 30 Hz using a Mixer Mill MM 400 (Retsch, Benelux Govatec, Aartselaar, Belgium). The metabolites were extracted by adding 800 μL of a 200 mM HCl solution to the homogenised samples and subsequently vortexed until thawed. After centrifugation (15 min, 13000 $\times g$, 4°C), 300 μL liquid supernatant aliquots were taken and 30 μL 200 mM NaH_2PO_4 (pH 5.6) was added. Samples were vortexed and ca. 200 μL 200 mM NaOH was added until pH 4-5 was reached, which was verified by pH indicator paper. For GSSG measurements, 100 μL extract was incubated with 1.3 μL 2-vinylpyridine (2-VP) for 30 minutes at room temperature. Subsequently, samples were centrifuged twice (15 min, 15000 $\times g$, 4°C) to precipitate 2-VP. Triplicate aliquots of 20

μL were added to plate wells containing 100 μL 200 mM NaH_2PO_4 – 10 mM EDTA buffer (pH 7.5), 50 μL dH_2O , 10 μL 10 mM NADPH and 10 μL 12 mM 5,5-dithiobis(2-nitro-benzoic acid). After the addition of 9 μL GR (20 U mL^{-1}) the increase in A_{412} was measured during 5 min. For total GSH (GSH), the same principle was used, but 10 μL untreated extract was used, and the amount of buffer was increased to reach 200 μL per plate well. Standards for GSSG (0-200 pmol) and GSH (0-1000 pmol) were run concurrently in the same plate as the triplicates.

Reduced GSH was calculated as the difference between total and oxidized GSH.

d. Gene expression analysis

Frozen root and shoot samples (50-100 mg) were grounded using a Mixer Mill MM 400 (Retsch, Benelux Govatec, Aartselaar, Belgium) for 3 min at 30 Hz prior to RNA extraction. RNA from root and shoot was extracted using Rneasy Plant Mini kit according to the manufacturers' instructions (Qiagen, Venlo, Netherlands) and the purity and concentration was tested with spectrophotometry at 230 nm, 260 nm and 280 nm on Nanodrop (Isogen Life Science). RNA was stored at -80°C . cDNA was synthesised using the QuantiTect Reverse Transcription Kit (Qiagen). For the comparability of RT-PCR results, the same amount of RNA (750 μg) was used in every cDNA synthesis reaction. Real-time PCR reactions (20s 95°C , 40 cycles of 3s at 95°C and 30s at 60°C), followed by the generation of a dissociation curve to check for specificity of amplification, were performed using SYBR green fluorescence on a 7500 Fast Real-Time PCR system (Applied Biosystems). In a 10 μL volume, 2.5 μL cDNA, 5 μL Fast SYBR Green Master Mix (Applied Biosystems), 0.3 μL forward primer, 0.3 μL and 1.9 μL Rnase-free water were added. Primers used are shown in tabel T1 and their efficiency was determined by the use of a standard curve that was constructed using a 4-fold dilution of a random sample. Only primers with an efficiency above 80% were withheld. Gene expression data was calculated with the $2^{-\Delta\text{Ct}}$ method (Pfaffl, 2004) and presented relative to control. Multiple reference genes were tested for normalisation (YSL8, SAND, ACT, EF1a, F-BOX, TIP-41, UBC, UBC10 and

At4G26410). GrayNorm software was used according to Remans et al. (2014) to select the best reference genes for every tissue (F-BOX, EF1a and YSL8 for roots; TIP-41, UBC and UBQ10 for leaves).

e. Statistical analysis

The statistical analysis was performed using the open source software package R (R i386 3.1.0, R Foundation for Statistical Computing, Vienna, Austria). The normal distribution and homoscedasticity of our data was tested with a Shapiro-Wilk and Bartlett test, respectively. Where needed, a transformation (logarithmic, inverse, square root, exponential) was applied. A two-way ANOVA was applied to identify any statistical differences between treatments and generations. When significant differences ($P < 0.05$) were found, a Tukey HSD test was applied to identify the specific differences between groups.

3. Results

a. Shoot and root fresh weight

Table 1: Fresh weight (FW) of roots and leaves and leaf surface of 21-day old *A. thaliana* exposed to 14 days gamma radiation in the first (P0) or second (S1) generation. Small letters indicate significant differences ($p < 0.05$) within the P0 generation between exposed and control plants, capital letters indicate significant differences ($p < 0.05$) within the S1 generation between exposed and control plants. Measurements are the average \pm SE of at least 50 replicates.

| | | Roots FW (mg) | Leaves FW (mg) | Leaf area (mm ²) |
|---------------|-----------|-----------------------------------|----------------------------------|----------------------------------|
| Generation P0 | Control | 19.54 \pm 1.16 ^{a, AC} | 66.84 \pm 1.41 ^{a, A} | 70.03 \pm 1.62 ^{a, A} |
| | 22 mGy/h | 28.87 \pm 2.19 ^{bc} | 88.82 \pm 2.94 ^b | 89.63 \pm 3.30 ^b |
| | 38 mGy/h | 23.4 \pm 1.36 ^{ab} | 88.35 \pm 2.78 ^b | 95.87 \pm 2.9 ^b |
| | 86 mGy/h | 29.31 \pm 2.09 ^c | 88.95 \pm 2.01 ^b | 85.19 \pm 2.23 ^b |
| | 457 mGy/h | 21.05 \pm 1.85 ^{ab} | 69.92 \pm 1.93 ^a | 67.82 \pm 2.33 ^a |
| Generation S1 | 22 mGy/h | 27.05 \pm 1.39 ^B | 89.34 \pm 2.56 ^B | 92.98 \pm 2.81 ^{BC} |
| | 38 mGy/h | 25.67 \pm 2.05 ^{ABC} | 90.28 \pm 2.06 ^B | 92.25 \pm 2.31 ^B |
| | 86 mGy/h | 23.73 \pm 1.64 ^{AB} | 84.68 \pm 2.03 ^B | 81.17 \pm 2.26 ^C |
| | 457 mGy/h | 17.36 \pm 0.97 ^C | 70.19 \pm 1.51 ^A | 68.45 \pm 1.91 ^A |

Upon gamma irradiation, an increase in root fresh weight of the P0 and S1 generation was observed for the first three dose rates compared to control conditions. Fresh weight was significantly higher compared to the control group in both generations after the treatment with the lowest dose rate. It was shown with a Tukey's HSD test that the overall root fresh weight of the P0 generation was significantly higher than the S1 generation. However, this could not be attributed to a specific gamma treatment. Leaf fresh weight increased significantly compared to control plants in both generations for the three lowest dose rates (table 1) and leaf area followed the same trend (table 1). At the highest dose rate, however, the leaf surface area and leaf fresh weight were again similar to control levels. The results of root and shoot fresh weight indicate a hormesis-like effect of gamma irradiation.

b. Antioxidative enzymes

To get an overview of the antioxidative response of gamma-irradiated *A. thaliana*, the activity of a number of antioxidative enzymes namely CAT, APX, GPX, and SPX (fig.1) were studied in two different generations after 14 days of irradiation at four different dose rates. CAT and APX are primarily involved in the control of H₂O₂ concentration, while GPX and SPX have been shown to function in cell wall biosynthesis (Fry, 1986; Goldberg et al., 1983).

The enzyme activities of CAT and APX were induced in the roots. For CAT, no differences were observed in the P0 generation, but activities were elevated in the S1 generation, with a significant transgenerational difference at the highest dose rate compared to the P0 generation. APX on the other hand showed a non-significant inducing trend at all dose rates, irrespective of exposures in the previous generation. In contrast with the induction of CAT and APX activities in the roots, activities of both enzymes in the shoots were below control levels after all four treatments. Although, all treatments in both generations resulted in an enzyme activity below control levels, the activity of CAT in the leaves followed an increasing trend. A significant difference between the lowest and highest dose rate was observed in the P0 generation.

GPX activity in the roots showed a significant upregulation in the P0 generation after exposure to the highest dose rate. However, this induction was already significant after exposure to 38 mGy/h or higher dose rates in the S1 generation. As such, significant transgenerational differences between the P0 and S1 were noted.

Transgenerational differences in enzyme activity were also observed for SPX in the leaves. In the P0 generation, significant dose-dependent increases in SPX activities were observed at the two highest dose rates. In the S1 generation, however, the SPX activity was significantly induced in all four treatments to a similar extent.

c. Glutathione concentration

Glutathione (GSH) is the principal low-molecular-weight thiol in plant cells. Reduced GSH can protect the cell by interacting with ROS, forming GSSG, the oxidized disulphide form of GSH (Noctor et al., 2012). In the roots (fig. 2A), exposure to 38 mGy/h or higher resulted in a significant dose-dependent increase in the concentrations of total glutathione in both generations as compared to the control. This trend was more pronounced in the S1 generation, which resulted in significant differences in total glutathione concentration between the treatment of 457 mGy/h and the two lowest dose rates (22 mGy/h and 38 mGy/h). No significant differences between irradiated and control plants could be detected in the leaves of the P0 generation at the level of glutathione concentration, nor redox state (fig. 2B). However, in the leaves of S1 generation plants, there was a significant increase in the total amount of glutathione at a dose rate of 457 mGy/h compared to 22 mGy/h. Overall, the same dose rate dependent trend of increasing total and reduced glutathione was observed for leaves and roots in both generations.

d. Gene expression

The response of gamma-irradiated *Arabidopsis* was also studied at the transcriptional level. The expression of three CAT genes (*CAT1*, *CAT2*, *CAT3*), two phenylalanine ammonia lyase genes (*PAL1*, *PAL2*), two glutathione synthase genes (*GSH1*, *GSH2*) and one glutathione reductase gene (*GR1*) gene were analysed. In Figure 3-5 the most significant results are presented, the other gene expression results can be found in supplementary material (S-table1 & S-table2).

H₂O₂ is the most abundant ROS in plant cells (Neill et al., 2002) and *A. thaliana* relies on several enzymes to control its intracellular concentration. CAT is one of these enzymes and the *A. thaliana* genome contains three isoforms of the CAT gene (*CAT1*, *CAT2* and *CAT3*). The relative transcriptional activities of the three isoforms are stacked in figure 3 in order to have a view on the total

transcriptional activity of *CAT* in leaves and roots. As *CAT1* has a low abundance in leaves (Hu et al., 2010; Mhamdi et al., 2010), the qRT-PCR results of *CAT1* are not taken into account in the total results of *CAT* in the leaves.

Total *CAT* transcription increased with increasing dose rates in both generations in the roots (fig. 3A). However, significant differences could only be detected in the P0 generation where the treatment with the highest dose rate showed a significant higher transcription compared to the treatment with the lowest dose rate. The biggest increase in transcription, relative to its own control, was seen for the isoform *CAT3* where transcription doubled at a dose rate of 38 and 457 mGy/h in both generations.

In the leaves (fig. 3B), total *CAT* transcription increased significantly in the S1 generation for the two lowest dose rates. The increase at a dose rate of 22 mGy/h was due to an elevation in *CAT2* transcription, while the increase at a dose rate of 38 mGy/h was the result of a multiplication of *CAT3* transcription. All the other treatments in both generations resulted in a non-significant elevation of total *CAT* transcription, but the ratio *CAT2*:*CAT3* was the same in both generations for the same treatments. Overall, the two isoforms, *CAT2* and *CAT3*, followed opposite trends in both generations. Transcription of *CAT2* decreased below control levels from the second dose on while transcription of *CAT3* was higher than its control in all conditions.

Gene expression of two isoforms of *PAL*, an enzyme that performs the first step in the pathway of lignin biosynthesis (Zhao & Dixon, 2011), was analyzed in the leaves (fig 4) and the roots (supplementary table 1) at the transcriptional level to further elucidate the possible role of lignin in the response to gamma stress.

The expression profile of *PAL* was similar for the two isoforms in both generations. Overall, a significant decrease of *PAL* expression with increasing dose rates was observed. However, differences

between generations were noticeable for *PAL1* expression, more specifically a significant reduction was observed at a dose rate of 457 mGy/h in the P0 generation, while this reduction was already significant at a dose rate of 38 mGy/h and higher in the S1 generation.

Finally, three genes important in the biosynthesis and homeostasis of glutathione were assessed. The expression profiles of these three genes in the leaves (fig. 5) were similar: expression was at control levels for the two lowest dose rates and below control levels for the highest dose rates. No significant effects were observed for *GSH1* transcript level. The *GSH2* transcript level followed the same trend as *GSH1*, but the S1 generation showed expression below control levels at all dose rates, with transcription at the two highest dose rates being significantly reduced.

4. Discussion

Differences in the response to gamma radiation induced by a difference in gamma exposure history over several generations of *A. thaliana* are not widely documented, as the majority of research in the field of radioecology is mainly focused on acute effects within one generation. However, due to a plant's sessile lifestyle, the next generation of a stressed plant will probably live under the same stress conditions as its mother plant. Therefore, adaptation to stress conditions over different generations might well be the best strategy of survival for plants. In order to better understand the effect of gamma radiation on the plant population and the ecosystem, it is thus necessary to study the stress response over multiple generations.

The aim of the present study was to investigate possible differences between generations in the oxidative stress responses of *A. thaliana* plants after exposure to different dose rates of gamma radiation in one or two consecutive generations. To achieve this, 7-day old *A. thaliana* plants were irradiated for 14 days for the first (P0) or second time (S1). Hence, S1 plants had a history of

exposure to elevated gamma radiation, while the P0 plants had never been exposed to enhanced gamma radiation before. Different endpoints were analyzed: growth, transcript levels and enzyme activities of different antioxidative enzymes, glutathione concentration and redox status and expression of glutathione-related genes. Comparing plants with or without a history of exposure to radiation enabled the possible detection of differences between generations in the response to gamma irradiation. From all antioxidative enzymes that were tested, differences between generations in activity were observed for CAT, SPX and GPX. No significant transgenerational differences between P0 and S1 were observed for GSH, plant growth and transcript levels.

By comparing the growth rate between generations and treatments, it was possible to see whether all the responses of the enzymatic and non-enzymatic defence mechanisms that were observed, resulted in advantages for the development of *A. thaliana*. The results on fresh weight of roots and leaves and the leaf area (table 1) demonstrated that exposure to gamma radiation has a transient positive effect on plant growth. The lowest tested dose rates of gamma irradiation always had a positive effect, and this effect was no longer observed at the highest dose rates, irrespective of the gamma exposure history (in both P0 and S1). An increased plant growth after exposure to gamma radiation at similar dose rates as the ones used in our experiment, is a documented phenomenon (Biermans et al., 2015; Kim et al., 2005; Maity et al., 2005).

Enzyme activities of CAT in the leaves did not respond to the different gamma exposures compared to the control, suggesting a minimal role for CAT in the antioxidative response in the leaves after gamma irradiation. This coincides with results of Vandenhove et al. (2010) and Vanhoudt et al. (2011; 2010), who also found no significant difference in the activity of CAT in *A. thaliana* following an entire lifetime or 72h of gamma irradiation. Contrastingly, a significant increase of foliar CAT activity was observed in *Stipa capillata* after chronic gamma irradiation (Zaka et al., 2002) and Vanhoudt et al. (2014) even reported a significant reduction in the activity of CAT in the roots of *A. thaliana* after a 7-day exposure. These contrasting results might be due to a completely different experimental setup in

the former, while in the latter plants of 14 days were used, in contrast to 7-days old plants in this study, and it is known that plant age has an influence on the response to gamma irradiation (Biermans et al., 2015).

Although no differences were observed for total CAT enzyme activity, a pattern was present for different CAT isoforms (CAT2, CAT3) at the transcriptional level (fig. 3B), with an increase in CAT3 and a decrease in CAT2 transcript levels with increasing dose rates. This resulted in several significant increases in total CAT transcript levels in the S1 generation, which was not measured at the total enzyme activity level. This pattern of CAT2 and CAT3 in the leaves was also observed by Zimmerman et al. (2006) at the level of enzyme activity and has been linked to leaf senescence; CAT2 activity decreases after bolting and CAT3 activity increases with plant age. The transcriptional decrease of CAT2 could also be important to sustain a certain concentration of H₂O₂ necessary for several developmental and environmental responses, as a link between CAT2 and APX has already been suggested (Zimmermann et al., 2006). are in contrast with

In contrast to results in the shoots, plants of the S1 generation showed a significant increase in CAT activity in the roots at the highest dose rate, while no effects were observed for CAT activity in the roots of the plants of the P0 generation following gamma exposure (fig. 1). This significant difference in enzyme activity between generations might indicate that the progeny of irradiated *A. thaliana* had a better defense reaction after a new gamma exposure and consequent H₂O₂ formation.

Transgenerational differences in enzyme activities were also observed for GPX in the roots and for SPX in the shoots (fig. 1), both peroxidases with cell wall modifying functions. Within generations, a general increase of enzyme activities with increasing dose-rates was observed, which coincides with results of enzyme activities of peroxidases after gamma irradiation presented by Zaka et al. (2002) in *Stipa capillata*, by Kim et al. (2005) in *Capsicum annuum* and by Wada et al. (1998) in *Nicotiana tabacum* and *Nicotiana debyeni*. Similar results on antioxidative enzyme activities have been observed in plants from which the seeds were irradiated with gamma radiation; an induction of peroxidases was observed which resulted in an enhanced tolerance to salt stress (Qi et al., 2015; Qi

et al., 2014). However, our results demonstrate for the first time that the activities of peroxidases are influenced by gamma exposure in the previous generation. It suggests that there is some transgenerational regulation, resulting in an enhanced activity of peroxidases in the next generation.

SPX and GPX are both linked to functions in cell wall development as they are involved in the polymerization of lignin precursors and the catalyzation of cross links in the cell wall (Fry, 1986; Goldberg et al., 1983). Changes in cell wall modifying enzymes have been observed for other stressors before. Cuypers et al. (2002) demonstrated an increased activity of GPX and SPX in metal stressed (copper and zinc) plants and GPX and SPX were also found to be vital in the antioxidative respons of *L. minor* upon exposure to heavy metals (cadmium and uranium) (Horemans et al., 2015). Research by Trentin et al. (2015) hinted towards a role for cell wall modifying enzymes in the response to oxidative stress by demonstrating that proteins related to cell wall remodelling were downregulated in *ggt1* (gamma-glutamyltransferase) mutants compared to WT. Gamma-glutamyltransferase is bound to the cell wall and takes part in the gamma-glutamyl cycle for extracellular glutathione degradation and recovery, and may be implicated in redox sensing and balance. It was also demonstrated that irradiation with UV-B results in the production of secondary compounds such as lignin (Yamasaki et al., 2007) and in the formation of a protective layer of cells, anatomically similar to wound-periderm, which is made up of cells with thicker cell walls with a higher lignin content (Nascimento et al., 2015). Others reported anatomical changes induced by UV-B include increase in epidermis en cuticle thickness, modifications that can provide an extra layer of protection against radiation (Fukuda et al., 2008; Yamasaki et al., 2007). Although further experiments are needed to confirm the combined transgenerational induction of SPX and GPX observed here, it might indicate that the increased formation of cell wall stiffening components is part of a plant's response to gamma irradiation. It would result in a better protection against stress and give extra structural stability to the cell (Carpita & Gibeaut, 1993).

In addition to the induction of enzyme activities, the concentration of the antioxidant glutathione was analyzed (fig 2). Glutathione is a tripeptide that plays an important role in several physiological processes (Gill et al., 2013; Xiang et al., 2001), the expression of stress-responsive genes (Mullineaux & Rausch, 2005) and developmental processes such as cell differentiation, pathogen resistance and enzymatic regulation (for review see Noctor et al. (2012)). Our results indicate that also non-enzymatic defence mechanisms respond to the gamma irradiation, which could result in better tolerance against the oxidative consequences of gamma irradiation. As such, after a 14-day exposure, the concentration of glutathione in the roots increased significantly with increasing dose rates in both generations, though concentration was higher in the S1 generation. A similar slight increase in glutathione concentration in fronds of *L. minor* plants chronically exposed to high gamma dose rates was recently reported by Van Hoeck et al. (2015). Taken together with the higher activity of CAT in the S1 generation in the roots, it seems that the response to gamma irradiation in *A. thaliana* is predominantly focused on ROS detoxification.

In conclusion, our results show dose rate dependent and transgenerational responses of *A. thaliana* after being irradiated with gamma radiation for two weeks for one or two successive generations. It was demonstrated that low dose rates of gamma radiation have a positive effect on the growth rate of *A. thaliana*. It would be interesting to see whether this positive but transient effect on growth persists in the next generations or if it disappears due to, for example, accumulated radiation damage to vital structures.

The transgenerational upregulation of peroxidases involved in lignin biosynthesis suggests that cell wall strengthening might be an important defense mechanism in the response to gamma stress. The transgenerational induction of these peroxidases, together with the strong induction of CAT in the roots of the S1 generation, seems to suggest there is a transgenerational induction of the ability to increase antioxidative mechanisms after encountering gamma radiation for a second time. This could allow for the progeny to deal more efficiently with excessive ROS, thus better withstanding the harsh conditions in which they will germinate, grow and set seed themselves.

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