Norwegian University of Life Sciences

Faculty of Biology

Department of Plant Sciences

Master Thesis 2015

60 credits

Effects of gamma radiation on growth and development in Arabidopsis thaliana

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Ås, 2015

ABSTRACT

Different types of abiotic stresses are known to have strong impact on morphological development of plants. Exposure of plants to ionizing radiation such as gamma radiation is potentially damaging, but effects may vary with species, radiation dose and life stage. Exposure of a biological system to gamma radiation may involve two types of effects within the cells, direct and indirect. Direct targets involve water, in which gamma radiation results in electron excitation or water radiolysis and further leads to chain reactions that produce secondary oxygen species (ROS). On the other hand, indirect effects of radiation affects the DNA helix, depending on the dose; it induces DNA breaks, which may lead to chromosomal and genomic abnormalities. In order to defend themselves, plants possess cell cycle checkpoints and systems repairing DNA damages.

In *A. thaliana* the transcription factor Long Hypocotyl 5 (HY5), which is crucial in photomorphogenic development and formation of flavonoids acting as antioxidants, plays a major role in light and UV signaling. The ubiquitin ligase Constitutive Photomorphogenesis 1 (COP1) is essential in controlling HY5 by degradation of HY5 in darkness in contrast to in light. In *A. thaliana* HY5 was shown to play a crucial role in stem elongation and flavonoid biosynthesis under UV-exposure and lowered temperature.

The purpose of this study was to investigate after effects of gamma radiation at the molecular, morpho-structural and physiological levels in the *A. thaliana* wild type (WT) L*er* and the *hy5* mutant treated with different gamma doses ranging from 21.6 to 90.7 Gy. This included evaluation of expression of the *RAD51 RECOMBINASE* (*RAD51*) and *TRANSAPARENT TESTA* (*TT4*) gene, which play roles in DNA double strand break repair and biosynthesis of flavonoids, respectively, in plants cells. In addition, High performance liquid chromatography (HPLC) analysis was also performed in order to study production of phenolic acids and flavonoids. Investigations on the physiological level included recording of: rosette leaf formation, leaf area, stem elongation and time to visible flower buds. As late as 51 days after the gamma exposure, relative transcript levels of *RAD51* were increased in the WT under the highest gamma exposure doses 90.7 Gy and 72.2 Gy, compared to the unradiated control, indicating that the up-regulation of *RAD51* by gamma radiation is quite persistent, while the *TT4* gene did not show any significant differences between treatments.

The total level of flavonoids in the WT displayed significant differences between the highest gamma dose (90.7 Gy) compared to the control, while the total level of phenolic acids did not differ between the dose s. At the physiological level, only small differences were observed between different gamma treatments compared to control, in both genotypes.

ACKNOWLEDGEMENT

First, I would like to thank my supervisors, Prof. Jorunn E. Olsen and co-supervisors Prof. YeongKyeong Lee and Prof. Ole C. Lind for all the knowledge they thought me, support and help in order to write my thesis. I also appreciate and thank Tone I. Melby and Linda Ripel for guiding and helping me with laboratory work. Many thanks to gardener Marit Siira that helped me with taking care of almost two thousand plants and Prof. Line Nybakken and Prof Knut A. Solhaug in helping me with flavonoid analysis and measurements of fluorescence. I appreciate also Prof. Dag A. Brede, PhD students Yetneberk A. Kassaye and Elisabeth L. Hansen in helping me with setting up the gamma radiation experiment.

I would also like to thank Prof. Jorunn E. Olsen, Prof Ole C. Lind and Prof. Dag A. Brede to attend the 5th Annual CERAD (Center for the Environmental Radioactivity) Meeting in Oslo and thus present my experimental results.

Most of all I would like to thank my dear parents Milena and Branislav Blagojevic in giving me support through all my years of education and for being always there for me. Special thanks to my brother Milan Blagojevic in supporting and encouraging me through this work.

I dedicate this thesis to my parents, Milena and Branislav.

ABBREVIATIONS

ATM	Ataxia Telangiectasia Mutated Pathway
ATR	Ataxia Telangiectasia and Rad3-related protein pathway
bZIP	basic Leucine Zipper protein
CHS	*CHALCONE SYNTHASE*, same as *TT4*
COP1	Constitutive Photomorphogenesis 1
DDR	DNA Damage Response
DSB	Double Strand Break
HPLC	High performance liquid chromatography
HY5	Long Hypocotyl 5
HYH	HY5 Homolog
HR	Homologous Recombination
NEHJ	Non-homologous End Joining
IR	Ionizing Radiation
RAD51	*RAD51 RECOMBINASE*
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
TT4	*TRANSPARENT TESTA*, same as *CHS*
WT L*er*	Wild type *Landsberg erecta*
PSII	Photosystem II

CONTENTS

1. INTRODUCTION

1.1 The effect of gamma radiation on plant cells

Since plants in natural and man-made ecosystems may be exposed to different types of ionizing radiation, either from natural sources or due to accidental releases, it is important to evaluate sensitivity of plants to such radiation of which gamma radiation is the most energetic and thus most damaging (Wi et al. 2007). Radioactive radiation can interact directly with water and cause excitations and ionizations resulting in production of free radicals, which in turn leads to production of secondary reactive oxygen species (ROS; Figure 1). The •OH free radical can be responsible for extensive cell damages, because it can react rapidly with all types of molecules: lipids, proteins and nucleic acids (Esnault et al. 2010). Thereafter, secondary reactions are produced where one of the most crucial ROS species is hydrogen peroxide (H_2O_2).

Whether a plant are strongly affected by gamma rays depends on the dose but also factors such as species, plant age, cultivars, physiology and the size and state of the plant genome (De Micco et al. 2011). For seeds differences between a dry or fresh seed also plays a major role in gamma sensitiveness. Not-fully developed and germinating seeds are more sensitive to gamma radiation than dry seeds since water content is higher and the embryo easier to reach by the structures affecting ion capacity (Qin et al. 2007). After the Chernobyl accident, it was shown that plants with hairy leaves such as *Cydonia oblonga* and *Mespilus germanica*, or old (lower) leaves of *Zea mays* with large surface, absorbed higher amount of radioactive elements (Sawidis, 1988). In response to ionizing radiation, *Arabidopsis thaliana* has been shown to express different genes depending on radiation type and dose. Previous studies revealed that acute gamma radiation rather affects genes related to nucleic acids, while chronic gamma radiation, has an impact on genes essential for plant flowering (Kovalchuk et al. 2007). According to Kovalchuk et al. 2000, exposing *A. thaliana* and *Nicotiana tabacum* to acute or chronic gamma rays, increase frequency of homologous recombination (HR). On the other hand, in higher plants DNA double strand breaks (DSBs) is suggested to be processed by non-homologous end joining (NHEJ; Britt, 1999). In addition, because of ionizing radiation some plants exhibit also loss of photosystem II (PSII) functionality (De Micco et al. 2011).

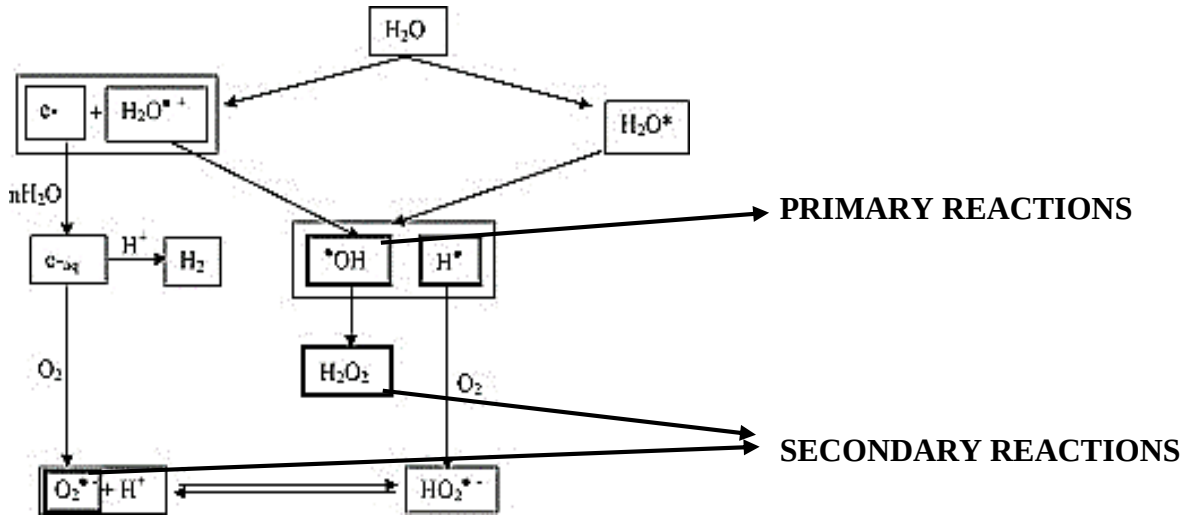


Figure 1. Gamma rays affecting target (H_2O) directly within a cell and causing excitation and ionization. This leads to production of primary and secondary reactions (free radical formation that negatively affect plants and other organisms; Esnault et al. 2010).

1.2 *A. thaliana* as a molecular model to study plant development

A. thaliana, which is a small weed plant belonging to the mustard family was already suggested to be used as an experimental model plant in the 1940s because of its ability to self-fertilize, small size and short generation time. *A. thaliana* can tolerate different environmental stresses and thus adapt to a various geographical areas. Later discoveries about *A. thaliana* such as possession of one of the smallest genomes (approximately 27 000 genes ~ 125 Mbp), among higher plants and the easiness of transformation and mutation, contributed to the fact of becoming an experimental model in molecular genetics from the 1980s. Further, the total number of genes is organized along five chromosomes, where each one is built up of specific sequences, approximately one gene per 5 kb (Koorneef and Scheres, 2001). Centromeric and telomeric regions consist of highly repeated elements (transposons), described as heterochromatin, while euchromatin represents genes coding for functional proteins.

Several studies have been performed in order to analyze how different light spectra are affecting plants adaptation and development. THE LONG HYPOCOTYL (HY5) transcription factor is known for its interaction with light responsive promoters and thus stimulating light controlled transcriptional activity (Chattopadhyay et al.1998). Thus, mutation in the HY5 gene affects plant cell elongation, cell proliferation and chloroplast development (Oyama et al. 1997).

Previous studies also observed that deficiency in HY5 affects flavonoid biosynthesis, which may be affected by gamma radiation.

Transcriptional regulation of chalcone synthase i.e. the type denoted *TRANSPARENT TESTA 4* (*TT4*; further described below) as the first enzyme involved in production of flavonoids, which act as antioxidants, was therefore chosen to be analyzed in WT and the *hy5* mutant after gamma radiation in order to observe whether there was after-effect on gamma rays and flavonoid biosynthesis. In addition, the *RAD51 RECOMBINASE* (*RAD51*) gene in *A. thaliana* is crucial in defense against gamma rays, because it is involved in the homologous DNA repair system. Therefore, effects of gamma rays on *RAD51* activation was analyzed in the present study (explained further below).

1.3 Hy5 advantages and disadvantages in defense against UV-B radaition

One of the crucial abiotic factors for plants is light, which is known as a source of energy and as a signal controlling growth and development. Plants uses different photoreceptor systems in order to coordinate their biological processes with the environmental conditions. In *A. thaliana* the most well-known photoreceptor systems are phytohcromes (phy), which include phyA-E (perceiving the red/far-red spectral region), cryptochromes (cry), which include cry1 and -2, phot1 and -2 (blue/UV-A spectral region) and the UV resistance locus 8 (UVR8) photoreceptor (UV-B spectral regions) (Ulm and Heijde, 2012). Transition of plants from light to darkness or vice versa has significant impact on the organism's further growth and the responses depend on a set of transcription factors.

One of the crucial proteins that promotes photomorphogenesis of young seedlings is HY5. It has been proposed to work as a positive regulator downstream of photoreceptor signaling pathway under hypocotyl elongation (Koorneef et al. 1980). In addition, earlier molecular analysis of HY5 in *A. thaliana* revealed that the HY5 gene encodes a basic leucine zipper protein (bZIP) localized in the nucleus and regulating development of roots and the hypocotyl (Oyama et al. 1997). During darkness, HY5 is targeted by the ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) for degradation by the proteasome complex, in contrast to during the light period when HY5 is allowed to accumulate and thus exert its effect as a transcription factor (Osterlund et al. 2000). Previous studies revealed that upon *A. thaliana* exposure to UV-B light, HY5 has an essential role in defense against oxidative damage.

In response UV –B *COP1* binds to the UV-B receptor UVR8 (Figure 2) HY5 expression is promoted which results in plant defense against UV-B light (Figure 3; Oravecz et al. 2006; Jenkins, 2014).

However, the mode of action of UV-B on HY5 appears to be complex since more degradation of HY5 was observed in *cop1* mutants than in the wild type (Jenkins 2014). In addition, Ulm et al. (2004) showed that HY5 as well as HYH, which interacts with HY5, are activated independently of phyA and phyB upon exposure to UV-B light.

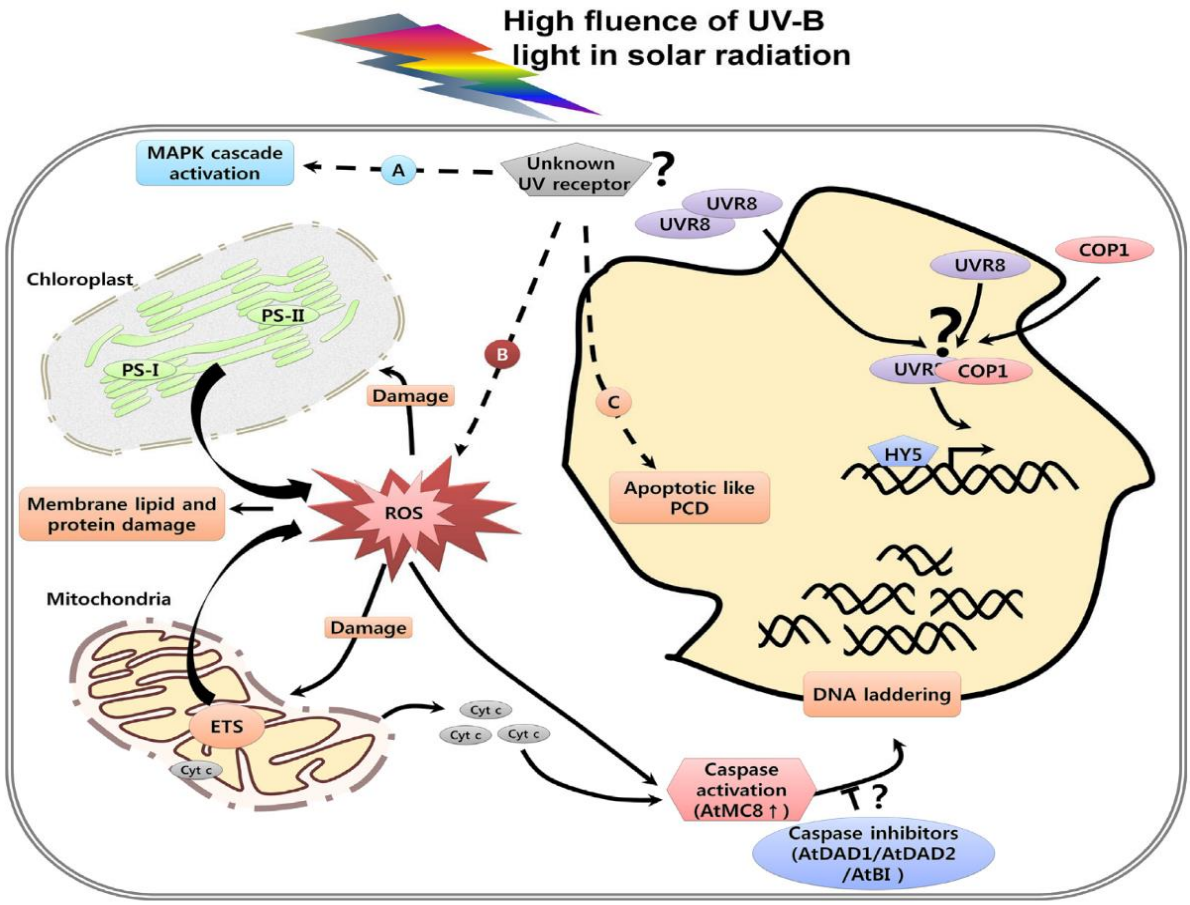


Figure 2. High-fluence UV-B light activates UVR8 pathway and cell death. Thereafter A) Mitogen-activated protein kinase (MAPK) gets activated and leads to regulation of programmed cell death (PCD); B) ROS gets released from chloroplast and mitochondria which causes membrane lipid and protein damage. This further lead to cytochrome c release and DNA laddering. *Dotted lines shows pathways stimulated by an unknown UV photoreceptor; "?" indicates unknown UV photoreceptor (Nawkar et al. 2013).

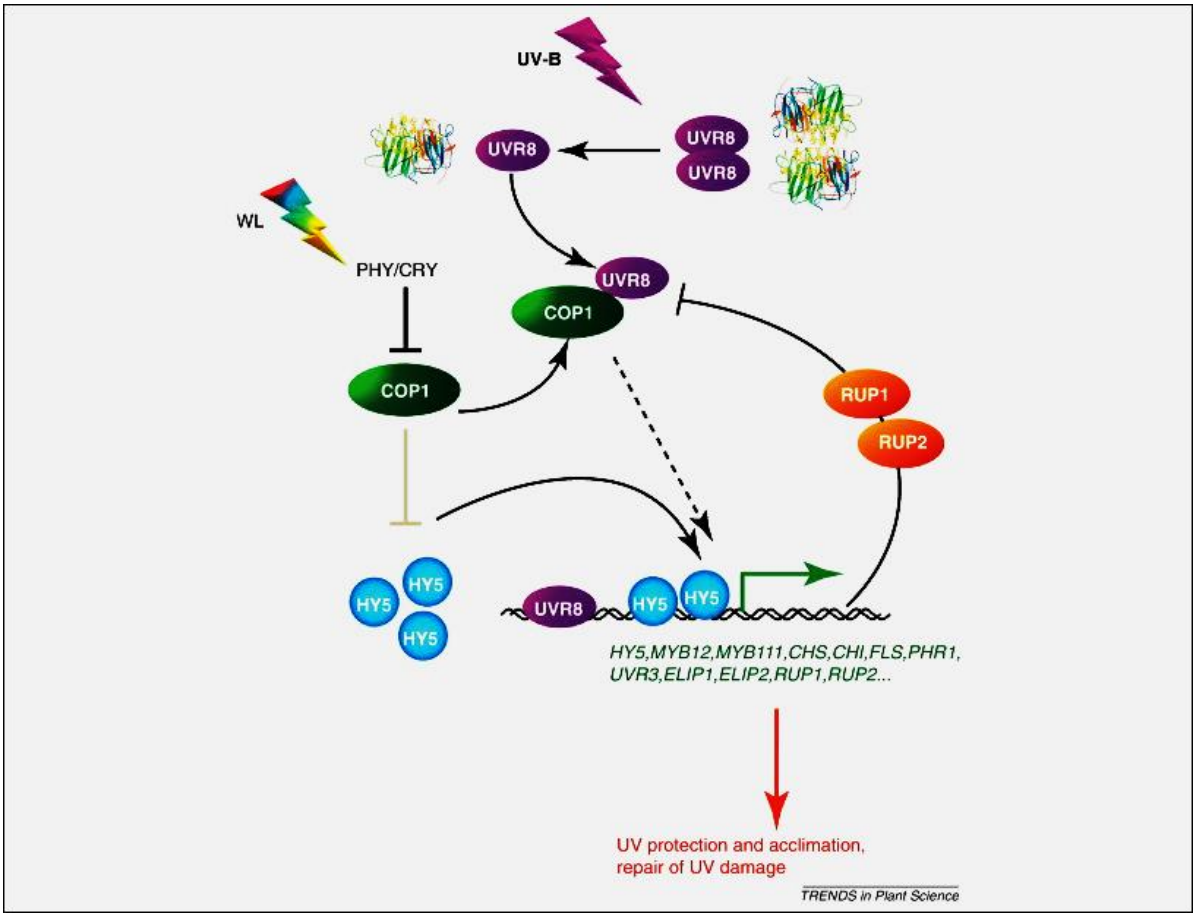


Figure 3. UVR8-mediated signalling. UVR8 is presented mainly as homodimer, which binds to COP1 under UV-B light and thus enhances HY5 transcription. The HY5 transcription factor gets stabilized. Thus UVB response genes are activated, which include genes encoding proteins crucial in UV protection (e.g. phenylpropanoid biosynthesis pathway, including CHS) and damage repair (e.g. UVR3). Abbreviations: CHI, CHALCONE ISOMERASE; CHS, CHALCONE SYNTHASE; CRY, cryptochrome; COP1, CONSTITUTIVELY PHOTOMORPHOGENIC 1; ELIP1 and ELIP2,EARLY LIGHT-INDUCIBLE PROTEIN 1 and 2; FLS, FLAVONOL SYNTHASE; HY5, ELONGATED HYPOCOTYL 5; MYB12 and MYB111, MYB DOMAIN PROTEIN 12 and 111; PHR1, PHOTOLYASE 1; PHY, phytochrome; RUP1 and RUP2, REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 and 2; UV-B, ultraviolet-B radiation; UVR3, UV REPAIR DEFECTIVE 3; UVR8, UV RESISTANCE LOCUS 8; WL, white light (Ulm and Heijde, 2012).

1.3 DNA Damage Response (DDR) in plants

An organism is constantly exposed to DNA damaging factors; therefore, it is crucial to possess DNA damage response system in order to sense and repair DNA damage. DDR represents a cluster of cellular networks, which are activated due to exposure to ionizing radiation (IR) and establish DNA repair, cell cycle arrest and apoptosis in order to remove a particular genetic material.

However, plants, like animals, contain many similar DNA damage response factors but are missing one of the important regulators, the p53 tumor suppressor that is crucial in preventing DNA damaging factors. Yoshiyama et al. (2013), suggest that suppressor of gamma response 1 (*SOG1*), a plant specific transcription factor, may play a crucial role in response to DNA damage.

DNA damage recognition involves the ataxia telangiectasia mutated pathway (ATM), activated by double strand break, and ataxia telangiectasia and Rad3-related protein pathway (ATR), activated by single strand DNA break. Yoshiyama et al. (2013), showed also that expression level of the gene encoding *RAD51*, a sensor involved in MRE11-RAD50-NBS1 (MRN) complex in ATM pathway, is immediately induced after plant exposure to DNA damage. Thus the gene encoding protein Breast cancer 1 (BRCA1), requires the MRN complex in order to enhance *SOG1* (Figure 4). It has also been identified that a wide range of genes gets upregulated after exposure to IR, whereas none is activated in the *sog1-1* mutant, which indicates that several transcriptional regulations are maintained through *SOG1* (Yoshiyama et al. 2009). In addition, *A. thaliana atm* mutants showed hypersensitivity to gamma radiation but no sensitivity under UV exposure (Garcia et al. 2003).

In addition, several earlier studies revealed that plants with large chromosomes are more sensitive to ionizing radiation, compared to plants with smaller chromosomes, which are more resistant. Species with predominantly acrocentric chromosomes showed greater sensitivity to ionizing irradiation than species with metacentric chromosomes. Moreover, increased degree of ploidy increases degree of resistance, thus plant cell are less sensitive to irradiation that animal cells, which rarely possess polyploidy (Sparrow and Woodwell, 1962).

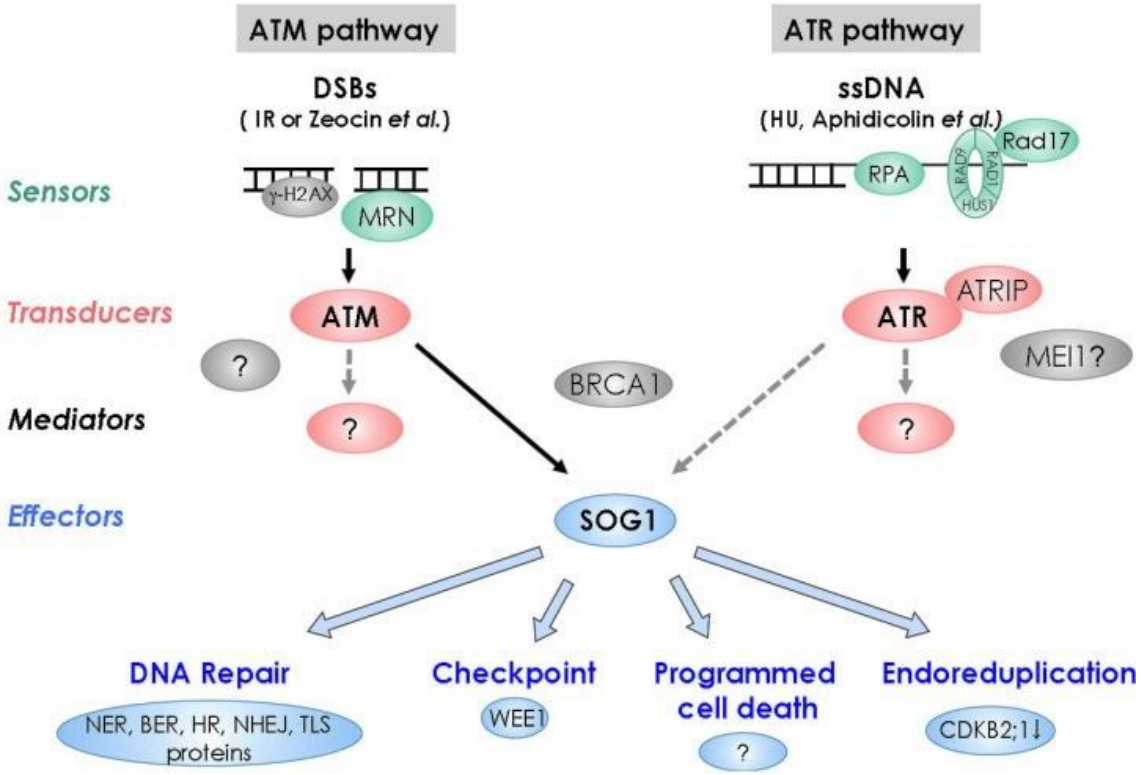


Figure 4. DNA damage response in plants. MRN complex involves the *RAD51* gene which further enhances SOG1 activation and thereby several pathways such as: DNA repair, Checkpoint, Programmed cell death and Endoreduplication. *Dotted lines indicate hypothetical situations (Yoshiyama et al. 2013).

1.5 Defense systems in plants against gamma rays

Overproduction of free radicals and other ROS species triggers plants self-defense, where paths of detoxifying enzymes are activated, such as peroxidases, ascorbate peroxidase, superoxide dismutases and glutathione reductase etc. (Esnault et al. 2010). Vanhoudt et al. (2014), illustrated that superoxide dismutase (SOD) and ascorbate peroxidase (APX) increased in *A. thaliana* in roots, after gamma exposure. In addition, chronic exposure of rice (*Oryza sativa*) or *A. thaliana* to caesium resulted in increased expression of genes involved in cell defense, stress response and detoxification (Rakwal et al. 2009; Sahr et al. 2005). Kim et al. (2007) discovered that 2165 gamma inducible and 1735 gamma repressible genes were activated 9 days after irradiation of *A. thaliana*. In addition, transcription of certain genes: *RAD51*, *BRCA1* and B type mitotic cyclin (*CYCB1*) were strongly induced within 8 hours after gamma radiation (Culligan et al. 2006).

1.6 Flavonoid biosynthesis as an essential factor in UV-B- and IR- defense

Flavonoids are plant secondary products that are present in epidermal cell layers of leaves and other tissues that are sensitive to UV light such as pollen and apical meristem (Shirley, 2002). A common chemical structure of flavonoids is three ring (C6-C3-C6) structure. Flavonoids can be divided in four major classes: anthocyanins, flavanols, flavanols and proanthocyanidins or tannins (Figure 5A). The have antioxidant activity play a crucial roles in plants defense against UV exposure and defense against phytopathogens, control of auxin physiology and male fertility (Petrussa et al. 2013). Flavonoid biosynthesis follows the phenylpropanoid pathway, where a set of enzymes represented in Figure 5B, is activated by UV-B and several other environmental conditions. One of the first enzymes to be activated in flavonoid biosynthesis is chalcone synthase (*CHS*) and chalcone isomerase (*CHI*). In *A. thaliana* there are three *CHS*-like genes representing a small gene family. One of them, At*CHS* (*TT4*) has been shown to be involved in flavonoid synthesis. Mutation in *AtCHS* lacks proanthocyanidin formation in the seed coat and a mutant of in this gene is named transparent testa (*tt4*; Shirley et al. 1995, Saito et al. 2013). Some earlier studies on *A. thaliana* showed UV-hypersensitive phenotypes when chalcone synthase is deficient (Li et al. 1993; Christie and Jenkins, 1996).

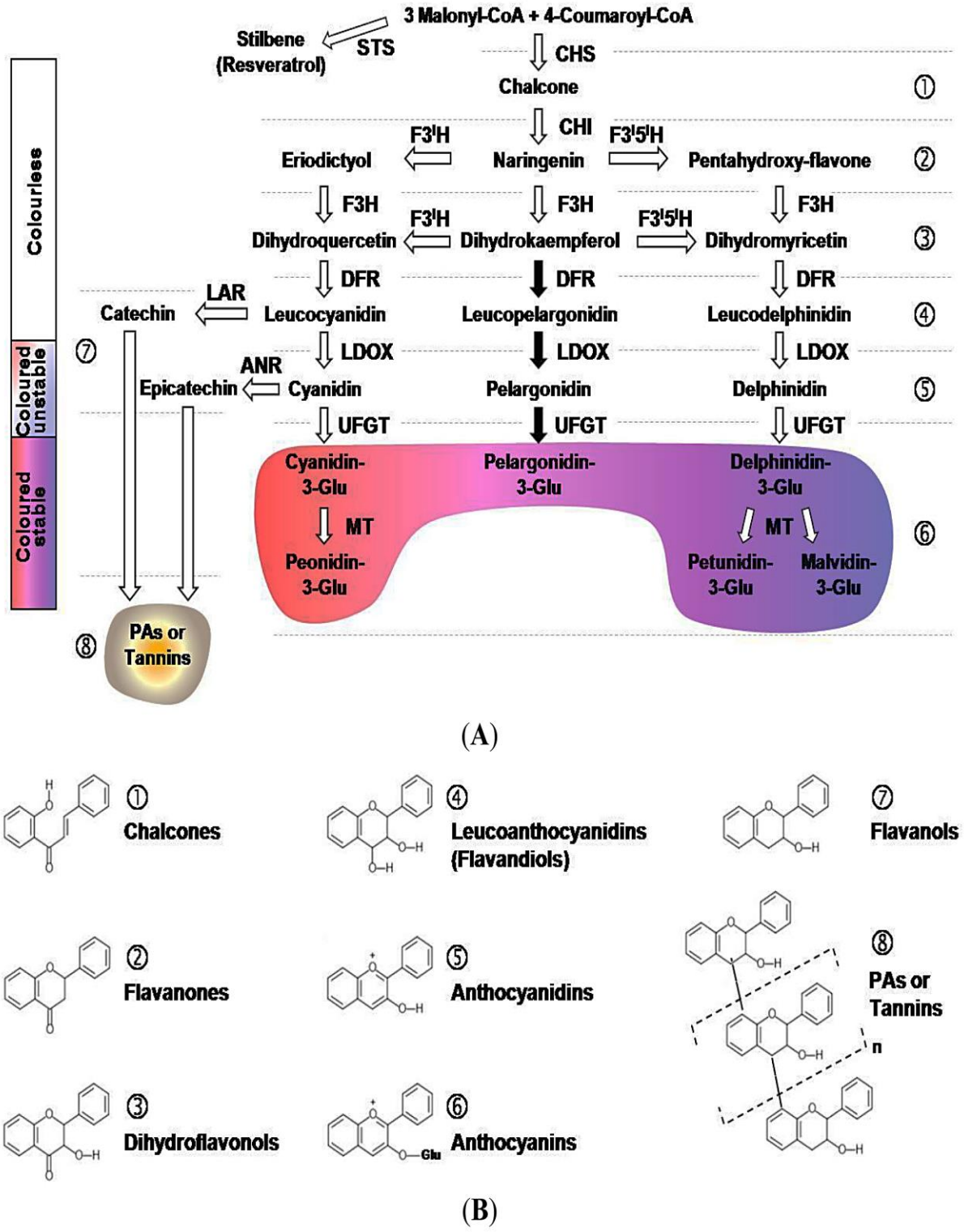


Figure 5. **(A)** Flavonoid biosynthesis pathway in plant cells with the enzymes involved: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol reductase; LDOX, leucoanthocyanidin oxidase; UFGT, UDP-glucose flavonoid 3-O-glucosyl transferase; MT, methyltransferase). Proanthocyanidins (PAs) synthesis branches off the anthocyanin pathway (LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; STS, stilbene synthase). **(B)** Chemical structures of different flavonoid groups (Petrussa et al. 2013).

1.6.1 High Performance Liquid Chromatography (HPLC) method

High Performance Liquid Chromatography is a method used to separate components from a mixture, determine their biological characteristics and thus analyze them. This method is used for different purposes such as identifying vitamin D in blood, drugs in urine, separation of compounds from complex biological structures and in analysis of pharmaceutical products. The HPLC method works on the same principle as paper chromatography, where mobile phase moves to solid stationary phase. In order to create a mobile phase, a pump moves the solvent through the chromatographic system. Furthermore, an injection system is needed to inject the probes into system by which the sample reaches the stationary phase and separation occurs. After being separated, the samples reaches a detector, moves through it and sends signals to a computer software (Figure 6).

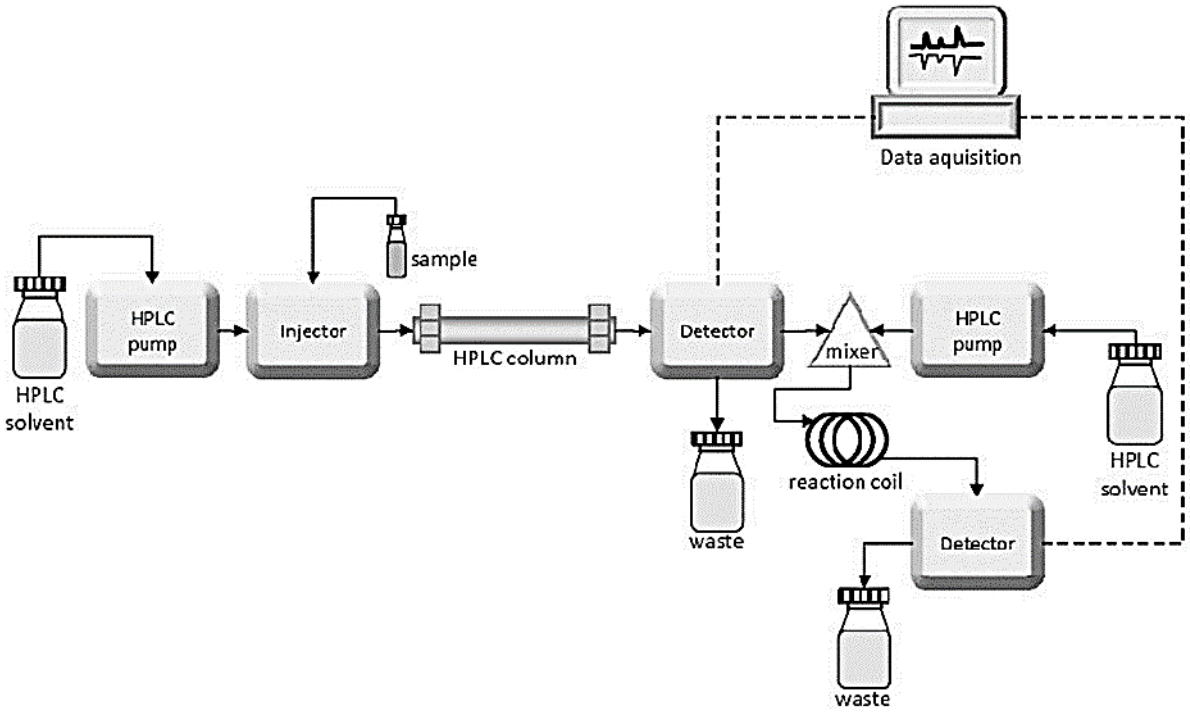


Figure 6. High Performance Liquid Chromatography method (Czaplicki, 2013).

2. AIMS OF THE STUDY

To study radiosensitivity in *A. thaliana* we exposed young seedling of two genotypes, WT L*er* and mutant *hy5* for gamma radiations, including treatments from 21.6 to 90.7 Gy. Aim of the study was to investigate dose-response relationship by testing out:

- Physiological structure, i.e. number of rosette leaves, leaf size, stem length and time to visible flower buds.
- Relative transcript levels of *RAD51 RECOMBINASE* (*RAD51*) and *TRANSPARENT TESTA* (*TT4*)
- Synthesis of phenolic compounds
- Production of reactive oxygen species (ROS)

3. MATERIALS AND METHODS

3.1 Plant materials and growth conditions

Arabidopsis thaliana (A. thaliana) WT L*er* and mutant *hy5* seeds were surface sterilized in Tween solution (1 mL of sodium hypochlorite solution, 9 mL of EtOH and two drops of Tween), rinsed five times in distilled water, and once in EtOH 96%. Sterilized seeds were placed on a filter paper for drying and then evenly sown on ½ MS medium (Duchefa Biochemie, Harleem, Netherland), 0.8% agar. In order to stratify the seed, petri dishes (5 cm in diameter, 2 cm height) were covered with aluminum foil and placed for 4 days at 4˚C. The stratified *A. thaliana* seeds were then germinated for 3 days under about 30 µmol m^{-2} s^{-1} irradiance (TL-D 58W/840 lamps, Phillips, Eindhoven, Netherlands) with the temperature set at 21˚C.

3.2 Gamma (^{60}Co source) radiation of plants

Gamma treatment included exposure of three days old seedlings with doses ranging from 0 to 90.7 Gy, using a ^{60}Co gamma source (THE NMBU LOW DOSE GAMMA RADIATION EXPOSURE FACILITY). Co-60 gamma irradiation source at CERAD/NMBU provides dose rate field from 2.5 Gy/h (at source) down to 300 µGy/h (Figure 8). The climate control conditions for the experimental hall were: 4-37 ˚C (+/- 1˚C), ca 50-300 lux with automatic dimer, 40- 65% (ScanClime) humidity and ventilation at 300 m^3/h (HEAPA filtered).

Two gamma treatment experiments were performed, where the first irradiation lasted for 72 h, while the second lasted for 168 h (Table 1). Petri dishes with seedlings on germination medium were placed vertically in front of the collimator (42.5 cm from radiation source and 0.5 mm from the collimator edge) divided in two rows (WT L*er* and *hy5* mutant). The first experiment had two rows and each contained four columns with five Petri dishes in each, while the second experiment included two rows containing five columns with four Petri dishes in each respective column (Figure 7). Gamma treatments in both experiments are shown in Table 1, as calculated in the middle of the Petri dishes at the different distances from the gamma source. During the radiation the room temperature was set at 20 ˚C with a 12 h daily light period with a photon flux density of about 55 µmol m^{-2} s^{-1} provided by High pressure Metal Halid lamps (HPI-T plus 250W lamps, Phillips, Eindhoven, The Netherlands). The irradiance was measured at the top of the petri dishes with a Li-Cor Quantum/Radiometer/Photometer (model LI-250, LI-COR, Lincoln, NE, USA). The red/far red-ratio was 3.5 (measured by a Sky Instruments, 660/730 sensor, Powys, Wales, UK) sensor. The lamps were placed 1.40 m above the surface of the uppermost Petri dishes with seedlings. During the radiation, as mentioned above, the Petri dishes were placed upon each other within each respective row. In order to insure more equal light intensity and gamma radiation effect, in the middle of each experiment the Petri dishes were rotated 180 degrees and the two upper and two lower dishes were interchanged. Control samples (12 petri dishes in three columns) were placed out of the radiation sector, protected by lead boxes and placed under light and temperature conditions as described for the radiated seedlings.

Table 1. Exposure of *A. thaliana* plants to gamma radiation using a ^{60}Co source. Each respective row (including four columns of Petri dishes with five dishes in each in 1st experiment and five columns of Petri dishes with four dishes in each in 2nd experiment, i.e. at different distances from the gamma source) absorbed a specific amount of the gamma radiation, represented both as dose rate (dose h^{-1}) and total dose.

	1st experiment		***2nd experiment***	
Row	**Dose h^{-1}**	**Total dose (72 h)**	**Dose h^{-1}**	**Total dose (168 h)**
1	0.55 Gyh^{-1}	39.6 Gy	0.54 Gyh^{-1}	90.72Gy
2	0.44 Gyh^{-1}	31.7 Gy	0.43Gyh^{-1}	72.2 Gy
3	0.36 Gyh^{-1}	25.9 Gy	0.35Gyh^{-1}	58.8Gy
4	0.30 Gyh^{-1}	21. 6 Gy	0. 29 Gyh^{-1}	48.7 Gy
5	-	-	0. 18 Gyh^{-1}	30.2 Gy

Figure 7. *A. thaliana* WT L*er* and *hy5* mutant seedlings in Petri dishes placed in front of a collimator and treated with gamma radiation from a ^{60}Co gamma source. Petri dishes were organized into two rows of columns (wild type vs *hy5* mutant). The figure represents the second experiment, containing five columns, while the first experiment contained four columns.

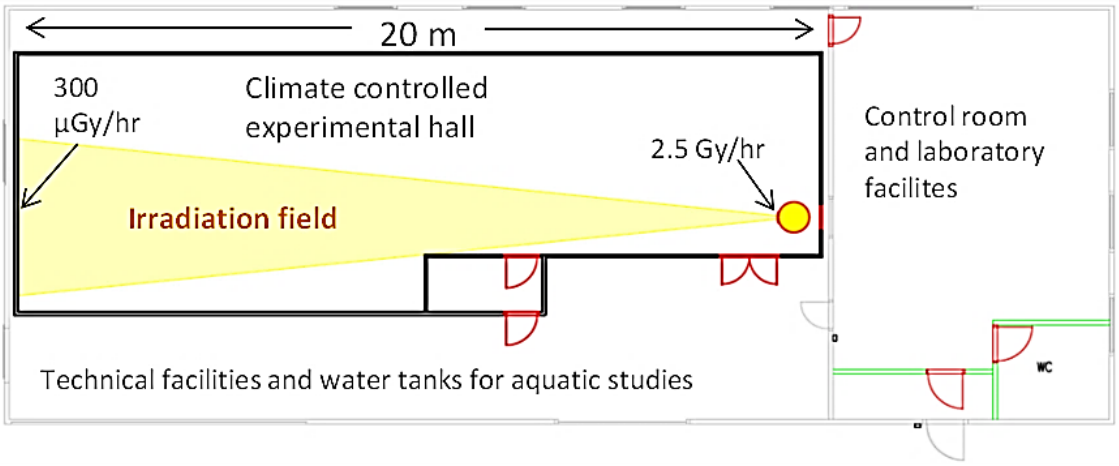


Figure 8. The NMBU low dose gamma radiation exposure facility (FIGARO), place where *A. thaliana* seedling were placed at different distanced from the gamma radiation source. Control plants (no radiation) were placed in the same room and shielded by lead.

3.3 ROS (Reactive Oxygen Species) staining of plant tissue

3.3.1 DAB (3, 3'- diaminobenzidin) staining of H_2O_2

Immediately after gamma exposure, 3-4 *A. thaliana* seedlings were placed in 1 ml pre-prepared aqueous solution of DAB (10% DAB (100 µL) diluted in 900 µl milliQ- H_2O) in order to test the plant tissue for its H_2O_2 content. Seedlings from each gamma treatment including unradiated control samples, were incubated in an Eppendorf tube for approximately 18 h. Thereafter the seedlings were boiled for 15 min in 96% EtOH at a temperature set at 100˚C and examined using microscope. Presence of H_2O_2 is indicated by a reddish-brown color, (Thordal-Christensen et al. 1997).

3.3.2 NBT (Nitro blue tetrazolium) staining super oxide anion

A similar procedure was followed for NBT staining of superoxide anion. Seedlings were incubated in darkness for 30 min in Nitro blue tetrazolium (Promega, Madison, USA). After incubation, seedlings were washed in H_2O in order to stop the reaction and thereafter boiled in 96% EtOH for 15 min at a temperature set at 100˚C. Microscopy analysis followed. Beyer et al. (1987) described super oxide anion presence as deposits of dark-blue insoluble formazan compounds.

3.4 Growth conditions and growth measurements after gamma treatment

Right after gamma treatment, plants were placed at the same conditions as before gamma exposure, about 30 µmol m^{-2} s^{-1} irradiance with the temperature set at 21˚C. The day after, plants were transferred to pots (12 cm diameter, 7 cm height) filled with S- soil (45% low moist peat, 25% high moist peat, 25% pelite and 5 % sand). Five plants per pot of *A. thaliana* WT L*er* and *hy5* mutant were cultivated in a growth chamber (Conviron, Growth Chambers, Controlled Environments Ltd, Winnipeg, Canada) with a daily light period at 12 h with a photon flux intensity at 50 µmol m^{-2} s^{-1} from fluorescent tubes (60W lamps, Phillips, Eindhoven, Netherlands). Temperature was set at 20 ˚C and red/ far-red ratio was adjusted to 1.7 with incandescent lamps (Osram, Munich, Germany) and the relative air humidity (RH) was adjusted to 78% corresponding to 0.56 kPa water vapour pressure deficiency. Change of irradiance followed after seven days, when light period was set at 8h with 100 µmol m^{-2} s^{-1} irradiance (Cool White 215W lamps, V. H. O, Ontario, Canada).

The idea was that since shorter daylight (and thus lower light sum) results in slower development that longer daylength, it might be easier to distinguish after-effects of different doses of gamma radiation given that these affects growth and development differently. Temperature, red/far-red ratio and RH remained the same. Growth recordings were performed for 20 plants from each treatment per gamma; number of rosette leaves, flower bud registrations, height of the inflorescence (bolting) stem, leaf length (petiole and lamina) and leaf width.

Number of rosette leaves (leaves >5 mm) was recorded at day 19, 24, 29 after gamma treatment for the first experiment and for the second experiment at day 18, 22 and 26 after gamma exposure, i.e. until the first flower bud was visible. Reproductive growth, recorded as time to visible flower buds, was registered each day, but visible flower buds were seen at day 32, 35 and 38 after the gamma irradiation treatment. Registration of reproductive growth for the second experiment followed at day 26, 29 and 33 after gamma treatment, i.e. until all plants had visible flower buds. Length of the bolting stem (distance from the base to the first flower on the stem) was measured at the end of the second experiment (51^{st} day after gamma radiation) by using a ruler. Leaf size parameters were measured for rosette leaf number six from the base of the plant by measuring the length and width of the leaf lamina and petiole length by using a digital slide caliper.

3.5 Chlorophyll fluorescence measurements

In order to obtain information about the photosystem II (PSII) efficiency, 28 days after the gamma treatment in experiment 2, pots were placed in the dark for 15 min. A modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany) was then used to measure optimal PSII efficiency by chlorophyll fluorescence. Measurements were performed on 35 plants from each gamma treatment. F_m and F_o were measured for dark adapted leaves using a saturating pulse of 0 6 s. Thereafter, Fv/Fm was calculated by $F_v/F_m = (F_m - F_o)/F_m$ (Stavang et al. 2010).

3.6 RNA extraction and gene analysis (RT-PCR)

For studies of gene expression plants (entire rosettes) were harvested into liquid nitrogen 51 days after the gamma treatment, and stored at -80 C until analyses. Total RNA was extracted 51 days after gamma treatment from the WT L*er* and mutant *hy5* leaves using the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA) following the manufacturers specification. In order to test RNA quality for further analyses an Agilent 2100 Bioanalyzer with an RNA 6000 NanoKit was used (Agilent technologies, Waldbronn, Germany).The resulting cDNA was diluted 1:5 and 2 µl were used in the quantitative amplification reaction for the samples *AtACTIN (ACT)* was used as internal standard for quantification of transcript levels of *AtRAD51* and *AtTT4*. qRT-PCR was performed in 20 µl with Platinum Quantitative PCR Supermix-UDG, SYBRGreen and using specific primers shown in Table 2. In order to investigate the transcript levels in the wild type and the *hy5* mutant and to compare different gamma treatments with control, cycle threshold (Ct) values were calculated for treated samples ($Ct\ target_{treated} - Ct\ reference_{treated\ sample}$) and calibrator sample ($Ct\ target_{calibrator} - Ct\ reference_{calibrator\ sample}$). Furthermore, the ΔΔCt value was calculated for each of the gamma treated samples ($\Delta\Delta Ct_{treated\ samples} = \Delta Ct_{treated\ sample} - \Delta Ct_{calibrator\ sample}$). In order to determine ratio of expression levels in *hy5* versus wild type and different gamma treatments for specific genes (*RAD51* and *TT4*) results were presented as fold difference in log_2 scale: Fold difference = Log_2 (RQ) = $-\Delta\Delta Ct$.

Table 2. Forward and reverse sequences for *A. thaliana* genes: *ACT, RAD51* and *TT4* used in qPCR method

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
AtACT	TCAGATGCCCAGAAGTCTTGTTCC	CCGTACAGATCCTTCCTGATATCC
AtRAD51	GCCTATGCGAGGGCGTATAA	CGAAAGCTCTCCCCTTCCAG
AtTT4	ACATGCCTGGTGCTGACTAC	CACGTGCTCCACGATTGTTC

3.7 Quantification of phenolic compounds from leaf material

65 days after exposing *A. thaliana* for different gamma treatments, plant material was collected in order to examine phenolic compounds. Before collection, the inflorescence stem was removed by using scalpel. Thereafter plants were freeze dried for 24 h and approximately 20 mg plant material was weight by micro scale (Mettler Toledo, Oslo, Norway). Each sample contained one plant and in total eight plants were analyzed separately for each respective treatment. To each sample a 2 ml Eppendorf tube, one stainless steel bead (5mm in diameter) was added. Thereafter 600 µl MeOH was added to each vial and the samples were homogenized for 30s in centrifuge at 6500rpm (Retsch, Haan, Germany). The vials where placed in an ice bath for 15 min and thereafter centrifuged for 3 min at max speed 15 000 rpm.

The supernatant was pour into a marked reagent vial (10 mL size) and procedure was repeated for each sample 4 times, without the 15 min on ice bath, leaving the debris colorless.

After collecting of the extracts, the MeOH was evaporated by use of a SpeedVac (SAVANT SC210A, Thermo Scientific, Weaverville NC, USA) vacuum centrifuge (Eppendorf tubes 15 ml) and the dried extracts were dissolved in 200 µl MeOH with the help of an ultrasound bath, and diluted with 200 µl Millipore-water. The liquid extracts were thereafter transferred by Pasteur pipette to a 1.5 ml Eppendorf vial and centrifuged. Thereafter, the extracts were poured into HPLC vials, capped and analyzed on HPLC.

2.7.1 High-performance liquid chromatography (HPLC) analysis

Phenolic compounds were analyzed by HPLC (Agilent, Series 1100, Germany). The different metabolites were separated by use of a 50 x 4.6 mm ODS Hypersil column (Thermo Fisher Scientific Inc, Waltham, MA, USA). The samples were eluted (flow rate 2 ml min-1) using a MeOH: water gradient from X-Y% (Nybakken et al. 2012). The total injection volume was 20 µl, and the column temperature was 30 ˚C. The identification of the phenolic compounds was based on retention times, UV spectra and comparison with those of commercial standards.

The chromatogram peaks were used to measure quantity of phenolic acids and flavonoid compounds. The following formula was used:

$$\text{Quantity} = \frac{A \; x \; RF \; x \; V \; dissolved}{V \; injected \; x \; Weight \; mg}$$

A = Area under the peak

RF = Response Factor

$V_{dissolved}$= 200µl MeOH and 200µl Millipore-water used in order to solve plant material

$V_{injected}$ = amount of sample injected, here 20µl

Weight = dry leaf material (mg), here total plant without bolting stem

3.8 Statistical analyses

For the recorded growth parameters, two way analyses of variance were done using ANOVA general linear model in Minitab statistical software (Minitab 17, Minitab Inc, PA, USA) for effects of the two factors, genotype and treatment ($p \leq 0.05$). In order to detect differences between means, Tukey's test was used. Regression analysis (Minitab 16, Minitab INC, PA, USA) was used for analyses of effect of different gamma doses on time to visible flower bud.

4. RESULTS

4.1 Experiment 1: Effects of gamma radiation (21.6-39.6 Gy) on WT L*er* and *hy5*

4.1.1 Effect of ^{60}Co gamma radiation on number of rosette leaves

In order to investigate effects of different gamma radiation doses on leaf formation in *A. thaliana WT* L*er* and the *hy5* mutant, the number of rosette leaves was recorded (Figure 9 and 10) 19, 24 and 29 days after gamma irradiation. Statistical analysis done for day 29 showed some significant differences with the highest dose showing significantly lower leaf number than the control. However, overall, the number of rosette leaves between different gamma treatments did not vary systematically with gamma dose in any of the genotypes.

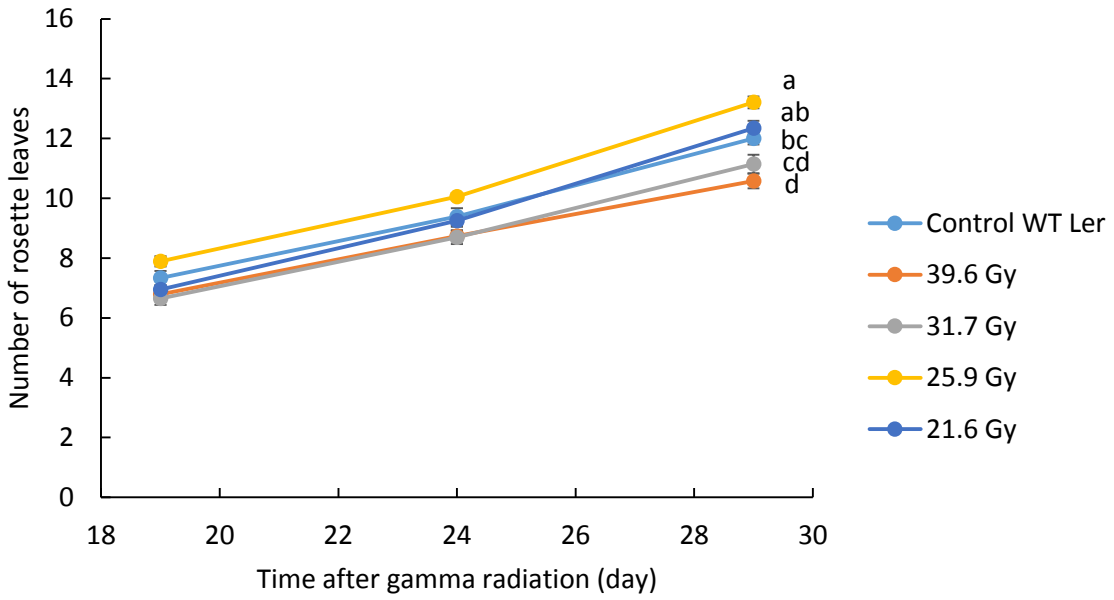


Figure 9. Number of rosette leaves in WT L*er A. thaliana* after exposure to ^{60}Co gamma radiation. Results are mean of 20 plants, ± SE for each treatment. Different letters indicate significant differences, while same letter showing no difference ($p \leq 0.05$).

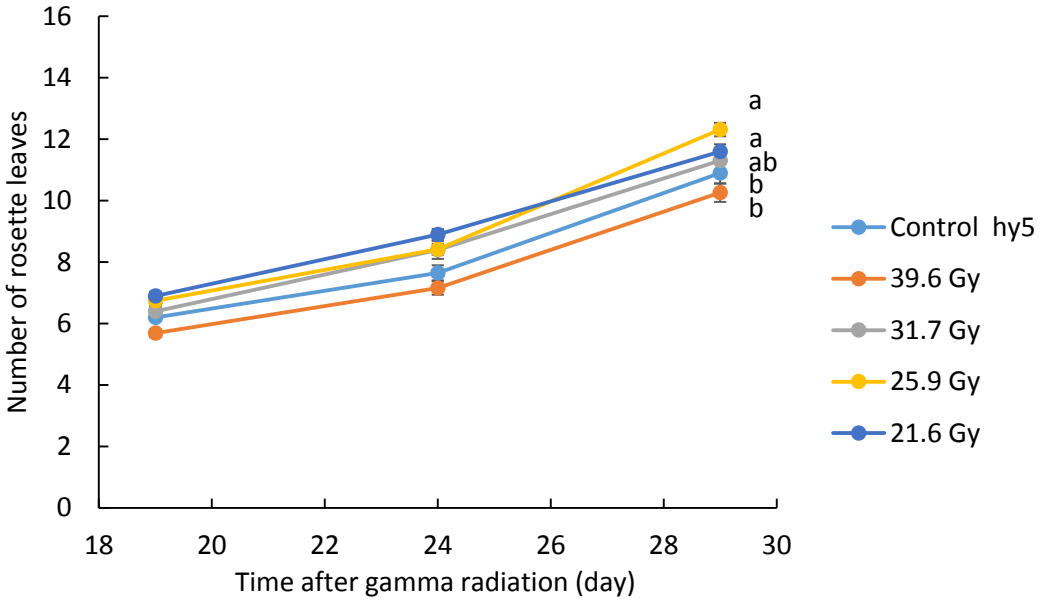


Figure 10. Number of rosette leaves in the *hy5* mutant in *A. thaliana* after ^{60}Co gamma radiation. Results are mean of 20 plants, ± SE for each treatment. Different letters indicate significant differences, while same letter show no difference ($p \leq 0.05$).

4.1.2 Effects of ^{60}Co gamma radiation on flower bud development

Number of rosette leaves at visible flower buds were recorded for *A.* thaliana WT and mutant *hy5* after gamma radiation (Figure 11). Statistical analysis exhibited small significant differences between control and the highest gamma treatments 39.6 and 31.7 Gy in *hy5* mutant. Control plants in *hy5* mutant exhibited higher amount of rosette leaves at visible flower buds (20%) compared to the highest gamma doses 39.6 and 31.7 Gy. On the other hand, WT plants did not show any major differences between different treatments.

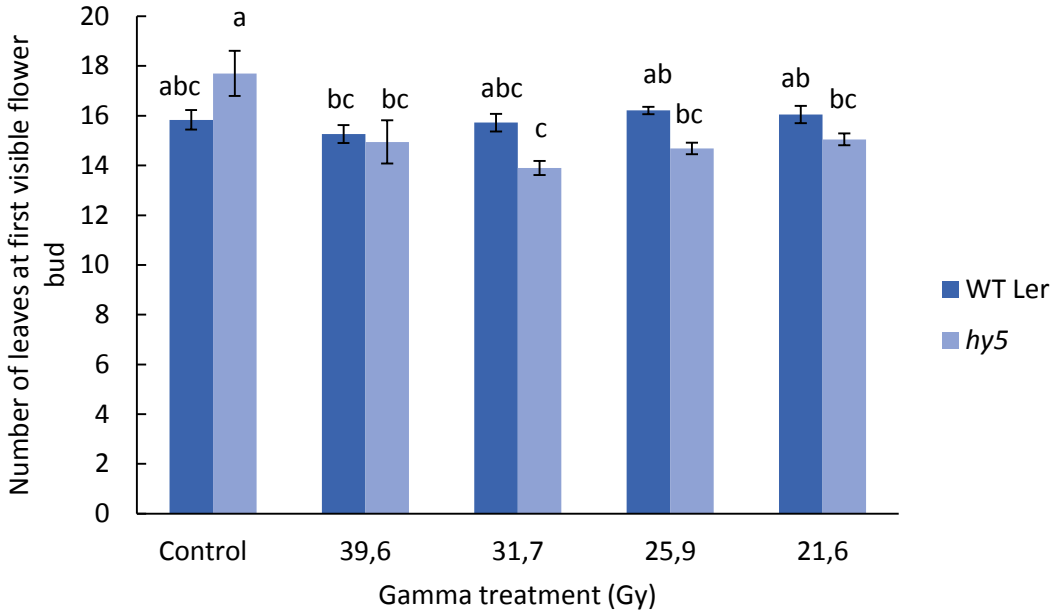


Figure 11. Number of rosette leaves at visible flower buds in *A. thaliana* WT and *hy5*. Results are mean of 20 plants, ± SE for each treatment. Different letters indicate significant differences, while same letters show no differences ($p \leq 0, 05$).

Percentage of flower buds in *A. thaliana* WT and mutant *hy5* was recorded at day 32, 35 and 38 after gamma radiation (Figure 12 and 14). In order to determine whether different gamma treatments exhibit significant differences, regression analysis for day 32 were done for both genotypes (Figure 13 and 15). The WT control then exhibited higher percentage of plants with flower buds (25%) compared to the highest gamma treatment (39.6 Gy; Figure 13). Since R-sq value for WT showed 29.6%, no major linear relationship between treatment and response. In mutant *hy5* no significant differences was observed between the control and the highest gamma treatments (Figure 15). R- sq value for *hy5* showed 11.9%.

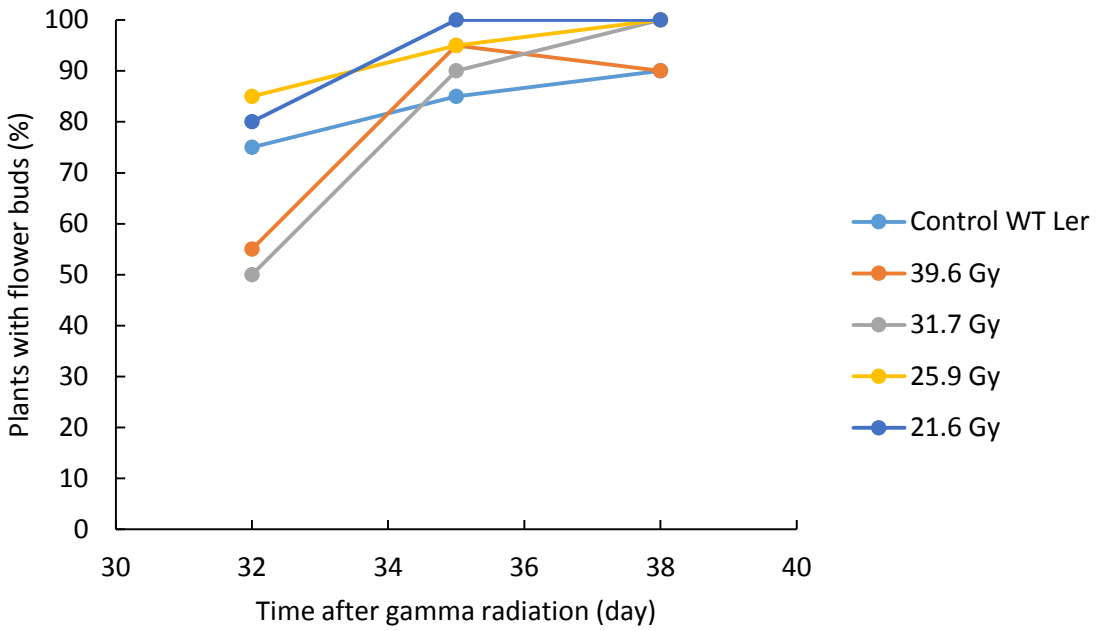


Figure 12. Number of rosette leaves at the first visible flower buds in *A. thaliana* WT after gamma treatment. Results are mean of 20 plants, ± SE for each treatment.

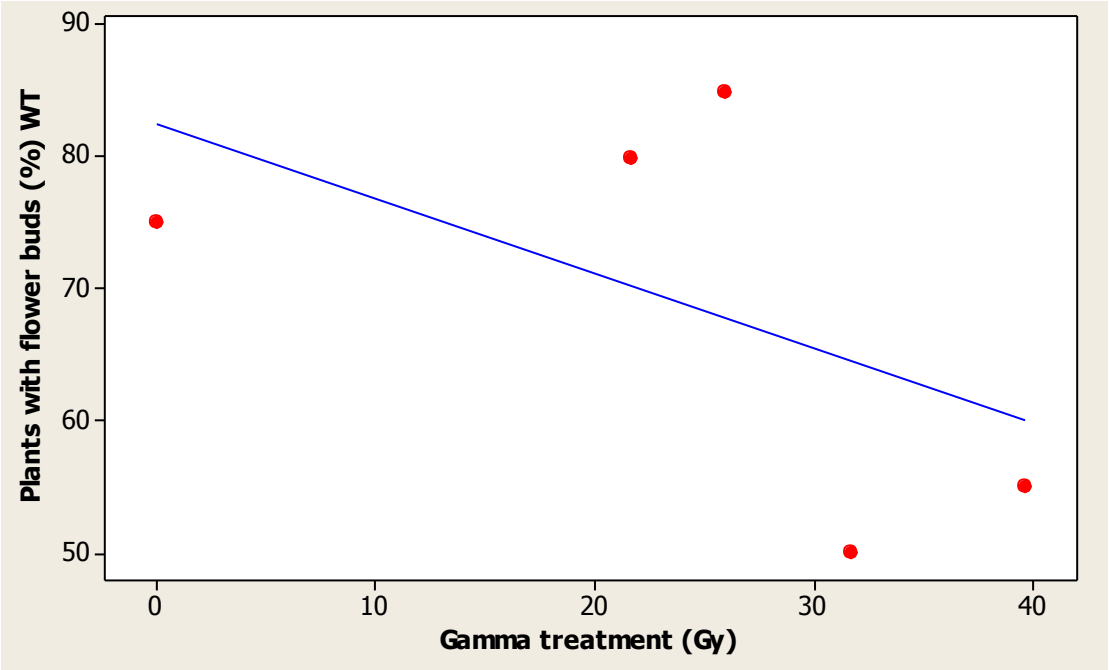


Figure 13. Regression analysis on plants with flower buds (%) in WT *A. thaliana* after gamma treatments. R-sq value: 29.6%.

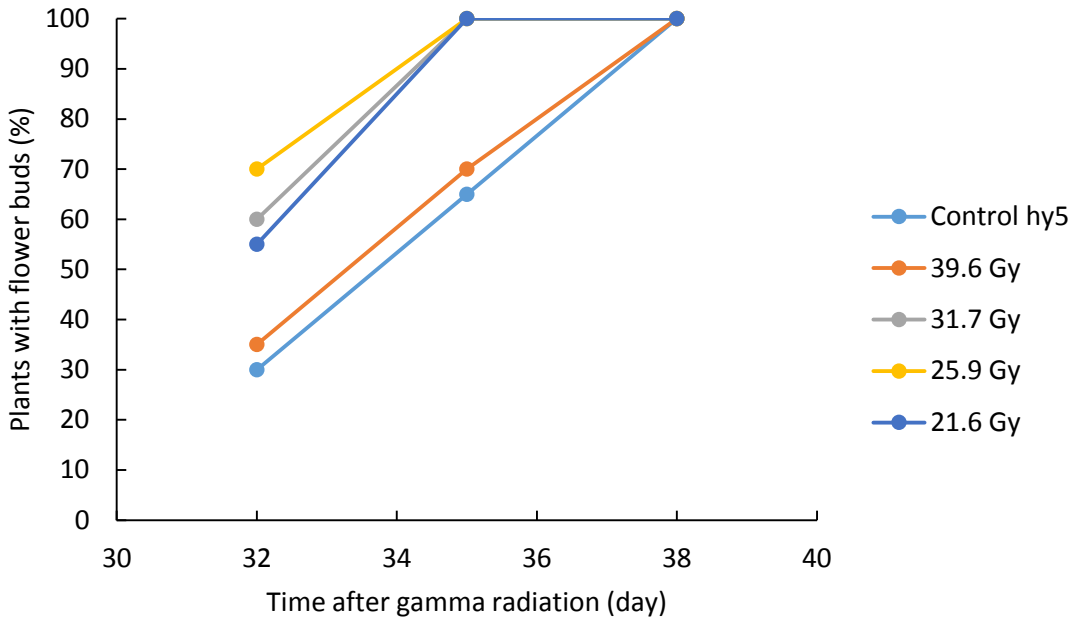


Figure 14. Number of rosette leaves at visible flower buds in *A. thaliana* mutant *hy5* after ^{60}Co gamma treatment. Results are mean of 20 plants, ± SE for each treatment.

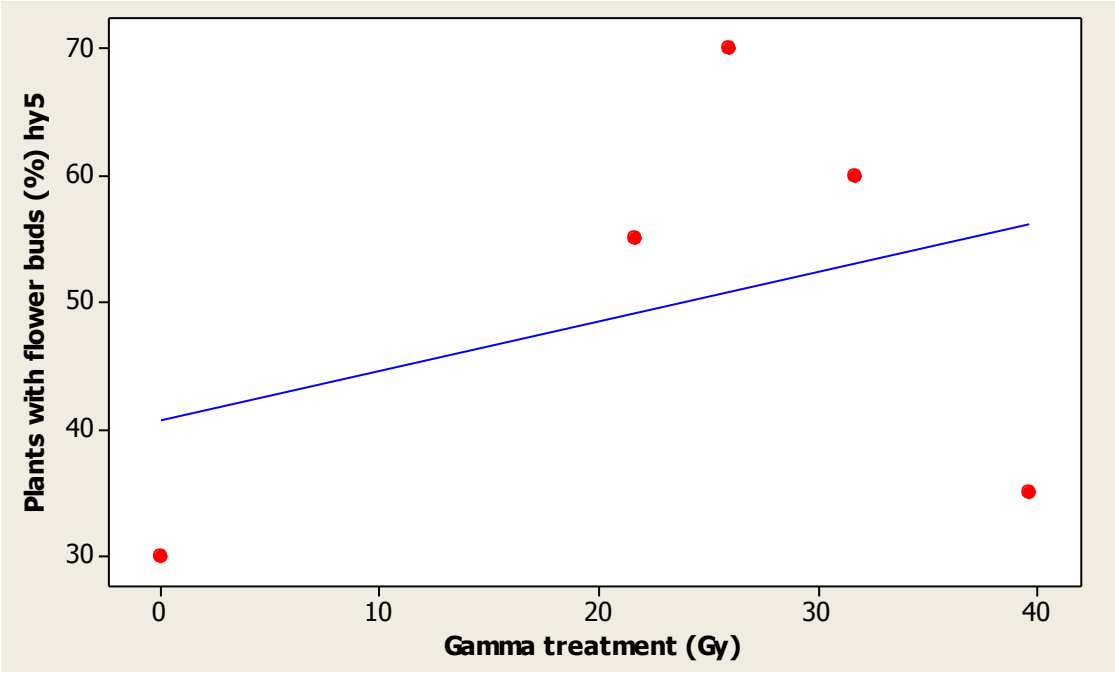


Figure 15. Regression analysis on plants with flower buds (%) in *hy5 A. thaliana* after gamma treatments. R-sq value: 11.9%.

4.1.3 Effects of ^{60}Co gamma radiation on bolting stem length

Final length of the bolting stem for both genotypes was measured 51 days after gamma exposure (Figure 16). Overall, there were no significant differences between the different gamma dose s and the control. For the *hy5* mutant the statistical test exhibited a significant difference between the lowest dose compared to the highest dose and control, but no relevant difference between the control and the highest dose (39.6 Gy).

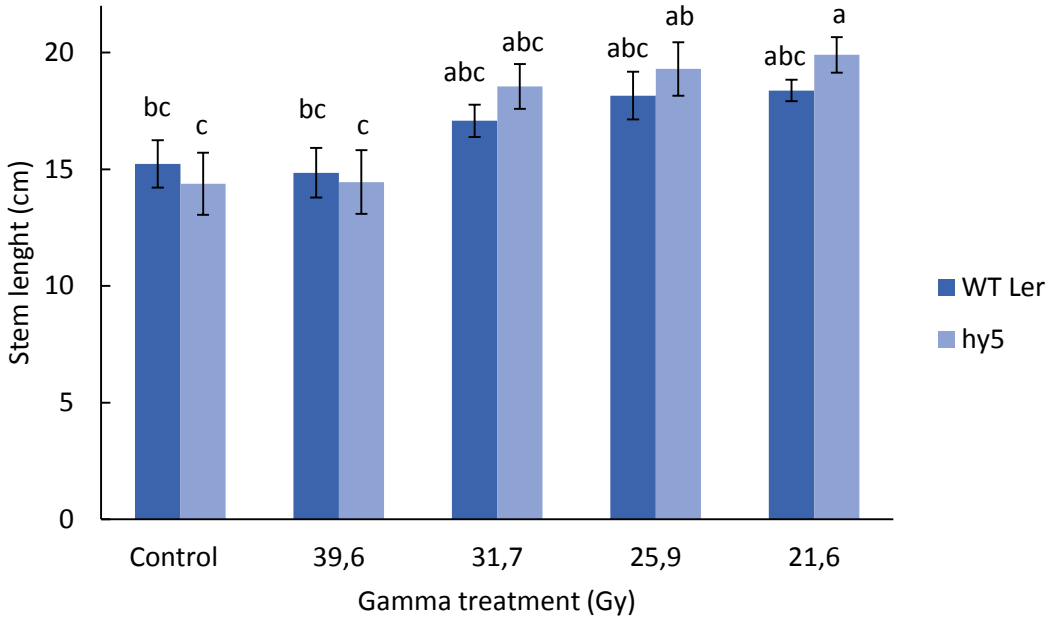


Figure 16. Final length of the bolting stem in *A. thaliana* WT and mutant *hy5* at 51st day after gamma radiation treatment. Results are mean of 20 plants with ± SE. Different letters indicate significant differences, while same letter denote no significant differences ($p \leq 0.05$).

4.2 Experiment 2: Effects of gamma radiation (30.2-90.7 Gy) on WT L*er* and *hy5*

4.2.1 Effect of ^{60}Co radiation on number of rosette leaves

Compared to the first experiment, the second experiment included gamma treatment with a longer exposure time, thus a higher gamma dose.

Comparing different gamma treatments with control in both genotypes (Figure 17 and 18), general linear model analysis showed no significant differences in number of rosette leaves at day 26 after gamma radiation.

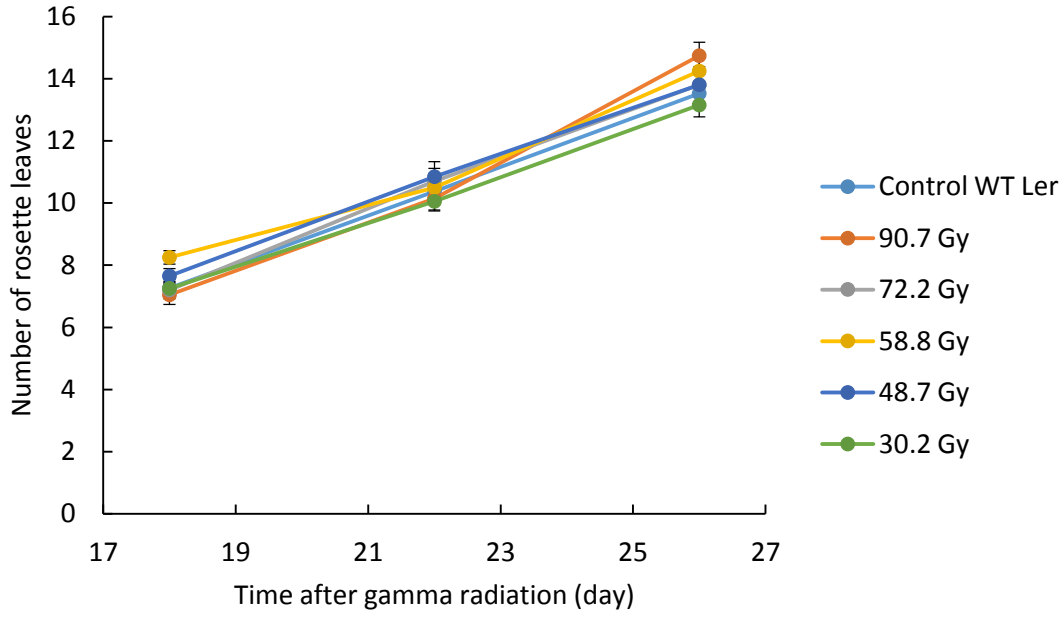


Figure 17. Number of rosette leaves in *A. thaliana* WT after different gamma radiation doses. Results are presented as mean of 20 plants, ± SE.

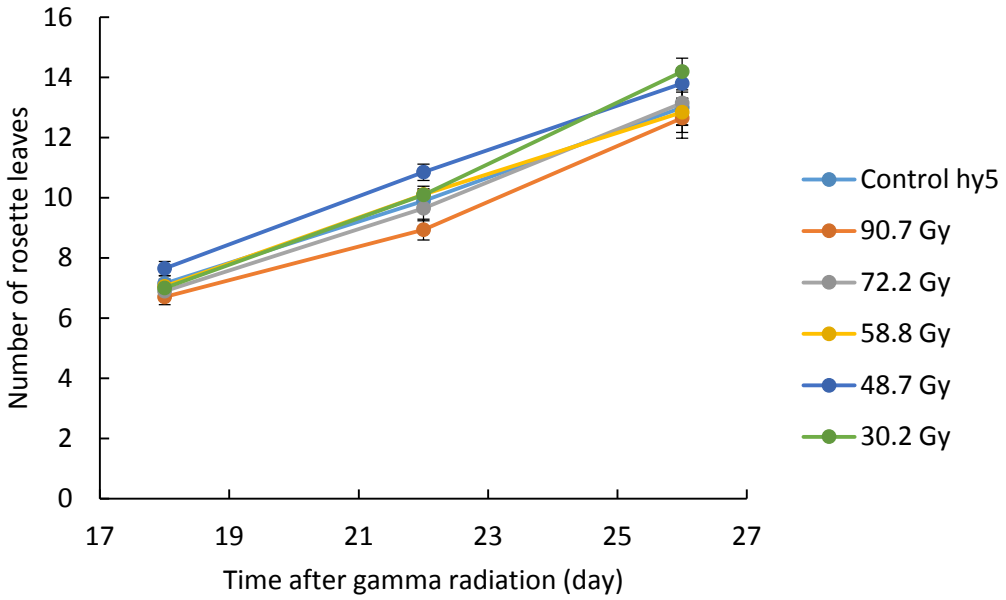


Figure 18. Number of rosette leaves in *A. thaliana* mutant *hy5* at day 18, 22 and 26 after gamma radiation. Results are mean of 20 plants with ± SE.

4.2.2 Effects of ^{60}Co gamma radiation on plant development at flowering stadium

Observations on number of rosette leaves at first visible flower buds were recorded after gamma treatment in *A. thaliana* WT L*er* and mutant *hy5* (Figure 19). By comparing control and the highest gamma dose (90.7 Gy) in each respective genotype, no significant differences were observed. On the other hand, comparing controls and the highest dose (90.7 Gy) between WT and *hy5* mutant, WT exhibited reduced number of leaves at first visible flower buds compared to *hy5*.

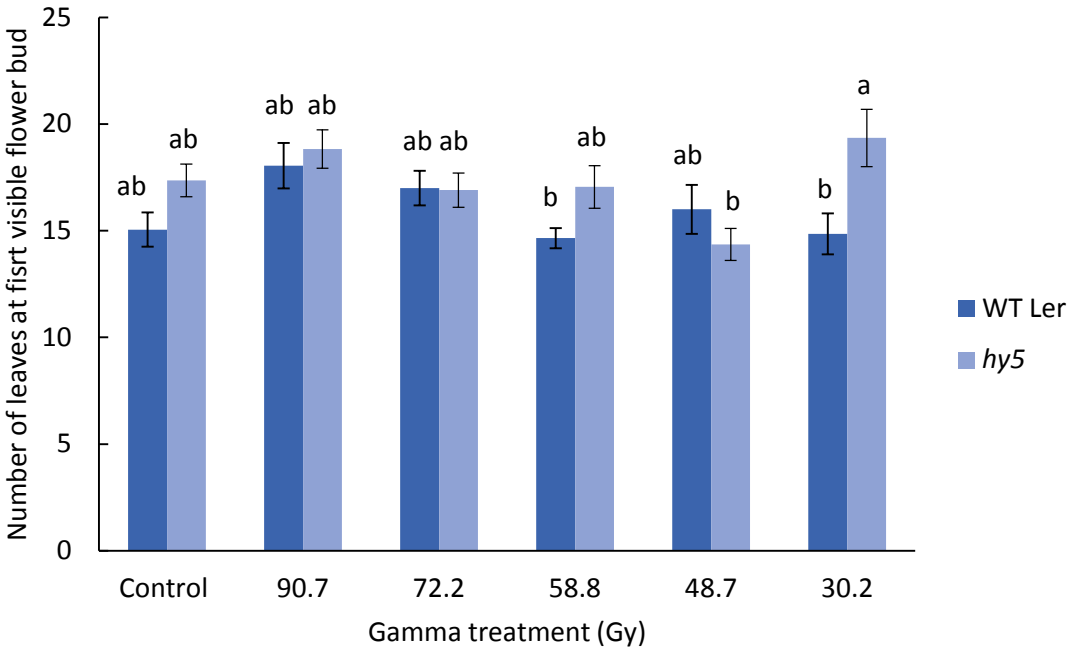


Figure 19. Number of rosette leaves at first visible flower buds in *A. thaliana* WT and mutant *hy5*. Results are showed as mean of 20 plants ± SE. Different letters indicate significant differences ($p \leq 0.05$).

Percentage of plants with flower buds were determined on day 26, 29 and 33 after gamma radiation for *A. thaliana* WT and mutant *hy5* (Figure 20 and 22). On 34th day after gamma radiation, pictures were taken of unradiated control plants and doses 90.7 and 48.7 Gy (Figure 24). Regression analysis for day 29 were done in order to investigate the gamma dose response relationship for each respective genotype (Figure 21 and 23). For the WT (Figure 20), the two highest doses, 90.7 Gy and 72.2 Gy differed significantly from the control at day 29. Ca 90% of the control plants had flower buds at day 29, while plants exposed for 90.7 Gy and 72.2 Gy had less number of plants with flower buds, around 40% (Figure 21). Generally, it appeared that flowering was delayed with increasing gamma dose (R-sq at 73.4%). The *hy5* mutant showed lowest percentage of plants at visible flower buds in treatment 90.7 Gy (Figure 22). In comparison with the *hy5* control (ca 30% plants with flower buds), no systematic difference between the gamma doses was observed. However, for the other gamma treatments, picture was less clear than for the WT since the control exhibited second lowest percent of plants with flower buds (about 30 %) at day 29 after radiation.

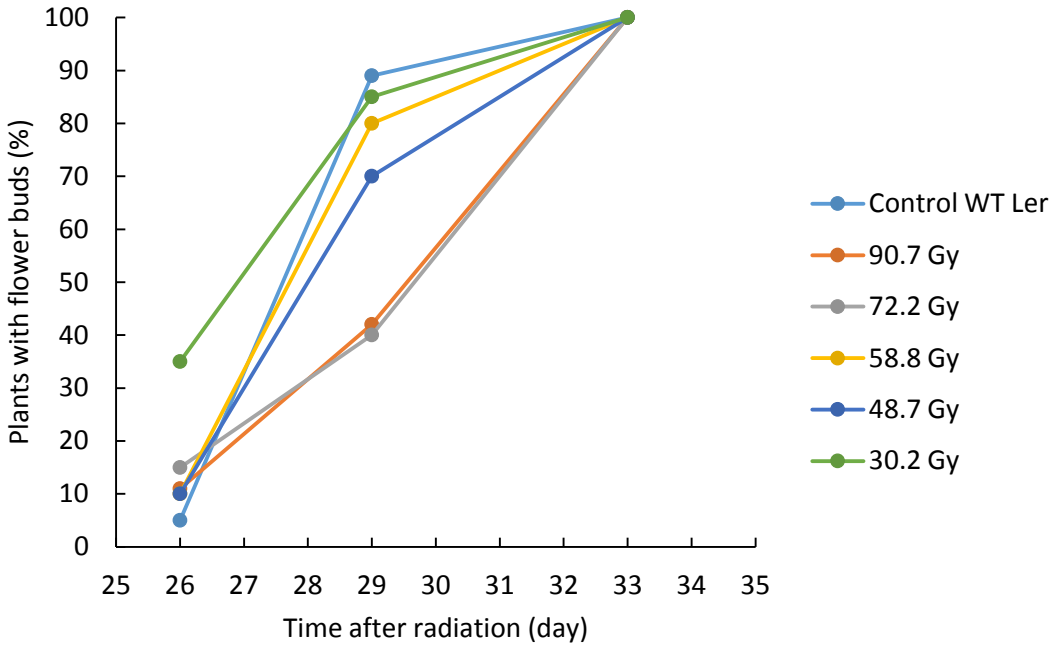


Figure 20. Percent *A. thaliana* WT L*er* plants with flower buds (%) after gamma exposure. Results are mean of 20 plants from each treatment.

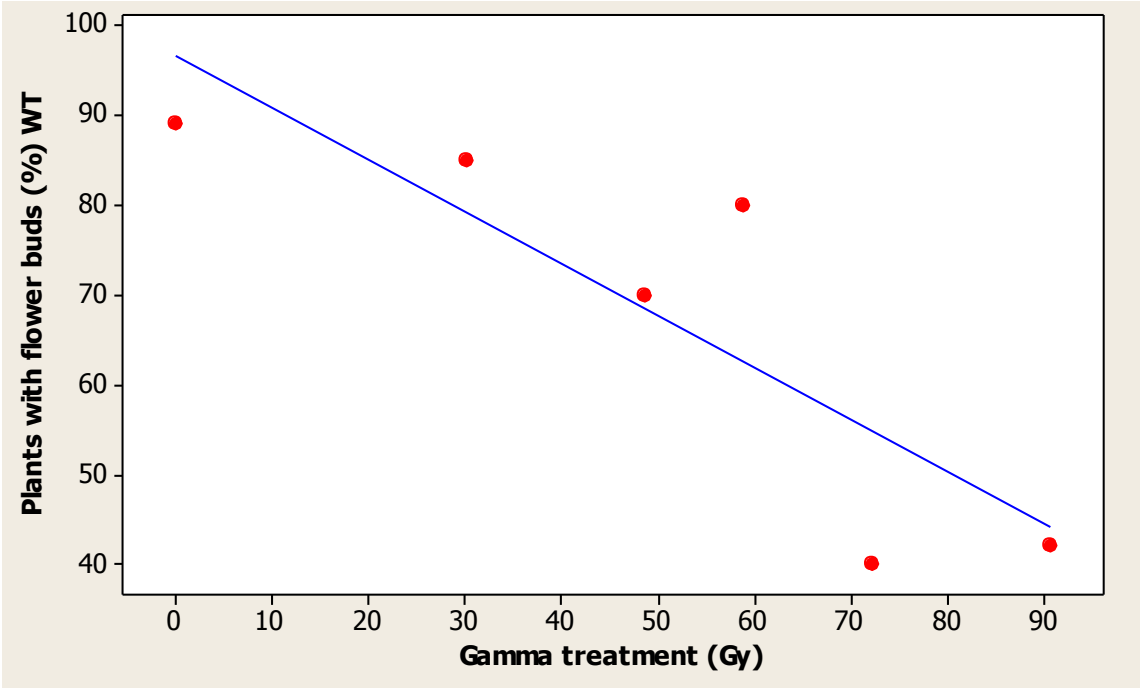


Figure 21. Regression analysis for *A. thaliana* WT L*er* at day 29 after gamma exposure. R-sq value: 73.4%.

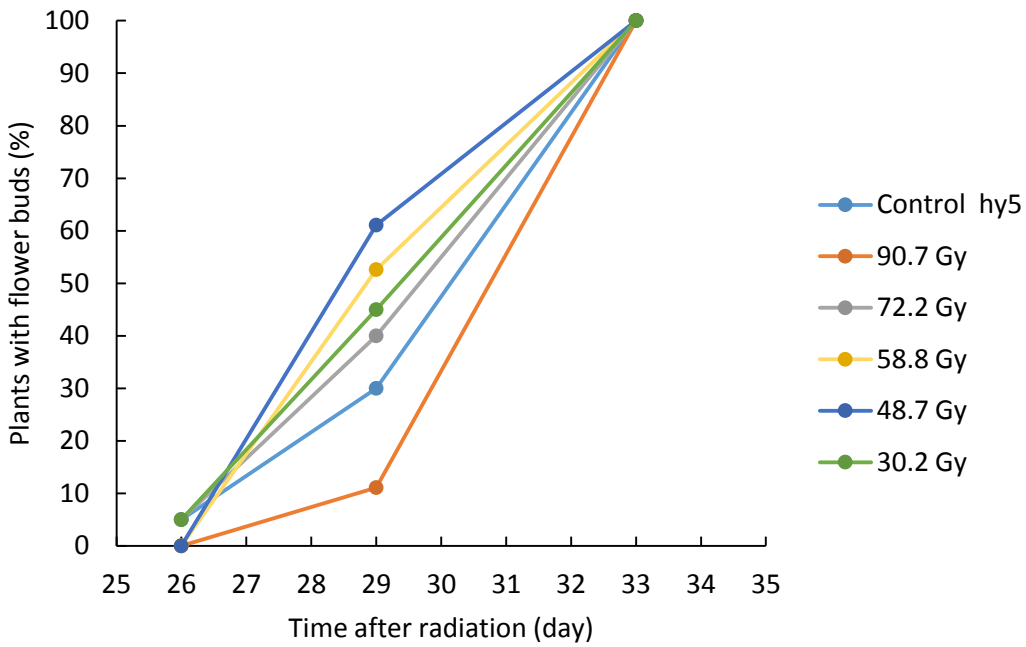


Figure 22. Percent *A. thaliana hy5* mutant plants with flower buds (%) after gamma exposure. Results are mean ± SE of 20 plants from each treatment.

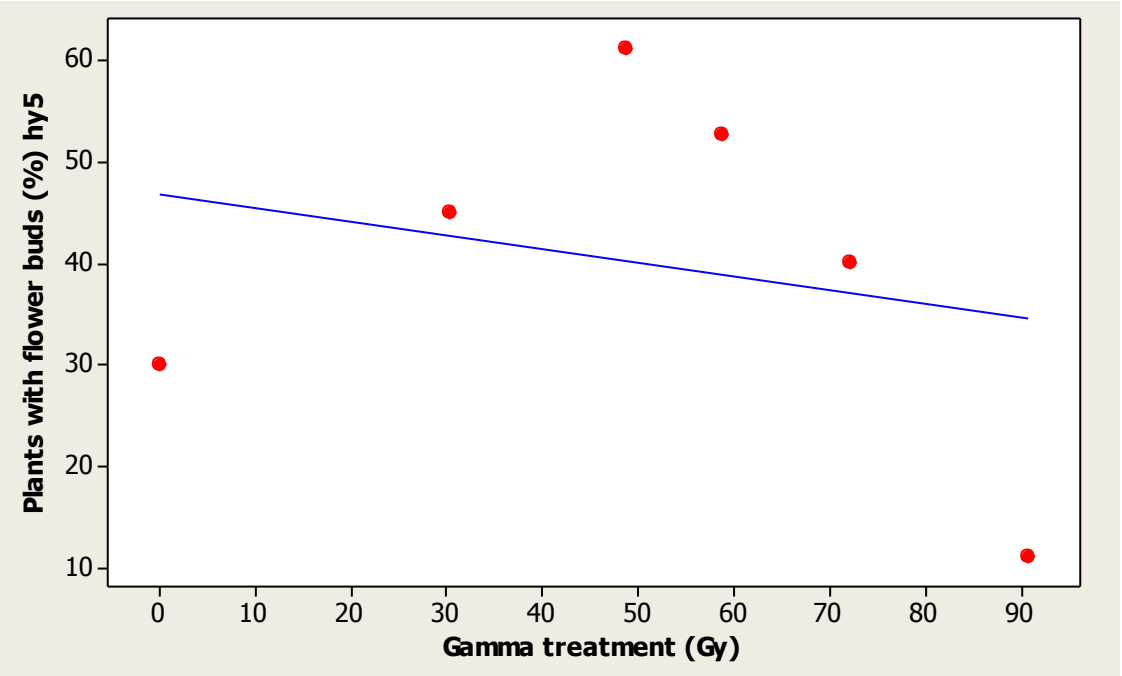


Figure 23. Regression analysis for *A. thaliana* hy5 at day 29 after gamma exposure. R-sq value: 6%.

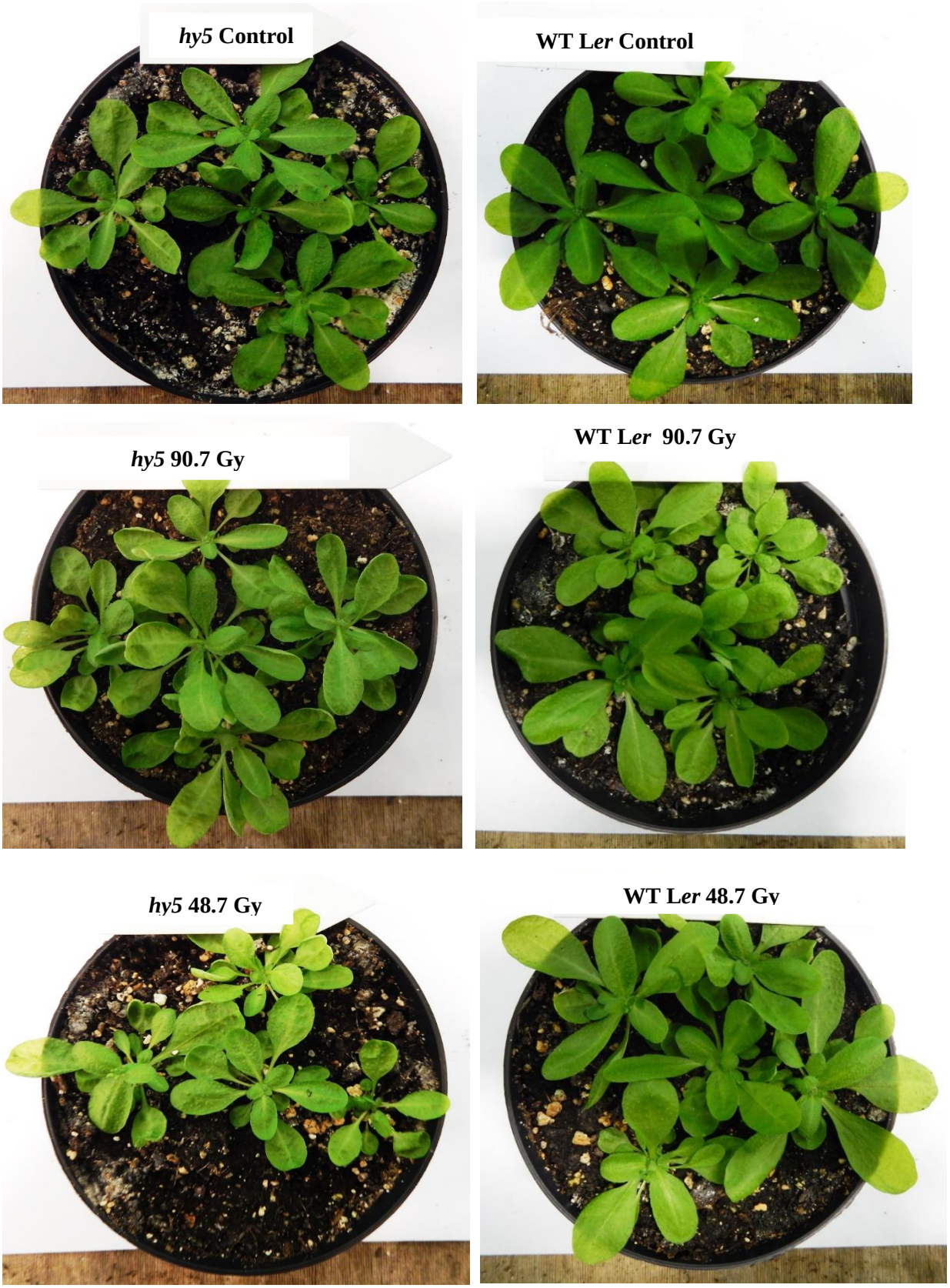


Figure 24. *A. thaliana hy5* mutant and WT 34 days after gamma treatment. Each pot contains five plants and represent specific gamma treatment.

4.2.3 Effects of ^{60}Co gamma radiation on bolting stem length

To investigate the effect of gamma radiation on length of the bolting stem in *A. thaliana* WT and mutant *hy5*, total length of the stem was measured 51 day after gamma radiation (Figure 25). Pictures of the unradiated control and respective doses: 90.7, 72.2, 58.8, 48.7 and 30.2 Gy were taken for both genotypes (Figure 26).

In WT length of the bolting stem was significantly shorter (35%) for the highest gamma dose (90.7 Gy) compared to the control, whereas the other doses did not differ significantly from the control. For the *hy5* mutant no significant differences were observed.

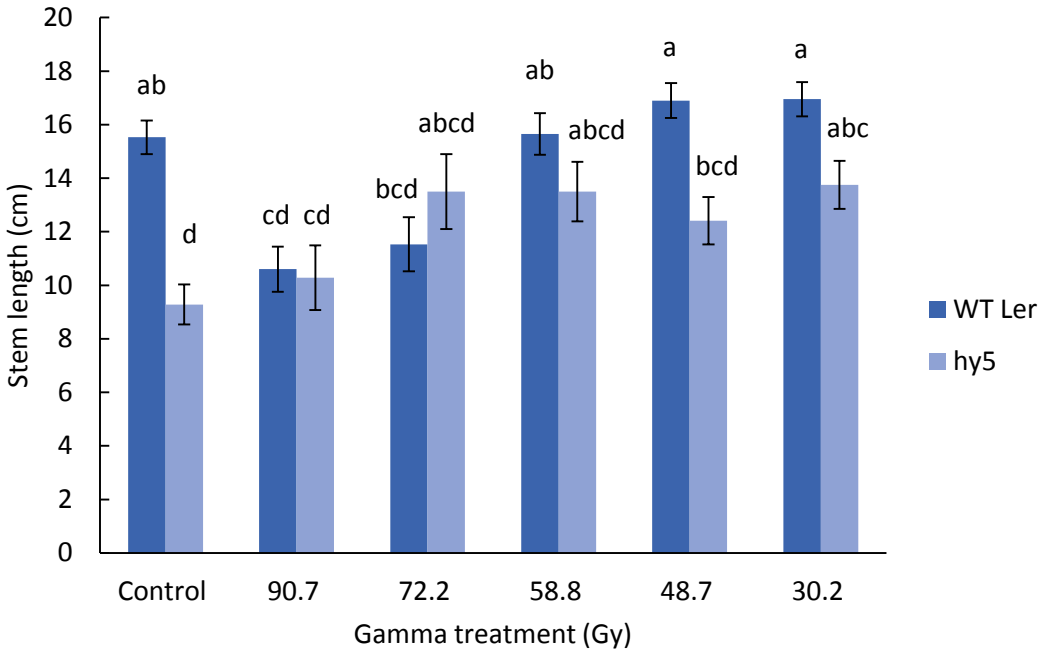
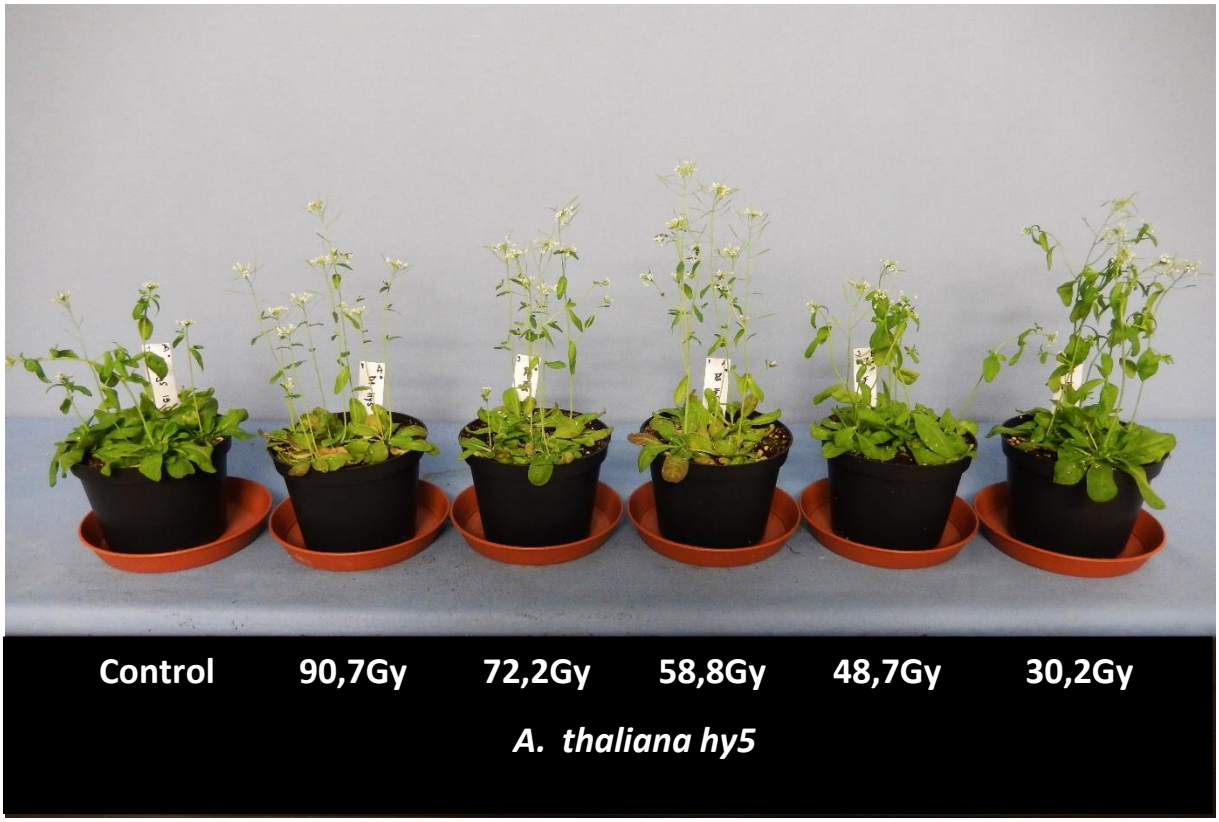


Figure 25. Length of the bolting stem for *A. thaliana* WT L*er* and mutant *hy5*. 51 day after gamma exposure. Results are presented as a mean of 20 plants with SE. Same letters represent no significant differences, while different letters differ significantly ($p \leq 0.05$).



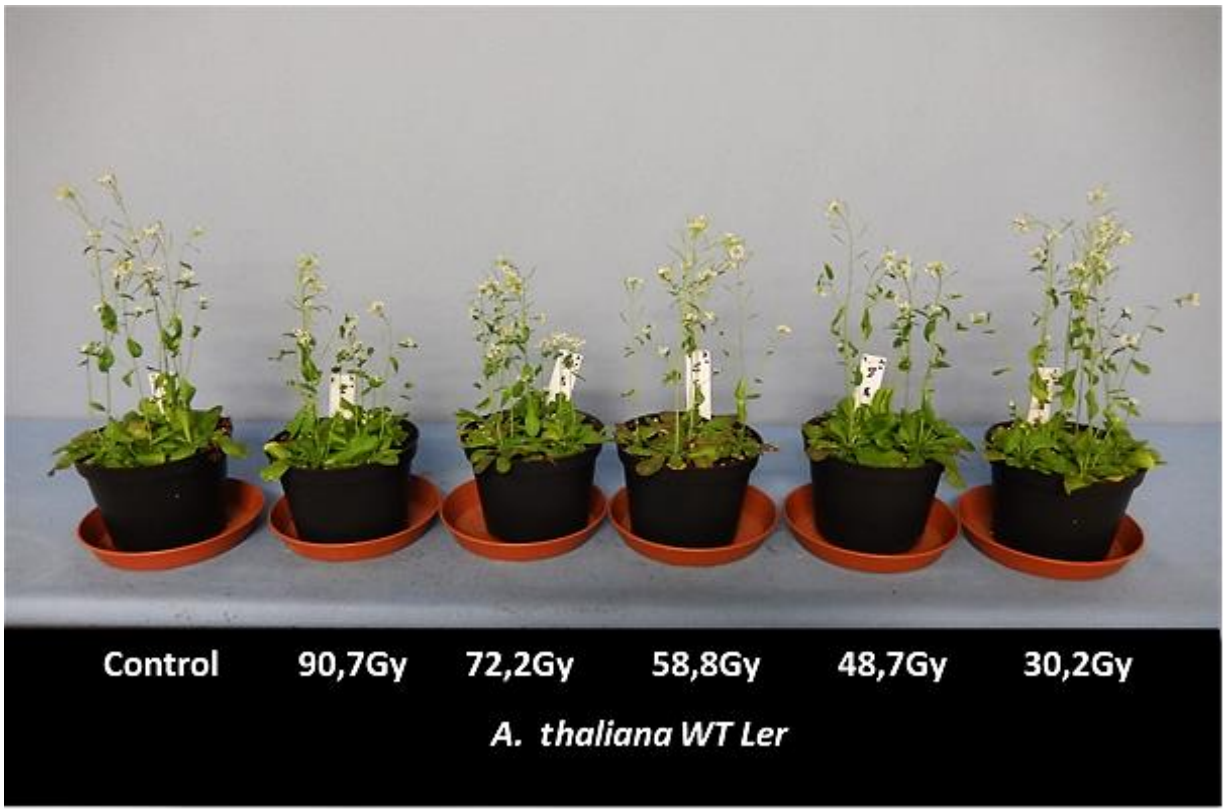


Figure 26. *A. thaliana* WT L*er* and *hy5* 51 days after gamma exposure. Each pot contains five plants.

4.2.4 Effect of ^{60}Co gamma radiation on leaf size

Leaf size (length and width) of lead 6 (fully extended leaf) counted from the base of the plant, was measured 51 days after gamma treatment on *A. thaliana* WT and mutant *hy5* (Figure 27 and 28). Lamina and petioles measurements gave a total leaf length. In the WT leaf width was slightly, but significantly lower at the highest gamma dose compared to the control (Figure 27). The *hy5* mutant exhibited significant reduction in leaf length at the two highest gamma doses compared to the other gamma treatments and control (Figure 28).

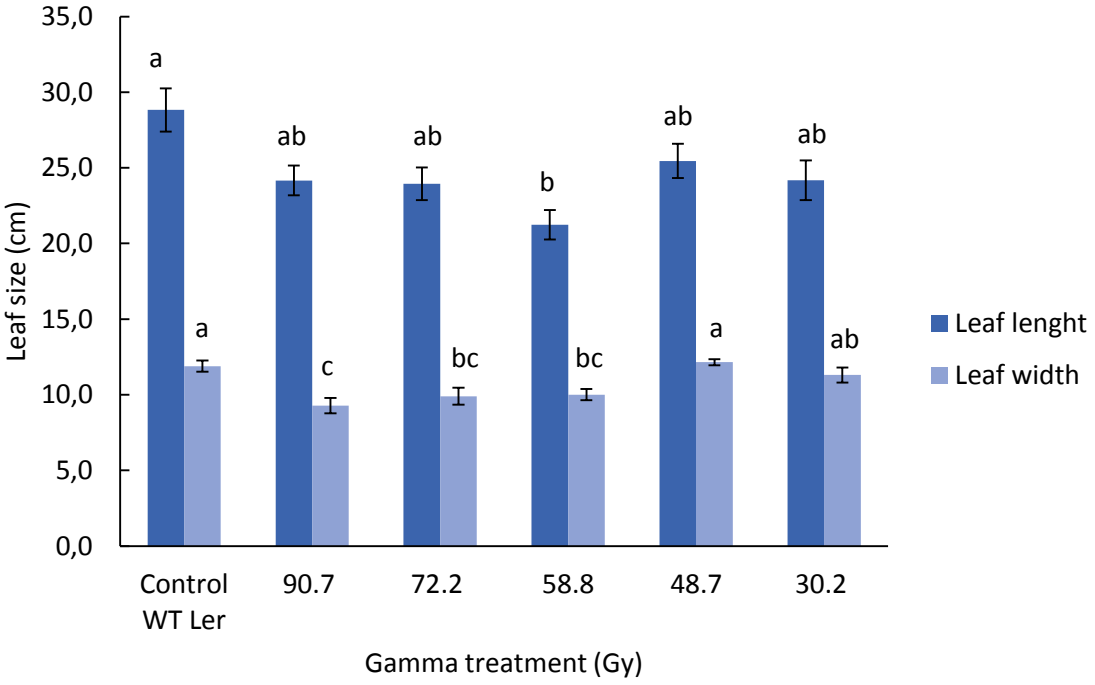


Figure 27. Leaf size (leaf length and –width) of *A. thaliana* in WT L*er*. Results are represented as a mean of 20 ±SE. Same letters show no significant differences, while different letters differ significantly ($p \leq 0.05$).

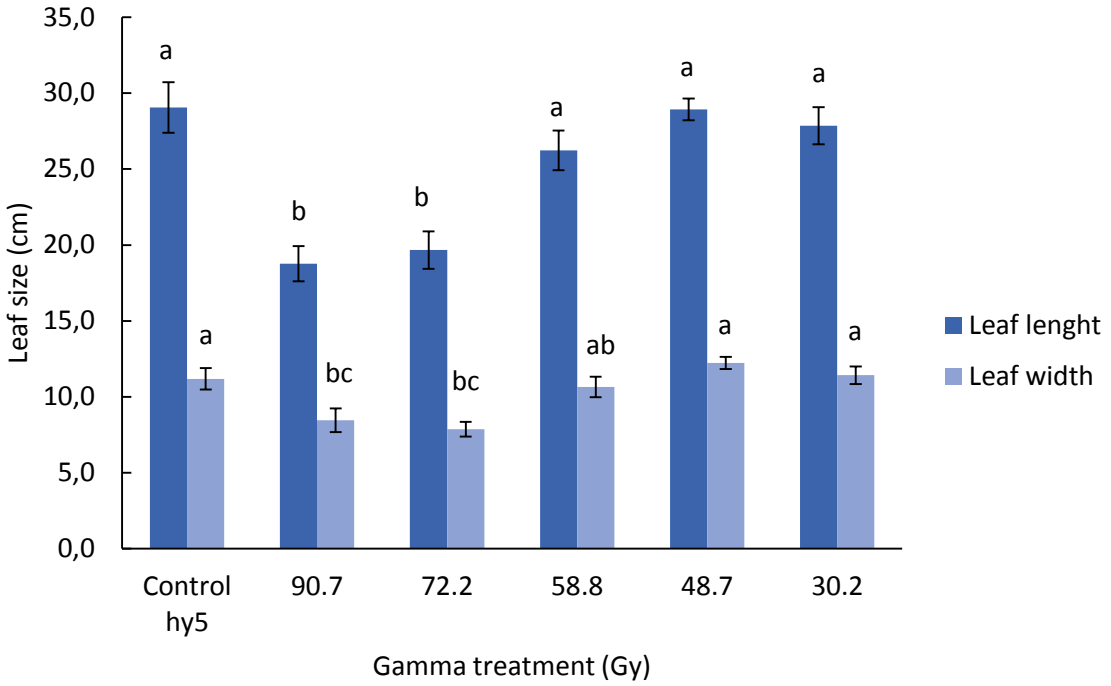


Figure 28. Leaf size (leaf length and –width) of *A. thaliana* in mutant *hy5*. Results are represented as a mean of 20 plants ±SE. Same letters show no significant differences, while different letters differ significantly ($p \leq 0.05$).

4.2.5 Effect of ^{60}Co Gamma radiation on relative gene expression: *TT4* and *RAD51*

Relative gene expression of *TRANSAPRENT TESTA* (*TT4*) and *RECOMBINASE RAD51* (*RAD51*) was analyzed by RT-PCR in the *A. thaliana* WT and *hy5* from experiment 2 (Figure 29 and 30). Plants tested for relative gene expression were exposed to the following treatments: 90.7 Gy, 72.2 Gy, 48.7 Gy and control.

No significant differences in transcript levels of *TT4* between different gamma treatments and control for any of the genotypes were observed, only a slight trend of increased expression with increasing gamma dose in the WT (Figure 29). On the other hand, relative expression level of *RAD51* (Figure 30) showed significant difference (70-50%) in the WT between the control and the highest gamma doses, 90.7 Gy and 72.2 Gy. In the *hy5* mutant, no significant differences in *RAD51* transcript levels were observed.

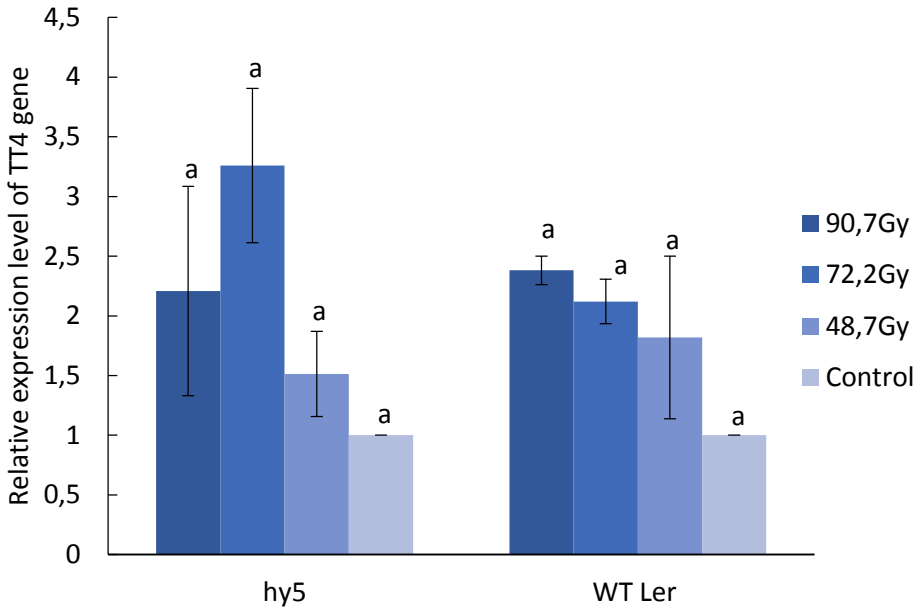


Figure 29. Relative expression level of the *TT4* gene in *A. thaliana* after gamma exposure of genotypes WT and *hy5*. Results are presented as mean of three samples consisting of three plants each. The transcript levels were normalized against actin and thereafter against the control within each genotype. All samples were analyzed in triplicate. Same letters represent no significant differences, while different letters differ significantly ($p \leq 0.05$).

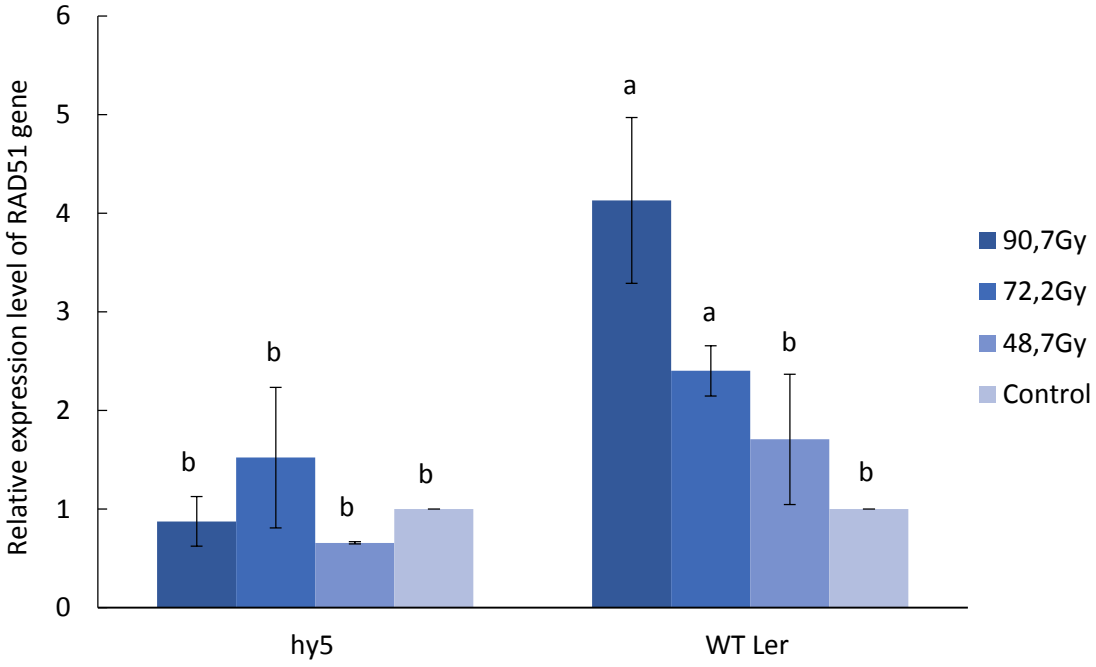


Figure 30. Relative expression level of the *RAD51* gene in *A. thaliana* after gamma exposure of genotypes WT and *hy5*. Results are presented as mean of three samples consisting of three plants each. The transcript levels were normalized against actin and thereafter against the control within each genotype. All samples were analyzed in triplicate. Same letters represent no significant differences, while different letters differ significantly ($p \leq 0.05$).

4.2.6 Effect of ^{60}Co gamma radiation on phenolic acids and flavonoid biosynthesis

HPLC analysis of phenolic acids and flavonoids was performed in the *A. thaliana* WT and *hy5* mutant for the gamma dose s: 90.7 Gy, 72.2 Gy, 48.7 Gy and control (Figure 31 and 32). Sum of the concentration of the phenolic acids, presented in Figure 31, indicates significant difference between control and the highest gamma treatment (90.7 Gy) in the WT. WT plants exposed to the highest gamma treatment, displayed 20% higher content of phenolic acids than control. For the *hy5* mutant there was a similar trend, although not statistically significant. The total flavonoid level, displayed in Figure 32, also differed significantly between the genotypes. The *hy5* mutant exhibited lower levels (50%) of flavonoids compared to the WT. In addition, by comparing amount of flavonoids only in WT, the control exhibited significantly higher concentration (25%) of flavonoid compounds compared to the highest gamma treatment (90.7 Gy).

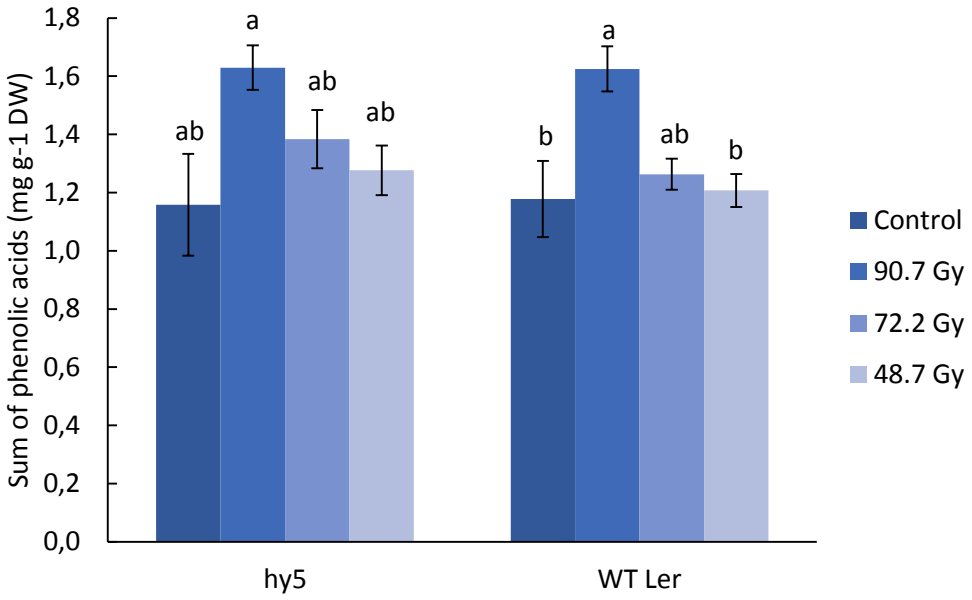


Figure 31. Sum of phenolic acids in *A. thaliana* WT and mutant *hy5*. Results are mean of eight samples each containing one plant ± SE. Equal letter are showing no significant differences, while different letters differ significantly ($p \leq 0.05$).

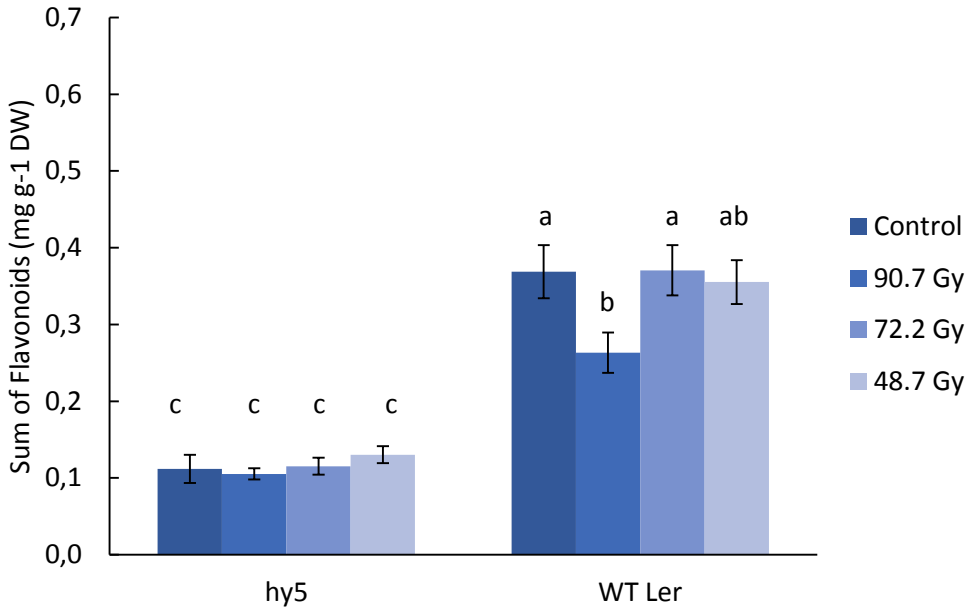


Figure 32. Sum of flavonoids in *A. thaliana* WT and mutant *hy5*. Results are mean of eight samples, each containing one plant ± SE. Equal letter are showing no significant differences, while different letters differ significantly ($p \leq 0.05$).

We identified 14 different phenolic compounds. The peak number shows specific phenolic compounds (Table 3). We identified four kaempferol-glycosides, the rest were tentatively identified as phenolic acids. With the equipment and standards available, further specification of phenolic acids was not possible.

Table 3. Different phenolic compounds detected at peak number by using HPLC method.

Peak number	**Compound**
1	Phenolic acid
2	Phenolic acid
3	Phenolic acid
4	Phenolic acid
5	Phenolic acid
6	Phenolic acid
7	Kaempferol-3galactoside
8	Kaempferol-3glucoside
9	Phenolic acid
10	Phenolic acid
11	Phenolic acid
12	Kaempferol-3arabinoside
13	Phenolic acid
14	Kaempferol-3rhamnoside

4.2.7 Effects of ^{60}Co gamma radiation on PSII system

Potential efficiency of the PSII system was measured for *A. thaliana* WT and the *hy5* mutant 28 days after exposure to the gamma doses: 90.7 Gy, 72.2 Gy, 58.8 Gy, 48.7 Gy, 30.2 Gy and control (Figure 33). In the *hy5* mutant, a small significant reduction of Fv/Fm was observed at the highest gamma dose (90.7 Gy) as compared to the control and the other treatments. In the WT, there were no significant differences between the treatments. In addition, genotypes differ significantly with the WT showing slightly higher Fv/Fm values compared to *hy5*.

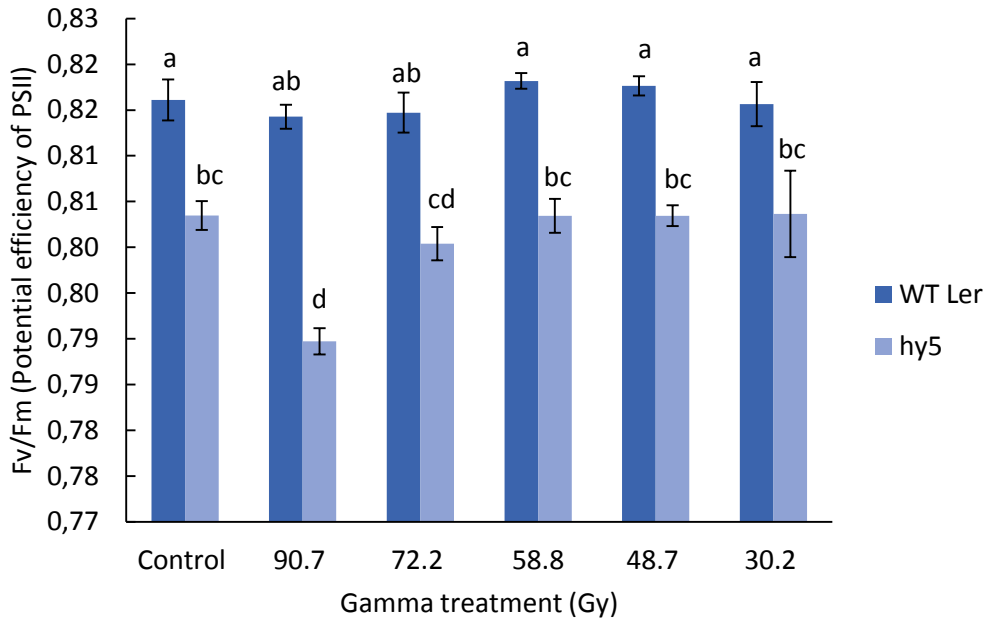


Figure 33. Potential efficiency of PSII in *A. thaliana* WT L*er* and mutant *hy5*. Results are represented, as mean of 35 plants ± SE. Equal letter are showing no significant differences, while different letters differ significantly ($p \leq 0.05$).

5. DISCUSSION

5.1 Effects of low gamma radiation

In order to examine dose-response relationship between gamma irradiation and morphological, physiological and molecular traits in *A. thaliana*, seedlings exposed to different gamma treatments. Two experiments were done, with the first experiment involving gamma radiation from 39.6 to 21.6 Gy (gamma exposure 72 h), and the second experiment involving gamma dose from 90.7 to 30.2 Gy (gamma exposure 168 h). The experiments involved two genotypes: WT L*er* and mutant *hy5*. After-effects of the gamma radiation on several parameters were tested: formation of rosette leaves, leaf size parameters, length of the inflorescence (bolting) stem, formation of flower buds, expression of *RAD51* and *TT4* genes and phenolic acid levels. ROS staining after gamma exposure was also performed.

5.2 Effects of gamma radiation on rosette leaf formation, flowering stage and bolting stem length

As mentioned earlier whether a plant gets affected by gamma rays depends on several factors such as species, plant age, cultivars, physiology and plant genome (De Micco et al. 2011). In our study, the number of rosette leaves after gamma exposure did not show any high differences between genotypes, nor between different gamma treatments in both experiment 1 (Figure 9 and 10) and 2 (Figure 17 and 18). This may be due to the plant's high robustness. At which growth stage a plant is exposed, probably plays a crucial role, and may be at least partly due to differences in water content at different development stages. As an example, a developing and a germinating seed is more sensitive to gamma radiation than a dry mature seed since water content is higher and embryo is easier to reach by the structures affecting ion capacity (Qin et al. 2007). Generally, gamma rays have a larger effect on an organism if it contains higher percentage of water, because primary reaction occurs by attacking water molecules (Figure 1). In experiment 1 on number of rosette leaves at first visible flower bud, statistical analysis exhibited small significant differences between control and the highest gamma treatments 39.6 and 31.7 Gy in *hy5* mutant. Thus, control plants in *hy5* mutant exhibited higher amount of rosette leaves at visible flower buds (20%) compared to the highest gamma doses 39.6 and 31.7 Gy, while WT plants did not show substantial differences between different gamma treatments. Also significant differences between WT and hy5 were not observed (Figure 11).

Experiment 2 thereby did not show significant differences in number of rosette leaves at first visible flower bud between different gamma treatments in WT and *hy5*. While comparing controls and the highest dose (90.7 Gy) between WT and *hy5* mutant, WT exhibited reduced number of leaves at first visible flower buds compared to *hy5* (Figure 18). Similar results are also observed in experiment 1. However, Wi et al. 2007, showed that after gamma treatment *A. thaliana*, chloroplasts in the cortical cells in the stem cell were damaged after 50 Gy gamma treatment. They also indicated that thylakoids were swollen and deformed, while mitochondria and nuclei after the same gamma exposure did not differ from the control. Organelle integrity and function was not investigated in our study, but might possibly at least to a certain extent explain differences in leaf size parameters.

The percentage of plants with flower buds in experiment 2 exhibited differences in the WT at day 29 after gamma treatment. Ca 90% of the control plants then had flower buds, while plants exposed to 90.7 Gy and 72.2 Gy had lower number of flower buds, with around 40% of the plants having flower buds (Figure 21). Thus in the WT the formation of flower buds seemed to be delayed with increasing dose of gamma treatment, compared to the control, which indicates that gamma treatment had impact on transition to reproductive development in WT plants.

Previous studies showed that gamma rays induced leaf trichome formation in *A. thaliana* but after a high gamma radiation: 1-3 kGy (Nagata, 1999). Vandenhout et al. 2010, showed that root fresh weight decreased in response to gamma irradiation (2336, 367 and 81 µGy h^{-1}). In addition, they showed that leaf and stem fresh weight were significantly reduced at the highest gamma treatment (2335 µGy h^{-1}) after 54 days of exposure. In our study leaf size did not differ significantly between overall in both genotypes between the highest gamma irradiation level and control (Figure 27 and 28). However, the *hy5* mutant exhibited reduction in leaf length (40 %) at the highest gamma treatment (90.7 Gy) compared to the *hy5* control. In contrast, WT plants only showed a slight, significant reduction in leaf width at the highest gamma dose. Previous studies on *Z. mays* after the Chernobyl accident showed that older leaves accumulated higher amount of radioactive elements and showed more damage (Sawidis, 1988). However, very young *A. thaliana* seedling like those in our study (3 days old), appears highly resistant to gamma radiation as the plants later did generally not show substantial differences in size parameters between different gamma treatments.

Our observations on the length of the bolting stem in experiment two, revealed only some differences while comparing control and the highest gamma treatment (90.7 Gy) in WT. Results showed a reduction of the stem length in dose 90.7 compared to the control.

Differences between the other gamma treatments could not be observed in the WT, and no differences in inflorescence stem length between the gamma doses were observed for the *hy5* mutant.

5.3 Effects of gamma radiation on relative gene expression: *RAD51* and *TT4*

Studies of relative gene expression of *A. thaliana* at 51 day after gamma treatment showed increased transcript levels of *RAD51* gene in the WT L*er*. (Figure 30). The *RAD51* expression increased with increasing gamma level and was substantially increased 70 % in plants exposed to the highest gamma treatment 90.7 Gy, compared to control. Earlier studies from Culligan et al. 2006, also showed high induction of *RAD51* within 8 h after gamma irradiation at 100 Gy in A. thaliana. Since gamma rays involve double strand break, *RAD51* plays a major role in plant response. Our study shows that the RAD51 up-regulation in *A. thaliana* can persist for a long time after the gamma exposure (51 days).

RAD51 works as a sensor (Figure 3), that recognizes gamma rays and further enhance a pathway, by enhancing certain genes, which involve genes involved in DNA repair or incomplete DNA replication. Gicquel et al. 2012, showed transcriptional regulation of the *RAD51* gene, which increased after only 2 h of gamma exposure at 10 Gy and 40 Gy. These results indicated induction of *RAD51* already after shorter period of gamma treatment, as well as exposure to a lower dose compared to our results. *AtRAD51* transcript level increased also after gamma treatment using a ^{137}Cs source (Doutriaux et al. 1998). Since *RAD51* enhances protein ATM, some studies showed that *atm* mutants are highly sensitive to gamma rays (Garcia et al. 2003). Kovalchuk et al. 1998, identified increased HR in *A. thaliana* after Chernobyl accident, which induced double strand breaks. However, expression of *RAD51 A. thaliana* under the highest gamma treatments in our studies, implies this protein as an essential factor in DNA repair. This is also in accordance with previous researches.

On the other hand, the *TT4* gene, involved in flavonoid synthesis, did not show significant differences between genotypes nor between different gamma treatments, only slightly trends of increased expression with increasing gamma dose. There was also no significant different pattern in TT4 transcript levels between the two genotypes (Figure 29. In addition studies done with UV-B radiation exhibited hypersensitivity of *tt4* mutants (Li et al. 1993).

5.4 Effects of low gamma irradiation on phenol acids/flavonoid synthesis and PSII system

Plant mutated in the HY5 gene are known for their inability of normal flavonoid synthesis, which might be of significant importance under gamma exposure. Plant defense system against gamma rays may indeed involve also flavonoids, which can protect the organism against the exposure due to their antioxidant activity.

Our HPLC analysis on phenolic compounds in experiment 2, showed some small differences between genotypes and different gamma treatments. Differences in sum of phenolic acids between control and different gamma treatments was observed in both genotypes. Plants exposed to the highest gamma dose (90.7 Gy) displayed 15-20% higher content of phenolic acids than the control plants (Figure 31). Furthermore, in the WT, the control exhibited higher content (25%) of flavonoid compounds compared to the highest gamma treatment (90.7 Gy). As expected, the *hy5* mutant showed decreased level of flavonoids (mostly kaempferol compounds; Figure 32) compared to the WT. The sum of the phenolic acids indicates significant differences between control and the highest gamma treatment (90.7 Gy) in the WT and a similar trend in the *hy5* mutant. As mentioned earlier, phenolic compounds play a crucial role in plants defense against UV-B radiation (Ulm and Heijde, 2012). Our results gave thereby increase in phenolic compounds at the highest dose (90.7) compared to the control, which indicates phenolic compounds as important factors in *A. thaliana* defense against gamma radiation. Measurement of the potential efficiency of PSII system in *hy5* mutant, showed a small significant reduction of Fv/Fm at the highest gamma dose (90.7 Gy) as compared to the control and the other treatments. In the WT, there were no significant differences between the treatments. On the other hand, genotypes differ significantly with the WT showing slightly higher Fv/Fm values compared to *hy5*.

5.5 ROS production response to gamma radiation

ROS can have a high damaging effect on cellular components, especially on membrane lipids. If peroxidation of membrane occurs, it may lead to functional defect of membranes (Weckx and Clijsters, 1996). In our study, right after gamma irradiation DAB staining was used in order to identify H_2O_2. According to previous studies, brown reddish color in cell components indicates H_2O_2 (Thordal-Christensen et al. 1997), but in our study no such staining was observed. As mentioned earlier *A. thaliana* is quit robust plant and may not show any H_2O_2 production since our experiment involved 21.6-90.7 Gy irradiation treatments.

In some earlier studies on pumpkin, that used chronic gamma treatment such as 1 kGy, the content of H_2O_2 increased in leaves (Wi et al. 2007). In addition, POD activity increased in corner middle lamella of parenchyma cells after gamma treatment in pumpkin. SOD activity increased as well in roots and leaves in *A. thaliana* after 58.8 Gy (Vanhoudt et al. 2014).

Also, the NBT staining method was used in order to detect superoxide anion. Earlier studies described detection of superoxide anion as dark-blue insoluble formazan compounds (Beyer et al. 1987), but our results did not show any differences between the treatments, maybe because of the robustness of the plants. Alternatively, the methods for ROS staining did not work as expected, and such studies should be repeated in new experiment.

6. CONCLUSION

Our results exhibited no consistent, significant differences of the formation of rosette leaves between different gamma treatments nor between WT and the *hy5* mutant. This indicates that young A. thaliana seedlings are highly resistant to gamma radiation from 21.6 Gy up to 90.2 Gy. The length of the bolting (inflorescence) stem showed only reduction in WT at the highest gamma treatment 90.7 Gy (35%) compared to control. Thus, this may indicate that perhaps even higher gamma radiation would have a greater impact on the inflorescence stem in *A. thaliana*. Gamma radiations in our experiment 1 on number of rosette leaves at first visible flower bud, exhibited only significant difference in *hy5* mutant comparing the highest dose (39.6; Figure) to the control. Reduction of rosette leaves at visible flower bud was observed in control and the highest gamma doses 39.6 and 31.7 Gy in comparison to the mutant *hy5*. Small similarities could also be observed in experiment 2. The percentage of plants with flower buds appeared to show systematic variation with gamma dose in the WT in the second experiment at day 29 after gamma treatment. Plants treated with dose 90.7 Gy and 72.2 Gy exhibited only 40% of plants with flower buds, compared to the control with 90%. This indicates that gamma radiation in our study prolonged the flower bud formation in the WT of *A. thaliana*. Relative expression of the *RAD51* gene increased in the WT with increasing gamma dose up to the highest dose 90.7 Gy. This implies this protein as an essential factor in DNA repair, which is in accordance to previous research. In addition, this result indicates that the up-regulation of the *RAD51* gene is persistent, at least for 51 days after the gamma radiation as shown in our experiment. In addition, flavonoid synthesis reduced in WT after gamma treatment 90.7 Gy which indicates that gamma rays have an impact on flavonoid biosynthesis.

7. SUGGESTIONS FOR FURTHER REASEARCH

In order to valuate radiosensitivity and dose-response relationships of different physiological processes and development stages of *A. thaliana* WT and *hy5* mutant it would be important to expose seedlings for higher doses of gamma radiation, and to expose also older plants at different development stages.

On the molecular level, *RAD51* exhibited higher expression at the highest gamma dose in WT even at day 51 after gamma radiation. It may be interesting to investigate its expression in plant cells also right after gamma treatment in order to improve the understanding of the regulation of this gene. Expression of *SOG1* would also be important to analyze, since it is an effector of checkpoint, DNA repair, apoptosis and endoreduplication and shown to correlate to *RAD51*.

The *TT4* gene, encoding chalcone synthase, did not show any variation between genotypes nor between different gamma dose at 51 day after gamma radiation, although a small reduction in flavonoid content was observed in the WT exposed to the highest gamma dose. Since *TT4* is the first enzyme in flavonoid biosynthesis, analysis also earlier and right after gamma treatment might be interesting.

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9. APPENDIX

Experiment 1.

Table 4. Number of Rosette Leaves versus treatment in WT Ler. R-Sq value is 43.60 %

	DF	F-value	P-value
Treatment	4	17,58	0.000
Error	91		
Total	95		

Table 5. Number of Rosette Leaves versus treatment in mutant *hy5*. R-Sq value is 19.14 %

	DF	F-value	P-value
Treatment	4	5.62	0.000
Error	95		
Total	99		

Table 6. Regression Analysis: Plants with flower buds in WT versus treatment. R-sq value is 29.6%.

Source	DF	F-value	P-value
Regression	1	1.26	0.343
Residual Error	3		
Total	4		

Table 7. Regression Analysis: Plants with flower buds in *hy5* versus treatment. R-sq value is 11.9%.

Source	DF	F-value	P-value
Regression	1	0.41	0.569
Residual Error	3		
Total	4		

Table 8. General Linear Model: Stem length versus Genotype*Treatment in WT and *hy5*. R-sq value is 17.06%.

	DF	F-value	P-value
Genotype	1	0.81	0.369
Treatment	4	8.96	0.000
Genotype*Treatment	4	0.61	0.659
Error	190		
Total	199		

Table 9. General Linear Model: Number of leaves at visible flower buds versus Genotype*Treatment in WT and *hy5*. R-sq value is 18.88%.

	DF	F-value	P-value
Genotype	1	3.35	0.069
Treatment	4	4.72	0.001
Genotype*Treatment	4	4.59	0.001
Error	182		
Total	191		

Experiment 2.

Table 10. General Linear Model: Number of leaves at flower buds versus Genotype*Treatment in WT and *hy5*. R-sq value is 11.41%.

	DF	F-value	P-value
Genotype	1	12.54	0.000
Treatment	5	2.21	0.054
Genotype*Treatment	5	1.15	0.334
Error	228		
Total	239		

Table 11. General Linear Model: Fluorescence versus Genotype*Treatment in WT and *hy5*. R-sq value is 76.42%.

	DF	F-value	P-value
Genotype	1	126.40	0.000
Treatment	5	3.94	0.004
Genotype*Treatment	5	1.88	0.114
Error	48		
Total	59		

Table 12. General Linear Model: Leaf size versus Genotype*Treatment in WT and *hy5*. R-sq value is 37.26%.

	DF	F-value	P-value
Genotype	1	8.86	0.003
Treatment	5	12.23	0.000
Genotype*Treatment	5	12.63	0.000
Error	220		
Total	231		

Table 13. General Linear Model: Stem length versus Genotype*Treatment in WT and *hy5*. R-sq value is 28.41%.

	DF	F-value	P-value
Genotype	1	19.68	0.000
Treatment	5	7.87	0.000
Genotype*Treatment	5	4.71	0.000
Error	210		
Total	221		

Table 14. Regression Analysis: Plants with flower buds in WT versus treatment. R-sq value is 73.4%.

Source	DF	F-value	P-value
Regression	1	11.02	0.029
Residual Error	4		
Total	5		

Table 15. Regression Analysis: Plants with flower buds in *hy5* versus treatment. R-sq value is 6%.

Source	DF	F-value	P-value
Regression	1	0.25	0.641
Residual Error	4		
Total	5		

Table 16. General Linear Model: Flavonoid content versus Genotype*Treatment in WT and *hy5*. R-sq value is 78.01%.

	DF	F-value	P-value
Genotype	1	183.61	0.000
Treatment	3	3.11	0.034
Genotype*Treatment	3	1.92	0.136
Error	56		
Total	63		

Table 17. General Linear Model: Phenolic acids content versus Genotype*Treatment in WT and *hy5*. R-sq value is 35.72%.

	DF	F-value	P-value
Genotype	1	2.22	0.142
Treatment	3	9.32	0.000
Genotype*Treatment	3	0.31	0.815
Error	56		
Total	63		

Table 18. General Linear Model: *RAD51* expression versus Genotype*Treatment in WT and *hy5*. R-sq value is 72.00%.

	DF	F-value	P-value
Genotype	1	15.09	0.001
Treatment	3	4.38	0.020
Genotype*Treatment	3	4.30	0.021
Error	16		
Total	23		

Table 19. General Linear Model: *TT4* expression versus Genotype*Treatment in WT and *hy5*. R-sq value is 52.29%.

	DF	F-value	P-value
Genotype	1	0.24	0.631
Treatment	3	4.81	0.014
Genotype*Treatment	3	0.96	0.436
Error	16		
Total	23		

Table 20. General Linear Model: Number of leaves at visible flower buds versus Genotype*Treatment in WT and *hy5*. R-sq value is 13.52%.

	DF	F-value	P-value
Genotype	1	2.86	0.016
Treatment	5	6.48	0.012
Genotype*Treatment	5	2.70	0.022
Error	219		
Total	230		

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