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Masterproef
Does prior exposure to uranium protect future generations of *Arabidopsis thaliana* plants to metal stress?

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Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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KNOWLEDGE IN ACTION

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LIST OF ABBREVIATIONS

•OH	Hydroxyl radical
2-VP	2-vinyl-pyridine
AP site	Apurinic/ Apyrimidinic site
APX	Ascorbate peroxidase
AsA	Ascorbate
BER	Base excision repair
CAT	Catalase
Cd	Cadmium
Cu	Copper
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DMSO	Dimethylsulfoxide
DSB	Double strand breaks
DTNB	5.5-dithiosbis(2-nitro-benzoic acid)
DTT	1,4-dithiothreitol
Fe	Iron
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HR	Homologous recombination
ICP-MS	Inductively coupled plasma – mass spectrometry
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
MMR	Mismatch repair
Mn	Manganese
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NOR	Naturally occuring radionuclides
$O_2^{•-}$	Superoxide
PVP	Polyvinylpyrrolidone
PX	Peroxidases
qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RT	Reverse transcriptase
SAZ	Syringaldazine
SOD	Superoxide dismutase
SPX	Syringaldazine peroxidase
SSB	Single strand breaks
TBA	Thiobarbituric acid
TBA-rc	Thiobarbituric acid reactive compounds
TCA	Trichloroacetic acid
U	Uranium
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
XOD	Xanthine oxidase
Zn	Zinc

ACKNOWLEDGEMENTS

SAMENVATTING

Uranium is een niet-essentieel radionuclide en zwaar metaal dat vrij voorkomt in het milieu in relatief lage concentraties. Antropogene activiteiten zoals uranium en fosfaat mijnbouw hebben bijgedragen aan de toename van dit metaal in de omgeving. Het is gekend dat blootstelling aan uranium een verstoorde groei en ontwikkeling kan veroorzaken in *A. thaliana* planten. Het is echter nog niet geweten welke onderliggende mechanismen betrokken zijn in de toxiciteit van uranium. In dit project hebben we daarom de hypothese opgesteld dat blootstelling aan uranium in een vorige generatie van *A. thaliana* planten kan beschermen tegen de effecten die geïnduceerd worden door blootstelling aan toekomstige metaalstress.

Om deze hypothese the onderzoeken, worden *A. thaliana* planten, afkomstig van zowel controle zaden als uranium-zaden, drie dagen blootgesteld aan verschillende concentrations cadmium (5 en 10 µM Cd) en uranium (25 en 50 µM U). De controle zaden zijn afkomstig van *A. thaliana* planten die nooit in aanraking zijn gekomen met metaalstress, terwijl de uranium-zaden afkomstig zijn van *A. thaliana* planten die gedurende hun groei heel de tijd zijn blootgesteld aan 5 µM U. Vervolgens worden de effecten van metaalblootstelling gemeten op enkele oxidative stress parameters en DNA herstel mechanismen. Bovendien wordt ook de transgenerationele stress respons onderzocht door de expressie van enkele DNA methylatie genen en het totale percentage DNA methylatie te meten. Hogere concentraties van cadmium en uranium zijn aanwezig in de wortels van de blootgestelde planten dan in de bladeren. Dit kan geassocieerd worden met de geobserveerde significante dalingen van de expressie van verschillende antioxidant, DNA herstel en DNA methylatie genen in de wortels van controle zaden. De wortels van uranium-zaden daarentegen vertonen een significante stijging van sommige antioxidant, DNA herstel en DNA methylatie genen na blootstelling aan cadmium en uranium. Er worden echter maar enkele significante verschillen gezien in de gen expressie in de bladeren en de verschillen die er zijn, zijn gelijkaardig voor zowel de controle als de uranium-zaden. De ernstigere effecten die gezien worden in wortels kunnen mogelijks verklaard worden door de lage translocatie van metalen van de wortels naar de bladeren, waardoor er lagere metaalconcentraties aanwezig zijn in de bladeren. De gemeten antioxidant enzym activiteiten en de antioxidant metaboliet concentraties vertonen daarenboven andere en minder significante resultaten in vergelijking met de transcriptionele effecten van de antioxidant genen in zowel wortels en bladeren. In dit project wordt nog eens bevestigd dat blootstelling aan cadmium en uranium schadelijke effecten veroorzaakt in wortels en bladeren van *A. thaliana* planten. Een vergelijking van de responsen geïnduceerd door beide metalen toont echter aan dat de mechanismen die aan de basis liggen van de stress responsen verschillend zijn voor cadmium en uranium. Onze resultaten tonen voor de eerste keer ook verschillen aan tussen planten van controle en uranium-zaden. Vooral de expressie van antioxidant, DNA herstel en DNA methylatie genen in de wortels verschilt sterk tussen beide zaadtypes. Dit duidt op de mogelijkheid dat blootstelling aan uranium in een vorige generatie kan leiden tot veranderingen in stress responsen in de volgende generatie, met name veranderingen die kunnen leiden tot een betere bescherming tegen metaalblootstelling.

ABSTRACT

Background: Uranium is a non-essential radionuclide and heavy metal that occurs naturally in the environment in relatively low concentrations. Anthropogenic practices such as uranium or phosphate mining have increased the disposal of this metal in the environment. It is known that exposure to uranium causes a disturbed growth and development of *A. thaliana* plants, however little information is known about the underlying mechanisms of its toxicity. We hypothesized that uranium exposure in a previous generation protects *A. thaliana* plants against future metal-induced stress.

Material & methods: *A. thaliana* plants were grown from control seeds (i.e. with no previous exposure to metal stress) and from seeds that were continuously exposed in their previous generation to 5 µM U (i.e. U-seeds). Both control *A. thaliana* seedlings and U-seedlings were grown hydroponically for 18 days, after which they were exposed to different concentrations of cadmium (5 or 10 µM Cd) and uranium (25 or 50 µM U) for three days. Subsequently, the effects on several oxidative stress-related parameters, DNA repair and transgenerational stress response, i.e. the degree of DNA methylation, were determined.

Results: Higher concentrations of Cd and U were found in the roots than in leaves. In roots, expression of different antioxidative, DNA repair and methylation genes was significantly downregulated in plants grown from control seeds. For U-seeds, however, significant upregulation of some antioxidative, DNA repair and methylation genes was seen for the high Cd- and U-conditions in roots. In contrast, only few significant differences were seen in gene expression in the leaves and significant differences that did occur, were in general similar for both control and U-seeds. In addition, the measured responses of the antioxidative enzyme capacities and the antioxidative metabolite concentrations were different and showed less significant alterations in comparison to the transcriptional responses of the antioxidative genes in both roots and leaves.

Discussion & conclusions: In our study, it was shown that exposure to Cd or U induces adverse effects in both roots and leaves of *A. thaliana* plants after three days. Although, different mechanisms seem to be involved in the effects on the oxidative stress response, DNA repair mechanisms and DNA methylation pathways induced by Cd and U in roots and leaves. The low root-to-shoot transfer of Cd and especially U can possibly explain the more severe effects seen in roots compared to leaves. In addition, our results show for the first time differences between plants from control and U-seeds, especially for the expression of antioxidant, DNA repair and methylation genes in roots. This indicates that changes can occur in the stress response in plants that were exposed to U in the previous generation and that metal exposure in a previous generation can possibly induce a protective abiltiy in the following generation.

1 INTRODUCTION

1.1 ENVIRONMENTAL POLLUTION

Environmental pollution has increased tremendously all around the world, with heavy metals being one of the most important contributors in terrestrial environments and in salt- and freshwater areas. Different heavy metals occur naturally in the environment in very low concentrations. However, various anthropogenic activities such as industries, agriculture and waste disposal have contributed to the current metal contamination as they are responsible for the release of heavy metal pollutants into the environment (1, 2). Because of their non-biodegradability and persistence, accumulation of those heavy metals in the environment can pose a major cause of many human health risks and serious ecological problems. In addition, metals released in the environment can be taken up by plants, which can cause a negative effect on plant growth and development (2, 3).

Besides their negative effect, metals also have a substantial role in the life processes of many organisms. Metals such as calcium, copper, iron, magnesium, potassium, sodium and zinc, are called essential metals because they serve as micronutrients and are used in maintaining the redox balance (4). On the other hand, many metals (e.g. lead, cadmium, mercury, silver, arsenic) are nonessential, which means that they have no nutritious value in organisms. These heavy metals can lead to toxic effects as they interact with essential components within the cell (5). At high concentrations, however, both essential and nonessential metals are toxic and responsible for damage to cell membranes, alterations in enzyme specificity, disruption of cellular functions, and damage to the structure of DNA (4).

In the present project, the stress responses induced by two toxic metals uranium (U) and cadmium (Cd) are investigated in *Arabidopsis thaliana* plants. It is already known that both elements have detrimental effects on human health and plant growth and development, which highlights the importance to gain a better insight into the underlying mechanisms of their toxicity. As plants cannot escape harmful environments, it is important that they can easily respond and adapt to recurring stressors. To investigate if adaptation from a previous exposure can be transmitted to the next generation, effects are studied in both control plants (i.e. plants with no prior history of metal exposure) and in U-plants (i.e. plants that were exposed to a low U concentration in their previous generation).

1.1.1 Uranium

Uranium is a heavy metal and radionuclide that occurs naturally in the environment. It can be found in soil, rocks, surface and underground water and air. Natural processes such as erosion, wind activity and volcanic eruptions can cause a redistribution of U in the environment. Anthropogenic practices like U mining, milling and the processing of materials containing naturally occurring radionuclides (NOR) can cause a local increase of U in the environment and has led to the presence of U contamination in many countries. There are three naturally occurring uranium isotopes, of which ^{238}U is the most abundant (6). Since ^{238}U has a very long half-life (4.47×10^9 year), the chemical toxicity of U is the primary environmental health hazard, while its radioactivity is of secondary concern (7).

U always occurs in combination with radioactive elements such as, thorium and radium, or non-radioactive contaminants such as Cd (8).

Effects of uranium in humans
As U is omnipresent, it can occur in trace amounts in various foods and in drinking water. Animals and humans are, therefore, always exposed to low concentrations of U. Uranium isotopes emit alpha particles which are characterized by rapid loss of their kinetic energy and small penetrating power. They are thus unable to penetrate human skin but do represent a great health hazard when humans are internally contaminated with U. Ingestion, aerosol inhalation and transcutaneous uptake via wounds are the three pathways by which U is taken up into the body (6, 9). More than 95% of U that enters the body is not absorbed, thus excretion of U can rapidly occur via faeces. The small portion of U that is absorbed into the bloodstream, will mostly be filtered by the kidney and excreted in the urine or will be distributed throughout the body to the bones and soft tissues. Accumulation of U in the kidneys can possibly cause renal dysfunction, which is characterized by cellular injury and tubular necrosis. Depending on the size of U particles, they can enter the bloodstream or can be retained in the lungs, where they can cause radiological or chemical damage as U decays into other radionuclides. Exposure to U is therefore also associated with an increased risk for lung cancer or chronic respiratory disease (6, 10).

Effects of uranium in plants
Plants are strongly affected when exposed to U. Even when metals, such as U and its decay products, are not essential for plant growth, the roots of plants can easily absorb these heavy metals from the soil solution. Soil parameters such as pH have an influence on the form of U and hence, can increase or decrease the total solubility of U and its uptake in plants. Subsequent to metal uptake via the roots, metal ions are further transported to the leaves. The U content, however, is generally greater in the roots than in the aboveground tissues indicating a limited transfer to the leaves. Therefore, it is to be expected that the toxicity effects of U will be more severe in roots as compared to the leaves (11). It is already known that U induces plant growth reduction, can lead to oxidative stress, and can interfere with the uptake and distribution of plant nutrients. In addition, leaf chlorosis and yellow turning roots are observed and studies have also showed significant changes in the expression and activity of different antioxidative enzymes and concentrations of antioxidative metabolites like ascorbate (AsA) and glutathione (GSH) in both roots and leaves of *A. thaliana* plants (11-13). Furthermore, U is also known to induce DNA damage, as discussed in section 1.3.2.

1.1.2   Cadmium
Cadmium is a non-essential metal that is naturally present in the environment. Anthropogenic sources such as power stations, heating systems, metal-working industries and urban traffic lead to higher Cd concentrations in the environment. Cadmium is an extremely significant pollutant because of its high toxicity and large solubility in water (14-16).

Effects of cadmium in humans
As Cd is present in soil, it can be taken up by crops and vegetables grown for human consumption and so be introduced into the food chain. As such, food is considered the main source of Cd intake for humans. Besides uptake via food, humans can also be exposed to Cd via the oral route when Cd is present as airborne particles. Cigarette smoking is another important source of Cd exposure

because one cigarette contains approximately 1 – 2 µg of Cd. The absorption of Cd in the lungs and gastrointestinal tract is not very high, but unlike U, Cd has a slow excretion rate from the body which leads to high accumulation of Cd in body tissues such as kidneys and liver, possibly leading to kidney tubular damage and renal failure. Further, Cd has been associated with bone damage such as osteoporosis, with lung disorders and it can increase the risk for cancer development in organs such as the lungs and kidneys (17-19).

Effects of cadmium in plants
Due to the high toxicity of Cd, most plants are already sensitive to low concentrations (20). Cadmium exerts several effects on morphology and physiology levels in plants, such as leaf roll, chlorosis and reduced plant growth. In addition, Cd exposure can disturb the water balance and the photosynthesis, and it can interfere with the uptake, transport and use of several essential nutrients (15, 16). The toxicity of Cd in plants is mainly due to induction of oxidative stress (21). Although Cd is a non-redox-active compound, it is able to induce oxidative stress indirectly. It stimulates oxygen free radical production by either replacing redox-active elements, such as Fe, or by decreasing the activity of enzymatic and non-enzymatic antioxidants (20, 22). In addition, like U, Cd is also genotoxic and is able to induce DNA damage in plants.

1.2   OXIDATIVE STRESS RESPONSES TO METAL EXPOSURE

It is already known that acute exposure of plants to high concentrations of U and Cd can induce oxidative stress (23). Under oxidative stress conditions, there is a disturbance of the balance between cellular pro- and antioxidants in favour of the pro-oxidants. This can lead to the enhanced concentration of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) (24). However, ROS are also produced at very low concentrations during various metabolic processes as they function as second messengers in various plant developmental processes. ROS thus have a dual role since they occur both as key regulators in biological processes, like growth, cell cycle, biotic and abiotic stress responses and as toxic by-products of aerobic metabolism which can lead to the oxidative destruction of cells (25). By oxidizing different biological molecules, like DNA, proteins and lipids, ROS are responsible for causing cellular damage.

Plants have developed an antioxidative defence system to manage the elevated ROS concentrations, so the signal transduction processes can proceed while the oxidative damage is limited. This defence system comprises a number of antioxidant enzymes and metabolites that are able to detoxify different ROS (13). Superoxide dismutase (SOD) is a first line of defence against ROS because of its capacity to invert $O_2^{\bullet-}$ into H_2O_2 and molecular oxygen. SOD relies on a co-factor for its antioxidative reaction. Based on their metal co-factor, three isoforms of SOD can be distinguished in various plant species, namely manganese SOD (Mn-SOD), iron SOD (Fe-SOD) and copper-zinc SOD (Cu/Zn-SOD). The different SOD enzymes can be found in different compartments of the cell (24, 26).
Catalase (CAT) and peroxidases (PX) are responsible for the elimination of H_2O_2. Catalases convert H_2O_2 into H_2O and O_2, while peroxidases need the oxidation of a co-substrate for the detoxification of H_2O_2 to H_2O (13, 27). Ascorbate peroxidases (APX) and glutathione peroxidases are present in plants and require AsA and GSH, respectively, as co-substrates for the neutralisation of H_2O_2. In

addition, AsA and GSH are two important antioxidant metabolites which can directly scavenge different ROS non-enzymatically. As GSH and AsA can be oxidized by ROS, the action of the AsA-GSH cycle is responsible for the reduction of oxidized GSH and AsA to reform GSH and AsA (Fig. 1).
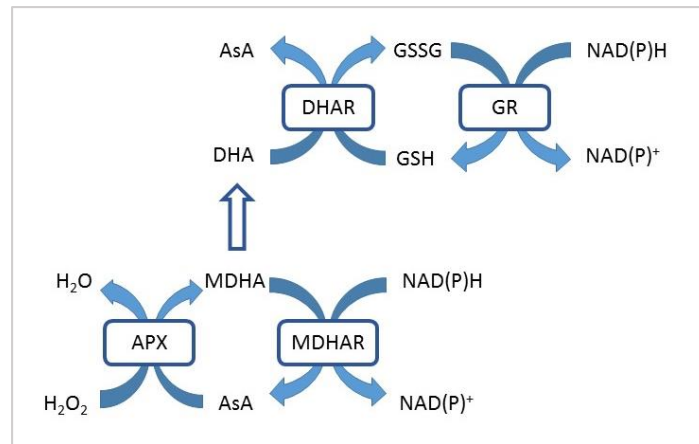
Figure 1. Ascorbate-Glutathione cycle (28, 29)*. The ascorbate (AsA)-glutathione (GSH) cycle is responsible for the regeneration of the reduced state of AsA and GSH when they become oxidized by the detoxification of H_2O_2. As H_2O_2 is detoxified by ascorbate peroxidase (APX) into H_2O, AsA is oxidized and dismutated into dehydroascorbate (DHA). Oxidation of GSH enables the regeneration of AsA, while subsequently, GSH is regenerated from the oxidized glutathione (GSSG) through oxidation of NADPH (Figure adapted from Foyer et al. 2011).*

In the AsA-GSH cycle, APX detoxifies H_2O_2 by the oxidation of AsA to monodehydroascorbate (MDHA), which can spontaneously dismutate into dehydroascorbate (DHA). Ascorbate can be regenerated via two routes: [1] by monodehydroascorbate reductase (MDHAR), which uses the reduction of NADPH as a driving force and [2] by dehydroascorbate reductase (DHAR), a process which is driven by the oxidation of GSH to oxidized glutathione (GSSG). Subsequently, glutathione reductase (GR) is responsible for the regeneration of GSH from GSSG by using NADPH as a reducing agent (23, 28).

1.3 DNA DAMAGE AND REPAIR

Besides the ability to induce oxidative stress responses in plants, Cd and U are also known to be genotoxic metals. Damage to DNA can appear in various forms, like mismatches, base alterations or deletions, and as single and double strand breaks (SSB and DSB). Since DNA damage can cause the reduction of protein synthesis, the destruction of cell membranes and damage to photosynthetic proteins, an accumulation of DNA damage can lead to a general deterioration of cell function and eventually cell death (30, 31). This, in turn, will negatively influence plant growth and development.

1.3.1 DNA repair mechanisms

As different endogenous and exogenous DNA-damaging factors constantly challenge the genomic integrity of organisms such as plants, it is crucial that they develop mechanisms for the maintenance of the genome integrity (32). Further, to prevent loss or incorrect transmission of genetic information from one generation to the next, it is essential to repair DNA damage. Therefore, a signal-transduction pathway is activated upon the detection of damaged DNA. Depending on the severity and type of damage, this will lead to cell cycle arrest to promote DNA repair mechanisms to restore the damage or to apoptosis of the damaged cell (30, 31). Different DNA repair mechanisms exist to repair the various types of DNA damage in plants (31).

Base excision repair (BER) is a process that is activated by recognition of damaged or modified bases and by SSB. Specific to BER is that it removes a damaged base rather than a nucleotide by a DNA glycosylase enzyme. DNA glycosylases cleave the N-glycosylic bond between the damaged target base and deoxyribose, which results in a free base and an apurinic or apyrimidinic (AP) site in the DNA strand. Subsequently, these AP sites have to be repaired by AP endonuclease or AP lyase activities. Finally, a ligase enzyme removes the nucleotide overhang that was replaced and rejoins the repaired DNA strand (33, 34).

In contrast to BER, nucleotide excision repair (NER) is a mechanism that removes a 24-32 base oligonucleotide containing the damaged product. NER can remove a variety of different DNA lesions, such as UV-induced photoproducts and DNA adducts that generate conformational changes in DNA. After detection and removal of the DNA lesions, the gap that remains, is filled by a DNA polymerase and DNA ligase is responsible for the ligation of the DNA strand (33, 35).

Mismatch repair (MMR) is mainly active to correct DNA replication errors like mismatches and inserted or deleted nucleotides. A larger part of the DNA strand that contains the DNA lesion is excised by an exonuclease. Afterwards, DNA polymerases and DNA ligases are activated to fill the gap and reseal the DNA ends (32, 33).

Two major pathways exist to repair DSBs: Homologous recombination (HR) and non-homologous end joining (NHEJ). The first mechanism uses an identical or homologous DNA sequence as template to repair the DSB. The repair is initiated by resecting the 3' end to form a long single-stranded DNA tail onto which a homologous substrate can bind and DNA synthesis can start. The newly synthesized DNA can then re-anneal with the other DNA strand and repair the break (32, 36). The NHEJ process does not require homologous sequences to repair DSBs but is able to rejoin the two end breaks. This mechanism is more prone to errors because it does not use homologous template sequences and therefore, degraded or inappropriate ends may be rejoined (36, 37).

1.3.2 Uranium and cadmium genotoxicity

While the induction of oxidative stress responses in plants after U exposure has been extensively studied, little information on the genotoxicity of U in plants is available (11, 13). Studies have shown that U can induce DNA damage to various animal species (e.g. zebrafish, rats, earthworms) (12, 38, 39). In addition, there are indications that U is also genotoxic in plants (40, 41). Uranium can cause DNA damage directly by interacting with the negatively charged DNA phosphate backbone or indirectly through oxidative stress, when U induces the formation of ROS. Damage to DNA can appear as SSB and DSB or as oxidative changes to bases (42). At low concentrations, U induces little cellular modifications and DNA damages that can be easily repaired. However, at high concentrations, DNA damage and cellular alterations are more severe and more difficult to restore (38).

Likewise to U, Cd is genotoxic and exposure to Cd leads to DNA damage in plants. More specifically, Cd can induce DNA strand breaks, DNA-protein crosslinks, oxidative DNA damage, chromosomal aberrations, dysregulation of gene expression resulting in enhanced proliferation, depressed apoptosis or altered DNA repair (22). The mechanism by which Cd induces DNA damage is an indirect manner. Cd exposure induces an increase in ROS formation, which in turn will lead to subsequent DNA damage (21, 43).

1.4 TRANSGENERATIONAL INHERITANCE AND EPIGENETICS

All organisms are influenced by different factors in their environment. Because plants cannot escape harmful environments, they have a great need to be able to respond and adapt to recurring stressors (44). Plants have therefore evolved some mechanisms that allow them to respond to changing conditions in the environment and enable them to persist in variable surroundings. One of the mechanisms that plants have, is called 'priming of defence'. By this mechanism, plants are able to adapt to hostile conditions in their environment by intensifying the responsiveness of their immune system upon a second exposure to the hostile signals, such as pathogens, insects or in response to abiotic stress. As such, plants can have a faster or stronger activation of the defence mechanisms when they are re-exposed to a certain stressor (45, 46), making a plant more resistant (47).

An important priming strategy is the induction of stable alterations in gene expression that do not involve changes of the DNA sequence itself. The expression of genes depends on the chromosomal structure, histone modifications and silencing mechanisms such as DNA methylation, miRNA's and siRNA's, so-called epigenetic changes. In the current project, the focus is on DNA methylation. Addition of methyl groups to DNA bases modifies the structure of DNA and subsequently alters their transcription, resulting in silencing of the gene expression. This process is critical for normal development in plants and animals. DNA methylation can be reversed by the action of enzymes, such as DNA glycosylases but also non-enzymatically during replication and cell division (48). Epigenetic alterations, such as DNA methylation, are considered essential for the differentiation and development of various organisms but they can also arise by different influences from the environment (49). For example, it was found that Chernobyl radiation-exposed *A. thaliana* plants have a significantly higher global DNA methylation level than control plants. Other stress conditions, like high salt and high or low temperatures, can also induce increased DNA methylation (50). Such hypermethylation can be an indication of an activated defence strategy of plants to prevent genome instability and to allow survival in extreme environments (51, 52).

Epigenetic changes can possibly be transmitted to the next generation (49). Also for plants, recent publications showed that in *A. thaliana* some defence responses, such as the adaptation of plants to Cd exposure, can be inherited transgenerational (46, 53-55). In addition, Boyko *et al*. (50) has shown that the progeny of stressed plants also exhibit changes in genome methylation and stress tolerance. As such, transgenerational epigenetic inheritance can be important in the adaptive properties of plants against environmental stressors such as abiotic stress. This could be of evolutionary importance as it might facilitate the selection of well-adapted genotypes in an adverse environment on the long term (56). Inheritance of epigenetic changes can make it possible that parents provide their progeny with an enhanced ability to tolerate certain stressful conditions to which the parents were exposed. As such, the offspring is more prepared for future stressful situations. On the contrary, first generation plants that are exposed to different biotic and abiotic environmental factors might also be responsible for a decreased tolerance to a future stress response in plants of a next generation. This can be due to limited resources within the plant as these resources are necessary for the plant to sufficiently cope with stress conditions (55).

1.5 AIM OF THE RESEARCH PROJECT

The overall hypothesis of this study is that **metal exposure in previous generations protects *Arabidopsis thaliana* plants against U- and Cd-induced stress**. The experiments within this project are performed on the plant species *A. thaliana*. This plant is a model organism for the study of cellular and molecular processes in plants and is therefore very suitable for our project. In addition, the plant is easy to grow, has a relatively short life cycle and is able to produce an abundant amount of progeny. The entire genome of *A. thaliana* has also been sequenced and annotated, making it an ideal plant for molecular analyses (57).

The first objective of the project is to determine if **long-term exposure of plants to U in a previous generation induces altered tolerance or sensitivity towards different abiotic stressors** (U and Cd). Both control and U-seed stocks are used to cultivate plants in a hydroponic system. The control seeds have never been exposed to metal stress before, while the U-seeds were continuously exposed to an environmentally relevant U concentration (i.e. 5 µM U) in their previous generation. After 18 days, plants are exposed to five different conditions: 0 µM Cd or U, 5 µM Cd, 10 µM Cd, 25 µM U and 50 µM U. These concentrations were chosen as it is known that mild stress is induced with the lower concentrations (5 µM Cd and 25 µM U), while the higher concentrations (10 µM Cd and 50 µM U) induce a higher stress-level in plants (13, 55). Subsequently, roots and leaves are harvested and biometrical parameters, including root and leaf fresh weight, are analysed. Metal uptake is determined using inductively coupled plasma mass spectrometry (ICP-MS). At molecular level, gene expression analysis of reactive oxygen species producing enzymes (e.g. NADPH oxidases) and antioxidant enzymes (e.g. superoxide dismutase) are performed via quantitative real-time polymerase chain reaction (qPCR). Next, the capacity of antioxidative enzymes and the concentration of antioxidative metabolites, such as GSH, are analysed spectrophotometrically. Furthermore, the thiobarbituric acid reactive compounds (TBA-rc) are determined as a measure for membrane damage.

In the second objective, **the difference in the occurrence and degree of DNA damage and repair in primed versus non-primed plants** is investigated at molecular level. Therefore, different enzymes involved in DNA repair and in the cell cycles (e.g. PARP1, KU80) are studied via qPCR.

While the differences in metal tolerance are studied in the two first objectives, the last objective focusses on mechanisms that lie at the basis of the transgenerational effects. This is done by looking at **the difference in the degree of DNA methylation**. Therefore, the global DNA methylation is analysed in all exposure conditions via ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). In addition, some enzymes important in the methylation process (e.g. MET1, CMT3) are analysed at gene expression level. This will increase our knowledge concerning the mechanisms related to transgenerational-induced effects in *A. thaliana* plants after heavy metal exposure.

2 MATERIALS AND METHODS

In this project, *A. thaliana* plants (ecotype Columbia) were grown from control seeds that have never been exposed to heavy metal stress and seeds continuously exposed to 5 µM U in their previous generation (i.e. U-seeds). Eighteen-day old plants were exposed to different concentrations of Cd (5 or 10 µM) or U (25 or 50 µM) for three days. Subsequently, the effects on several oxidative stress-related parameters, DNA repair and the global methylation level were determined.

2.1 PLANT HYDROCULTURE AND METAL EXPOSURE

Two different seed stocks of *A. thaliana* were used: control seeds that have never been exposed to metal stress before, and U-seeds that were exposed continuously to 5 µM U in their previous generation. The seeds were transferred to tubes that were filled with 0.6% agar in a low phosphate Hoagland solution as described by Vanhoudt *et al*. (58). The tubes were placed into a PVC cover on top of a container filled with 1.35 L of distilled water. Each cover contained 36 tubes, from which half contain control seeds and the other half were filled with U-seeds. The plants were grown in a growth chamber with the following germination conditions: 14 hours photoperiod (light intensity of 165 µmol.m^{-2}s^{-1}) with day/night temperatures of 22°C/18°C, and a constant relative humidity of 65%. One week after sowing, the boxes were filled with Hoagland HP solution (1000 µM KNO$_3$, 300 µM Ca(NO$_3$)$_2$•4H$_2$O, 200 µM MgSO$_4$•7H$_2$O, 100 µM NH$_4$H$_2$PO$_4$, 1.6 µM FeSO$_4$•7H$_2$O, 0.78 µM Na$_2$-EDTA•2H$_2$O, 4.6 µM H$_3$BO$_3$, 0.91 µM MnCl$_2$•4H$_2$O, 0.032 µM CuSO$_4$•5H$_2$O, 0.055 µM H$_2$MoO$_4$ and 0.076 µM ZnSO$_4$•7H$_2$O) and an air supply was added to the containers. The Hoagland HP solution was replaced twice a week. At day 18, plants were exposed to different U or Cd concentrations for three days: 0 µM U/Cd, 5 µM Cd, 10 µM Cd, 25 µM U and 50 µM U. The pH of all conditions was adjusted to the pH value of the control conditions, i.e. pH 5.5. The nutrient solution that was used during the exposure was Hoagland LP because of its lower phosphate content (25 µM) which enables a better U availability to plants as discussed in Vanhoudt *et al.* (58). On day 21, plants were harvested. Leaf and root fresh weight was determined before they were snap-frozen in liquid nitrogen and stored at -80°C for further analyses.

2.2 CADMIUM AND URANIUM ANALYSIS

The amount of Cd and U was determined in root and leaf samples and also in samples of the Hoagland LP solution after contamination. Immediately after harvesting, roots were washed twice for 10 min with 1 mM Pb(NO$_3$)$_2$ and then once for 10 min with distilled water to exchange surface-bound U or Cd. Subsequently, root and leaf samples were dried at 65°C for at least one week. The oven-dried samples were then incinerated in a muffle furnace at 550°C. After cooling down to room temperature, 1 mL of 0.1 M HCl was added to all samples in order to dissolve the plant material. To ensure complete digestion of the plant material, the samples were placed on a heated sand bath for approximately 30 min. Subsequently, samples were filtered with Acrodisc 25 mm syringe filters with 0.45 µm HT Tuffryn to exclude any remaining particles. The U and Cd concentrations in the samples were determined via ICP-MS at the lab of Soil and Water Management (KU Leuven, Leuven, Belgium).

2.3 DETERMINATION OF GENE EXPRESSION WITH REAL-TIME QUANTITATIVE PCR

2.3.1 RNA extraction

RNA was extracted from frozen root and leaf samples by following the protocol of RNeasy plant mini kit (Qiagen, Venlo, The Netherlands). Briefly, root and leaf samples were disrupted under frozen conditions by using 3 chrome shredder beads and the Retsch Mixer Mill MM400. Subsequently, RNA was extracted with a column-based extraction method. Impurities were pelleted and by applying different washing steps, purified RNA plant extract was eluted in 60 µL RNase-free water. The RNA quantity and purity were determined using the Nanodrop® ND-1000 Spectrophotometer (Isogen Life Science, De Meern, The Netherlands). The integrity of RNA was checked via gel electrophoresis on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Afterwards, RNA samples were stored at -80°C.

2.3.2 cDNA synthesis

An input of 1 µg RNA was used for cDNA synthesis in a total volume of 20 µL. The synthesis was performed in two steps. First, all RNA samples were treated with a DNAse-step using TURBO DNA-free Kit (Ambion, Thermo Fisher Scientific, Breda, The Netherlands). A mastermix containing 1/10th volume 10x Buffer and 0.25 µL DNase was added to each sample. After incubation for 25 min at 37°C, 2 µL DNase Inactivation Reagent was added and samples were incubated 2 min at room temperature. After centrifugation for 1.5 min at 10 000 rpm, a pellet containing the DNase Inactivation Reagent is formed. The supernatant was collected for the synthesis of cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). In each sample, 10 µl of the reverse transcriptase (RT) mastermix was added, consisting of 4 µL of 5x PrimeScript buffer, 1 µL RT enzyme, 1 µL oligo(dT) primers, 1 µL random hexamers and 3 µL of RNase-free water. cDNA synthesis was carried out on a PCR gradient thermal cycler (Techne TC-500; Techne, Ramsey, MN, USA) with a PCR thermal cycling program of 15 min at 37°C and 5 s at 85°C. Afterwards, cDNA samples were diluted 10 times in RNA-free H_2O and then stored at -20°C until further use.

2.3.3 Real-time quantitative PCR

Quantitative PCR was performed with the ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA), using SYBR Green chemistry in a total volume of 10 µL, containing 2.5 µL cDNA sample, 5 µL Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.3 µL forward primer, 0.3 µL reverse primer and 1.9 µL RNase-free H_2O. The PCR amplifications were performed at following cycling conditions: 15 min at 95°C, 40 cycles of 15 s at 94°C, 30 s at 50-60°C and 30 s at 72°C. Primers used for gene expression analysis are given in Table 1. Multiple reference genes were used for leaf (*UBQ10* (AT4G05320)*, YLS8* (AT5G08290*) and *UBC* (AT5G25760)) and root normalization (*UBQ10, SAND* (AT2G28390) and *YLS8*). The expression stability of the reference genes was evaluated with GrayNorm (59). Gene expression data was calculated relative to the control treatment using the $2^{-\Delta Ct}$ method and the data was normalized to a normalisation factor based on the expression level of the multiple reference genes.

Table 1. Overview of the primers (forward and reverse) used for gene expression analysis of the reference genes and genes of interest with RT-qPCR.

Gene	Forward primer	Reverse primer
REFERENCE GENES		
UBQ10	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
UBC	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
SAND	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
YLS8	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
PRO-OXIDATIVE GENES		
LOX1	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC
LOX2	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC
RBOHC	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
RBOHD	TATGCATCGGAGAGGCTGCT	TAGAGACAACACGTTCCCGGG
RBOHF	GGTGTCATGAACGAAGTTGCA	AATGAGAGCAGAACGAGCATCA
ANTIOXIDATIVE GENES		
CAT1	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
CAT2	AACTCCTCCATGACCGTTGGA	TCCGTTCCCTGTCGAAATTG
CAT3	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA
APX1	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
GR1	CTCAAGTGTGGAGCAACCAAAG	ATGCGTCTGGTCACACTGC
MSD1	ATGTTTGGGAGCACGCCTAC	AACCTCGCTTGCATATTTCCA
CSD1	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
CSD2	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
CSD3	GTTGTTGTGCATGCGGATCC	CACATCCAACTCTCGAGCCTG
FSD1	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
FSD2	TTGGAAAGGTTCAAGTCGGCT	CATTTGCAACGTCAAGTCTATTCG
FSD3	AACGGGAATCCTTTACCCGA	TGTCTCCACCACCAGGTTGC
DNA REPAIR GENES		
PARP1	TGCATTGGGAGAAATACATGAGC	CCGAGCCCTTTGGTCGAG
PARP2	ATCGGAGGTGATTGATCGGTATG	AAATCATGAGGTATCACTGTGTAGAACTCT
KU80	CTTCTTCCAGCACAACTCCTCAA	CTACGCATCGCAGGACCTACAT
LIG4	GATGTATCGG ATATCAAGGGCA	GAATGGGACCGAGGCACG
RAD51	GTCCAACAACAAGACGATGAAGAA	AACAGAAGCAATACCTGCTGCC
POLGAMMA1	AAACTGGACGCTTATCGGCTAG	TGACGGATTTTGTACCGATCTTT
MND1	AACGAGATGGTACAATTTGCTGA	CGACTGGTGAGCAACTTCAAT
KRP2	GGAATAAGTTGTTGGAATGTTCTATGAAGT	AACCCACTCGTATCTTCCTCCAC
DMC1	ATGAAGACGAAGATCTATTTGAGATGATT	CTTGTACGTTTTTCACATCTCCTGC
METHYLATION GENES		
CMT3	ACAAAGATCCCACAACGCCATTTC	TGATTGTGAACCTGACGCTTCATC
DRM2	ATCTAGCTGGTGTAGCCGTGAC	AACCTCGTCTGAGAAGCCCATC
MET1	CCAGTAGATTTCGCTTCTCAACGG	AGTGGTCTCTTCTTTCGCTTCGC

2.4 ANALYSIS OF ENZYME CAPACITIES

Root and leaf samples were grounded under frozen conditions with the Retsch Mixer Mill MM400 using chrome beads. A spatula tip of polyvinylpyrrolidone (PVP) was added before shredding to absorb polyphenols, which can possibly interfere with the enzymatic reactions. Each sample was extracted in 1.5 mL extraction buffer (pH 7.8) containing 0.1 M TRIS, 1 mM Na_2-EDTA and 1 mM 1,4-dithiothreitol (DTT). After centrifugation, the supernatant extract solution was used to measure the enzymatic capacities. The capacities of six antioxidative enzymes were determined spectrophotometrically at 25°C by using the PowerWave HT plate reader (BioTek).

The capacity of CAT was assessed in 96-well UV-plates. Each well of the plate contained 10 µL of plant extract and 190 µL of H_2O_2 solution, consisting of 49 mM H_2O_2 diluted in a 0.1 M KH_2PO_4 buffer (pH 7.0). The decrease in absorbance was monitored kinetically at 240 nm.

To determine the capacity of guaiacol peroxidase (GPX) 10 µL of sample extract, 140 µL of 0.1 M KH_2PO_4 buffer (pH 7.0) and 50 µL of guaiacol mastermix, consisting of 90 mM guaiacol and 163 mM H_2O_2 solution, mixed on a 1:1 ratio were added to each well of a 96-well plate. The appearance of tetraguaiacol was then measured spectrophotometrically at 436 nm.

For the determination of the syringaldazine peroxidase (SPX) capacity, 20 µL plant extract was first added to each well in a 96-well UV-plate. Subsequently, 155 µL 0.1 M TRIS buffer (pH 7.5), 20 µL 98 mM H_2O_2 and 5 µL syringaldazine (SAZ) were added. The capacity of SPX was determined by measuring the appearance of oxidized SAZ at 530 nm.

The APX capacity was determined by monitoring the decrease of AsA at 298 nm. A 96-well UV-plate was therefore filled with 18 µL of plant sample, 155 µL of a 0.1 M HEPES-1 mM EDTA buffer (pH 7.0) and 27 µL ascorbate mastermix, consisting of 30 mM Na- Ascorbate and 20 mM H_2O_2.

For the determination of the SOD capacity, blanks must be made and measured as the SOD measurement relies on an inhibition reaction. For the blanks, 5 µL xanthine oxidase (XOD), 135 µL 50 mM KH_2PO_4 buffer (pH 7.8) and 60 µL SOD mastermix consisting of 1 mM Na_2-EDTA, 0.5 mM xanthine and 0.1 mM cytochrome C in KH_2PO_4 buffer, were added to the wells of a 96-well plate. The other wells contained, besides 5 µL XOD, 130 µL KH_2PO_4 buffer and 60 µL SOD mastermix, also 5 µL plant extract. The extent of cytochrome C reduction was monitored kinetically at 550 nm. Cytochrome C is reduced by $O_2^{•-}$. SOD inhibits this reduction of cytochrome C by scavenging $O_2^{•-}$. Because the blanks do not contain any SOD enzyme capacity, they were used to calculate the inhibition reaction of SOD in the plant extracts.

To determine the capacity of GR, 28 µL plant extract, 165 µL 0.1 M TRIS- 1 mM EDTA buffer (pH 8) and 7 µL GR mastermix, containing 82 mM GSSG and 6 mM NADPH, were added to a 96-well UV-plate. The decrease of NADPH, which is used for the reduction of GSSG, was measured spectrophotometrically at 340 nm.

Afterwards, the protein content of all samples was measured to determine the enzyme capacities in terms of units per mg protein content. The protein content was measured spectrophotometrically at 750 nm using a 96-well plate. This protein assay is based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent, which leads to a blue colour development with maximum absorbance at 750 nm.

2.5 METABOLITE ANALYSIS

A plate-reader assay was used to measure oxidized and reduced forms of GSH spectrophotometrically in root and leaf samples. Samples were shredded under frozen conditions with the Retsch Mixer Mill MM400 after addition of 8 Zirconia beads to each sample. By adding 800 µL of 200 mM HCl, the samples were extracted via an acidic protocol. After centrifugation for 15 min at 13 500 rpm and 4°C, 300 µL sample aliquots were taken and neutralized. This acidic extraction requires a neutralization with 200 mM NaOH to obtain a pH 4-5 at which the metabolites can be measured. This extract was used for the measurement of the total GSH concentrations. To measure GSSG, 100 µL of the sample extract supernatant was incubated with 1.3 µL 2-vinyl-pyridine (2-VP) during 30 min at room temperature in order to mask all free thiol groups.

A standard range was prepared for both GSH and GSSG from a 10 mM GSH stock and 10 mM GSSG stock, respectively, in 200 mM NaH_2PO_4 (pH 5.6) buffer. The standards for GSH ranged from 0 pmol to 1000 pmol and from 0 pmol to 200 pmol for GSSG.

For both measurements, a mastermix was made containing 200 mM NaH_2PO_4 – 10 mM EDTA (pH 7.5), dH_2O, 10 mM NADPH, GR and 12 mM 5.5-dithiobis(2-nitro-benzoic acid) (DTNB) in dimethylsulfoxide (DMSO). For GSH, 10 µL of sample or standard was added to the wells of a 96-well plate and for GSSG, 20 µL was added. In total, each well contained 200 µL for the measurement of the reduction of DTNB to an absorbing molecule by GSH. This increase in reduced DTNB was measured at 412 nm.

2.6 DETERMINATION OF LIPID PEROXIDATION

Thiobarbituric acid reactive compounds (TBA-rc), such as malondialdehyde (MDA), were measured in *A. thaliana* leaves to have an indication on the amount of membrane damage. Leaf samples of approximately 100 mg were grounded in the Retsch Mixer Mill MM400 for 3.5 min at 30 Hz. Homogenisation of the samples was obtained by adding 1 mL of 0.1% Trichloroacetic acid (TCA). After 10 min centrifugation at 13 000 rpm and 4°C, 400 µL supernatant was diluted 3.5 times in 1 mL 0.5 % 2-Thiobarbituric acid (TBA) in 20 % TCA. The samples were then incubated for 30 min at 80°C and immediately cooled down in an ice bath afterwards. To pellet the TBA precipitate, a centrifugation step of 5 min at 13 500 rpm was performed. The absorbance of the supernatant was determined spectrophotometrically via a plate-reader assay at 532 nm and was corrected by subtraction of non-specific absorption at 600 nm. The MDA concentration was calculated according to the law of Lambert-Beer (ε = 155 mM-1 cm-1) taking into account the fresh weight and the dilutions made.

2.7 DETERMINATION OF DNA METHYLATION

To measure the DNA methylation, DNA was extracted from frozen root samples by following the protocol of ZR Plant/Seed DNA MiniPrep kit (Zymo Research Corporation, Irvine, CA, USA). In short, samples were harvested in ZR BashingBead Lysis tubes and shredded under frozen conditions with the Retsch Mixer Mill MM400. Fast-spin column technology was used to remove impurities and two wash steps were performed before purified DNA plant extract was eluted in 50 µL DNA Elution Buffer. Subsequently, the eluted DNA was filtered to remove any remaining polyphenolics. Afterwards, the

Nanodrop® ND-1000 Spectrophotometer (Isogen Life Science, De Meern, The Netherlands) was used to determine the DNA quantity and purity. DNA samples were stored at -80°C until further use.

An input of 900 ng DNA was used for the measurement of DNA methylation in a total volume of 25 µL. Subsequently, 3.5 µL mastermix, containing 2.5 µL 10x DNA degradase reaction buffer and 1 µL DNA degradase plus enzyme, was added to each sample. DNA degradation was carried out on a PCR gradient thermal cycler (Techne TC-500;Techne, Ramsey, MN, USA) by incubating the samples 2 h at 37°C and 20 min at 70°C, respectively to activate and inactivate the DNA degradase enzyme. The enzymatically digested DNA samples were inserted in the UPLC-MS/MS apparatus at the unit Seperation and Conversion Technologies (VITO, Mol, Belgium) to determine the percentage of global DNA methylation of the root samples.

2.8 STATISTICAL ANALYSIS

The free software package R (version 3.2.2) was used for statistical analysis. Roots and leaves were analysed separately. Two-way ANOVA was used to determine effects of the treatment (U or Cd), seed type and treatment x seed type interaction effect. Statistical differences in group means were determined with a post-hoc Tukey Kramer adjustment for multiple comparisons. Normal distribution of the data was tested with the Shapiro-Wilk test, and homoscedasticity was evaluated with the Bartlett's test. To obtain normally distributed data, it was sometimes necessary to implement logarithmic or square root transformations of the data. If the data could not achieve a normal distribution, a non-parametric Kruskal-Wallice test was carried out with a post-hoc Wilcoxon rank sum test.

3 RESULTS

To investigate if metal exposure in a previous generation can protect future generations of *A. thaliana* plants against metal-induced stress, plants grown from control and U-seeds were exposed for three days to different metal concentrations: 0 µM Cd or U, 5 µM Cd, 10 µM Cd, 25 µM U or 50 µM U. The control seeds come from *A. thaliana* plants that have never been exposed to heavy metal stress, while U-seeds are seeds which are derived from *A. thaliana* plants that were exposed continuously to 5 µM U. The effects on processes such as oxidative stress, DNA repair and DNA methylation were studied within and between the two seed types in root and leaf samples separately.

3.1 GROWTH RESPONSES

After exposure for three days to Cd or U, the fresh weights of both root and leaf samples were measured. Samples were also harvested at T_0, the time point at which the samples were exposed to different Cd and U concentrations (i.e. day 18). This enabled us to determine the growth responses of roots and leaves after metal exposure for three days (Fig. 2).
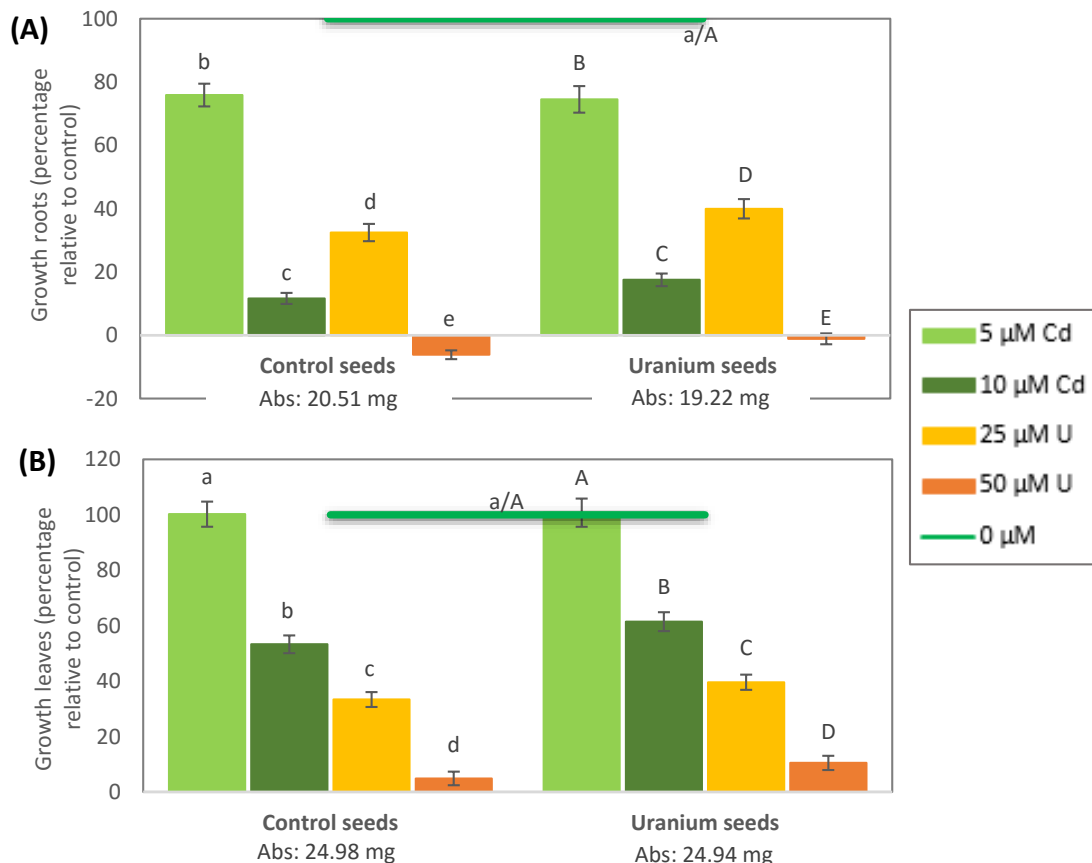
Figure 2. Relative growth of roots (A) and leaves (B) of A. thaliana plants from control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The growth of metal-exposed roots and leaves is represented as the percentage relative to their own control which was set to 100. The absolute values of the control conditions are set out in the figures and in Supplementary Table 1. Values represent the relative mean ± SE of at least 50 biological replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for control seeds ($p<0.05$). Different capital letters indicate a significant difference for uranium seeds ($p<0.05$).

The results show that metal exposure caused a significant decrease in growth in the roots of both control and U-seeds in all conditions (Fig. 2A). In addition, the higher metal concentrations (e.g. 10 µM Cd and 50 µM U) induce a stronger decrease in growth than the lower metal concentrations. The highest U concentration even shows a negative growth relative to the control condition of 0 µM. In comparison to the roots of U-seeds, growth is more decreased after exposure to 10 µM Cd, 25 µM U and 50 µM U in roots grown from control seeds (Fig. 2A).

Similar to roots, growth in leaves also has a decreasing trend with increasing metal concentrations (Fig. 2B). All conditions, except the leaves exposed to 5 µM Cd, are significantly decreased in growth relative to the controls. In addition, the leaves from U-seeds show slightly more growth after three days exposure than those from the control seeds (Fig. 2B).

In both roots and leaves, no significant differences are observed for the same metal condition between control and U-seeds (Fig.2).
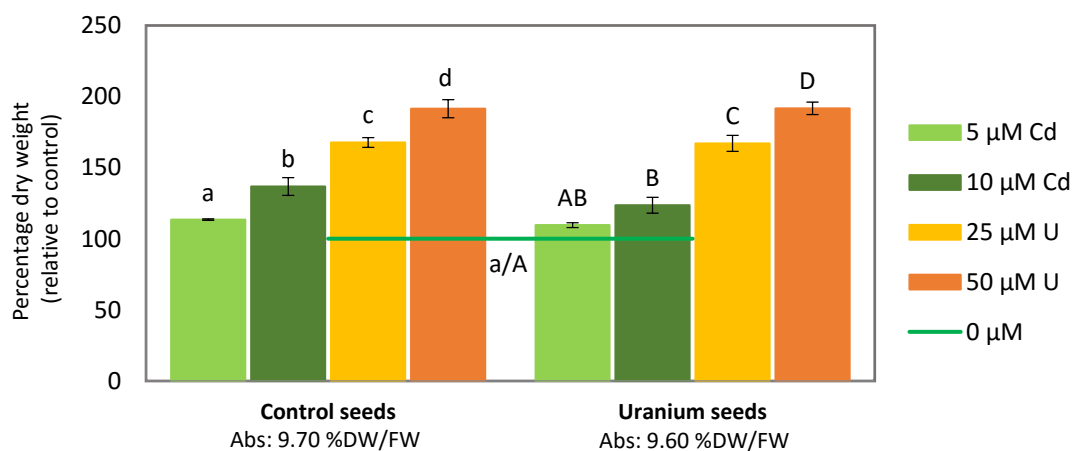
Figure 3. Percentage dry weight to fresh weight (%DW/FW) in leaves of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The percentages of metal-exposed leaves are represented relative to their own control which was set to 100. The absolute values of the control conditions are set out in the figure and in Supplementary Table 1. Data represent the mean ± SE of 4 biological replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for the control seeds (p<0.05). Different capital letters indicate a significant difference for the uranium seeds (p<0.05).

In addition to the fresh weights, dry weights of the leaves were also determined in order to obtain the percentage of dry weight of leaves. As shown in Figure 3, exposure to a higher metal concentration results in a higher percentage of dry weight to fresh weight (%DW/FW). Significant increases in % DW/FW are seen after exposure to 10 µM Cd, 25 and 50 µM U. However, no significant differences are observed between the leaves of control and U-seeds (Fig. 3). So while metal exposure induces a decreased fresh weight and growth in leaves (Fig. 2), exposure to Cd and U results in increasing dry weight (Fig. 3). An increased %DW/FW also suggests wilting of the plants.

3.2 CADMIUM AND URANIUM CONTENT

The amount of Cd and U taken up by the plants from the metal-contaminated Hoagland solution was determined by measuring the Cd and U concentrations in roots and leaves using ICP-MS.

In Table 2, Cd and U contents in roots are represented. The uptake of both metals in roots is higher in control than in U-seeds. However, only the Cd uptake after exposure to 10 µM Cd in control seeds shows a significantly higher Cd-amount regarding to the 10 µM Cd condition in U-seeds (Table 2).

For the control seeds, the amount of Cd and U that was taken up into the roots increases greatly with exposure to increasing Cd and U concentrations. The uptake of Cd after exposure to 10 µM Cd is more than two times the amount taken up from the Hoagland solution containing 5 µM Cd. In U-exposed roots, Cd uptake is not detectable because the levels of Cd in these roots are insignificant as compared to the Cd-exposed roots. The amount of U taken up from the Hoagland solution containing 25 µM U is more than three times less the U concentration after exposure to 50 µM U. The uptake of U in the Cd-exposed roots is also negligible compared to U-uptake after exposure to U (Table 2).

The same response is observed for Cd and U uptake in the roots from U-seeds (Table 2). The uptake of Cd and U is significantly higher for the higher exposure conditions (i.e. 10 µM Cd and 50 µM U) compared to the lower exposure conditions (i.e. 5 µM Cd and 25 µM U). The amount of Cd in U-exposed roots and U in Cd-exposed roots is again not detectable compared to the concentrations of Cd and U uptake, respectively (Table 2).

Exposure to 10 µM Cd shows a significantly different Cd uptake between the roots of control and U-seeds. The amount of Cd that was taken up after 10 µM Cd-exposure in control seeds is significantly higher compared to the amount of Cd taken up in uranium seeds after exposure to 10 µM Cd (Table 2).

Table 2. Metal concentrations (µg/g DW) in roots of A. thaliana plants from control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. Data represent the mean ± SE of at least 4 biological independent replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for the control seeds (p<0.01). Different capital letters indicate a significant difference for the uranium seeds (p<0.01). An asterisk (*) indicates a significant difference between the roots of control and uranium seeds for the same condition (p<0.01). N.D.: not detectable.

Control seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
Cd (µg/g DW)	N.D.	1 101.60 ± 39.26[a]	2 871.56 ± 168.41[b*]	N.D.	N.D.
U (µg/g DW)	N.D.	N.D.	N.D.	17 962.83 ± 1 672.52[a]	59 758.99 ± 9 335.60[b]
Uranium seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
Cd (µg/g DW)	N.D.	989.13 ± 58.60[A]	2 216.01 ± 110.63[B*]	N.D.	N.D.
U (µg/g DW)	N.D.	N.D.	N.D.	16 567.16 ± 1 173.34[A]	46 112.18 ± 6 392.97[B]

The Cd and U concentrations taken up in the leaves are represented in Table 3. Similar to the results within the roots, the amount of Cd- and U-uptake also increases with increasing Cd and U concentrations (Table 3). For the leaves of control seeds, only a slight increase of Cd concentration is observed for the 10 µM Cd condition compared to the exposure condition of 5 µM Cd. Uptake of Cd after exposure of to 25 and 50 µM U is negligible in comparison to Cd uptake after Cd-exposed leaves. The amount of U taken up after exposure to 50 µM U is more than twice the amount of U absorbed after 25 µM U exposure. Furthermore, the concentrations of U in Cd-exposed leaves are not detectable as these concentrations were very low (Table 3).

In the leaves grown from U-seeds, exposure to 10 µM Cd has almost double the amount of Cd taken

up in the leaves compared to exposure to 5 µM Cd (Table 3). As seen within the control seeds, the Cd-uptake in U-exposed leaves is insignificant and non-detectable. The U-uptake in plants grown from U-seeds is similar to the uptake of U in control seeds (Table 3).

Both U and Cd concentrations are lower in leaves than in roots but especially the amount of U is very low in leaves as compared to the amount observed in roots.

Table 3. Metal concentrations (µg/g DW) in leaves of A. thaliana plants from control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. Data represent the mean ± SE of at least 4 biological independent replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for the control seeds (p<0.05). Different capital letters indicate a significant difference for the uranium seeds (p<0.05). N.D.: not detectable.

	Control seeds				
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
Cd (µg/g DW)	N.D.	714.59 ± 99.91^a	724.84 ± 27.46^a	N.D.	N.D.
U (µg/g DW)	N.D.	N.D.	N.D.	7.49 ± 0.90^a	16.48 ± 2.31^b
	Uranium seeds				
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
Cd (µg/g DW)	N.D.	490.83 ± 22.34^A	886.57 ± 73.36^B	N.D.	N.D.
U (µg/g DW)	N.D.	N.D.	N.D.	7.19 ± 0.65^A	16.31 ± 2.62^{AB}

3.3 METAL-INDUCED OXIDATIVE STRESS-RELATED RESPONSES

To investigate the effects on the oxidative stress response induced by metal-exposure on *A. thaliana* plants, four parameters were determined in roots and/or leaves of plants exposed to Cd or U concentrations: [1] expression of selected pro- and antioxidative genes, [2] antioxidative enzyme capacities, [3] antioxidative metabolite concentrations and [4] amount of lipid peroxidation. In order to determine the effect of long-term exposure of plants to U in a previous generation, plants were grown from both control and U-seeds and the effects of these parameters were investigated in and between both seed types.

3.3.1 Expression of pro- and antioxidative genes

The expression of several selected pro- and antioxidative genes was determined in *A. thaliana* plants grown from control and U-seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. Results of the gene expression measurements in roots are presented in Table 4. The metal-exposed conditions of both control and U-seeds are shown relative to their controls.

The expression of several **pro-oxidative genes** is significantly affected after exposure to Cd and U in the roots of control and U-seeds (Table 4). For control seeds, expression levels of lipoxygenase 1 (*LOX1*) are significantly increased after exposure to 5 and 10 µM Cd. An increasing trend for the Cd conditions is also seen for respiratory burst oxidase homolog F (*RBOHF*), while after exposure to 25 and 50 µM U gene expression of both *LOX1* and *RBOHF* is decreased. In contrast, exposure to 10 µM Cd, 25 and 50 µM U significantly decreases *RBOHC* levels.

Similar to the control seeds, a significant increase of *LOX1* expression levels is also seen for the Cd conditions in plants grown from U-seeds (Table 4). In addition, exposure to 10 µM Cd significantly increases the expression of *RBOHF*. Unlike in control seeds, *LOX1* and *RBOHF* levels in U-seeds show an increasing trend after exposure to U.

In addition, significant differences in gene expression are observed between the same conditions of

the two different seed types. Exposure to 10 µM Cd results in a significant increase of the expression of all three pro-oxidative genes in the roots of U-seeds compared to those from control seeds. Also, *RBOHC* levels are significantly increased in roots from U-seeds after exposure to 25 and 50 µM U and the same is seen for *RBOHF* levels at 25 µM U (Table 4).

Exposure to Cd and U also significantly alters the expression of several **antioxidative genes** in the roots of control and U-seeds (Table 4). In roots of control seeds, the expression of catalase (*CAT2*) is significantly decreased in all metal-exposed conditions in a concentration-dependent manner. In contrast, other catalase isoforms (i.e. *CAT1* and *CAT3*) have expression levels with an increasing trend. Exposure to both Cd and U results also in a decrease of ascorbate peroxidase (*APX1*) levels, however, only the decrease after exposure to 10 µM Cd is significantly lower than the control condition. The expression levels of Cu/Zn superoxide dismutases (*CSD1, CSD2* and *CSD3*) are all significantly influenced by exposure to 10 µM Cd in a negative manner. In addition, *CSD1* expression is also significantly lower after exposure to 5 µM Cd. Metal exposure affects the different Fe superoxide dismutase (*FSD*) isoforms differently as *FSD1* and *FSD2* levels show a more increasing trend and expression of *FSD3* is rather decreasing. While *FSD1* levels are significantly increased after exposure to the Cd conditions, the expression of *FSD2* is significantly increased after exposure to 25 and 50 µM U. However, compared to the control condition, expression of *FSD1* is highly increased in all metal-exposed conditions but no clear effect of Cd on *FSD2* could be observed. Furthermore, exposure to 10 µM Cd and 50 µM U results in a significant decrease of *FSD3* expression levels in roots from control seeds.

For the U-seeds, a significant increase in *CAT1* expression is seen after exposure to the highest Cd and U concentrations (Table 4). The same is observed in the gene expression of *CAT3.* As is seen in roots of control seeds, the levels of *CAT2* expression in the roots of U-seeds show a similar decreasing trend, however not significantly compared to the control condition. Exposure to 10 µM Cd results in a significant increase in the expression of the glutathione reductase (GR) gene. The expression of *CSD1* and *CSD2* in roots of U-seeds is also negatively influenced by exposure to the different metals. A significant decrease is only seen for the Cd conditions, more specifically after 5 µM Cd, *CSD1* levels are significantly decreased, while *CSD2* levels show a significant decrease after 10 µM Cd. As seen within the control seeds, the gene expression of *FSD1* is very high compared to the control conditions, but only exposure to 10 µM Cd results in a significant increase in roots of U-seeds. The levels of *FSD3* are significantly decreased in roots of U-seeds after exposure to Cd and U, except for the decrease that is seen when a concentration of 25 µM U was applied. The effect of metal exposure on the expression of manganese superoxide dismutase 1 (*MSD1*) is very small in roots from both control and U-seeds as no significant change is observed compared to the control conditions (Table 4).

Significant differences are also seen within the same antioxidative genes in the roots between the two seed types (Table 4). Exposure to 10 µM Cd results in a significant change of the gene expression levels of *CAT2*, *CAT3*, *APX1*, *GR1*, *CSD3* and *MSD1*, where the expression is higher in U-seeds than control seeds for each gene. In addition, the expression of *APX1, CSD3* and *MSD1* is also significantly higher after exposure to 25 or/and 50 µM U in roots from U-seeds compared to control seeds. Only *FSD2* shows a significantly lower expression for U-seeds after exposure to 25 µM U (Table 4).

Table 4. Expression levels of pro- and antioxidative genes in roots of *A. thaliana* plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The expression in metal-exposed roots is represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in Supplementary Table 1. Values are the mean ± SE of at least 3 biological replicates. Significance levels (Two-way ANOVA) are relative to the controls (0 µM) and indicate an increased or decreased expression, respectively (= p < 0.05; = p < 0.01; = p < 0.05; = p < 0.01). Bold and underlined values indicate a significant difference between the roots of control and uranium seeds for the same metal condition (p<0.05). LOX: lipoxygenase; RBOH: respiratory burst oxidase; CAT: catalase; APX: ascorbate peroxidase; GR: glutathione reductase; CSD: copper/zinc superoxide dismutase; FSD: iron superoxide dismutase; MSD: manganese superoxide dismutase.

ROOTS	Control seeds					Uranium seeds				
	0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U	0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U
PRO-OXIDATIVE GENES										
LOX1	1 ± 0.46	3.69 ± 0.67	4.82 ± 1.36	0.92 ± 0.11	0.65 ± 0.07	1 ± 0.29	4.24 ± 1.46	17.55 ± 2.47	1.99 ± 0.18	1.83 ± 0.44
RBOHC	1 ± 0.07	0.81 ± 0.14	0.29 ± 0.08	0.17 ± 0.07	0.11 ± 0.01	1 ± 0.07	1.13 ± 0.27	1.74 ± 0.32	0.80 ± 0.30	0.49 ± 0.08
RBOHF	1 ± 0.29	1.78 ± 0.22	1.71 ± 0.43	0.49 ± 0.08	0.70 ± 0.05	1 ± 0.21	1.47 ± 0.30	5.39 ± 0.84	1.28 ± 0.05	1.63 ± 0.41
ANTIOXIDATIVE GENES										
CAT1	1 ± 0.28	1.17 ± 0.19	1.84 ± 0.52	1.1 ± 0.31	1.85 ± 0.27	1 ± 0.22	1.13 ± 0.24	3.78 ± 0.45	2.09 ± 0.47	3.69 ± 1.08
CAT2	1 ± 0.05	0.48 ± 0.04	0.28 ± 0.04	0.33 ± 0.05	0.14 ± 0.02	1 ± 0.13	0.67 ± 0.09	0.94 ± 0.14	0.68 ± 0.19	0.49 ± 0.10
CAT3	1 ± 0.20	1.42 ± 0.19	2.19 ± 0.12	2.15 ± 0.39	2.72 ± 0.45	1 ± 0.14	1.28 ± 0.16	4.88 ± 0.75	2.38 ± 0.25	4.09 ± 0.91
APX1	1 ± 0.09	0.82 ± 0.09	0.36 ± 0.10	0.63 ± 0.10	0.73 ± 0.10	1 ± 0.15	0.74 ± 0.08	1.06 ± 0.15	1.42 ± 0.09	1.59 ± 0.35
GR1	1 ± 0.19	1.02 ± 0.11	0.95 ± 0.23	0.40 ± 0.06	0.63 ± 0.080	1 ± 0.17	0.89 ± 0.13	2.83 ± 0.32	1.02 ± 0.06	1.40 ± 0.39
CSD1	1 ± 0.12	0.30 ± 0.08	0.28 ± 0.04	0.43 ± 0.06	0.57 ± 0.07	1 ± 0.12	0.22 ± 0.07	0.36 ± 0.05	0.45 ± 0.12	0.97 ± 0.33
CSD2	1 ± 0.10	0.39 ± 0.11	0.10 ± 0.01	0.36 ± 0.03	0.42 ± 0.06	1 ± 0.14	0.45 ± 0.15	0.19 ± 0.06	0.51 ± 0.16	0.74 ± 0.12
CSD3	1 ± 0.09	0.65 ± 0.06	0.25 ± 0.04	0.64 ± 0.09	0.90 ± 0.06	1 ± 0.14	1.05 ± 0.17	0.66 ± 0.06	1.64 ± 0.08	2.14 ± 0.68
FSD1	1 ± 0.10	24.63 ± 4.38	21.98 ± 5.69	11.58 ± 5.41	6.94 ± 1.10	1 ± 0.17	14.87 ± 2.47	23.17 ± 3.01	11.45 ± 4.12	8.21 ± 2.5
FSD2	1 ± 0.49	1.95 ± 0.65	0.70 ± 0.06	3.53 ± 0.51	3.20 ± 0.82	1 ± 0.17	0.83 ± 0.12	0.40 ± 0.06	1.48 ± 0.22	1.43 ± 0.21
FSD3	1 ± 0.09	0.95 ± 0.08	0.33 ± 0.05	1.00 ± 0.09	0.63 ± 0.06	1 ± 0.08	0.66 ± 0.05	0.43 ± 0.04	0.87 ± 0.07	0.55 ± 0.01
MSD1	1 ± 0.12	0.94 ± 0.07	0.74 ± 0.04	0.80 ± 0.06	0.69 ± 0.20	1 ± 0.04	0.92 ± 0.08	1.22 ± 0.08	1.09 ± 0.06	1.37 ± 0.33

The expression levels of the selected pro- and antioxidative genes in leaves are presented in Table 5. In leaves, the gene expression levels show less significant changes within and between both seed types in comparison to the roots. Nevertheless, exposure to Cd and U also induces significant alterations in the expression of several **pro-oxidative genes** in the leaves of control and U-seeds. However, in contrast to the roots, changes in gene expression levels measured in metal-exposed leaves are similar for both seed types. The expression of *LOX1* and *LOX2* is significantly increased after exposure to 10 µM Cd in leaves of both control and U-seeds. In addition, exposure to 25 µM U significantly increases *LOX2* levels in leaves from U-seeds. The expression levels of *RBOHF* shows a slight increase in response to Cd exposure, while exposure to U results in downregulation. However, a significant decrease of *RBOHF* levels is only seen after exposure to 25 µM U. Leaves of both control and U-seeds show a similar expression pattern for *RBOHF* (Table 5).

Exposure to Cd and U also significantly alters the expression of several **antioxidative genes** in leaves of control and U-seeds (Table 5). In general, responses in leaves from U-seeds are highly similar to those observed in control seeds. While *CAT1* and *CAT2* expression both show a decreasing trend, the expression of *CAT3* is increased after exposure to the Cd and U concentrations, except after exposure to 5 µM Cd, expression levels remain unchanged. In leaves of both control and U-seeds, exposure to all four metal conditions results in a significantly decreased expression of *CSD1* and *CSD2.* Leaf *APX1*, *FSD2*, *FSD3* and *MSD1* expressions are also downregulated by exposure to Cd and U in both seed types, however, not significantly. Exposure to 25 and 50 µM U results in a deceased expression of *GR1*, while exposure to Cd concentrations only shows a slight change (Table 5).

Table 5. Expression levels of pro- and antioxidative genes in leaves of A. thaliana plants of control and uranium seeds exposed to 0 μM, 5 or 10 μM Cd, 25 or 50 μM U for three days. The expression in metal-exposed leaves is represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in Supplementary Table 1. Values are the mean ± SE of 4 biological replicates. Significance levels (Two-way ANOVA) are relative to the controls (0 μM) and indicate an increased or decreased expression, respectively (■ = $p < 0.05$; ■ = $p < 0.01$; ■ = $p < 0.05$; ■ = $p < 0.01$). LOX: lipoxygenase; RBOH: respiratory burst oxidase; CAT: catalase; APX: ascorbate peroxidase; GR: glutathione reductase; CSD: copper/zinc superoxide dismutase; FSD: iron superoxide dismutase; MSD: manganese superoxide dismutase.

LEAVES	Control seeds					Uranium seeds				
	0 μM	5 μM Cd	10 μM Cd	25 μM U	50 μM U	0 μM	5 μM Cd	10 μM Cd	25 μM U	50 μM U
PRO-OXIDATIVE GENES										
LOX1	1 ± 0.25	1.17 ± 0.23	4.20 ± 0.93	0.91 ± 0.22	1.21 ± 0.13	1 ± 0.15	1.73 ± 0.56	6.00 ± 1.25	1.19 ± 0.21	0.96 ± 0.18
LOX2	1 ± 0.25	2.16 ± 0.44	5.38 ± 1.10	2.98 ± 0.42	3.61 ± 0.45	1 ± 0.27	2.12 ± 0.48	5.62 ± 1.13	4.21 ± 0.86	3.20 ± 0.45
RBOHF	1 ± 0.13	1.85 ± 0.57	1.64 ± 0.08	0.31 ± 0.07	0.43 ± 0.04	1 ± 0.24	1.08 ± 0.44	1.24 ± 0.17	0.28 ± 0.06	0.30 ± 0.05
ANTIOXIDATIVE GENES										
CAT1	1 ± 0.19	0.46 ± 0.12	0.85 ± 0.23	0.34 ± 0.08	0.52 ± 0.10	1 ± 0.11	0.36 ± 0.05	1.03 ± 0.31	0.46 ± 0.11	0.40 ± 0.06
CAT2	1 ± 0.21	0.75 ± 0.04	0.36 ± 0.04	0.37 ± 0.05	0.23 ± 0.03	1 ± 0.13	0.62 ± 0.08	0.37 ± 0.05	0.48 ± 0.05	0.20 ± 0.02
CAT3	1 ± 0.07	0.92 ± 0.19	2.92 ± 0.58	2.33 ± 0.46	2.34 ± 0.37	1 ± 0.08	1.03 ± 0.20	2.53 ± 0.39	1.84 ± 0.12	1.39 ± 0.27
APX1	1 ± 0.20	0.60 ± 0.18	0.75 ± 0.15	0.73 ± 0.17	0.71 ± 0.12	1 ± 0.19	0.43 ± 0.10	0.59 ± 0.06	0.86 ± 0.22	0.64 ± 0.11
GR1	1 ± 0.24	1.08 ± 0.26	1.15 ± 0.19	0.47 ± 0.08	0.51 ± 0.04	1 ± 0.16	0.75 ± 0.15	1.03 ± 0.11	0.51 ± 0.11	0.43 ± 0.04
CSD1	1 ± 0.09	0.14 ± 0.04	0.13 ± 0.02	0.30 ± 0.11	0.46 ± 0.03	1 ± 0.03	0.16 ± 0.03	0.11 ± 0.02	0.34 ± 0.10	0.33 ± 0.07
CSD2	1 ± 0.28	0.09 ± 0.02	0.03 ± 0.01	0.18 ± 0.07	0.20 ± 0.02	1 ± 0.24	0.08 ± 0.02	0.03 ± 0.01	0.23 ± 0.07	0.18 ± 0.04
CSD3	1 ± 0.26	1.23 ± 0.21	0.97 ± 0.05	1.23 ± 0.24	1.19 ± 0.03	1 ± 0.12	0.96 ± 0.16	1.13 ± 0.07	1.84 ± 0.30	1.57 ± 0.28
FSD1	1 ± 0.20	0.90 ± 0.14	1.41 ± 0.26	1.04 ± 0.54	0.41 ± 0.12	1 ± 0.25	0.89 ± 0.10	1.42 ± 0.17	1.56 ± 0.72	0.60 ± 0.20
FSD2	1 ± 0.37	0.89 ± 0.30	0.32 ± 0.11	0.34 ± 0.12	0.29 ± 0.09	1 ± 0.16	0.78 ± 0.27	0.37 ± 0.07	0.50 ± 0.09	0.37 ± 0.07
FSD3	1 ± 0.14	0.98 ± 0.06	0.93 ± 0.03	0.78 ± 0.14	0.59 ± 0.10	1 ± 0.18	0.92 ± 0.23	0.55 ± 0.05	0.70 ± 0.10	0.47 ± 0.11
MSD1	1 ± 0.16	0.98 ± 0.14	0.86 ± 0.08	0.56 ± 0.06	0.71 ± 0.07	1 ± 0.09	0.94 ± 0.11	0.96 ± 0.02	0.89 ± 0.21	0.67 ± 0.10

3.3.2   Antioxidative enzyme capacities

The capacity of six antioxidative enzymes SOD, CAT, GR, APX, GPX and SPX was spectro-photometrically determined in both roots and leaves samples from *A. thaliana* grown from control and U-seeds. The protein content of all samples was also measured in order to represent the enzyme capacities in U/mg protein. The results of the enzyme measurements in roots and leaves are shown in Table 6 and Table 7, respectively. The response of enzyme capacities to metal exposure is different in both organs and between both seed types.

In roots, no significant differences in the capacities of all six measured enzymes are seen after exposure to Cd or U in control seeds (Table 6). In contrast, metal exposure in roots of U-seeds did cause significant alterations in the enzyme capacities of SOD, APX and GPX. Their capacity is increased after exposure to all four metal conditions, however only exposure to 10 µM Cd, 25 and 50 µM U results in significant increases. In general, the capacity of all enzymes is higher in roots from U-seeds than control seeds. In addition, both APX and GPX capacities show a concentration-dependent increase in both seed types. The other enzymes, however, are not affected by metal exposure in a specific manner. Between roots from control and U-seeds, exposure to 5 µM Cd is significantly different for the SOD capacity (Table 6).

Table 6. Antioxidative enzyme capacities (U/mg proteins) in roots of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The enzyme capacities in metal-exposed roots are represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in Supplementary Table 1. Values are the mean ± SE of at least 3 biological replicates. Significance levels (Two-way ANOVA) are relative to the control conditions and indicate an increased capacity (= $p < 0.05$; = $p < 0.01$). Bold and underlined values indicate a significant difference between the roots of control and uranium seeds for the same metal condition ($p<0.05$). SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; APX: ascorbate peroxidase; GPX: guaiacol peroxidase; SPX: syringaldazine peroxidase.

Control seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.22	**0.64 ± 0.04**	1.01 ± 0.15	0.69 ± 0.11	1.27 ± 0.15
CAT	1 ± 0.28	0.98 ± 0.14	0.80 ± 0.17	0.82 ± 0.19	0.63 ± 0.07
GR	1 ± 0.44	1.06 ± 0.43	1.27 ± 0.69	1.01 ± 0.43	0.90 ± 0.42
APX	1 ± 0.19	0.77 ± 0.06	1.41 ± 0.14	0.97 ± 0.08	1.99 ± 0.55
GPX	1 ± 0.12	1.19 ± 0.13	1.86 ± 0.40	1.24 ± 0.21	1.49 ± 0.13
SPX	1 ± 0.16	0.73 ± 0.08	0.77 ± 0.13	0.80 ± 0.09	0.85 ± 0.14
Uranium seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.08	**1.37 ± 0.14**	1.65 ± 0.06	1.73 ± 0.19	1.83 ± 0.19
CAT	1 ± 0.05	1.17 ± 0.14	0.88 ± 0.10	1.01 ± 0.13	0.60 ± 0.10
GR	1 ± 0.5	1.55 ± 0.56	1.55 ± 0.76	1.73 ± 0.56	1.78 ± 0.69
APX	1 ± 0.16	1.19 ± 0.08	2.34 ± 0.15	1.56 ± 0.15	2.11 ± 0.24
GPX	1 ± 0.11	1.48 ± 0.21	2.37 ± 0.35	1.62 ± 0.18	1.91 ± 0.33
SPX	1 ± 0.04	0.84 ± 0.08	0.72 ± 0.05	1.14 ± 0.12	0.83 ± 0.07

The enzyme capacities are differently altered in leaves of control and U-seeds in comparison to roots (Table 7). The capacities of SOD, CAT and APX are not significantly affected by Cd or U exposure in the leaves of both seed types. Exposure to 10 µM Cd results in a significant increase of GPX capacity in control seeds and of SPX capacity in U-seeds. The enzyme capacity of GR is increased after

exposure to U in a concentration-dependent manner in leaves from both seed types. However, only exposure to 50 µM U in leaves of U-seeds results in a significant increase. In addition, GR capacity is significantly different upon exposure to 5 µM Cd between leaves of control and U-seeds. No other great differences are seen in the other enzyme capacities between the leaves of both seed types (Table 7).

Table 7. Antioxidative enzyme capacities (U/mg proteins) in leaves of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The enzyme capacities in metal-exposed leaves are represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in Supplementary Table 1. Values are the mean ± SE of 4 biological replicates. Significance levels (Two-way ANOVA) are relative to the control conditions and indicate an increased capacity (▇ = $p < 0.05$). Bold and underlined values indicate a significant difference between the leaves of control and uranium seeds for the same metal condition ($p<0.05$). SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; APX: ascorbate peroxidase; GPX: guaiacol peroxidase; SPX: syringaldazine peroxidase.

	Control seeds				
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.08	1.19 ± 0.02	1.24 ± 0.25	0.97 ± 0.06	0.79 ± 0.06
CAT	1 ± 0.07	0.99 ± 0.07	1.11 ± 0.06	1.10 ± 0.15	0.89 ± 0.07
GR	1 ± 0.16	**0.86 ± 0.14**	0.99 ± 0.10	1.18 ± 0.09	1.55 ± 0.19
APX	1 ± 0.19	1.00 ± 0.21	1.82 ± 0.19	1.31 ± 0.31	1.31 ± 0.30
GPX	1 ± 0.15	1.13 ± 0.09	1.95 ± 0.22	1.02 ± 0.04	1.18 ± 0.14
SPX	1 ± 0.19	1.19 ± 0.15	1.78 ± 0.20	1.10 ± 0.09	0.74 ± 0.10
	Uranium seeds				
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.06	1.49 ± 0.16	1.23 ± 0.24	0.90 ± 0.07	1.07 ± 0.14
CAT	1 ± 0.02	0.95 ± 0.09	1.13 ± 0.08	1.20 ± 0.01	1.11 ± 0.10
GR	1 ± 0.10	**1.83 ± 0.25**	1.60 ± 0.16	1.41 ± 0.29	1.94 ± 0.23
APX	1 ± 0.08	1.73 ± 0.37	1.99 ± 0.17	1.23 ± 0.22	1.54 ± 0.28
GPX	1 ± 0.10	1.13 ± 0.16	1.79 ± 0.50	0.81 ± 0.07	1.14 ± 0.07
SPX	1 ± 0.09	1.32 ± 0.21	1.91 ± 0.34	0.89 ± 0.08	0.93 ± 0.13

The enzyme capacities were also set out in accordance to the fresh weights of the samples instead of the protein content. These results are shown in Supplementary Tables 2 and 3 for roots and leaves, respectively. The enzyme capacities showed different significant increases, especially after exposure to 10 µM Cd and 50 µM U in roots of both control and U-seeds (Supplementary Table 2) compared to the results of the roots shown in U/mg protein (Table 6). In leaves, less significant alterations were measured in comparison to the roots (Supplementary Table 3).

3.3.3   Antioxidative metabolite concentrations
The concentrations of the oxidized and reduced forms of the antioxidative metabolite GSH were measured spectrophotometrically in both roots (Fig. 4) and leaves (Fig. 5) exposed to Cd and U. The total amount of GSH was measured, as were the concentrations of GSSG and GSH separately and the percentage of reduced GSH. In both organs, the different metabolite concentrations are very similar between control and U-seeds. The amount of total and reduced GSH is higher in leaves than in roots, while the amount of GSSG and the percentage of reduced GSH is the same in both roots and leaves.

In roots, reduced GSH concentrations are not significantly affected by any of the exposure conditions in both control and U-seeds. Moreover, the same is seen for the total GSH concentrations after all

four metal exposures in the plants grown from U-seeds (Fig. 4). A significant increase of the total GSH concentrations is seen after exposure of the roots from control seeds to 10 µM Cd. Oxidized glutathione concentrations are increased after exposure to 10 µM Cd, 25 and 50 µM U, however they are only significant in comparison to the 5 µM Cd condition, which is lower than the control condition of the control seeds. For U-seeds, exposure to 5 µM Cd results in a decrease and exposure to 10 µM Cd, 25 and 50 µM U in an increase, which is similar in the control seeds. A significant increase in GSSG concentrations is observed only after exposure to 25 µM U in roots of U-seeds (Fig. 4).

The percentage of reduced GSH levels shows a decreasing trend in both seed types. However, only exposure to 10 µM Cd and 25 µM U results in a significant decrease of the percentage of reduced GSH levels in roots of U-seeds (Fig. 4).
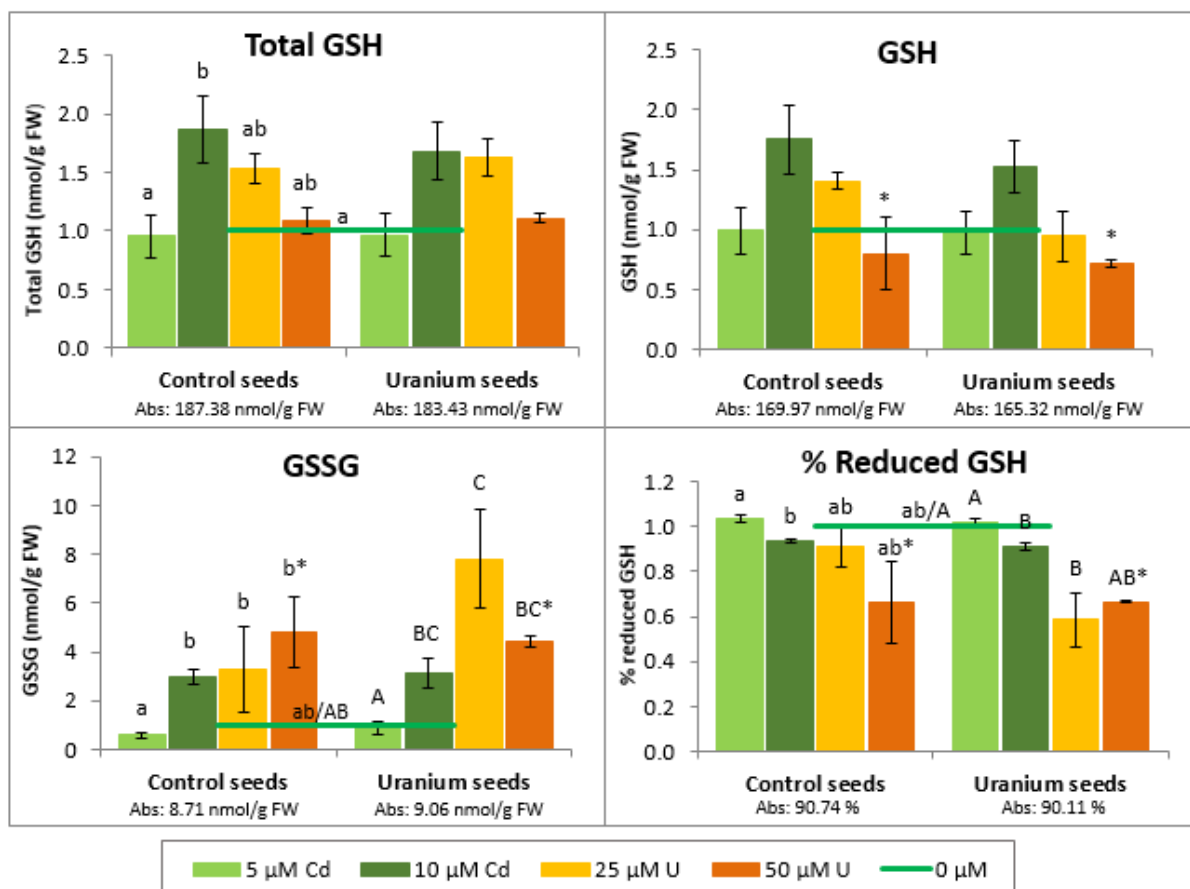
Figure 4. Glutathione concentrations (nmol/g FW) in roots of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The antioxidative metabolite concentrations of metal-exposed roots are represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in the figures and in Supplementary Table 1. Data represent the mean ± SE of at least 3 biological replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for the control seeds (p<0.05). Different capital letters indicate a significant difference for the uranium seeds (p<0.05). An asterisk (*) indicates that data is derived from only 2 biological replicates. GSH: glutathione (reduced form); GSSG: glutathione disulfide (oxidized form); Total GSH: addition of reduced and oxidized glutathione; % Reduced GSH: percentage of reduced GSH to total GSH.

In leaves, exposure to 10 µM Cd significantly increases the total and reduced GSH concentrations in both control and U-seeds (Fig. 5). The other metal conditions results in a decrease of total and reduced GSH concentrations. Oxidized glutathione concentrations, in contrast, are significantly lower after exposure to 5 and 10 µM Cd in both seed types and higher in response to U-exposure. The percentage reduced GSH is significantly increased after exposure to Cd in control seeds, while

exposure to 50 µM U results in a significant decrease. For U-seeds, a significant decrease is also seen after exposure to 25 and 50 µM U. Furthermore, significant differences are observed in the percentage levels of reduced GSH for the same exposure conditions between control and U-seeds. The percentage of reduced GSH is significantly higher in control seeds after exposure to 5 and 10 µM Cd and 25 µM U than in U-seeds (Fig. 5).
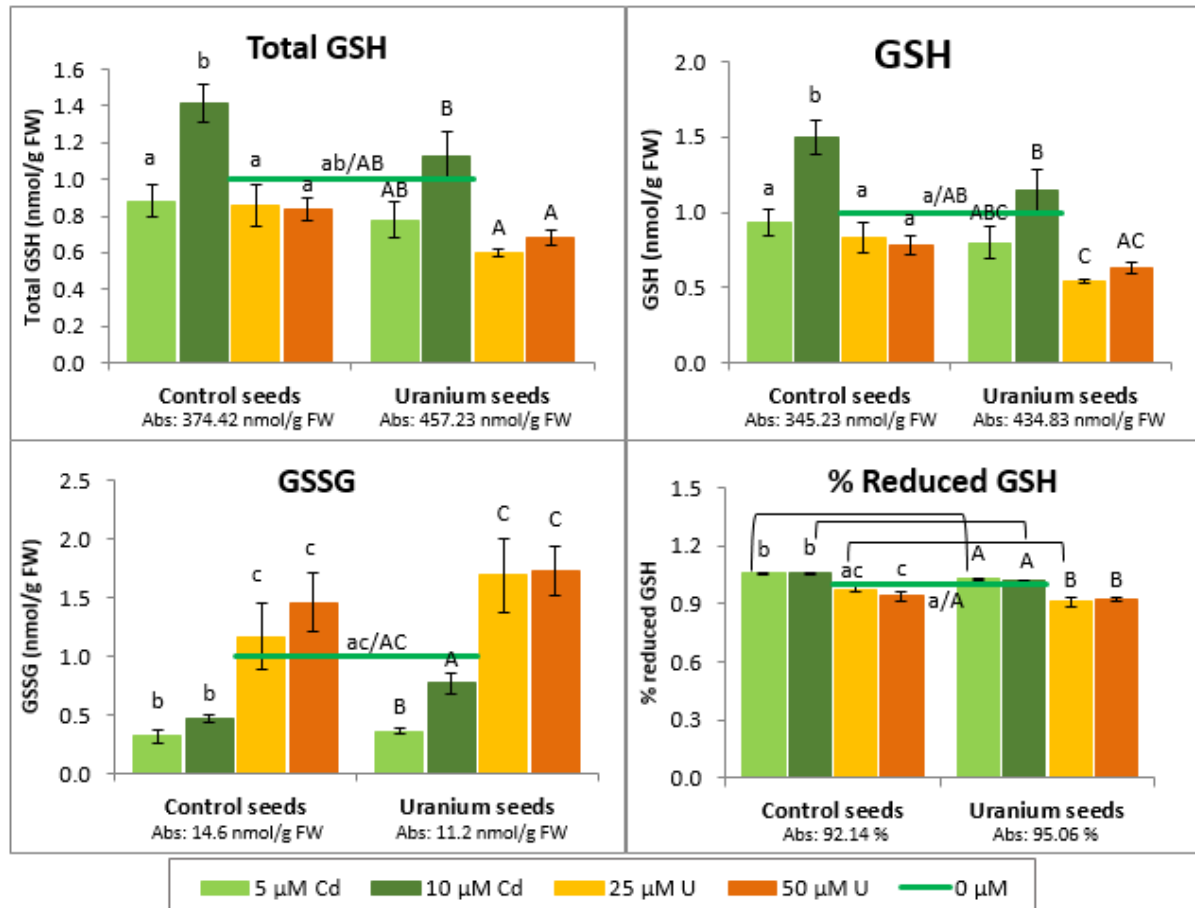
Figure 5. Glutathione concentrations (nmol/g FW) in leaves of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The antioxidative metabolite concentrations of metal-exposed leaves are represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in the figures and in Supplementary Table 1. Data represent the mean ± SE of 4 biological replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for the control seeds (p<0.05). Different capital letters indicate a significant difference for the uranium seeds (p<0.05). Brackets indicate significant differences between the same metal condition of different seed types (p<0.05). GSH: glutathione (reduced form); GSSG: glutathione disulfide (oxidized form); Total GSH: addition of reduced and oxidized glutathione; % Reduced GSH: percentage of reduced GSH to total GSH.

3.3.4 Amount of lipid peroxidation

Thiobarbituric acid reactive compounds (TBA-rc) are measured in *A. thaliana* leaves to have an indication of the amount of membrane damage induced by metal-exposure. The amount of lipid peroxidation is shown in Figure 6 as TBA-rc concentrations relative to the control. A significant increase of TBA-rc levels is seen after exposure to 10 µM Cd, 25 and 50 µM U in the leaves of both control and U-seeds (Fig. 6). Exposure to U results in higher TBA-rc concentrations than exposure to Cd. The TBA-rc levels are not significantly increased after exposure to 5 µM Cd compared to the control conditions. The amount of lipid peroxidation is smaller in leaves from U-seeds than in leaves from control seeds for all four metal conditions (Fig. 6).
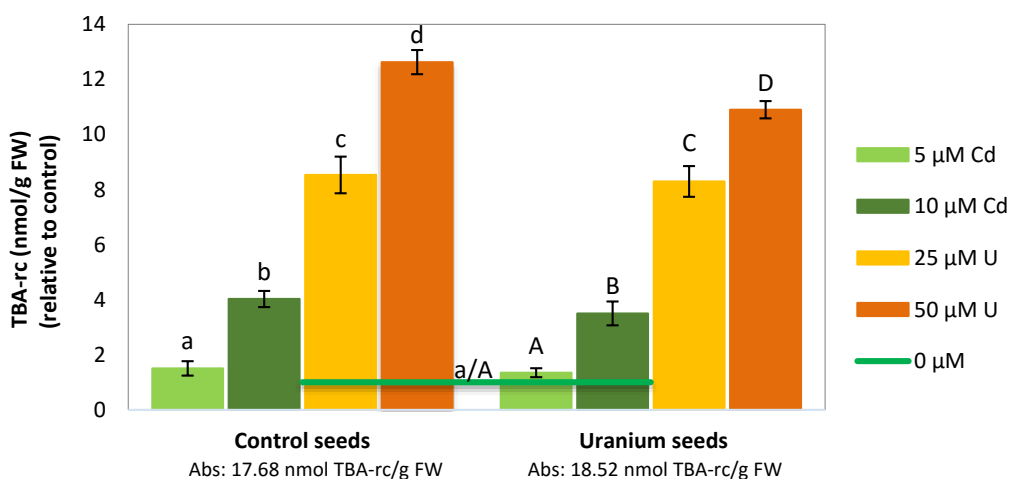
Figure 6. Lipid peroxidation measurements (nmol TBA-rc/g FW) in leaves of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The level of lipid peroxidation was based on the amount of TBA-reactive compounds (TBA-rc). The TBA-rc levels of metal-exposed leaves are represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in the figures and in Supplementary Table 1. Data represent the mean ± SE of 4 biological replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for the control seeds ($p<0.05$). Different capital letters indicate a significant difference for the uranium seeds ($p<0.05$).

3.4 EXPRESSION OF DNA REPAIR GENES AFTER METAL EXPOSURE

To investigate the difference in the occurrence and degree of DNA damage and repair in primed versus non-primed plants, the expression of several **DNA repair genes** was determined in *A. thaliana* plants grown from control and U-seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. In both roots and leaves from control and U-seeds, results of the metal-exposed conditions are presented relative to their controls.

Table 8 shows the results of the expression of genes involved in DNA repair pathways in roots. The expression of several DNA repair genes is significantly affected after exposure to Cd and U in the roots of both seed types. The expression levels of genes involved in **base excision repair** are, in general, decreased after exposure to all metal conditions (Table 8). For control seeds, Poly(ADP-ribose) polymerase 1 *(PARP1)* is significantly decreased after exposure to 10 µM Cd, while exposure to 10 µM Cd, 25 and 50 µM U results in a significant decrease of polymerase gamma 1 (*POLGAMMA1*) levels. A decreasing trend is also seen for the two genes involved in **non-homologous end joining.** Exposure to 10 µM Cd, 25 and 50 µM U significantly decreases expression of *KU80* in control seeds. Expression of ligase 4 (*LIG4*) is also decreased after exposure to both Cd and U, however not significantly in comparison to control. The three genes of **homologous recombination** have different expression patterns. Expression of RAD51 recombinase (*RAD51*) decreases after exposure to Cd, while exposure to U results in an increase. Meiotic nuclear divisions 1 (*MND1*) gene has no significantly changed expression after metal-exposure. Exposure to 10 µM Cd and 25 µM U induces a significant decrease in the expression of DNA meiotic recombinase 1 (*DMC1*). The expression of cyclin-dependent kinase inhibitor 2 (*KRP2*), which is involved **inhibition of the cell cycle**, is also decreased after exposure to all four metal conditions, however exposure to 5 µM Cd did not show a significant decrease (Table 8).

ROOTS	Control seeds					Uranium seeds				
	0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U	0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U
BASE EXCISION REPAIR (BER)										
PARP1	1 ± 0.03	0.99 ± 0.13	0.34 ± 0.05	0.65 ± 0.06	0.72 ± 0.09	1 ± 0.16	0.98 ± 0.15	0.73 ± 0.13	1.08 ± 0.12	1.60 ± 0.46
PARP2	1 ± 0.16	1.08 ± 0.12	0.51 ± 0.07	0.95 ± 0.25	0.48 ± 0.05	1 ± 0.27	1.50 ± 0.42	1.45 ± 0.41	2.30 ± 0.46	1.82 ± 0.50
POLGAMMA1	1 ± 0.13	0.79 ± 0.16	**0.19 ± 0.03**	**0.37 ± 0.06**	**0.47 ± 0.06**	1 ± 0.11	0.86 ± 0.12	**0.77 ± 0.10**	**1.05 ± 0.06**	**1.34 ± 0.23**
NON-HOMOLOGOUS END JOINING (NHEJ)										
KU80	1 ± 0.09	0.65 ± 0.10	**0.33 ± 0.03**	**0.56 ± 0.05**	**0.54 ± 0.06**	1 ± 0.07	0.83 ± 0.11	**1.13 ± 0.10**	**1.20 ± 0.09**	**1.32 ± 0.19**
LIG4	1 ± 0.23	0.87 ± 0.26	**0.54 ± 0.19**	0.49 ± 0.13	0.34 ± 0.04	1 ± 0.17	0.99 ± 0.24	**1.67 ± 0.28**	1.08 ± 0.07	0.62 ± 0.06
HOMOLOGOUS RECOMBINATION (HR)										
RAD51	1 ± 0.08	0.89 ± 0.09	**0.34 ± 0.04**	**1.23 ± 0.08**	**0.91 ± 0.09**	1 ± 0.07	1.03 ± 0.10	**0.70 ± 0.13**	**2.35 ± 0.16**	**2.01 ± 0.40**
MND1	1 ± 0.18	1.22 ± 0.24	0.35 ± 0.09	0.83 ± 0.18	1.04 ± 0.29	1 ± 0.08	1.08 ± 0.11	0.54 ± 0.08	1.49 ± 0.10	1.45 ± 0.07
DMC1	1 ± 0.08	1.25 ± 0.15	**0.53 ± 0.07**	**0.51 ± 0.05**	0.74 ± 0.15	1 ± 0.02	1.24 ± 0.19	**1.23 ± 0.08**	**0.87 ± 0.09**	2.27 ± 0.95
CELL CYCLE INHIBITOR										
KRP2	1 ± 0.18	0.56 ± 0.06	**0.16 ± 0.03**	**0.35 ± 0.06**	**0.40 ± 0.01**	1 ± 0.07	0.60 ± 0.06	**0.42 ± 0.03**	**1.04 ± 0.08**	**1.13 ± 0.35**

In contrast to control seeds, less significant changes are observed in the expression of DNA repair genes in roots of U-seeds (Table 8). Only *RAD51* levels are significantly increased after exposure to 25 and 50 µM U and expression of *KRP2* is significantly decreased upon exposure to 10 µM Cd. In addition, the expression levels of DNA repair genes in roots of U-seeds are generally higher compared to control seeds. Significant differences are also seen between the gene expression levels of both seed types in roots. Expression of *POLGAMMA1* is significantly higher in roots of U-seeds after exposure to 10 µM Cd, 25 and 50 µM U. These exposure conditions also result in a significant higher expression of *KU80*, *RAD51* and *KRP2* in roots from U-seeds. *LIG4* levels are also significantly higher in U-seeds after exposure to 10 µM Cd. Exposure to 10 µM Cd and 25 µM U results in a significant difference in the expression of *DMC1* as the expression levels are higher in the roots of U-seeds than in the control seeds as well (Table 8).

The expression levels of the DNA repair genes in leaves are represented in Table 9. These results show no great statistical differences within and between the different genes in leaves from control and U-seeds (Table 9). For control seeds, exposure to 5 µM Cd significantly increases the expression of *PARP2*. Expression of the other two genes involved in **base excision repair**, e.g. *PARP1* and *POLGAMMA1*, also shows an increase upon exposure to 5 µM Cd, however not significantly. In contrast, exposure to U results in all three base excision repair genes in a decrease in gene expression. The expression levels of the **homologous recombination** genes are, in general, decreased after exposure to all four metal concentrations. In addition, exposure to 10 µM Cd significantly decreases expression of *DMC1*, while *RAD51* levels are also significantly affected by exposure to 50 µM U (Table 9). In contrast to the roots, no significant alterations are seen in the expression of genes involved in **non-homologous end joining** and **cell cycle inhibition** in the leaves of control seeds.

Similar to the control seeds, gene expression levels in leaves of U-seeds show no great significant changes (Table 9). Only expression of *PARP2* is significantly increased after exposure to 5 µM U and *RAD51* levels are significantly decreased after exposure to 50 µM U in leaves of U-seeds. In addition, similar expression patterns of the other DNA repair genes are seen for both seed types and no significant differences between control and uranium seeds are observed (Table 9).

Table 9. Expression levels of DNA repair genes in leaves of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The expression in metal-exposed leaves is represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in Supplementary Table 1. Values are the mean ± SE of 4 biological replicates. Significance levels (Two-way ANOVA) are relative to the controls and indicate an increased or decreased expression, respectively (■ = $p < 0.05$; ■ = $p < 0.05$; ■ = $p < 0.01$). *PARP*: poly(ADP-ribose) polymerase; *POLGAMMA1*: polymerase gamma 1; *KU80*: KU80 homolog; *LIG4*: ligase 4; *RAD51*: RAD51 recombinase; *DMC1*: DNA meiotic recombinase 1; *MND1*: meiotic nuclear divisions 1; *KRP2*: cyclin-dependent kinase inhibitor 2.

LEAVES	Control seeds						Uranium seeds				
	0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U		0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U
BASE EXCISION REPAIR (BER)											
PARP1	1 ± 0.24	1.19 ± 0.14	0.69 ± 0.06	0.71 ± 0.08	0.66 ± 0.07		1 ± 0.16	1.16 ± 0.32	0.72 ± 0.08	0.68 ± 0.07	0.56 ± 0.10
PARP2	1 ± 0.20	4.58 ± 0.52	1.27 ± 0.16	0.58 ± 0.10	0.45 ± 0.12		1 ± 0.19	5.48 ± 2.26	1.23 ± 0.21	0.53 ± 0.13	0.37 ± 0.11
POLGAMMA1	1 ± 0.24	1.52 ± 0.30	1.27 ± 0.07	0.74 ± 0.10	0.77 ± 0.08		1 ± 0.13	1.51 ± 0.41	1.50 ± 0.18	0.91 ± 0.18	0.68 ± 0.10
NON-HOMOLOGOUS END JOINING (NHEJ)											
KU80	1 ± 0.13	1.08 ± 0.09	1.22 ± 0.10	0.87 ± 0.09	0.99 ± 0.09		1 ± 0.09	0.84 ± 0.13	0.98 ± 0.09	0.88 ± 0.11	0.78 ± 0.10
LIG4	1 ± 0.29	0.77 ± 0.26	0.96 ± 0.38	0.56 ± 0.14	0.97 ± 0.25		1 ± 0.18	0.68 ± 0.20	0.80 ± 0.11	0.65 ± 0.19	0.45 ± 0.08
HOMOLOGOUS RECOMBINATION (HR)											
RAD51	1 ± 0.19	2.11 ± 0.23	0.60 ± 0.08	0.57 ± 0.06	0.37 ± 0.08		1 ± 0.15	1.88 ± 0.47	0.53 ± 0.07	0.64 ± 0.11	0.29 ± 0.07
MND1	1 ± 0.14	1.71 ± 0.29	0.64 ± 0.10	0.62 ± 0.17	0.68 ± 0.12		1 ± 0.17	1.86 ± 0.59	0.74 ± 0.06	0.78 ± 0.11	0.49 ± 0.07
DMC1	1 ± 0.24	0.40 ± 0.08	0.30 ± 0.03	0.62 ± 0.04	0.69 ± 0.11		1 ± 0.13	0.41 ± 0.10	0.41 ± 0.08	0.85 ± 0.17	0.53 ± 0.11
CELL CYCLE INHIBITOR											
KRP2	1 ± 0.19	1.02 ± 0.09	1.17 ± 0.03	1.17 ± 0.10	1.14 ± 0.09		1 ± 0.13	1.17 ± 0.22	1.33 ± 0.11	1.67 ± 0.17	1.04 ± 0.18

3.5 DNA METHYLATION

Besides investigating the effects of metal exposure onto oxidative stress and DNA repair parameters, the difference in the degree of DNA methylation was also studied in roots and leaves of *A. thaliana* plants. First, the expression of selected genes involved in the methylation process was analysed. In addition, we looked at the global DNA methylation levels in metal-exposed roots.

3.5.1 Expression of genes involved in methylation

Similar to the expression profiles of oxidative stress-related and DNA repair genes, the expression of several **methylation genes** was also determined in *A. thaliana* plants grown from control and U-seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. Results of these gene expression measurements are shown in Tables 10 and 11 for roots and leaves, respectively. All four metal exposures are presented relative to their control conditions in control as well as U-seeds.

As shown in Table 10, several significant differences are seen in roots of both control and U-seeds upon metal exposure. In control seeds, exposure to 10 µM Cd results in a significant decrease in the expression of *CMT3* and *MET1*. In addition, *MET1* levels are also significantly decreased upon exposure to 50 µM U. While the expression of *CMT3* and *MET1* is decreased after exposure to both Cd and U, the expression levels of *DRM2* remain unchanged or show a slight increase.

In U-seeds, however, other significant changes are observed (Table 10). Exposure to 25 and 50 µM U results in an increase of the expression levels of all three tested DNA methylation genes. Furthermore, expression of *DRM2* is significantly increased after exposure to 5 and 10 µM Cd, while exposure to Cd leads to a decrease in expression levels of both *CMT3* and *MET1*.

Significant differences are also observed in gene expression between roots of control and U-seeds (Table 10). For *CMT3* expression levels, exposure to U results in a higher gene expression in U-seeds compared to control seeds. The same is seen for the expression levels of *DRM2* and *MET1* but moreover, the expression of these genes is also significantly elevated in roots of U-seeds upon exposure to 10 µM Cd (Table 10).

In leaves, different significant changes are observed compared to gene expression levels in roots (Table 11). In leaves of control seeds, expression of *CMT3* and *MET1* is decreased after exposure to Cd and significantly decreased after exposure to U. The gene expression levels of *DRM2* are increased upon metal exposure and furthermore, exposure to 10 µM Cd significantly increased gene expression of *DRM2* in leaves of control seeds. The same significant differences are seen in the gene expression profiles of *CMT3, DRM2* and *MET1* in leaves of U-seeds (Table 11). As the expression levels are similar in both seed types, no significant changes are observed between the leaves of control and U-seeds (Table 11).

Table 10. Expression levels of DNA methylation genes in roots of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The expression in metal-exposed roots is represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in Supplementary Table 1. Values are the mean ± SE of at least 3 biological replicates. Significance levels (Two-way ANOVA) are relative to the controls and indicate an increased or decreased expression, respectively ([ ] = $p < 0.05$; [ ] = $p < 0.01$; [ ] = $p < 0.05$; [ ] = $p < 0.01$). Bold and underlined values indicate a significant difference between the roots of control and uranium seeds for the same metal condition ($p<0.05$). *CMT3*: chromo-methyltransferase 3; *DRM2*: domains rearranged methyltransferase 2; *MET1*: DNA (cytosine-5)-methyltransferase 1.

ROOTS	Control seeds						Uranium seeds				
	0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U		0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U
DNA METHYLATION GENES											
CMT3	1 ± 0.06	0.94 ± 0.13	0.19 ± 0.03	**0.84 ± 0.06**	**0.67 ± 0.07**		1 ± 0.07	0.93 ± 0.12	0.57 ± 0.06	**1.68 ± 0.08**	**1.71 ± 0.22**
DRM2	1 ± 0.16	2.12 ± 0.50	**0.97 ± 0.12**	**1.37 ± 0.07**	**1.03 ± 0.19**		1 ± 0.08	2.35 ± 0.40	**3.86 ± 0.75**	**3.70 ± 0.26**	**4.17 ± 1.02**
MET1	1 ± 0.08	0.67 ± 0.14	**0.24 ± 0.03**	0.53 ± 0.05	**0.49 ± 0.07**		1 ± 0.12	0.73 ± 0.13	**0.93 ± 0.10**	1.16 ± 0.09	**1.37 ± 0.13**

Table 11. Expression levels of DNA methylation genes in leaves of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The expression in metal-exposed leaves is represented relative to their own control which was set to 1. The absolute values of the control conditions are set out in Supplementary Table 1. Values are the mean ± SE of 4 biological replicates. Significance levels (Two-way ANOVA) are relative to the controls and indicate an increased or decreased expression, respectively ([ ] = $p < 0.05$; [ ] = $p < 0.01$; [ ] = $p < 0.05$; [ ] = $p < 0.01$). *CMT3*: chromo-methyltransferase 3; *DRM2*: domains rearranged methyltransferase 2; *MET1*: DNA (cytosine-5)-methyltransferase 1.

LEAVES	Control seeds						Uranium seeds				
	0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U		0 µM	5 µM Cd	10 µM Cd	25 µM U	50 µM U
DNA METHYLATION GENES											
CMT3	1 ± 0.14	0.89 ± 0.11	0.55 ± 0.03	0.39 ± 0.05	0.28 ± 0.03		1 ± 0.08	0.95 ± 0.24	0.56 ± 0.07	0.38 ± 0.06	0.20 ± 0.04
DRM2	1 ± 0.24	1.27 ± 0.19	2.47 ± 0.22	1.37 ± 0.19	1.32 ± 0.14		1 ± 0.12	1.16 ± 0.21	2.08 ± 0.25	1.71 ± 0.27	1.29 ± 0.25
MET1	1 ± 0.22	0.62 ± 0.08	0.51 ± 0.03	0.36 ± 0.05	0.33 ± 0.04		1 ± 0.14	0.73 ± 0.18	0.53 ± 0.06	0.36 ± 0.06	0.25 ± 0.02

3.5.2 Degree of global DNA methylation

Based on the results of the methylation gene expressions, global methylation levels are only measured in root samples as in roots greater significant differences are observed in and between both seed types. Figure 7 shows the percentage of methylation that was present in the roots after exposure to 5 and 10 µM Cd and 25 µM U. The metal exposures are presented relative to their controls in roots of both control and U-seeds. Methylation data of the 50 µM U condition is not available because only low amounts of DNA could be collected from the root samples exposed to 50 µM U. The low DNA concentrations resulted in a methylation measurement that was close to the detection limit of the UPLC-MS/MS and therefore, the results are unreliable.

In control seeds, exposure to 5 and 10 µM Cd results in a decreased methylation percentage relative to the control while methylation levels after exposure to 25 µM U are increased (Fig. 7). The results seen in roots of U-seeds are similar to those in control seeds as Cd-exposure again results in a decrease and exposure to U in a significant increase. Moreover, the increase of the percentage of methylation after 25 µM U-exposure is higher in roots of U-seeds compared to the control seeds. However, no significant differences are observed between the metal exposures of both seed types (Fig. 7).
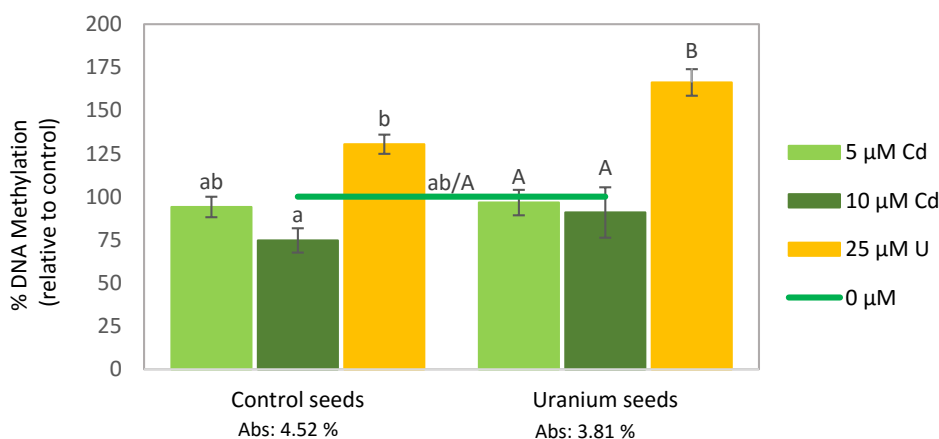
Figure 7. Total methylation level (%) of DNA from roots of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The percentage methylation of metal-exposed roots are represented relative to their own control which was set to 100. The absolute values of the control conditions are set out in the figure and in Supplementary Table 1. Data represent the mean ± SE of 4 biological replicates. Two-way ANOVA was used to determine significant effects. Data points with different small letters are significantly different for the control seeds (p<0.05). Different capital letters indicate a significant difference for the uranium seeds (p<0.05).

4 DISCUSSION

Environmental pollution due to metal contamination has become more prominent and a worldwide problem. The excess release of heavy metals into the environment due to anthropogenic activities has contributed to metal contamination in both terrestrial and aquatic areas (60). This contamination can have severe negative effects on different organisms in the environment. Plants, as such, are able to take up different metals from contaminated soils and water, thereby introducing them into the food chain, which can lead to harmful health effects not only in plants, but in other organisms as well (2, 5). This highlights the importance to gain more insight into the effects of metal toxicity on different organisms.

In this study, the effects of metal contamination (i.e. exposure to Cd and U) on oxidative stress, DNA repair and DNA methylation are investigated in *A. thaliana* plants. In addition, effects are studied in both control plants (i.e. plants with no prior history of metal exposure, called here control seeds) and in U-plants (i.e. plants that were grown from seeds that were continuously exposed to 5 µM U in their previous generation, called here U-seeds) to determine if transmission of adaptive responses can occur onto the next generation.

4.1 EFFECTS OF EXPOSURE TO CD OR U ON *A. THALIANA* ROOT AND LEAF GROWTH AND METAL UPTAKE

In this project, we firstly determined the root and leaf growth of plants exposed to Cd or U for three days to investigate the toxicity of the used metal concentrations. In general, a decrease in growth was observed after exposure to all four metal exposure conditions in both roots and leaves (Fig. 2). The higher metal concentrations (i.e. 10 µM Cd and 50 µM U) are known to induce a higher stress level in plants (13, 55). This is evidenced here as exposure to the high Cd and U concentrations resulted in a lower growth in roots compared to exposure to the lower Cd and U concentrations (Fig. 2A). In addition, even a decrease in root biomass was seen after exposure to 50 µM U. This could possibly be explained by the higher %DW/FW (Fig. 3) that indicates wilting of the plants due to a disturbed water balance or to increased cell damage. Similar results have been observed before in *A. thaliana* plants after exposure to higher U concentrations (61). The occurrence of the negative growth after exposure to 50 µM U was only seen in roots, not in leaves. This is probably because, in the treated plants, roots were in direct contact with U in the solution, while exposure in leaves depends more on the metal transfer from roots to leaves.

Results of the metal uptake showed that Cd and U uptake is much greater in the roots than in the leaves (Tables 2 and 3). As expected, this can indicate that more severe toxicity effects occur in the roots as compared to the leaves. Plus, the higher U concentrations in roots ensure that direct damage to e.g. membranes is more prominently present in roots. Due to the low root-to-shoot transfer of U, signalling from the roots rather than direct toxicity effects of U has been suggested to result in the observed decrease in leaf growth (62). Although the amount of Cd present in the leaves was more than 50 times higher than the amount of U, exposure to U seems to be more toxic for *A. thaliana* plants (Table 3) as leaf growth was significantly more decreased after exposure to U than after Cd-exposure (Fig. 2B).

The percentage of growth was slightly higher in both roots and leaves of U-seeds than in control

seeds (Fig. 2). However, because no significant differences were observed between control and U-seeds, this finding cannot yet support our hypothesis that previous metal exposure can protect plants in the next generation to metal stress.

To verify the amount of Cd and U that was added in our experiment, the metal concentrations were measured in the Hoagland LP solution collected immediately after contamination. The data showed that the measured concentrations corresponded well with the predetermined concentrations of 5 and 10 µM Cd or 25 and 50 µM U. This was accurate for all metal conditions, except for the 50 µM U condition, where measurements indicated a total U concentration of approximately 60 µM instead of 50 µM. This is probably due to a technical error as it is possible that more U was added to the Hoagland solution.

In addition, the amount of Cd and U was also determined in root and leaf samples. Table 2 shows that higher Cd and U concentrations in the Hoagland solution led to higher uptake of the metal concentrations in roots. These results are in agreement with results of previous studies. As such, Cuypers *et al.* (63) observed similar concentration-dependent increases in root and leaf Cd concentrations in *A. thaliana* plants that were exposed to 5 µM and 10 µM Cd for 24 h. Vanhoudt *et al.* (61) also reported comparable results after exposure to U.

In leaves, U uptake also showed a concentration-dependent increase in both control and U-seeds. However, the results of Cd uptake in the leaves from control seeds were unexpected as the Cd concentrations were almost the same after exposure to 5 µM Cd and 10 µM Cd (Table 3). This is in contrast with our expectations but is unlikely to be a biological effect as no previous studies observed the same outcome.

Compared to U concentrations in roots, the amount of U transferred onto the leaves was very low as the translocation factor from roots to leaves was approximately 0.0004 for both 25 and 50 µM U conditions (Table 3). This very low root-to-shoot transfer for U was already observed by Vandenhove *et al.* (64), Vanhoudt *et al*. (65) and Saenen *et al.* (13). In contrast, Cd showed a higher root-to-shoot translocation (translocation factor of approximately 0.44) than U but the largest amount of Cd was still retained in the roots. These results were also in accordance with findings from studies of Vanhoudt *et al.* (65) and Cuypers *et al.* (63).

As already mentioned, the growth of roots and leaves was slightly higher in U-seeds compared to growth in control seeds (Fig. 2). This can correspond to the amount of Cd and U that is taken up since in general, the uptake of Cd and U concentrations in roots and leaves were also lower in U-seeds than in control seeds. The uptake of metal concentrations was thus slightly altered in plants grown from U-seeds, which can be an indication that these plants are already less sensitive towards metal stress due to the metal exposure in their previous generation. However, these differences were not significant and are thus not yet conclusive.

4.2 EFFECTS OF EXPOSURE TO CD OR U ON OXIDATIVE STRESS MECHANISMS AT THE TRANSCRIPT LEVEL

As it is known that both Cd and U induce oxidative stress in plants, we investigated the effects of exposure to different concentrations of Cd or U on several oxidative stress-related parameters in *A. thaliana* plants grown from control or U-seeds. This gives us more insight into the molecular

mechanisms that underlie the oxidative stress response induced by metal exposure and it takes part in finding an answer to our first research question if long-term exposure of plants to U in a previous generation induces an altered tolerance or sensitivity towards different abiotic stressors.

Firstly, gene expression of three selected pro-oxidative genes was analysed in roots exposed to 5 µM Cd, 10 µM Cd, 25 µM U or 50 µM U (Table 4). Uranium can directly induce ROS formation non-enzymatically through Fenton and Haber-Weiss reactions because it is a redox-active metal (66). Cadmium, on the other hand, is a non-redox active metal that uses indirect mechanisms to increase the ROS concentration. For example, Cd exposure can cause inhibition of the antioxidant defence system, activation of ROS-producing enzymes or induction of enzymatic lipid peroxidation (67). *RBOHC* and *RBOHF* are two plasma membrane bound NADPH oxidases, which are known to be an important source of ROS production in plants under abiotic stress, as they catalyse the formation of $O_2^{\bullet-}$ (68). Our results showed no significant increase in gene expression of *RBOHC* and *RBOHF* after exposure to U in roots of both control and U-seeds (Table 4). Moreover, U exposure resulted in a significant downregulation of *RBOHC* levels in roots of control seeds. Expression levels of *RBOHF* were also significantly decreased after exposure to U in leaves (Table 5). This seems to indicate that the NADPH-mediated oxidative burst plays no important role in ROS production in *A. thaliana* roots exposed to U. LOX is another pro-oxidative enzyme that can lead to the production of ROS, such as $O_2^{\bullet-}$ (66). Similar to the findings of Saenen *et al.* (68), expression of *LOX1* was also not significantly affected by U-exposure in *A. thaliana* roots and leaves. In contrast, gene expression of *LOX2* was increased after exposure to U in leaves of plants grown from both control and U-seeds (Table 5). Moreover, exposure to 25 µM U resulted in a significant upregulation of *LOX2* levels. This overexpression of *LOX2* levels in leaves after exposure to metals is often seen (66, 69). It thus seems that besides LOX2, the pro-oxidative NADPH oxidases and LOX1 enzyme are not involved in the U-induced stress responses.

Exposure to Cd, however, resulted in significant increases of *LOX1* expression in roots of both control and U-seeds and expression of *RBOHF* was also significantly increased after exposure to 10 µM Cd in roots grown from U-seeds (Table 4). These results are similar to those from the studies of Cuypers *et al*. (63) and Remans *et al.* (69) that also investigated the effects of Cd exposure on pro-oxidative gene expression levels in *A. thaliana* plants. In leaves, expression levels of *LOX1* and *LOX2* pro-oxidative genes also showed a significant increase after exposure to 10 µM Cd. Exposure to Cd also resulted in an upregulation of *RBOHF* expression levels in leaves, however not significantly (Table 5). So, while our results show that Cd can induce oxidative stress by enhancing the transcription of several pro-oxidative genes, we cannot conclude the same for oxidative stress induced by U-exposure. Moreover, in both Cd-exposed leaves and roots, we observed that the expression of pro-oxidative genes is generally higher in U-seeds than in control seeds (Tables 4 and 5), which can indicate that the observed effects are more enhanced in primed than non-primed plants. These are as such the first indication that the underlying mechanisms of metal-induced oxidative stress can thus probably differ in plants exposed to metals in a previous generation.

To counteract the toxicity of oxidative stress that is induced by exposure to Cd or U, plants are able to activate an antioxidative defence system of ROS-scavenging enzymes (e.g. SOD, CAT, APX) and antioxidative metabolites (e.g. AsA and GSH) (66). In general, exposure of roots and leaves to 5 or 10 µM Cd similarly affected antioxidative gene expression as reported before by Remans *et al.* (69)

and Smeets *et al.* (70), who exposed *A. thaliana* plants to different Cd concentrations and indicated that Cd has an influence on the antioxidative defence system. The effects of U-exposure on antioxidative gene expression in roots and leaves of *A. thaliana* plants from our project are comparable to the results reported by Vanhoudt *et al.* (25, 71) and Saenen *et al*. (66) in studies that investigated U-induced oxidative stress responses in *A. thaliana* roots and leaves.

SOD enzymes are ROS-scavenging enzymes that constitute the first line of defence because they dismutate $O_2^{\bullet-}$ to H_2O_2 (66). At transcriptional level, the gene expression of the different measured SOD isoforms was affected differently by U and Cd in roots and leaves (Tables 4 and 5). Gene expression levels of *CSD1* and *CSD2* were downregulated upon exposure to Cd and U in both roots and leaves grown from control and U-seeds. In contrast, *CSD3* levels were decreased after exposure to Cd and U in roots of control seeds, while exposure to 5 µM Cd, 25 and 50 µM U resulted in an upregulation in the roots of U-seeds (Table 4). In leaves, expression of *CSD3* was also increased after exposure to Cd and U in both seed types, however not significantly (Table 5). The down-regulation of *CSD* expression was generally stronger after exposure to Cd than U. The reduction in gene expression levels observed for *CSD1* and *CSD2* were confirmed by results of Saenen *et al.* (68) for U exposure and by Smeets *et al.* (70) for *A. thaliana* roots under Cd stress. These results were linked to the involvement of miRNA398 in the regulation of SOD responses. Under metal stress, miRNA398 expression is induced, which leads to a downregulation of *CSD* expression (68). In roots, the decreased *CSD1/2* expression was compensated by an increased expression of *FSD1*. Especially, exposure to Cd resulted in significantly higher *FSD1* expression than exposure to U (Table 4). This ensures the maintenance of the scavenging of $O_2^{\bullet-}$. The expression of *CSD1/2* was also significantly downregulated in leaves from control and U-seeds (Table 5). However, this decreases was not compensated by an increased *FSD1* expression in leaves. Saenen *et al.* (66) and Vanhoudt *et al.* (61) have reported that exposure to U disturbs nutrient uptake and distribution of several nutrients, including Fe, in *A. thaliana* plants. The same was seen after Cd exposure by Cuypers *et al.* (63). A significant decrease in leaf Fe content was observed upon metal exposure, which can explain the lack of compensation by *FSD1* because limited Fe conditions are responsible for declined *FSD* expressions. Since no significant differences were seen in the expression of the different SOD isoforms between both seed types of the two organ types, the decreased expression of both *CSD* and *FSD* can lead to a decreased capacity to scavenge $O_2^{\bullet-}$ in leaves grown from primed as well as non-primed seeds.

The gene expression of *CAT,* an enzyme involved in detoxification of H_2O_2, was influenced the same by Cd and U in roots of both control and U-seeds. The *CAT* expression profiles were also similarly affected by Cd and U in leaves from control and U-seeds. In roots, expressions of *CAT1* and *CAT3* were overall increased after exposure to Cd or U (Table 4). The same was seen for the expression levels of *CAT3* in leaves, while *CAT1* expression levels were decreased after exposure to Cd and U in leaves (Table 5). Saenen *et al.* (13) confirmed these results after exposure to 25 and 50 µM U in *A. thaliana* leaves. The higher expression of *CAT1* and *CAT3* in roots of both control and U-seeds, indicate a higher potential to detoxify H_2O_2 upon metal-exposure. In contrast, gene expression levels of *CAT2* were significantly decreased in roots and leaves. This decrease in *CAT2* expression was also observed before by Saenen *et al.* (13, 68) in roots and leaves after exposure to U and by Cuypers *et al.* (63) after Cd-exposure. Zimmermann *et al.* (72) showed that this down regulation of *CAT2* gene expression is involved in producing an elevated H_2O_2 level in the regulatory mechanisms during

senescence. As Cuypers *et al.* (63) already indicated, Cd can thus induce early senescence. In addition, our results possibly indicate that U can also induce early senescence as it is responsible for a decreased *CAT2* gene expression as well.

No differences were observed between the expression levels of *CAT* in the roots and leaves between control and U-seeds. Therefore, no real conclusions can be made about the possible enhanced involvement of CAT in the detoxification of H_2O_2 in primed plants compared to non-primed plants.

The AsA-GSH cycle is another pathway involved in detoxification of H_2O_2 in plants. APX and GR are two enzymes which play a role in the AsA-GSH cycle. APX detoxifies H_2O_2 by the oxidation of AsA to MDHA, while GR is responsible for the reconversion of GSSG to GSH (28). Expression of both *APX1* and *GR1* was differently affected by Cd and U in roots of control and U-seeds. While *APX1* and *GR1* were decreased after exposure to Cd and U in roots from control seeds, their gene expression was increased upon exposure to 10 µM Cd, 25 or 50 µM U in roots from U-seeds. In leaves, expression of *APX1* was decreased after exposure to all metal conditions in control as well as U-seeds. Gene expression levels of *GR1* were upregulated in response to Cd, while exposure to U resulted in a decreased expression in the leaves of control seeds. In leaves from U-seeds, expression of *GR1* was generally decreased. As APX is an important scavenger of H_2O_2, a decreased *APX1* expression can indicate a decreased H_2O_2 detoxification. The same accounts for GR1 as it is important in the recycling of GSH, which subsequently scavenges H_2O_2 and can react non-enzymatically with other ROS (68). A downregulated *GR1* gene expression can therefore also indicate a lower ability to recycle GSH and thus H_2O_2 detoxification. However, our results show that Cd and U exposure to roots from U-seeds do increase *APX1* and *GR1* gene expression, indicating an enhanced H_2O_2 detoxification in primed plants. Nevertheless, changes in gene expression do not always lead to the expected changes in protein concentrations and enzyme capacities. Therefore, we also investigated the capacity of some antioxidant enzymes and the amount of GSH, an important antioxidant metabolite, in roots and leaves from control and U-seeds, as will be discussed in section 4.3.

4.3   Effects of exposure to Cd or U on mechanisms of the oxidative stress response

Besides investigating the effect of Cd- and U-exposure on the oxidative stress response at the transcriptional level, we also examined the capacities of several antioxidative enzymes, looked at the concentrations of the antioxidative metabolite GSH and determined the amount lipid peroxidation. Despite significant changes in transcription of SOD isoforms in roots and leaves (Tables 4 and 5), no significant effects of exposure to Cd or U were seen on overall SOD capacity in roots and leaves of control seeds (Tables 6 and 7). In roots of U-seeds, on the other hand, exposure to U resulted in a significant increase in SOD capacity. In addition, a significant increase was also seen in the SOD enzyme capacity after exposure to 5 µM Cd in roots of U-seeds in comparison to control seeds. So, although this difference between roots of control and U-seeds was not clearly seen at transcriptional level, the SOD capacity is clearly higher upon metal exposure in primed plants. This could indicate that roots of primed plants have an increased capacity to scavenge superoxide radicals because of their higher SOD capacity.

While metal exposure significantly affected expression levels of all *CAT* isoforms in both roots and leaves, no significant changes were observed in the overall enzyme capacity of CAT (Tables 6 and

7). In addition, no great changes were seen in the CAT capacity between roots and leaves of control and U-seeds. This is, however, not unusual since multiple steps lie between the transcription of genes and the activity of the corresponding enzymes.

The capacity of different peroxidases (GPX, SPX and APX) was measured in both roots and leaves and it was shown that these enzymes were affected differently by metal exposure. GPX capacity is increased after exposure to Cd and U in roots and leaves of control and U-seeds (Tables 6 and 7). Moreover, exposure to 10 µM Cd significantly increased GPX capacity in roots of U-seeds, while 10 µM Cd significantly affected GPX capacity in the leaves of control seeds. In addition, capacity of SPX was decreased in roots, but increased in leaves, expect after exposure to U. The increase of GPX and SPX after exposure to Cd was also observed by Cuypers *et al*. (63), indicating the importance of H_2O_2 scavenging in the Cd-induced oxidative stress response. Horemans *et al.* (73) also observed elevated levels of SPX after exposure to U in *Lemna minor* plants. Since SPX is known to be involved in cell wall lignification, an increase in its capacity might therefore indicate that cell wall lignification plays an important role in both Cd- and U-induced stress in plants. An increase in SPX capacity can thus possibly limit the cellular uptake of Cd and U. In contrast to our results, Horemans *et al.* (73) found that GPX was strongly induced by Cd but inhibited by U in *Lemna minor*. Although Saenen *et al.* (68) supports our results as they also observed an increased GPX activity in response to U exposure in roots of *A. thaliana*. Increased GPX activity indicates an important role of H_2O_2 scavenging upon exposure to Cd and U and can also play a role in the reduction of free cellular Cd and U as GPX is also involved in cell wall lignification.

As already mentioned, the AsA-GSH cycle can also play an important role in the scavenging of H_2O_2 under Cd and U stress. This is shown in our results as the enzyme capacities of both APX and GR were generally increased after exposure to Cd or U (Tables 6 and 7). Although this is in contrast with our results of the measured gene expressions, where *APX1* and *GR1* expression levels were only significantly increased in response to 10 µM Cd, 25 and 50 µM U in the roots of U-seeds (Table 4). These findings, however, can again be explained by the different steps that are included between the actual gene expression and the eventual, corresponding enzyme capacities. The increase in APX and GR transcription and activity seen in roots of U-seeds, could possibly be involved in a defence mechanism against metal-induced increases of H_2O_2. More specifically, the increased enzyme capacity of APX and GR seems to indicate an important role for the AsA-GSH cycle in Cd- and U-induced stress responses. However, if actual H_2O_2 levels were measured in addition, a more definite conclusion could be made. Measurement of AsA levels could also provide additional information on the defence mechanisms involving APX.

The increased capacity of GR that is observed after Cd and U exposure in roots of U-seeds, indicates that the roots try to keep GSH in its reduced state so subsequently, DHA will be reduced into AsA. This is however in contradiction with our results of GSH measurements, where no significant increase of GSH concentrations is seen in roots (Fig. 4). Furthermore, exposure to U resulted in decreasing GSH levels, while GSSG concentrations were increased. The total amount of GSH, which is the addition of the reduced GSH and oxidized GSSG concentrations, was only significantly increased after exposure to 10 µM Cd in the roots of control seeds. Especially exposure to 50 µM U, resulted in a decreased amount of GSH and additionally, a decrease in the percentage reduced GSH was also observed, which can be a reflection of the cellular damage caused by 50 µM U exposure (Fig. 4).

Saenen *et al*. (13) has also observed lower GSH concentrations in response to high U concentrations. In addition, exposure to different abiotic stresses has been found to cause similar lower GSH concentrations in several plant species (74, 75). This decrease can possibly be related to a decreased GSH synthesis in the roots. However, expression levels of enzymes involved in GSH biosynthesis should therefore first be investigated. Our results show no change in GSH concentrations after exposure to 5 µM Cd (Fig. 4). The higher Cd-exposure condition resulted in a significantly increased amount of total GSH, which can confirm the higher GR activity present in the roots. No great significant differences are seen between the roots of control and U-seeds, except for the significant increase of GSSG levels seen after exposure to 25 µM U in roots of U-seeds (Fig. 4). The increase in total GSH observed in the roots of U-seeds after 25 µM U is therefore probably a result of these increasing GSSG levels as the GSH levels were slightly decreased. However, since GR capacity is not significantly altered after exposure to 25 µM U in the roots of U-seeds, this can possibly explain the lower GSH levels and higher GSSG levels (Table 6).

Similar to the roots, exposure to 10 µM Cd resulted in a higher level of total and reduced GSH as well as lower total GSH and reduced GSH levels after exposure to 50 µM U in the leaves of control and U-seeds (Fig. 5). In addition, exposure to 25 µM U also caused decreasing levels of total and reduced GSH in leaves. Significant differences were seen in the percentage of reduced GSH between the leaves of control and U-seeds after exposure to all four metal conditions, except for 50 µM U (Fig. 5). The percentage of reduced GSH was higher after exposure to Cd or U in the leaves of control seeds compared to the leaves of U-seeds, which indicates that higher reduced GSH levels were present in comparison to the total amount of GSH. These results are also in contrast with the GR capacity observed in leaves of control and U-seeds as the GR capacity is higher in the leaves of U-seeds than control seeds (Table 7). Because our results showed a more increased enzyme capacity of APX and GR in roots and leaves of U-seeds, this could indicate a more important role for the AsA-GSH cycle in Cd- and U-induced stress responses in primed plants. However, based on the GSH determinations, it could adversely also be concluded for the roots and leaves of non-primed plants. Further research thus is needed to determine the effect of priming of plants on the importance of AsA and GSH under metal stress.

The level of lipid peroxidation in leaves was based on the amount of TBA-rc, such as MDA, in leaves as a measure for membrane damage. A concentration-dependent increase of lipid peroxidation was seen after exposure to Cd and U in the leaves of both control and U-seeds (Fig. 6). Studies form Smeets *et al.* (76), Cuypers *et al.* (63) and Saenen *et al.* (13) also reported an increased lipid peroxidation in response to exposure to Cd and U, respectively. The increased lipid peroxidation levels indicate an altered membrane integrity and functionality, which can cause leakage of nutrients from the cell. The increasing amount of lipid peroxidation can also attribute to the explanation of the increasing dry weight levels seen in Figure 3. Exposure to higher metal concentrations, leads to more lipid peroxidation (Fig. 6) which can cause wilting of the plants and is therefore responsible for the higher %DW/FW in leaves (Fig. 3). An enhanced H_2O_2 level can additionally play a role in lipid peroxidation as it can convert fatty acids into toxic lipid peroxides, which in turn are responsible for destroying biological membranes. An increase in LOX activity can also lead to enhanced lipid peroxidation. In our project, we saw that the increase of lipid peroxidation was generally larger in the leaves of control seeds than of U-seeds. This can possibly point out that plants grown from U-

seeds have an increased antioxidative defence system or that metal exposure in primed plants results in a lower induction of oxidative damage.

4.4 EFFECTS OF EXPOSURE TO CD OR U ON DNA REPAIR MECHANISMS AT THE TRANSCRIPT LEVEL

Within this project, the effects of exposure to 5 or 10 µM Cd, 25 or 50 µM U on DNA repair were investigated at the transcriptional level in *A. thaliana* plants grown from control and U-seeds. Therefore, the expression of several genes involved in important DNA repair pathways was determined in roots and leaves (Tables 8 and 9). Based on their involvement in several DNA repair pathways, the measured genes can be subdivided into four classes.

The first class of genes encode enzymes involved in the BER pathway. This pathway is responsible for the replacement of damaged bases by undamaged bases by recognition and removal of the damaged base, followed by incision and gap filling and finally, sealing of the repaired DNA strand (33). The expression of the genes involved in BER was differently affected by metal exposure between the two seed types in roots. While all three genes (i.e. *PARP1*, *PARP2* and *POLGAMMA1*) were downregulated upon exposure to Cd or U in control seeds, exposure to U resulted in upregulation of their gene expression in U-seeds (Table 8). In addition, *PARP2* levels are also upregulated after exposure to 5 and 10 µM Cd. This is in accordance with findings from Doucet-Chadbeaud *et al.* (77), who showed that both *PARP1* and *PARP2* are induced by DNA breaks, while *PARP2* is also induced by different kinds of environmental stresses such as exposure to heavy metals in *A. thaliana.*
In leaves, these differences were not observed. Furthermore, expression levels of all measured BER genes were the same between leaves of both control and U-seeds (Table 9). In both seed types, only exposure to 5 µM Cd significantly increased the expression of *PARP2,* which can also be explained as an effect of heavy metal exposure.

The next two classes of DNA repair genes that we measured, were both involved in the repair of DSBs in plants. Repair of DSBs can occur either by HR, a pathway that uses a homologous DNA sequence in the genome as a template for repair, or NHEJ, which rejoins DNA ends in a random manner (36). Similar to the genes involved in BER, NHEJ-related genes showed a downregulation after metal exposure in the roots of control seeds but an upregulation was observed in roots of U-seeds upon exposure to 10 µM Cd, 25 or 50 µM U (Table 8). In addition, a similar expression profile was also found for HR-related genes in roots. In general, metal exposure resulted in a downregulation of HR gene expression in roots of control seeds, while exposure to U mostly upregulated expression of HR genes in roots grown from U-seeds (Table 8). Exposure to all four metal conditions resulted in a general but insignificant downregulation of NHEJ genes in leaves from both control and U-seeds (Table 9). The decrease in HR gene expression levels in the roots of control seeds was also seen in the leaves from control seeds (Table 9). While HR genes are rather downregulated upon U-exposure in leaves from U-seeds compared to the roots. This effect can be assigned to the low root-to-shoot transfer of U that results in low concentrations of U in the leaves and therefore less harmful effects.

The final class of DNA repair genes that was investigated included a gene that was involved in inhibition of the cell cycle. When errors occur in the DNA strand, inhibition of the cell cycle is necessary to repair DNA before the errors undergo replication and cause more damage (78). In roots, exposure to all four metal conditions resulted in a downregulation of *KRP2* gene levels of control

seeds (Table 8). In contrast, gene expression of *KRP2* was slightly upregulated after exposure to U in roots from U-seeds, while Cd-exposure still led to a significant downregulation. Gene expression of *KRP2* did not show any significant differences in leaves from control and U-seeds (Table 9).

In conclusion, a three day exposure to Cd generally resulted in a decrease in the expression of DNA repair genes in the roots from control seeds, although with a few exceptions (i.e. *PAPR2, LIG4* and *MND1)*. This downregulation of DNA repair genes upon Cd-exposure was reviewed in a study of Bertin and Averbeck (79), who proposed that inhibition of DNA repair is a major factor in the Cd genotoxicity. As for Cd, a similar decreasing response was also observed for most DNA repair genes after exposure to U in control roots. Although this has not been described in literature before, this can be taken as an indication that U genotoxicity also involves inhibition of DNA repair. However, additional studies are needed to verify these results.

In contrast to the downregulation seen upon U-exposure in roots from control seeds, the upregulation that was observed after exposure to U in U-seeds can have two explanations: [1] higher upregulation of DNA repair genes is a response to more DNA damage or [2] a greater ability of the plants to repair more DNA damage. To determine which of the two explanations is the most accurate, it is necessary to measure the amount of DNA damage present is both organ types. For example, measurement of γ-H2AX can determine the amount of DSBs and can thus give an indication of the amount of DNA damage that is caused by metal exposure (80). Furthermore, the higher expression of DNA repair genes that is seen in U-seeds compared to control seeds can possibly indicate an altered tolerance of primed plants to metal exposure. However, repetition of this research is necessary to confirm these findings.

In contrast to the roots, only marginally differences were found in the expression of several DNA repair genes in the leaves and this for both Cd and U and for both seed types. This can be related to the lower Cd and U concentrations in the leaves compared to concentrations found in the roots. As such, this also suggests that DNA damage is not induced through root-to-shoot signalling but by the direct presence of the metal in the tissue.

It remains however important to keep in mind that changes in gene expression are not always reflected by changes in protein concentrations and/or enzyme activities. Therefore, investigation of the effects of Cd and U exposure on DNA repair should also be performed on these levels.

4.5   EFFECTS OF EXPOSURE TO CD OR U ON DNA METHYLATION AT THE TRANSCRIPT LEVEL

As a start to investigate the possible mechanisms that lie at the basis of possible transgenerational effects, we chose to look at total DNA methylation levels in roots and leaves. Because cytosine DNA methylation is considered an epigenetic silencing mechanism that is involved in many important biological processes, such as control of genomic imprinting and regulation of gene expression, investigating the effect of Cd and U exposure on DNA methylation seemed a good starting point to gain more insight in the transgenerational mechanisms involved in *A. thaliana* (81)*.*

First, the expression of three genes involved in DNA methylation was determined in roots and leaves of *A. thaliana* plants grown from control and U-seeds exposed to 5 or 10 μM Cd, 25 or 50 μM U for three days. We focused on three methyltransferase genes that are involved in catalysing the addition and removal of methyl groups to 5-cytosines. While MET1 is responsible in maintaining the CG methylation, DRM2 and the plant-specific CMT3 are two methyltransferases responsible for

methylation at non-CG sites (81, 82).

The gene expression profiles of *CMT3*, *DRM2* and *MET1* were very different between both seed types in both tissue organs. In the roots of control seeds, especially exposure to 10 µM Cd resulted in a significant decrease of *CMT3* and *MET1* expression (Table 10). In contrast, gene expression of *CMT3* and *DRM2* was significantly upregulated after U-exposure in roots of U-seeds. In addition, Cd-exposure significantly increased gene expression of *DRM2* as well. In *A. thaliana* leaves of both control and U-seeds, exposure to 10 µM Cd significantly increased *DRM2* expression, while U-exposure resulted in a significantly downregulated gene expression of *CMT3* and *MET1* (Table 11). Our results thus showed highly variable methylation gene expression profiles in roots and leaves of both seed types. The downregulation of *CMT3* expression after Cd exposure was also seen in the study of Ou *et al*. (82), which showed that heavy metal stress, like exposure to Cd, can significantly alter the cytosine methylation patterns of different genes in rice. In contrast to our results however, they observed upregulation of *MET1* and *DRM2* gene expression. Ou *et al.* (82) concluded that disruption of the expression of these methylation genes due to heavy metal stress appears to be the cause for changed DNA methylation patterns. The upregulated changes that we see in the roots of U-seeds in comparison to the roots of control seeds can possibly be due to stress-induced epigenetic variation and inheritance in plants. Our results also suggest that in primed plants, methylation at non-CG sites plays a greater role in cytosine methylation compared to CG methylation since the expression levels of *CMT3* and *DRM2* were increased. Studies of Boyko *et al*. (50, 83) also suggested that progeny of plants exposed to various abiotic stresses, such as high salt exposure, exhibit changes in the different methylation patterns. It is however also possible that loss of 5-methyl-cytosines does not depend on the activity of these methyltransferase enzymes, but that it is an effect of exposure to heavy metals on DNA itself. It is known that abiotic stresses cause an increased production of ROS and that ROS, subsequently, can cause DNA damage such as DSBs. If damage to the DNA strand then interferes with the capability of DNA to act as an acceptor for methyl groups, this can eventually result in loss of methylation (82, 84). Therefore, no concrete conclusions can be drawn from our DNA methylation expression results yet and more research is still necessary to investigate which mechanisms are involved in transgenerational effects.

4.6 EFFECTS OF EXPOSURE TO Cd OR U ON GLOBAL DNA METHYLATION LEVELS

Besides looking at the transcriptional level of several methylation genes, we also determined the percentage of global DNA methylation in roots from control and U-seeds exposed to 5 or 10 µM Cd, 25 or 50 µM U for three days. In general, the total amount of DNA methylation that we observed in roots exposed to Cd or U was low (Fig. 7). The percentage of DNA methylation showed a slightly decreasing trend after exposure to Cd in roots of control and U-seeds in a concentration-dependent manner. The higher Cd concentration showed a more decreased amount of DNA methylation than exposure to 5 µM Cd. In contrast, the global DNA methylation percentage was increased in roots of control seeds and significantly increased in roots of U-seeds after exposure to 25 µM U. A significant increase was also seen after exposure to 50 µM U, however due to the low DNA concentrations that could be extracted from those roots, the results of the global DNA methylation measurements were close to the detection limit and therefore unreliable. As was reported before by Saenen *et al.* (68), one of the deleterious effects expected after prolonged exposure to high U concentrations in plants

is DNA and RNA degradation. Since only low DNA amounts could be extracted from roots exposed to 50 µM U, this can indicate that U exposure affects the cell viability. Although it would thus be very interesting to repeat the measurements of methylation in the roots exposed to 50 µM U because of the increasing trend that is seen after exposure to 25 µM U, we will probably not be able to extract more intact DNA from those samples. Our results indicate a difference in the effect of Cd-exposure on global DNA methylation levels and the effect of U-exposure. However, other studies showed a significantly higher global methylation level due to exposure to abiotic stress conditions (50, 85, 86). Increased methylation percentages after metal stress can indicate that plants have activated their defence mechanisms to prevent genome instability. In primed plants this can possibly lead to enhanced tolerance to metal stress which enables them to survive better in harmful environments.

5 CONCLUSION AND SYNTHESIS

Within this project, effects of exposure to Cd and U on the oxidative stress response, DNA repair and DNA methylation were determined in U-primed and non-primed *A. thaliana* plants. The two toxic metals U and Cd were chosen as it is known that both can negatively affect human health and plant growth and development. In addition, both metals frequently co-occur in polluted areas. Because plants are immobile organisms that cannot escape harmful environments, they are in great need of the ability to respond and adapt to recurring stressors. To investigate if effects caused by a prior exposure can be transmitted and possibly protect future generations of *A. thaliana* plants, the effects were studied in plants grown from control seeds (i.e. with no previous exposure to metal stress) and plants grown from U-seeds (i.e. seeds derived from plants that were continuously exposed to 5 µM U). Both control *A. thaliana* seedlings and U-seedlings were grown hydroponically for 18 days, after which they were exposed to 5 or 10 µM Cd or 25 or 50 µM U for three days.

Our results showed a slight difference in the growth of roots and leaves from control seeds compared to those from U-seeds as growth of roots and leaves of control seeds was more decreased upon metal exposure. This can be related to the higher amount of Cd and U that is seen to be taken up in roots and leaves of control seeds. As these changes, however, were not significant, a new study should be set up to confirm our findings.

Several differences in the oxidative stress parameters were observed between Cd- and U-exposed roots and leaves of control seeds compared to U-seeds. A higher expression of pro-oxidative genes was found in both roots and leaves from U-seeds, especially after exposure to Cd. Enhancement of the transcription of several pro-oxidative genes can therefore probably play a greater role in the induction of oxidative stress in primed plants than in non-primed plants. In contrast, plants grown from U-seeds also exhibited a greater ability for H_2O_2 detoxification as the gene expression levels and capacities of APX and GR were increased. Moreover, the increase in APX and GR enzyme capacities can also imply that the AsA-GSH cycle plays a more important role in Cd- and U-induced stress responses in primed than in non-primed plants. However, the higher GR capacity was not reflected in the observed amounts of GSH. In addition, the SOD capacity in roots of U-seeds was clearly higher upon metal exposure, which can indicate that primed plants could have an increased capacity to scavenge $O_2^{\bullet-}$. A significant increase of lipid peroxidation was seen after exposure to Cd and U in leaves of both control and U-seeds, but the increase was generally larger in the leaves of control seeds. So although Cd and U both induce oxidative stress in primed and non-primed plants, the mechanisms underlying the oxidative stress response seem to be altered by prior metal exposure in the previous generation. Hence, it can be stated that the results of this study indicate that priming of plants in a previous generation results in less oxidative stress and a better protection of plants when the progeny was exposed to Cd and U.

It was shown that exposure to U also caused downregulation of DNA repair genes, similar as Cd-exposure in roots of control seeds. In contrast, exposure to U resulted in an upregulation of DNA repair gene expression in roots of U-seeds. Furthermore, the higher expression of DNA repair genes seen in roots of U-seeds further supports our hypothesis as this indicates that metal exposure in a previous generation can alter the tolerance against new stressors in primed plants. However, further investigation is still required, especially to get a better insight in the cause for the upregulation of

DNA repair genes. Research into the amount of DNA damage present in the tissues can provide such additional information.

Interestingly, the observed effects in this project were generally more pronounced in the roots than in the leaves. This can be explained by the lower Cd and U concentrations that were translocated to the leaves compared to the roots. As such, this possibly suggests that effects such as oxidative stress and DNA damage are induced by the direct presence of the metals in the tissues instead of induction through root-to-shoot signalling.

The DNA methylation genes showed substantial differences in expression in both roots and leaves of control as well as U-seeds. While the methylation genes were mostly downregulated after exposure to Cd and U in the roots of control seeds, they were generally upregulated in the roots of U-seeds upon metal exposure. Primed plants thus seem to have undergone changes that possibly lead to altered methylation patterns. In leaves, however, no significant differences in expression between both seed types was seen but exposure to Cd resulted in increased gene expression while exposure to U caused significant decreases in methylation gene expression. These effects in the leaves of control and U-seeds are therefore probably more due to induction through root-to-shoot signalling than caused by the amount of metal present in the leaves.

Despite the general downregulation of DNA methylation gene expression levels in roots of control seeds and upregulation in roots of U-seeds, the total methylation level of DNA was quite similar between the roots of both seed types. A different response was only seen between the two metal exposures as Cd-exposure resulted in a decreased methylation level and exposure to U caused an increase in global DNA methylation. Further research is necessary to confirm that U-exposure does lead to a higher total DNA methylation level and that increasing methylation levels indicate a higher activation of the plants defence mechanisms.

In conclusion, Cd and U induce adverse effects in both roots and leaves of *A. thaliana* after three days of exposure. However, it seems that different mechanisms underlie the effects on the oxidative stress response, DNA repair mechanisms and DNA methylation pathways induced by Cd and U in roots and leaves. Although for a number of parameters similar results were observed between roots and leaves of control and U-seeds upon exposure to Cd or U, some evidence was found that indicates a protective ability of metal exposure in a previous generation. This altered tolerance in primed plants should therefore be further investigated, for instance by also investigating DNA damage, AsA measurements, more DNA methylation genes, specific DNA methylation (e.g. differences in the patterns of CG, CHG or CHH methylation) instead of global DNA methylation or by using multiple generation seedlings instead of seedlings derived from only one generation.

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SUPPLEMENTARY DATA

1. Supplementary Table 1

Supplementary Table 1. Absolute values of all control conditions of both roots and leaves of A. thaliana plants grown from control and uranium seeds exposed to 0 μM, 5 or 10 μM Cd, 25 or 50 μM U for three days.

ROOTS OF *A. THALIANA*			LEAVES OF *A. THALIANA*		
Relative growth (mg)			*Relative growth (mg)*		
	Control seeds	**Uranium seeds**		**Control seeds**	**Uranium seeds**
	20.51 ± 0.89	19.22 ± 0.87		24.98 ± 0.98	24.94 ± 1.22
Percentage Dry weight to Fresh weight (%)			*Percentage Dry weight to Fresh weight (%)*		
	Control seeds	**Uranium seeds**		**Control seeds**	**Uranium seeds**
	4.57 ± 0.30	4.37 ± 0.33		9.70 ± 0.24	9.60 ± 0.13
Expression levels of pro-oxidant genes			*Expression levels of pro-oxidant genes*		
	Control seeds	**Uranium seeds**		**Control seeds**	**Uranium seeds**
LOX1	0.14 ± 0.06	0.11 ± 0.06	**LOX1**	0.14 ± 0.03	0.13 ± 0.02
RBOHC	1.63 ± 0.11	0.87 ± 0.06	**LOX2**	0.17 ± 0.04	0.18 ± 0.05
RBOHF	0.38 ± 0.22	0.33 ± 0.14	**RBOHF**	0.45 ± 0.06	0.63 ± 0.15
Expression levels of antioxidant genes			*Expression levels of antioxidant genes*		
	Control seeds	**Uranium seeds**		**Control seeds**	**Uranium seeds**
CAT1	0.55 ± 0.15	0.45 ± 0.10	**CAT1**	0.57 ± 0.11	0.60 ± 0.07
CAT2	1.52 ± 0.08	0.94 ± 0.12	**CAT2**	1.72 ± 0.36	1.45 ± 0.18
CAT3	0.40 ± 0.08	0.36 ± 0.10	**CAT3**	0.34 ± 0.03	0.45 ± 0.03
APX1	1.72 ± 0.15	1.32 ± 0.20	**APX1**	1.48 ± 0.30	1.54 ± 0.29
GR1	0.85 ± 0.16	0.68 ± 0.11	**GR1**	0.85 ± 0.21	0.90 ± 0.14
CSD1	1.70 ± 0.20	1.58 ± 0.19	**CSD1**	1.79 ± 0.17	1.74 ± 0.04
CSD2	2.01 ± 0.21	1.20 ± 0.17	**CSD2**	1.65 ± 0.47	0.84 ± 0.20
CSD3	1.57 ± 0.15	0.97 ± 0.13	**CSD3**	1.00 ± 0.26	0.79 ± 0.10
FSD1	0.04 ± 0.005	0.09 ± 0.02	**FSD1**	0.54 ± 0.11	0.68 ± 0.17
FSD2	0.22 ± 0.11	1.03 ± 0.18	**FSD2**	0.88 ± 0.33	1.27 ± 0.20
FSD3	1.03 ± 0.10	1.69 ± 0.14	**FSD3**	0.87 ± 0.12	1.07 ± 0.20
MSD1	1.54 ± 0.19	1.55 ± 0.06	**MSD1**	1.25 ± 0.20	1.16 ± 0.10
Antioxidative enzyme capacities (U/mg protein)			*Antioxidative enzyme capacities (U/mg protein)*		
	Control seeds	**Uranium seeds**		**Control seeds**	**Uranium seeds**
SOD	127.01 ± 27.91	80.59 ± 6.12	**SOD**	34.85 ± 2.85	35.50 ± 2.30
CAT	0.04 ± 0.02	0.03 ± 0.002	**CAT**	0.11 ± 0.01	0.10 ± 0.002
GR	0.05 ± 0.02	0.03 ± 0.02	**GR**	0.02 ± 0.003	0.01 ± 0.001
APX	1.60 ± 0.31	1.07 ± 0.17	**APX**	0.94 ± 0.18	0.80 ± 0.06
GPX	13.29 ± 1.64	11.07 ± 1.21	**GPX**	0.22 ± 0.03	0.28 ± 0.03
SPX	8.22 ± 1.34	6.86 ± 0.26	**SPX**	0.43 ± 0.08	0.46 ± 0.04
Glutathione concentrations (nmol/g FW)			*Glutathione concentrations (nmol/g FW)*		
	Control seeds	**Uranium seeds**		**Control seeds**	**Uranium seeds**
Total GSH	187.38 ± 12.91	183.43 ± 6.30	**Total GSH**	374.42 ± 29.15	457.23 ± 32.42
GSH	169.97 ± 11.74	165.32 ± 6.01	**GSH**	345.23 ± 28.39	434.83 ± 31.80

Left table:

	Control seeds	Uranium seeds
GSSG	8.71 ± 1.36	9.06 ± 0.46
% GSH	90.74 ± 1.40	90.11 ± 0.48
Expression levels of DNA repair genes		
	Control seeds	Uranium seeds
PARP1	1.65 ± 0.05	1.34 ± 0.21
PARP2	0.88 ± 0.14	0.46 ± 0.12
POLG1	1.63 ± 0.21	1.05 ± 0.12
KU80	1.64 ± 0.15	1.09 ± 0.08
LIG4	1.13 ± 0.25	1.03 ± 0.17
RAD51	0.91 ± 0.07	0.74 ± 0.05
MND1	1.01 ± 0.18	1.07 ± 0.09
DMC1	1.14 ± 0.09	0.94 ± 0.02
KRP2	1.55 ± 0.12	0.99 ± 0.07
Expression levels of DNA methylation genes		
	Control seeds	Uranium seeds
CMT3	1.52 ± 0.10	1.08 ± 0.08
DRM2	0.57 ± 0.09	0.34 ± 0.03
MET1	1.78 ± 0.15	1.12 ± 0.14
Total DNA methylation level		
	Control seeds	Uranium seeds
	4.52 % ± 0.21	3.81 % ± 0.41

Right table:

	Control seeds	Uranium seeds
GSSG	14.6 ± 1.79	11.2 ± 1.94
% GSH	92.14 ± 0.89	95.06 ± 0.87
Lipid peroxidation (nmol TBA-rc/g FW)		
	17.68 ± 1.24	18.52 ± 1.28
Expression levels of DNA repair genes		
	Control seeds	Uranium seeds
PARP1	0.95 ± 0.23	0.94 ± 0.15
PARP2	0.17 ± 0.03	0.16 ± 0.03
POLG1	0.77 ± 0.19	0.68 ± 0.09
KU80	0.94 ± 0.12	0.24 ± 0.10
LIG4	0.76 ± 0.22	1.29 ± 0.24
RAD51	0.62 ± 0.12	0.59 ± 0.09
MND1	0.50 ± 0.07	0.52 ± 0.09
DMC1	1.61 ± 0.38	1.34 ± 0.18
KRP2	0.89 ± 0.17	0.74 ± 0.09
Expression levels of DNA methylation genes		
	Control seeds	Uranium seeds
CMT3	1.08 ± 0.16	1.09 ± 0.09
DRM2	0.47 ± 0.11	0.51 ± 0.06
MET1	1.44 ± 0.32	1.38 ± 0.19

2. Supplementary Table 2

Supplementary Table 2. Antioxidative enzyme capacities (U/g FW) in roots of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The enzyme capacities in metal-exposed roots are represented relative to their own control which was set to 1. Values are the mean ± SE of at least 3 biological replicates. Significance levels (Two-way ANOVA) are relative to the control conditions and indicate an increased or decreased capacity, respectively (⬜ = $p < 0.05$; 🟩 = $p < 0.01$; 🟥 = $p < 0.01$). Bold and underlined values indicate a significant difference between the roots of control and uranium seeds for the same metal condition ($p<0.05$). SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; APX: ascorbate peroxidase; GPX: guaiacol peroxidase; SPX: syringaldazine peroxidase.

Control seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.15	0.93 ± 0.08	1.98 ± 0.09	1.31 ± 0.14	1.83 ± 0.07
CAT	1 ± 0.23	1.35 ± 0.10	1.43 ± 0.07	1.26 ± 0.18	0.87 ± 0.04
GR	1 ± 0.46	1.37 ± 0.56	1.86 ± 0.83	1.38 ± 0.49	1.25 ± 0.6
APX	1 ± 0.13	1.03 ± 0.09	2.61 ± 0.26	1.52 ± 0.11	2.49 ± 0.22
GPX	1 ± 0.09	1.55 ± 0.15	3.19 ± 0.12	1.86 ± 0.15	1.96 ± 0.15
SPX	1 ± 0.11	**0.95 ± 0.04**	**1.36 ± 0.02**	**1.22 ± 0.01**	1.10 ± 0.07

Uranium seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.08	0.92 ± 0.06	1.51 ± 0.15	1.22 ± 0.12	1.51 ± 0.09
CAT	1 ± 0.06	0.98 ± 0.08	1.06 ± 0.10	0.91 ± 0.08	0.62 ± 0.04
GR	1 ± 0.49	1.21 ± 0.43	1.64 ± 0.78	1.57 ± 0.59	1.58 ± 0.49
APX	1 ± 0.05	1.02 ± 0.06	2.71 ± 0.14	1.42 ± 0.06	2.27 ± 0.12
GPX	1 ± 0.11	1.13 ± 0.09	2.57 ± 0.27	1.35 ± 0.09	1.83 ± 0.15
SPX	1 ± 0.05	**0.64 ± 0.03**	**0.80 ± 0.07**	**0.94 ± 0.03**	0.82 ± 0.07

3. Supplementary Table 3

Supplementary Table 3. Antioxidative enzyme capacities (U/g FW) in leaves of A. thaliana plants of control and uranium seeds exposed to 0 µM, 5 or 10 µM Cd, 25 or 50 µM U for three days. The enzyme capacities in metal-exposed leaves are represented relative to their own control which was set to 1. Values are the mean ± SE of 4 biological replicates. Significance levels (Two-way ANOVA) are relative to the control conditions and indicate an increased or decreased capacity, respectively. (■ = $p < 0.05$; ■ = $p < 0.01$; ■ = $p < 0.05$). Bold and underlined values indicate a significant difference between the leaves of control and uranium seeds for the same metal condition ($p<0.05$). SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; APX: ascorbate peroxidase; GPX: guaiacol peroxidase; SPX: syringaldazine peroxidase.

Control seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.06	1.06 ± 0.04	1.18 ± 0.20	0.96 ± 0.02	0.85 ± 0.05
CAT	1 ± 0.08	0.88 ± 0.05	1.07 ± 0.05	**1.08 ± 0.12**	0.96 ± 0.08
GR	1 ± 0.15	0.78 ± 0.15	0.96 ± 0.07	1.17 ± 0.07	1.68 ± 0.22
APX	1 ± 0.18	0.92 ± 0.22	1.78 ± 0.22	1.28 ± 0.26	1.39 ± 0.27
GPX	1 ± 0.13	1.01 ± 0.08	1.87 ± 0.16	1.02 ± 0.07	1.25 ± 0.11
SPX	1 ± 0.19	1.06 ± 0.12	1.71 ± 0.13	1.10 ± 0.12	0.79 ± 0.10
Uranium seeds					
	0 µM	*5 µM Cd*	*10 µM Cd*	*25 µM U*	*50 µM U*
SOD	1 ± 0.01	1.23 ± 0.11	1.06 ± 0.15	1 ± 0.07	1.17 ± 0.14
CAT	1 ± 0.04	0.77 ± 0.04	0.98 ± 0.04	**1.33 ± 0.04**	1.20 ± 0.05
GR	1 ± 0.08	1.49 ± 0.13	1.41 ± 0.12	1.59 ± 0.35	2.17 ± 0.30
APX	1 ± 0.05	1.41 ± 0.28	1.75 ± 0.13	1.37 ± 0.26	1.65 ± 0.19
GPX	1 ± 0.10	0.93 ± 0.16	1.51 ± 0.31	0.89 ± 0.05	1.27 ± 0.15
SPX	1 ± 0.08	1.08 ± 0.18	1.65 ± 0.19	0.99 ± 0.06	1.01 ± 0.09

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2016**

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Voor akkoord,

Lauwers, Elsa

Datum: **8/06/2016**