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Faculteit Industriële ingenieurswetenschappen

master in de industriële wetenschappen: biochemie

Masterthesis

Oxidative stress response in Arabidopsis thaliana after exposure to uranium: the role of glutathione

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Nick Janssen

Scriptie ingediend tot het behalen van de graad van master in de industriële wetenschappen: biochemie

Gezamenlijke opleiding UHasselt en KU Leuven

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UHASSELT KU LEUVEN

Deze masterproef werd geschreven tijdens de COVID-19 crisis in 2020. Deze wereldwijde gezondheidscrisis heeft mogelijk een impact gehad op de opdracht, de onderzoekshandelingen en de onderzoeksresultaten.

Foreword

This master thesis is written in the frame of my graduation of the corporated education industrial engineer biochemie of the KULeuven and the UHasselt on the campus Diepenbeek, in task of the research group BIS of SCK CEN. This master thesis provided the opportunity to bring the knowledge, that I acquired during the years of education, to practise. During this process I had the ability to build up new skills for the future. Due to the unanticipated consequences of the corona virus (crisis and lockdown) the planned analyses changed a lot. The first part of my work describes the original plan and analyses. Because of the circumstances during the extended lockdown a RNA sequencing dataset was provided by SCK CEN where I was responsible for the biological interpretation which is described in the second part. This work was new for me with a lot of challenges along the way. But that is what made it interesting. Without the occasionally challenge a man can't grow to his full potential. So despite the consequences of the corona virus, it was a very pleasant opportunity to work on 'my own research'.

I could never have written this master thesis without the necessary help I got. First of all I want to thank my mentor from the research group BIS, doctor Eline Saenen, for the pleasant accompaniment and feedback. Thereafter I want to thank my second mentor from UHasselt, engineer Liesbet Pauls, for all the help and feedback. Both of you thank you for always making time for me when I had a problem or wanted to talk. Thank you for sharing your expertise, for the encouragement and support. You helped my bring this work to a good end. I would also like to thank my fellow student and colleges in SCK CEN that I met during my internship. Thank you all for your help, the good welcome and the nice talks.

My gratitude also goes to my family and friends. Thank you for all the support. You gave me the distraction I needed in the free evenings or random moments during these long days of work. I am very thankful for all the encouragements I received through the good and bad days. Last but not least thank you to everyone that believed in my every single day of this journey.

Nick.

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Abbreviations

Ag	Zilver
As	Arsenic
AsA	Ascorbate
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BIS	Biosphere impact sudies
bp	Base pair
BSO	Buthionine sulfoximine
Ca	Calcium
CAR	Cartenoid
CAT	Catalase
Cd	Cadmium
cDNA	Complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
C_T	Treshold values
Cu	Copper
Cys	Cysteine
DEG	Differentially expressed genes
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DTNB	5,5'-dithiobis(2-nitro-benzoic acid)
DU	Depleted uranium
ER	Endoplasmic reticulum
FDR	False discovery ratio
gDNA	Genomic DNA
Glu	Glutamic acid
Gly	Glycine
GO	Gene ontology
GPOX	Glutathione-dependent peroxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GRx	Glutaredoxin
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione transferase
GSTF	GST class phi
GSTL	GST class lambda
GSTT	GST class theta
GSTU	GST class tau
GSTZ	GST class zeta
He	Helium
HP	High phosphate
ICP-MS	Indutively coupled plasma mass spectrometry

LFC	Log fold change
LP	Low phosphate
MAPEG	membrane associated proteins in eicosanoid and glutathione metabolism
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
Mg	Magnesium
MT	Metallothioneins
NADPH	Nicotinamide-adenine-dinucleotidefosfaat
OPDA	Oxylipin (15Z)-12-oxophyto-10,15-dienoic acid
PC	Phytochelatins
PCS	Phytochelatins synthase
POD	Peroxidase
PRx	Peroxiredoxins
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative PCR
SAZ	Syringaldazine
Se	Selenium
SOD	Superoxide dismutase
SPX	Syringaldazine peroxidase
TBA	2-thiobarnituric acid
TCA	Trichloroacetic acid
TCHQD	Tetrachlorohydroquinone dehalogenases
TNT	2,4,6-trinitrotoluene
TOC	Tocopherol
TRx	Thioredoxin
U	Uranium
VPD	2-vinylpyridine
WT	Wild type
Zn	Zinc

Abstract

Anthropogenic activities have caused pollution of various compartments of our environment with heavy metals, including uranium. Although it is a non-essential element, uranium can easily be taken up by plants where it can cause toxic effects, including oxidative stress. Plants have developed an antioxidative defence mechanism to counteract this stress. Glutathione (GSH) is an important antioxidant in this mechanism with three major function: redox homeostasis, metal homeostasis and detoxification. Although several studies have investigated the urani-um-induced stress responses, the role of GSH during uranium stress is not completely known. The present study aimed to further investigate the role of GSH in Arabidopsis thaliana during uranium exposure. Therefore seed-lings were cultivated for 18 days in a hydroponic setup with Hoagland nutrient solution, followed by three days of exposure to different uranium concentrations, ranging from 0-50 µM. After RNA extraction, the complete transcriptome of the plants was sequenced using the Illumina HiSeq2000 platform. Due to limited root-to-shoot trans-location and the limited timeframe, only the data of the roots were processed during this internship. Uranium exposure clearly disturbed the sulfur metabolism by causing a sulfur starvation response. No differentially ex-pressed genes related to glutathione synthesis where found, indicating no increased GSH biosynthesis. However, it seems that plants are trying to regulate the sulphur starvation response by providing cysteine for its essential functions as structural role in proteins by breaking down glucosinolates. Based on the differentially expressed genes, the results indicate that GSH probably don't play an important role in detoxifying reactive oxygen species via the AsA-GSH cycle under uranium stress, since genes related to this cycle were not significantly affected. In addition, no indications for the synthesis or presence of phytochelatins were found. However, uranium disturbs the homeostasis of multiple metals among which Fe. Finally, there seems to be an important role for the role for glutathione-S-.transferases in the detoxification of uranium and ROS during uranium exposure.

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Abstract in Dutch

Door antropogene activiteiten zijn verschillende compartimenten van ons milieu vervuild met zware metalen, waaronder uranium. Hoewel het een niet essentieel element is, kan uranium gemakkelijk worden opgenomen door planten waar het toxische effecten waaronder oxidatieve stress kan veroorzaken. Planten hebben een antioxidatief afweermechanisme ontwikkeld om deze stress tegen te gaan. Glutathion (GSH) is een belangrijk antioxidant in dit mechanisme met drie belangrijke functies: redoxhomeostase, metaalhomeostase en detoxificatie. Hoewel verschillende studies de door uranium veroorzaakte stressreacties hebben onderzocht, is de rol van GSH tijdens uraniumstress niet volledig bekend. Deze studie was bedoeld om de rol van GSH in *Arabidopsis thaliana* tijdens blootstelling aan uranium verder te onderzoeken. Daarom werden zaailingen 18 dagen gekweekt in een hydrocultuuropstelling met Hoagland voedingsoplossing, gevolgd door drie dagen blootstelling aan verschillende uraniumconcentraties, variërend van 0-50 µM. Na RNA-extractie werd de sequentie van het volledige transcriptoom van de planten bepaald met behulp van Illumina Hiseq2000-platform. Door de beperkte wortel naar scheut translocatie en het beperkte tijdsbestek werden tijdens deze master thesis alleen de gegevens van de wortels verwerkt. Blootstelling aan uranium verstoorde het zwavelmetabolisme duidelijk door een zwavelgebrekreactie te veroorzaken. Differentieel tot expressie gebrachte genen gerelateerd aan de glutathionsynthese werden niet gevonden, wat wijst op geen verhoogde GSH-biosynthese. Het lijkt er echter op dat planten deze zwavelgebrekreactie proberen te reguleren door cysteïne te leveren voor zijn essentiële functies als structurele rol in eiwitten door afbraak van glucosinolaten. Gebaseerd op de differentieel tot expressie gebrachte genen, geven de resultaten aan dat GSH waarschijnlijk geen belangrijke rol speelt in de detoxificatie van reactieve zuurstofsoorten (ROS) via de AsA-GSH cyclus onder uraniumstress, aangezien genen die verband houden met deze cyclus niet significant werden beïnvloed. Bovendien waren er geen indicaties voor de synthese of aanwezigheid van fytochelatines. Uranium verstoort echter de homeostase van meerdere metalen waaronder ijzer. Ten slotte lijkt er een belangrijke rol te zijn voor glutathion-S-transferasen bij de detoxificatie van uranium en ROS tijdens blootstelling aan uranium.

Chapter 1

Introduction

1.1 Context

SCK CEN is a nuclear research center located in Belgium. It is a world leader in the nuclear sector in the field of scientific research, services and educations. One of SCK CEN research groups is the Biosphere Impact Studies (BIS), which focusses, amongst others, on the effects from radionuclides on plants.

Uranium is a naturally occurring heavy metal and radionuclide. There are three natural isotopes of uranium, namely ^{238}U, ^{235}U and ^{234}U of which ^{238}U is the most abundant. Table 1 lists the characteristics of these isotopes [1]. Due to the long decay half-life of ^{238}U and its low specific activity, there is a greater risk for chemical toxicity than radiological toxicity [1].

Table 1: Characteristics of uranium isotopes in natural uranium [1, p. 94]

Isotope	Half-life (years)	Relative mass (%)	Specific activity (kBq/g)
U-238	4,47*10^9	99,3	12,455
U-235	7,04*10^8	0,72	80,011
U-234	2,46*10^5	0,006	231*10^6

As uranium is a primordial element, the average concentration in the Earth's surface is 1.7 ppm. However, anthropogenic activities such as uranium mining and milling, phosphate industry and metal mining and smelting contaminated large areas with uranium [2].

Uranium can easily be taken up by plants, where it mainly accumulates in the roots with limited roots to shoots translocation [3]. Regarding the toxicity, it is already known that uranium causes an oxidative stress response in plants [4]. Oxidative stress is a situation where the balance between the production and elimination of reactive oxygen species (ROS) is disturbed. These ROS include superoxides $O_2^{\circ-}$, HO_2°, hydroxyl radical OH°, hydrogen peroxide H_2O_2, peroxyl radicals RO°,ROO°. ROS are also by-products during normal cell metabolism [5]. However, under normal conditions ROS production is low, while under stress conditions the production is enhanced. The disturbance between ROS production and scavenging can lead to damage to proteins, lipids, polysaccharides and DNA which in turn can eventually lead to cell death [6].

Plants have antioxidative defence systems to prevent accumulation of free metal ions and neutralize excessive ROS [7]. These antioxidative defence mechanisms can both be enzymatic with superoxide dismutase, catalase, peroxidases or metabolites as ascorbate (AsA) and glutathione (GSH) [6] [8].

GSH is a tripeptide (γ-L-glutamyl-L-cysteinylglycine, also indicated as a pseudopeptide) synthesized from γ-glutamate, cysteine and glycine. It has an important role in the metal homeostasis, antioxidative defence mechanisms and signal transduction. GSH contains a free thiol group on cysteine which has a high affinity for metals. It is also used as substrate for the phytochelatin biosynthesis. Consequently, phytochelatin can be used for metal detoxification [9]. Also, GSH is incorporated in the AsA-GSH cycle, which is the most important antioxidant cycle in plants. This cycle links, among other, AsA with GSH. GSH can be linked to xenobiotics, with the help of glutathione-S-transferases (GSTs), resulting in a detoxification of these xenobiotics. Due to these characteristics, GSH is a key component in the antioxidative defence system.

1.2 Problem definition

It is already known that GSH is an important factor in the antioxidative defence system. But what is its function in the oxidative stress response when *Arabidopsis thaliana* is exposed to uranium?

To find the answer to this main question, the project will work with two types of *Arabidopsis thaliana*, one wild type (WT) and a mutant (cad2-1) that has a lower GSH level (explained later). So this main question can be split in some smaller questions, all under the conditions of oxidative stress induced by uranium:

- Does GSH has an influence on the U uptake?
- Does GSH has an influence on the expression of other genes?
- Does GSH effect the lipid peroxidation?
- Does GSH effect the enzyme activity?
- Does GSH effect the growth?

1.3 Research objectives

The main goal of this research is to investigate the functions of glutathione in the oxidative stress response of *Arabidopsis thaliana* when they are exposed to uranium.

First of all, the literature study will explain the mechanisms oxidative stress and the antioxidative defence mechanism. Thereafter it will explain the known functions of GSH in these mechanisms.

To obtain insight into the function of glutathione, two types of *Arabidopsis thaliana* will be investigated. As mentioned before two types of *Arabidopsis* thaliana will be used in this research. The first type is a wild-type (WT), the second type is a *cad2-1* mutant which has a defect in gamma-glutamylcysteine synthetase, the first enzyme of the biosynthesis of GSH. Because of this defect the cad2-1 mutant has only 30% of the wild-type GSH level.

Results obtained for the wild-type and the cad2-1 mutant will be compared on their: gene expression (PSC1, MT2a, GSH1, GSH2, GSTs, miR408, OX1-5,CSD1/2), uranium uptake, enzymatic activity (GST, antioxidative enzymes) and lipid peroxidation. Based on the comparison, the role of GSH in *Arabidopsis thaliana* exposed to uranium can be elucidated.

1.4 Materials and methods

In this project, *Arabidopsis thaliana* plants will be used. These type of plants are used because they have a short lifecycle, need little space, a lot of mutants are available and have a little genome. Seeds will be sterilized (1 min, 0.1% bleach) and sown in a hydroponic setup [10]. After 18 days, plants will be exposed to uranium (0 and 25 µM U). After 3 days of exposure, roots and leaves will be harvested and frozen at -80°C for further analyses.

Different analyses will be carried out:

- Gene expression.

The following genes will be investigated: PCS1, MT2a, GSH1, GSH2, GSTs (GSTU25), miR408 (MA15), OX1-5 and CSD1/2. To measure the gene expression, RNA will be extracted from root and leaf samples using the QIAGEN RNeasy plant mini kit [11]. The RNA will be converted to cDNA with reverse-transcriptase followed by a RT-qPCR [12]. This technique is able to monitor the amplification reaction by using fluorescence. Thus it has the capacity to measure the amount of a specific RNA.

- Uranium uptake

The uranium uptake will be determined by digesting dried plant material in 1M HCl using an ICP-MS. [3] The samples will be calcinated using a muffle furnace [13].

- GSH concentration

The glutathione concentration can be determined using a spectrophotometric method with a plate reader. This method uses a Corning 96well UV-transparent plate and a multiscan spectrum variable wavelength plate reader. First of all, the total glutathione (GSH+GSSG) will be measured. This measurement relies on the reduction of 5,5'-dithiobos(2-nitro-benzoic acid) (DTNB, Ellman's reagent) which is GR-dependent. The amount of GSSG can be measured by adding 2-vinylpyridine (VPD) which complexes GSH. To end with, the amount of GSH is equal to the first measurement minus the second [14] [15].

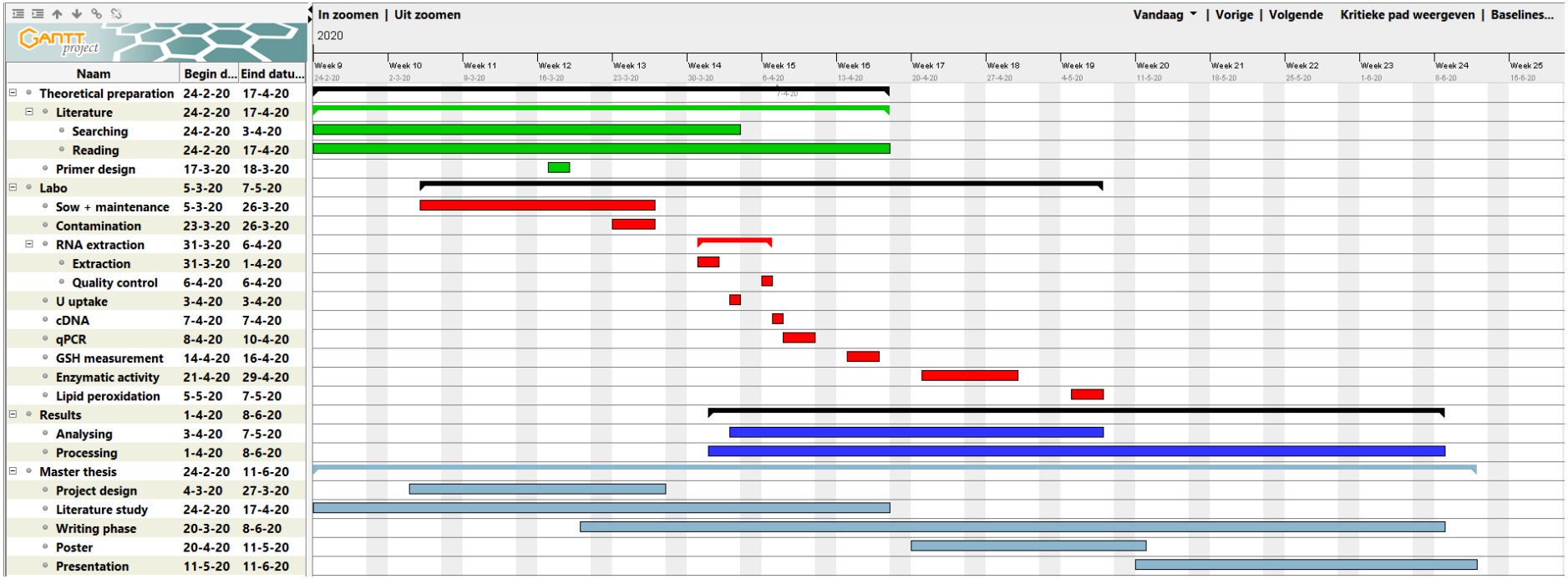
- Enzymatic activity

Thereafter, the enzymatic activity of six enzymes will be measured using a spectrophotometric method, namely GST, Antioxidative enzyme: superoxide dismutase(SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR) and ascorbate peroxidase (APX)) [16]. SOD is responsible for the conversion of $O_2^{\circ-}$ in to H_2O_2 and O_2. CAT converts H_2O_2 into H_2O and O_2. POD is also able to decompose H_2O_2, but it will need to oxidize a co-substrates while catalysing this reaction. GR is capable of regenerating GSH. Finally, APX can oxidize ascorbate, as such catalysing the first step of the H_2O_2 scavenging pathway. Plants are dependent on those antioxidant enzymes to metabolize H_2O_2 and $O_2^{\circ-}$ [17].

- Lipid peroxidation

An indication of lipid peroxidation can be obtained based on thiobarbituric acid (TBA) reactive compounds mainly malondialdehyde (MDA) using a plate-reader assay [2] [18].

1.5 Grant table



Naam	Begin d...	Eind datu...
Theoretical preparation	24-2-20	17-4-20
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Poster	20-4-20	11-5-20
Presentation	11-5-20	11-6-20

Figure 1: Grant table

1.5 Preview

This master's thesis contains two more chapters. Chapter two is a literature study which provides the knowledge about uranium, oxidative stress, the antioxidative defence mechanism and glutathione necessary for this research. Chapter three is the materials and methods, that are used to investigated the role of glutathione during oxidative stress conditions in *Arabidopsis thaliana* after exposure to uranium.

Chapter 2

Literature study

During this master's thesis the role of glutathione in the oxidative stress response of *Arabidopsis* thaliana after exposure to uranium is investigated. The literature study of this thesis starts with a description of uranium. Hereafter, the ROS production, signalling and damage mechanisms are described. The literature study ends with an overview of the importance of glutathione (GSH) in the antioxidative defence system.

2.1. Uranium

As mentioned before, uranium is a natural occurring radionuclide with an average concentration of 1.7 ppm in the earth's surface. This is the result of anthropogenic activities such as U mining and milling, phosphate mining and heavy metal mining have caused contamination of large areas with uranium by means of improper disposal of the radioactive waste material [19].

Uranium is a radionuclide and heavy metal with three naturally occurring isotopes, ^{238}U, ^{235}U and ^{234}U. Natural occurring uranium is a mixture of those three isotopes with ^{238}U as the dominant one, Besides the natural occurring uranium there exist other forms such as enriched uranium which contains higher amounts (>20%) of ^{235}U what is used for nuclear energy. When the biggest part of ^{235}U is removed, the residue is called depleted uranium (DU) [20]. The natural occurring isotopes of uranium only emit alpha particles. But isotopes of uranium can decay to other radioactive elements which emit beta and gamma radiation [1]. An alpha particle consist of two neutrons and two protons, it is an equivalent to a ^{4}He. They are generated by the transmutation of a mother nucleus to a daughter nucleus. Because of their 'large' size, they rapidly lose their kinetic energy and thus have only a small penetrating power but a high ionisation capacity.

The chemical properties of uranium are all the same due the same number of protons (92). But as illustrated in the Table 1 the radiological properties are different [1]. Because of the long decay half-life ^{238}U forms a greater risk with its chemical toxicity than its radiological toxicity [1] [3] [19] [20]. The effects of uranium toxicity are mainly studied on animal species and man, but for plants there is less information.

The U uptake of plants depends of the form of uranium. Soluble compounds are faster incorporated than oxides. This makes the aqueous uranyl ion a fast incorporated form of U and thereby one of the forms with the highest cause to chemical toxicity [2]. The uranium that is taken up by plants will mainly accumulate in the roots, only a limited amount will be transferred to the shoots [3] [21].

U can cause effects on both macroscopic and cellular level. On macroscopic level it cause reduced production in biomass and growth by affecting the nutrient profile. It is known that after exposure to U roots become stunted and will turn yellow and leaves will show chlorosis. On cellular level, uranium affect permeability of membranes, enzyme capacities and interact with several macromolecules such as nucleic acids and proteins. A feasible explanation for this effect can be that uranyl can replace Ca^{2+} and Mg^{2+} and it interacts with phosphate moieties of the cell wall. Besides these effects, heavy metals including U induce oxidative stress. During this condition there is a disturbance between the elimination and production of ROS, causing an accumulation of ROS. This disrupts the cellular redox balance. This imbalance is termed oxidative stress. The accumulation of ROS can cause cell death by damaging cellular compounds, DNA lesion and mutations which leads to irreparable dysfunctions [2] [6].

2.2. Oxidative Stress

2.2.1. ROS production

O_2 itself is relatively unreactive. However, during metabolic processes, energy or electrons can be transferred to O_2. This will lead to the production of ROS, which are essentially partially reduced forms of O_2 [22].

Some examples of ROS are: superoxide $O_2^{\cdot}$, hydroxyl radical $OH^{\cdot}$, perhydroxy radical $HO_2^{\cdot}$, hydrogen peroxide H_2O_2, alkoxy radical $RO^{\cdot}$ and peroxy radical $ROO^{\cdot}$. Figure 2 provides a schematic overview of the production of different ROS. These ROS are unavoidable, they are a natural by-product of the plants metabolism. However, under normal conditions the production of ROS is low. Non-redox-active metals can induce oxidative stress in two ways. Firstly these metals activate enzymes like NADPH oxidases and lipoxygenases, which are pro-oxidative enzymes. Secondly they can indirectly alter the cellular redox state by targeting components of the respiratory chain [6].

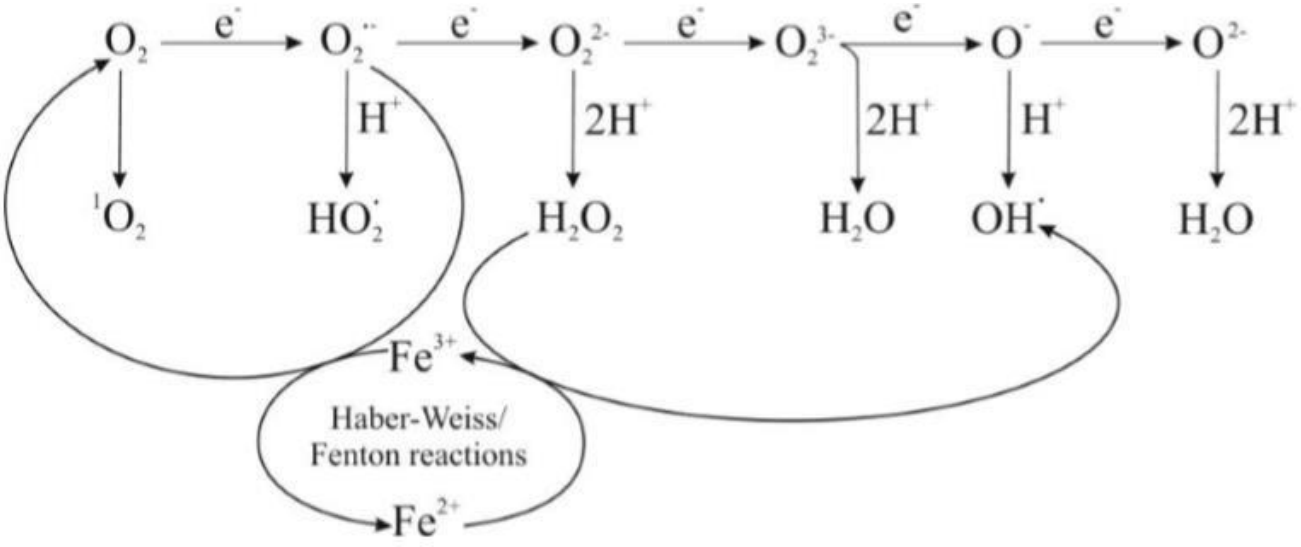

Figure 2: Overview of the ROS production [5, p. 712]

The production of ROS can be enhanced by the presence of stresses, e.g. metal stress. In the presence of redox-active metals (e.g. Fe^{2+} and Cu^{+}) H_2O_2 form very reactive OH° through the Fenton and Haber-Weis reaction shown below [23].

$$Fe^{3+} + O_2^{\circ -} \rightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\circ}$$
$$\_\_\_\_\_$$
$$O_2^{\circ -} + H_2O_2 \rightarrow OH^{\circ} + OH^{-} + O_2$$

ROS are mainly generated in the mitochondria, plasma membrane, endoplasmic reticulum (ER), chloroplasts and peroxisomes. In these subcellular compartments ROS are produced by reduction of O_2 to $O_2^{\circ -}$. Next, these superoxides are converted to hydrogen peroxide by SOD or, in the presence of redox-active metals, they can form hydroxyl radicals through the Fenton and Harber-Weiss reaction as mentioned above. Besides this, the mitochondria, chloroplasts and peroxisomes produce superoxides as by-products of their normal metabolism. In the ER ROS could facilitate the transfer of Ca^{2+} at the ER-mitochondria interface. H_2O_2 can hereby also diffuse out of the ER, bypass the protection from mitochondrial SOD (located in the matrix) and attack neighbouring mitochondria membranes [5].

2.2.2. ROS and damage

ROS cause damage to DNA, lipids and proteins. The oxidized DNA bases can cause functional problems if they are not quickly removed at critical positions. These changes in DNA cause genetic mutations, alterations in transcription, a decreasing fidelity of RNA and DNA polymerase and changes in protein conformation [23]. To prevent those problems, cells have multiple repair systems for damaged DNA bases.

Due to the critical role of lipids in membranes both functional and structural damage to lipids have multiple consequence. First of all there is the possibility for lipid peroxidation which is an important cause of cell damage. This process is illustrated in the Figure 3.

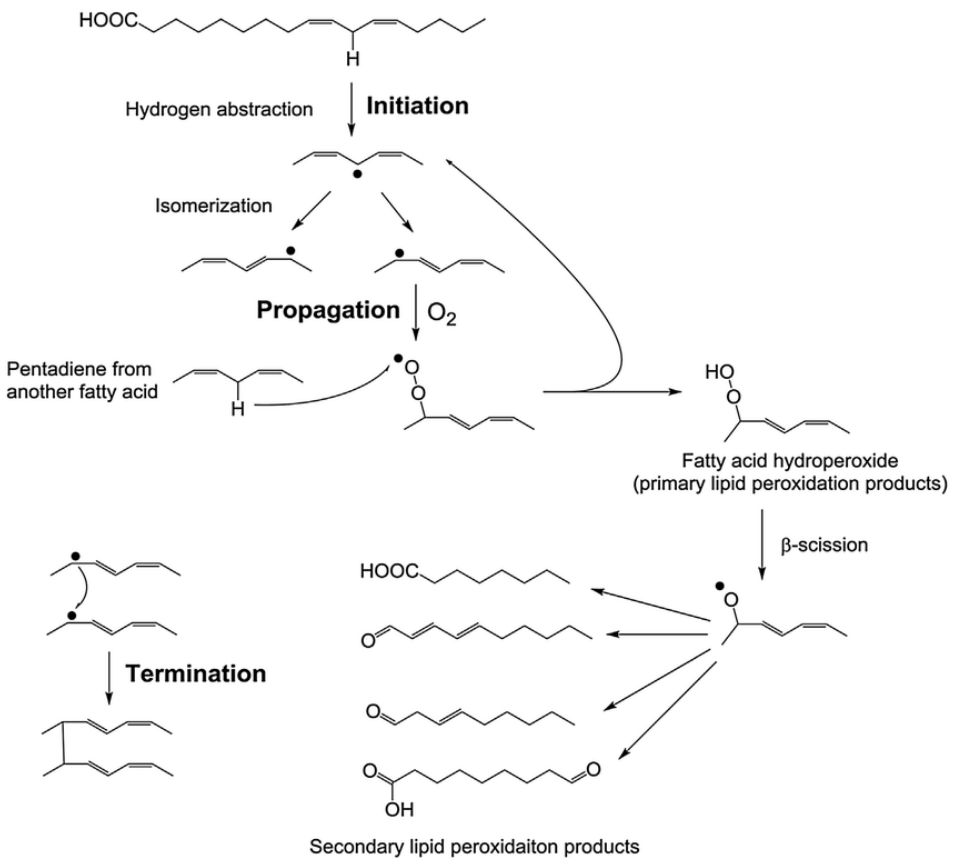

Figure 3: Schematic overview of lipid peroxidation [24]

Lipid peroxidation is a chain reaction which can inactivate ion channels and enzymes, damage membrane proteins or change the permeability of membranes. By removing a hydrogen atom from the methylene group between two non-conjugated double bounds, which are easy targets for free radicals, this chain reaction is initiated. The removal of a hydrogen atom results in the formation of a new radical species that interact with oxygen. This radical can subsequentially abstract another hydrogen atom from another fatty acids which results again in a radical and a lipid hydroperoxide. Secondly some oxidized fatty acids have the capacity to affect signalling pathways that induce the apoptotic form of cell death. Several studies have reported this connection between apoptosis and ROS, unfortunately the exact mechanism remains unknown [5] [23].

Finally there is the damage to proteins. By damaging proteins a range of reactive and stable products are formed. An example of a reactive product that is formed are protein hydroperoxides. When transition metal ions react with the formed protein hydroperoxides, they produce more radicals. These radicals can alter the function of the protein. It is more efficient for the cell to either prevent these products or to remove them by proteolysis than to repair the damaged protein. As a result protein repair systems are absent in the cell [23].

2.2.3. ROS and signalling

Like mentioned before, ROS are natural by-products of the cell metabolism, however at low concentrations. At these low concentrations, ROS can act as secondary messengers associated with the activation of stress responses and defence pathways, while at high concentrations they initiate cell death.

ROS can affect the cell metabolism and plant growth by interfering and/or utilizing signalling molecules which influence the plants stress response [5]. It is known that hydrogen peroxide induces the accumulation of stress hormones like salicylic acid and ethylene [13]. In addition, ROS also act as secondary messenger in different signalling pathways, for example differentiation and the regulation of immunity [19] [23].

Besides this, ROS are able to affect antioxidant enzymes, calcium signalling, ion transporters, various kinases such as c-jun N terminal kinase and mitogen activated protein kinase and also genes that are related to cell growth [19] [23]. ROS are involved in signalling due to their ability of reversible post-translational modification of proteins (thiol oxidation) [22].

2.3. Antioxidative defence mechanisms

Plants have three main mechanisms to defend themselves to oxidative stress. This master's thesis will only focus on the antioxidative defence mechanism. In this mechanism the plant will use integrated systems of enzymatic and metabolites antioxidants which have the ability to scavenge ROS, without itself undergoing conversion to a destructive radical [9]. The production and scavenging of ROS is in delicate balance. This is thought to be supported by the ROS gene network. In *Arabidopsis* this network consist of 152 genes [5].

Enzymatic mechanisms includes catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), syringaldazine peroxidase (SPX) and superoxide dismutase (SODs) [5]. The metabolites include ascorbic acid (AA), glutathione (GSH), tocopherols (TOCs) and CAR. Besides this, there are other enzymes needed for the regeneration and activation of the antioxidants such as dehydroascorbate reductase (DHAR) and glutathione reductase (GR) [5] [17]. The table below provides an overview of the most important components of the both enzymatic and non-enzymatic antioxidants systems.

Table 2: Overview of the antioxidative systems with their subcellular localization and ROS-scavenging [5, p. 713]

Enzymatic antioxidants	Reaction catalyzed	Enzyme code number	Subcellular localization
CAT	$2H_2O_2 \rightarrow O_2 + 2H_2O$	1.11.1.6	Per, Gly, and Mit
APX	$H_2O_2 + AA \rightarrow 2H_2O + DHA$	1.11.1.11	Cyt, Per, Chl, and Mit
GPX	$H_2O_2 + DHA \rightarrow 2H_2O + GSSG$	1.11.1.7	Chl, Cyt, Mit, and ER
SOD	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow 2H_2O_2 + O_2$	1.15.1.1	Cyt, Chl, Per, and Mit
MDHAR	$2MDHA + NADH \rightarrow 2AA + NAD^+$	1.6.5.4	Chl, Mit, and Cyt
DHAR	$DHA + 2GSH \rightarrow AA + GSSG$	1.8.5.1	Chl, Mit, and Cyt
GR	$GSSG + NADPH \rightarrow 2GSH + NADP^+$	1.6.4.2	Cyt, Chl, and Mit
Non-enzymatic antioxidants			
AA	Substrate for APX. Detoxifies H_2O_2		Chl, Cyt, Mit, Per, Vac, and Apo
GSH	Substrate for various POXs, GSTs and GR. Detoxified H_2O_2, other hydroperoxidases and toxic compounds		Chl, Cyt, Mit, Per, Vac, and Apo
TOCs	Protects membrane lipids from peroxidation, detoxifies lipid peroxides, and quenching $^1O_2$		Membranes
CARs	Quench $^1O_2$. Photosystem assembly, key components of the light harvesting complex, precursors for abscisic acid (ABA)		Chl, chromoplast, elaioplast, and amyloplast
Flavonoids	Can directly scavenge H_2O_2 and $OH^{\cdot}$		Vac

Abbreviations: $^1O_2$: singlet oxygen; AA: ascorbic acid; Apo: apoplast; APX: ascorbate peroxidase; CARs: carotenoids; CAT: catalase; Cyt; cytosol; Chlo: chloroplast; DHA: dehydroascorbate; DHAR: dehydroascorbate reductase; ER: endoplasmic reticulum; Gly: glyoxisomes; GPX: guaiacol peroxidase; GR: glutathione reductase; GSH: glutathione; GSSG: oxidized glutathione; GSTs: glutathione-*S*-transferases; H_2O: water; H_2O_2: hydrogen peroxide; MDHA: monodehydroascorbate; MDHAR: monodehydroascorbate reductase; Mit: mitochondria; O_2: oxygen; $O_2^{\cdot-}$: superoxide radical; $OH^{\cdot}$: hydroxyl radical; Per: peroxisomes; POXs: peroxidases; SOD: superoxide dismutase; TOCs: tocopherols; Vac: vacuole.

2.3.1. Enzymatic antioxidative defence system

The first line of defence against ROS is superoxide dismutase (SOD). These enzymes will convert $O_2^{\circ-}$ to H_2O_2, the reaction is shown in Table 2. Based on their metal co-factor these enzymes can be distributed in three groups: manganese SOD (MnSOD), iron SOD (FeSOD) and copper/zinc SOD (CuZnSOD). The different isoforms catalyse all the same reaction, but at different locations in the cell. MnSOD is mainly located in the mitochondria, FeSOD in the chloroplast and CuZnSOD occurs in the chloroplast, cytosol and peroxisomes [5] [7].

The next line of defence are CAT and peroxidases (POD) which are both H_2O_2 scavengers. CAT prevent the formation of OH° by converting 2 molecules H_2O_2 to H_2O [6] [7]. Besides its activity during oxidative stress CAT also removes hydrogen peroxide in peroxisomes during photorespiration, beta-oxidation of fatty acids and purine catabolism. In *Arabidopsis* three isoforms of CAT are present: CAT1, CAT2 and CAT3. Each of the isoforms are separately regulated and expressed [5].

In *Arabidopsis* POD occurs in different forms, there is APX, GPX and SPX. They all catalyse the conversion of hydrogen peroxide to water, with each a specific donor. First of all APX has its function in the chloroplast and cytosol of the plant. It uses ascorbate as hydrogen donor to form H_2O and monodehydroascorbate (MDHA). APX exists in three isoforms, one membrane-bounded form and two cytosolic forms. The membrane-bounded form instantly catches superoxide and subsequently convert it at the membrane surface. Besides its role in H_2O_2 scavenging it also controls the electron transport in the AsA-GSH cycle (2.4.1.2). Just like SOD, APX occurs in both thylakoid-bounded and soluble form in the chloroplast [5]. Despite the fact that CAT and APX are both H_2O_2 scavengers, they differ in affinity for H2O2. CAT has a lower affinity but a higher reaction speed when compared to APX. In contrary to APX, CAT has no need for a substrate to decompose H_2O_2. This suggest that CAT is responsible for removing the excess ROS whereas APX is better in the fine-tuning of ROS concentrations which are needed as secondary messenger [6].

Secondly the enzymatic oxidative mechanism consists of guaiacol peroxidase. This enzyme contains a heme group. Just like SPX this is a nonspecific donor peroxidase that can use several aromatic substrates to reduce H_2O_2 in the vacuole, cell wall, cytosol and extracellular space. Because GPX is both active in the intra- and extra-cellular space it is considered to be a key antioxidant. Besides this diverse site of activity it is a very important stress enzyme. The amount of GPX is a potential way of indicating the intensity of the stress and this makes GPX a useful biomarker. GPX has also a role in the biosynthesis of lignin [5].

2.3.2. Non-enzymatic antioxidative defence system

Besides enzymes, there are also some low mass antioxidants important in ROS scavenging, i.e. ascorbate (AsA), glutathione (GSH), tocopherols (TOCs) and carotenoids (CARs) *[5] [25]*. First of all there is AsA which is also known as vitamin C *[26]*. AsA is a very powerful antioxidant that under physiological conditions occurs for 90% in its reduced form in chloroplast. In the aqueous phase ascorbic acid is considered one of the main ROS-detoxifying metabolites due to its ability to donate electrons to diverse enzymatic and non-enzymatic reactions. OH°, $^1O_2$ and O2°- are

directly scavenged by AsA (shown in Figure 7) *[25]*. With the help of APX it is capable of reducing hydrogen peroxide to water. AsA and GSH are related through the AsA-GSH cycle, which will be explained in 2.4.1.2.

Glutathione is an important factor in these systems with both a function as ROS scavenger and as a redox buffer to maintain a balance in redox state of the cell [6]. When acting as an antioxidant, GSH will be oxidized to glutathione disulphide (GSSG). GSH will further be discussed in more detail in 2.4.

Next there is the antioxidant tocopherol (also known as vitamin E) which is only synthesized by plants. It is a lipophilic antioxidant and part of the vitamin E group. In a stress-tolerant plant there is an increased level of tocopherol present *[9]*. TOCs in the membrane of chloroplast are known to protect lipids by reacting with oxygen. Due to their ability to repair oxidizing radicals and by doing so preventing the propagation step of lipid peroxidation as shown in Figure 4, they are called chain-breaking antioxidants. TOC prevent this step by donating a hydrogen atom in the water-membrane interface. By doing so it forms a tocoperoxyl radical which can be regenerated by reaction with GSH or AsA *[5]*.

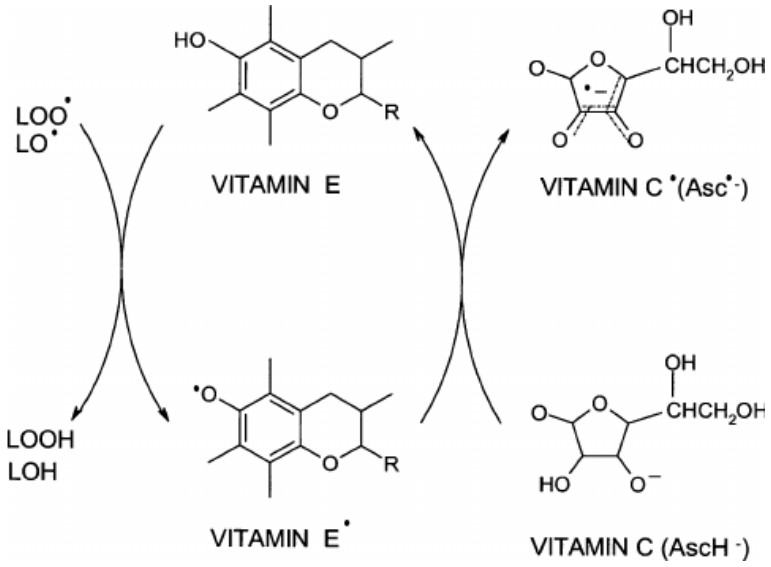

Figure 4: Prevention of lipid peroxidation by TOCs [27, p. 47]

Finally, carotenoids are lipophilic compounds, like TOC. They can be found in photosynthetic and non-photosynthetic tissue. CARs are better known as antenna molecules due to their ability to absorb light in the range of 450-570 nm. They also detoxify diverse forms of ROS. This ability makes sure that the photosystem is protected in multiple ways against ROS. First of all they can terminate the chain reaction of lipid peroxidation. Secondly they are able to scavenge $^1O_2$ and converting their energy in to heat. Third they can avoid the generation of those $^1O_2$ molecules by reacting with excited chlorophyll. As last they can remove the excessive energy in to the xanthophyll cycle [5].

Phenolic compounds such as tannins, flavonoids, hydroxycinnamate esters and lignin are metabolites which are present in generous amounts in plant tissue. Due to their ability to delocalize, stabilize unpaired electrons, chelate transition metal ions and reactivity as electron

donor they have antioxidative properties and the capacity to scavenge free radicals. For example flavonoids decrease the fluidity of the membranes by modifying the lipid packing. This will hinder the lipid peroxidation and free radicals [5].

2.4. Glutathione (GSH)

GSH is a pseudopeptide which is synthesized in two ATP-dependent steps from three amino acids in the chloroplast and cytosol. The biosynthesis starts with the uptake of sulphur in the form of sulphate. Sulphur will be used for the formation of cysteine, which is one of the three amino acids needed for the production of GSH. In the next step a amide bound between cysteine and glutamate will be formed catalysed by glutamylcysteine synthase (GSH1). Then glutathione synthetase (GSH2) adds the third amino acid glycine. The structure of GSH is presented in Figure 5.

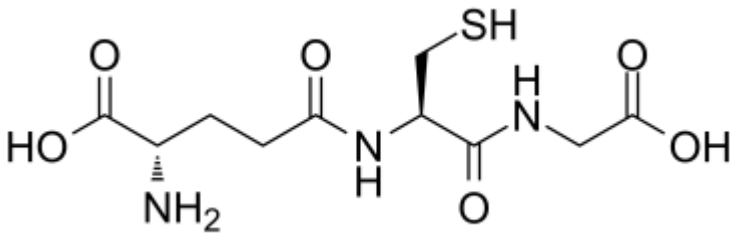

Figure 5: GSH structure [28]

Genome sequencing of *Arabidopsis* learned that both genes for the enzymes are singly encoded. Therefore a mutation in one of both genes is lethal for the plant [6]. GSH-deficient mutants are preferred to investigate the role of GSH instead of knock-out mutants. Another way to investigate the importance is by using buthionine sulfoximine (BSO) which is a GSH1 inhibitor [6] [25]. GSH is needed for multiple functions like redox homeostasis, metal homeostasis and detoxification through glutathione S transferase (GST) [29]. Figure 6 provides a schematic overview of the most important functions of GSH.

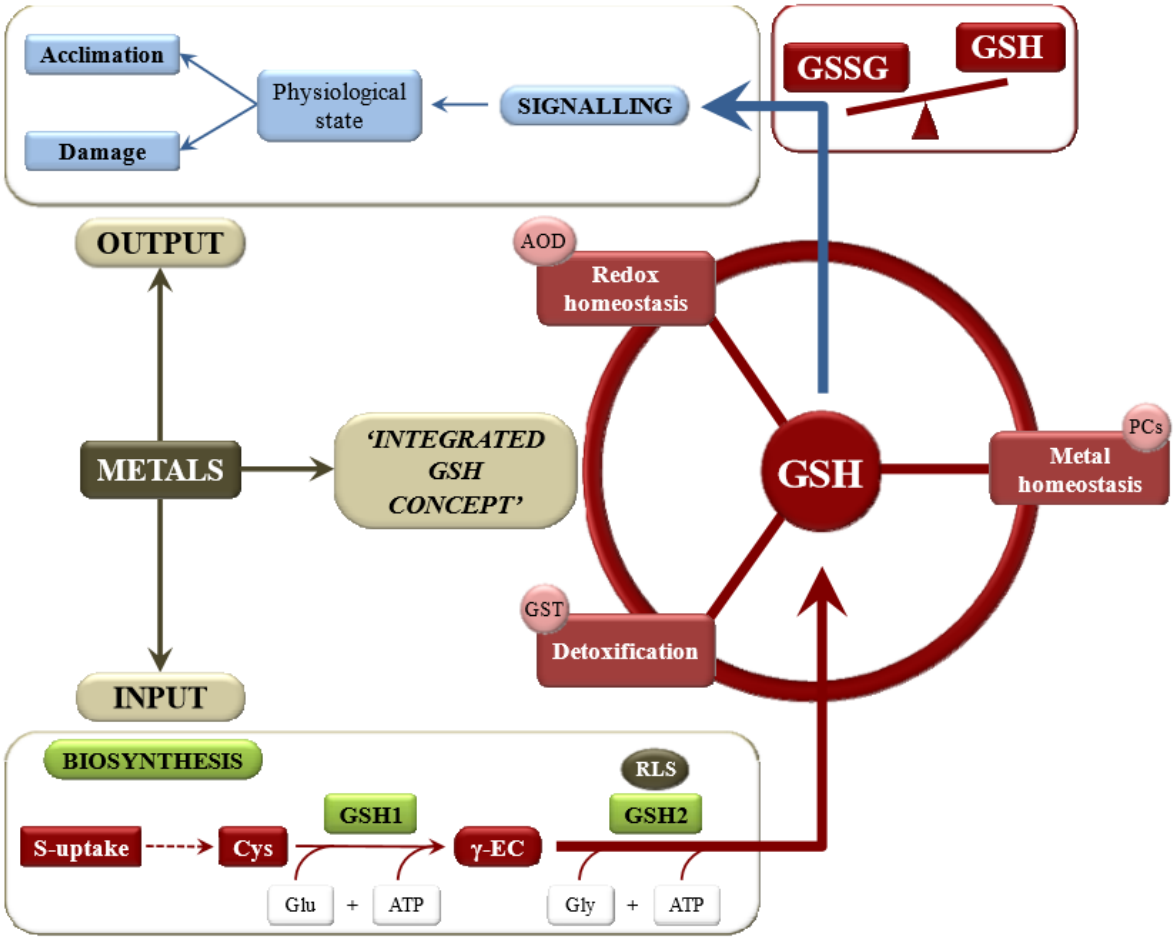

Figure 6: Overview of the most important functions of GSH [6, p. 3162]

2.4.1. Redox homeostasis

Plants need internal redox control systems to maintain a reduced state. This is necessary for the electrochemical gradient that plants use for their electron flow [6] [25]. This includes a continuous flow of electrons to molecular oxygen, resulting in superoxide. Superoxide and H_2O_2 are also formed by various enzymatic reactions and can be used for further production of ROS. The cellular redox state is controlled by antioxidants such as AsA, GSH and tocopherols (see X.X). GSH is the most occurring and thereby important one of these antioxidants [30]. GSH can metabolise H_2O_2 (i) by direct oxidation of GSH , (ii) through the AsA-GSH cycle and (iii) through the redoxin cycle.

2.4.1.1. Direct oxidation of GSH

The thiol group of cysteine in GSH can donate an electron to H_2O_2 or other ROS. Doing so, GSH becomes reactive, but rapidly forms GSSG by reacting with a second reactive GSH. To maintain the cellular redox state GSH needs to be regenerated from GSSG. This is a NADPH dependent regeneration catalysed by glutathione reductase (GR) [6].

2.4.1.2. AsA-GSH cycle

This cycle takes places in the mitochondria, chloroplast, cytosol, peroxisomes and apoplasts. It is the most important antioxidants cycle in plants [9]. It is essential for the antioxidative defence as well as their normal metabolism. For optimal function of this cycle there is need for a highly reduced pool of AsA and GSH. The AsA-GSH cycle, which is presented in Figure 7, consists of the reduction and oxidation of both AsA and GSH. By doing so APX keeps the capacity of reducing H_2O_2 to H_2O [6].

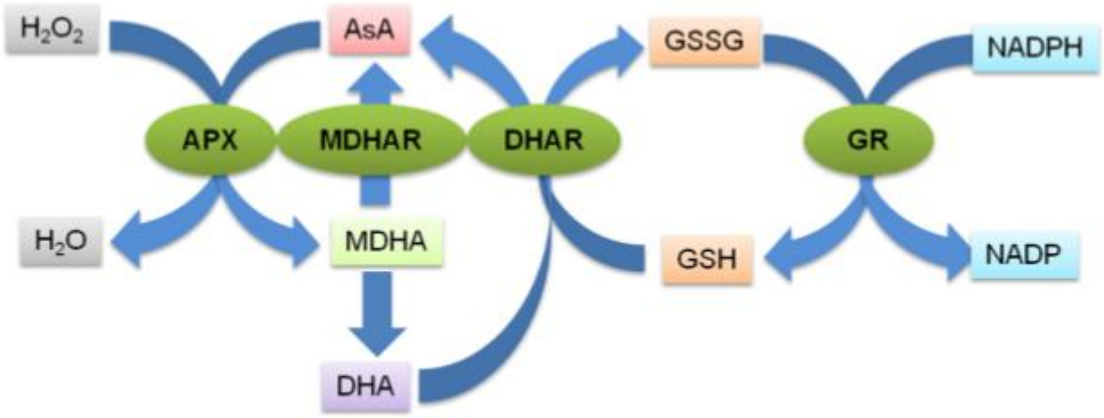

Figure 7: AsA-GSH pathway [25, p. 2]

The detoxification of H_2O_2 by APX catalyses the oxidation of AsA whereby MDHA is generated. The second step is either a non-enzymatic disproportionation DHA or a reduction back to AsA by MDHAR. DHA, in turn, can be reduced to AsA with the help of DHAR using GSH as a reductant. This will lead to the formation of GSSG. Finally, GSH will be regenerated from GSSG in a NADPH dependent reaction catalysed by GR [5] [6] [25] [31].

2.4.1.3. Redoxin cycle

During stress conditions the redoxin cycle protects proteins with a thiol group against irreversible oxidation. The thiol groups of cysteine are an active site for ROS. They can be oxidized and form disulphide bonds. The redoxin cycle, shown in Figure 8, consists of three thiol redox enzymes peroxiredoxin (PRx), glutaredoxin (GRx) ,which are both NADPH dependent, and thioredoxin (TRx). In this cycle H_2O_2 is reduced to H_2O in a reaction that is catalysed by PRx. In turn PRx can be regenerated by thiols from GRx, TRx or GSH [6].

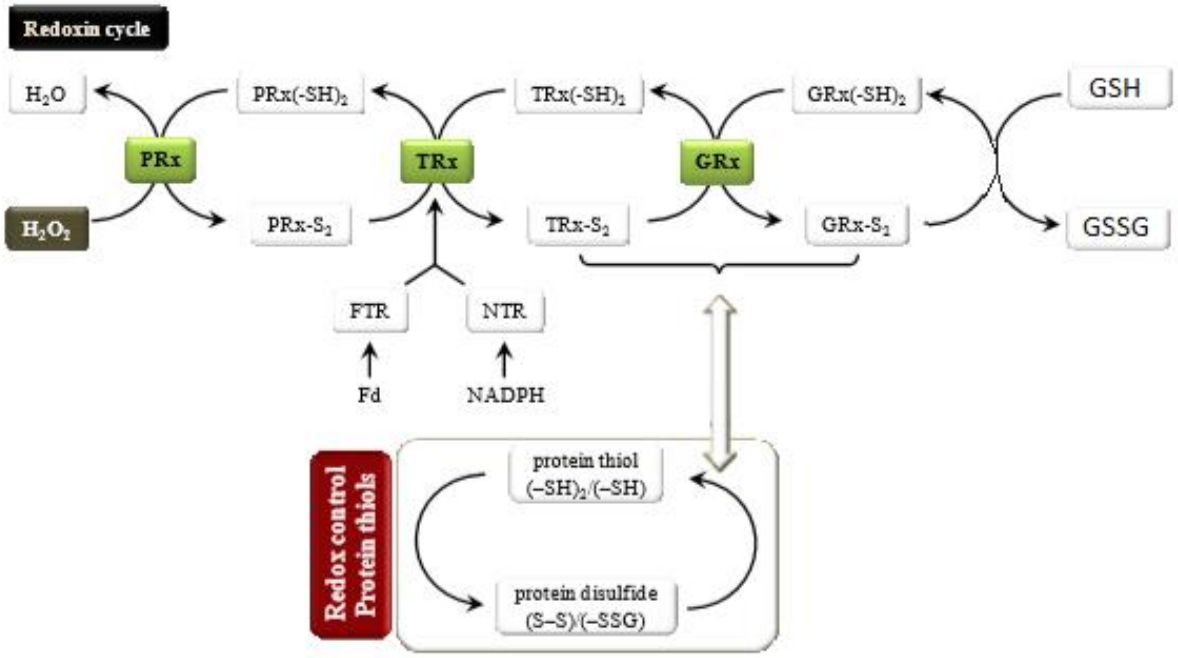

Figure 8: Schematic overview of the redoxin cycle [6, p. 3148]

2.4.2. Metal homeostasis

Metal ions are necessary for the proper functioning of biological systems. But in high concentrations they are toxic. To prevent accumulation of metal ions to toxic levels and to avoid to big fluctuations in their concentrations, the cell appeals for a metal homeostasis network. This network consists of two mechanisms which bind metals. The first mechanism is one with specific chaperones, the second one are chelators that neutralize the excess of free metal ions. Under normal conditions, essential metals are delivered to their cellular sit of action by chaperones. During metal stress the expression of chaperones is also enhanced to prevent damage of free metals. GSH, phytochelatins (PC) and metallothioneins (MT) are some of these chelators which bind and sequester the ligand-metal complexes [6] [32].

Most transition metals can chelate with the thiol group of cysteine by a redox reaction leading to a reduction of the metal and oxidation of cysteine. Since the reduced metals can form hydroxyl radicals through the Fenton reaction, as described before [6] [32], it is important to keep the free cysteine concentration low. This is done by the formation of GSH, where the cysteine amino group is blocked and the cell can contain a high GSH concentration. In addition GSH protect other proteins containing cysteine residues by the formation of complexes and sequestration from the delicate sites of the cell.

PC is a next important chelator and is formed out of GSH catalysed by phytochelatin synthase (PCS). PC occurs as a $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ structure where 'n' can variate between 2 and 11 [33]. Because GSH is the substrate in this biosynthesis, *Arabidopsis* mutants that are GSH deficient are also PC deficient. Under controlled conditions there is no production of PC. The enzyme needed for this biosynthesis is activated by an increased concentration of metals due to their metal-specific binding site [6] [32] [34]. But some metals that induce this biosynthesis can not be used

for chelation [33]. Because PC possess more cysteine residues their metal binding capacity is higher than this of GSH. Depending on the metal that is present in excessive amount the PC's length and concentration can variate [35]. PC form complexes with metals like Ag, Cu, As, Zn, Cd and Se. From all metals that are known to induce the production of PC, the PC-Cd complex is the strongest. In the cytosol this complex is present in a low molecular weight form. By moving to the vacuole PC decreases the cytosolic Cd-concentration [33] [35]. In this way the toxic metal can not interact with cellular compartments that are active in the metabolism [36].

Another cysteine rich protein with the ability to chelate metals are MT. These MT are divided in categories by their cysteine arrangement. Since MT are not produced from GSH, they will not be further discussed in this thesis. [33].

2.4.3. Detoxification

When *Arabidopsis thaliana* is under metal stress, it induces many glutathione transferases (GST). These GSTs have a major role in the detoxification of the cell. GSH can directly detoxify the cell at the same way as PC, by chelation of a metal ion. This chelation between GSH and a metal ion is catalysed by GST [32]. In this way GSH protects other cysteine-rich proteins against the loss of their functions [6] [32]. After this conjugation the conjugates will either be exported by membrane ATP-dependent pumps from the cell or be sequestered into the vacuole [37].

GST is a multifunctional enzyme, besides the detoxification by GSH conjugation it also functions as: GSH transferase, GSH dependent isomerase, GSH dependent peroxidase, GSH dependent oxidoreductase and a non-enzymatic carrier protein [31] [32] [38] [39]. Based on DNA array data it is known that GSTs belong to the most responsive genes in the field of stress and chemical signalling [38].

Arabidopsis has 55 genes for these GSTs of which 52 of them are transcribed and 41 have GSH dependent catalytic activities [38]. All GST contain two characteristic sites, a GSH binding site (G-site) and a hydrophobic site (H-site) [32] [38] [39]. They are divided in to 8 families based on the similarity of their catalytic active site residue. Like illustrated in Figure 9 these families can be split into three groups, a plant specific, a not-plant specific and an animal specific group. The animal specific GSTs will not be further discussed in this thesis.

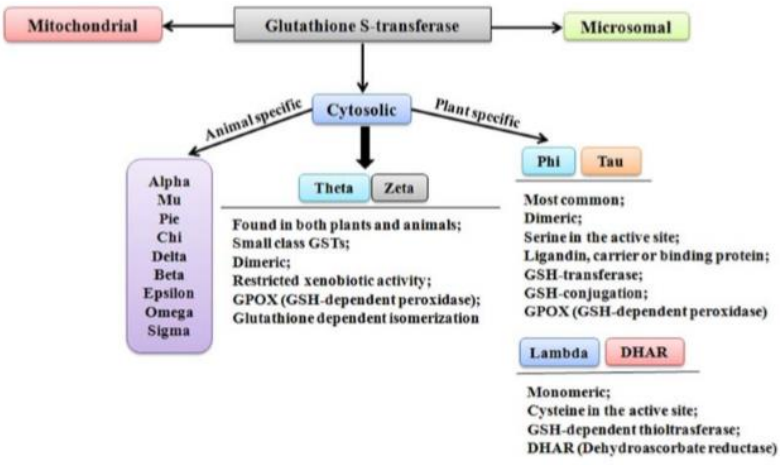

Figure 9: GST classes [37, p. 3]

2.4.3.1. Classes

- Theta

The GST theta class (GSTT) is not plant specific. *Arabidopsis* comprehends three clustered genes for this class, namely GST1, 2 and 3. This class contains a serine residue in their active site. Each of them has a C-terminal peroxisome targeting signal and a high pI (pH 8.9-9.5).

GSTTs in both plant and man show activity toward xenobiotic substrates. They reduce hydroperoxides to alcohols with the use of GSH like shown in Figure 10. This makes them efficient as glutathione-dependent peroxidases (GPOX).

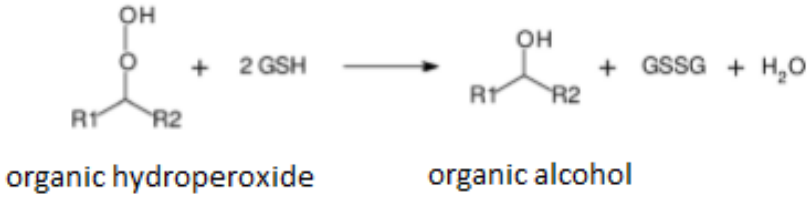

Figure 10: GSTTs involved reaction [38, p. 9]

GSTT will be active as GPOX towards both artificial substrates and endogenous fatty acids oxidation products. Large amounts of hydrogen peroxide are generated by the peroxisomal metabolism. Hydrogen peroxide will cause oxidative damage when it accumulate. It is thought that peroxisomal GSTTs can use these hydrogen peroxides as a substrate [38].

- Zeta

Zeta GSTs (GSTZs) are not plant specific. The *Arabidopsis* contains two genes for the GST zeta class. One of them, GSTZ1, is transcribed at a significant level while the other one is presumed as a pseudogene. Just like GSTT the GSTZ class also has a serine residue in their active site. GSTZ occurs in a dimer structure with a H-site which is more polar than the average GST.

GSTZs can use GSH catalytically rather then as a substrate, which is quite unusual for GSTs. GSTZs catalyse the isomeration of maleylacteoacetate to fumarylacetoacetate, an important step in tyrosine catabolism. In this addition reaction, activated GSH will bind to the cis double bond of maleylacetoacetate, which causes a free rotation. This is followed by the elimination of GSH resulting in the formation of fumarylacetoacetate with a trans double bond. Besides this, GSTZs are also involved in the GSH dependent dehalogenation of dichloroacetic acid to glyoxylic acid. Both reactions are shown in Figure 11 *[38]*.

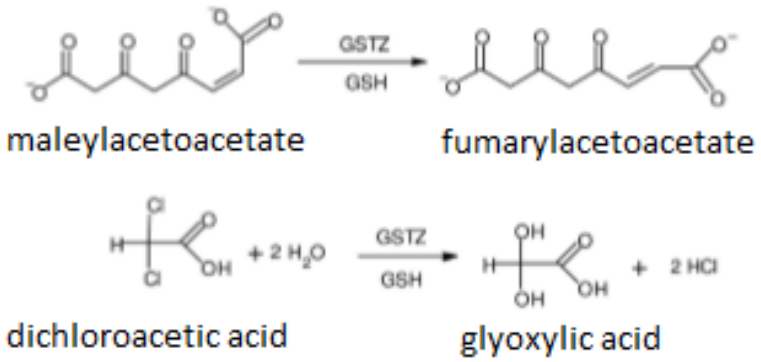

Figure 11: GSTZs involved reactions [38, p. 5]

- TCHQD

Just like the GSTT and GSTZ, the tetrachlorohydroquinone dehalogenases (TCHQDs) class is not plant specific. TCHQD is a rather unusual class of GSTs encoded in only one gene. Their sequence homology with prokaryotic enzymes, gives them the capacity to metabolise chlorinated xenobiotics, what makes this class unusual. But due to the serine residue in their active site it is thought that they are able to catalyse the standard reactions of GSTs. The TCHQD in *Arabidopsis* is about 25 amino acid residues longer in the middle and has a larger c-terminal extension than in other plant sequences [38].

- MAPEG

The last not plant specific class are the membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG). This class is single encoded in *Arabidopsis* and is phylogenetically unrelated to other GST classes. On hydrophobic substrates these enzymes can function as GSH dependent transferases and peroxidases. MAPEG have a low activity to 1-chloor-2,4-dinitrobenzeen (CDNB) [38].

- Dehydroascorbate reductases (DHARs)

The first plant specific class that will be discussed is dehydroascorbate reductases (DHARs). This class has 5 genes in the *Arabidopsis* of which only three are transcribed. The transcribed genes are DHAR1, 2 and 3. DHAR4 is a pseudogene that encodes an inactive enzyme, DHAR5 refers to a region that is untranscribed. This class is present in a monomeric structure and can be found in every subcellular compartment where the AsA-GSH cycle takes place.

In contradiction to the most GSTs which have a serine or tyrosine residue in their active site, DHARs have a cysteine residue. Therefore they can not stabilise the thiolate anion of GSH, and are not able to catalyse GSH conjugations. Instead they form a disulphide group as part of their catalytic mechanism. Like illustrated in Figure 12, DHARs reduce dehydroascorbate (DHA) to AsA in the AsA-GSH cycle. At the same time they oxidize reduced GSH to GSSG. This ability makes them very important during oxidative stress conditions as was mentioned in Figure 7 *[38]*.

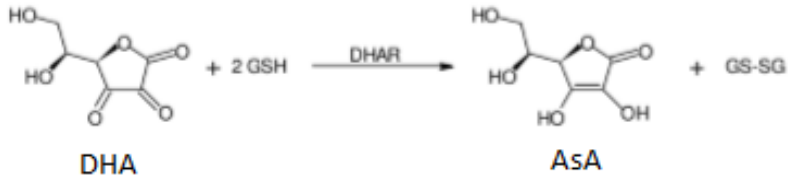

Figure 12: DHARs involved reaction [38, p. 8]

- Lambda

This plant specific class is present in three genes in the *Arabidopsis* plant. There are some differences between these genes. First off all GSTL1 and 3 can be found in the cytosol, while GSTL2 is suggested to be found in both cytosol and chloroplast due to its N-terminal peptide. Secondly GSTL1 and 2 are present in a monomeric structure. The last difference is based on transcription, in contrary to GSTL2 and 3 which have a constitutive expression, GSTL1 is highly stress inducible.

The GST lambda class (GSTLs) resembles to the DHAR class. GSTLs have also a cysteine at their active site, thus they are bonded to the same limitations as DHARs. It is generally assumed that the GSH-dependent reduction of small molecules is catalysed by GSTLs, as shown in Figure 13, however their true substrate remains unknown *[38]*.

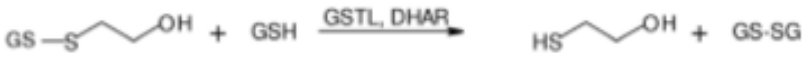

Figure 13: GSTLs involved reaction [38, p. 8]

- Phi

The GST phi class (GSTFs) is plant-specific. The observed knock-out lines for GSTFS showed a lack of phenotype which suggests that the individual enzymes are non-essential for the normal growth. Nevertheless small changes were observed in the metabolite level that can be associated to a decreased tolerance for oxidative stress.

Arabidopsis contains 13 GSTFs that are numbered from GSTF2 till 14. The numbering starts from two because GSTF1 is disregarded for its origin. In the reference genome of *Arabidopsis* the gene for GSTF1 is not present. However similar sequence were found in enzymes of fungi and amoebae. This could suggest that the sequence didn't originally occur in *Arabidopsis thaliana,* but that is was transferred from a co-cultivated pathogen to the plant. The gene for GSTF4, 5, 6, 7 are found at chromosome 1 as a tight cluster. GSTF9 and 10 can be found on chromosome 2 as a tandem array. The other GSTFs are occupant as singletons.

The transcript of GSTF2 is strongly inducible by oxidative stress, it is also the most studied GSTF for *Arabidopsis* in the area of location, crystal structure, biochemical properties and interaction with flavonoids. Knock out experiments of GSTF2 resulted in alterations in stress resistance, flowering time and shoot regeneration. GSTF8 has a N-terminal signal peptide that targets the chloroplast. But the majority of GSTF8 is spliced in a way that the signal peptide is removed and

the GSTF remains in the cytosol. Like shown in Figure 14 it catalyses the conjugation between GSH and oxylipin (15Z)-12-oxophyto-10,15-dienoic acid (OPDA) [38].

Figure 14: GSTFs involved reaction [38, p. 10]

- Tau

The last plant specific class is the Tau GST (GSTUs). This class contains 28 genes and is thereby the largest class of GSTs. All of these genes except four can be found in clusters. The Tau class is divided in three clades. The first one contains GSTU1 to 10 and are mainly root expressed, but GSTU3 and 4 can widely be distributed. GSTU1 to 7 form the biggest cluster and can be found on chromosome 2. It is assumed that whole this clade is present in the cytosol. GSTU5 and 7 are expressed as active enzymes. The difference between them is that GSTU5 auxin responsive, which will be explained later, and GSTU7 is stress responsive. The second clade consists of GSTU 11 till 18. The third clade comprehends the remaining ones, GSTU19 to 28. The GSTU19 is the best studied GST of its class [38]. Its expression is enhanced by the exposure of *Arabidopsis* to herbicide safeners. The protein expressed by GSTU19 is an abundant in *Arabidopsis*, it has an important contribution in the conjugating activity of 1-chloro-2,4-dinitrobenzene (CDNB) [38]. GSTU19 is more abundant in the roots than in shoots. *Arabidopsis* shows a strongly induced transcription of GSTU24 when exposed to TNT or other xenobiotics. Nevertheless types without this gene have the similar response as the wild type.

Almost all the GSTUs of *Arabidopsis* have the capacity to selectively bound to fatty acid derivatives. This specific binding suggest a physiological role for the GSTUs, probably in intercellular transport. Some GSTUs are auxin-responsive genes, this means that, for unknown reason, they are abundant in growing tissue [38].

2.4.4. Role of GSH under metal stress

There have been many studies about the role of GSH in *Arabidopsis thaliana* under different metal stresses. Plants adapt their GSH level in response to this stress. One of these metal stresses is cadmium. Cadmium has a very high affinity for thiol groups which makes GSH and PC very important components. First off all Cadmium exposure increases the transcription of genes for GSH synthesis and activates PC production. The chelating capacity is increased by this PC production. PC will help in the detoxification of Cadmium by chelating and sequestration into the vacuole [7] [29].

Besides this function GSH has also his role in the GSH/GSSG ratio and AsA-GSH cycle. In attempt to keep the cellular redox state the activity of GR is enhanced. Despite the increased GSH production and GR activity, the amount of reduced glutathione is decreased due to the great production of PC [7] [29].

High concentrations of Zn will enhance the production of GSH and cause a decrease in GR activity in both the leaves and roots. As result of this increased synthesis, the GSSG/GSH ratio will decrease and more GSH will be available for a function in the antioxidative defence mechanism. In the roots an activation of PCS will lead to a reduction of free GSH and thus an increase in the GSSG/GSH ratio. The lower amount of free GSH will cause a disturbance in the AsA-GSH cycle [40].

Under uranium stress, an increased activity of GR and APX in the roots have been reported which may indicate that the AsA-GSH cycle has an important role in the H_2O_2 scavenging under uranium stress. By doing this the roots try to ensure the reduction of DHA to AsA. In the leaves of the *Arabidopsis* plant there was no increase in GR and APX production when exposed to uranium. Instead there was an increase in AsA and GSH. But this increase was only transient. At exposure to a low uranium concentration (till 25µM) the leaves could regulate the oxidative stress by increasing the antioxidative defence mechanisms. But at exposure to a higher uranium concentration this defence was not sufficient and the mechanisms collapsed [3] [4].

For uranium induced stress in *Arabidopsis thaliana* studies have been executed in areas as GSH concentration and PC production. In contrast to most metals, uranium stress is not able to activate PCS. Thereby there will be no PC produced to chelate and sequester uranium (Horemans et al., unpublished results). Other functions of GSH during uranium exposure remain unstudied.

2.5. Conclusion

Uranium is able to induce oxidative stress, a condition at which there is a disturbance between the production and elimination of ROS. These ROS can damage DNA, proteins and lipids. The antioxidative defence mechanism is one of three ways a plant can defence itself against the accumulation (and damage) of these ROS. It consist of both enzymatic and metabolic antioxidants, GSH is one of these metabolic antioxidants.

GSH is a tripeptide that contains cysteine, glutamate and glycine. It can be used as substrate for the synthesis of PC and has functions in the redox homeostasis, metal homeostasis and detoxification.

A lot of research is done about oxidative stress in *Arabidopsis thaliana* during metal exposure. But when it comes to the role of GSH during uranium exposure, there has only been research to the GSH concentration and the possibility of PC synthesis (which was not present). This master's thesis will study the remaining functions of GSH during these conditions.

Chapter 3

Materials and methods

This chapter describes the materials and methods used to answer our research question "what is the role of glutathione in the oxidative stress response of *Arabidopsis thaliana* after exposure to uranium?", starting with the cultivation of *Arabidopsis thaliana* plants. Two types of *Arabidopsis thaliana* were cultivated, a wild-type and a *cad2-1* mutant. Like mentioned before the *cad2-1* mutant has a defect in gamma-glutamylcysteine synthetase. This is the first enzyme in the biosynthesis of GSH. The defect in this enzyme causes that the mutant has only 30% of the wild type GSH level. The analyses that will be described are: gene expression, uranium uptake, GSH concentration, enzymatic activity and lipid peroxidation.

3.1. Cultivation of *Arabidopsis thaliana* plants

This project started with the cultivation of *Arabidopsis thaliana* in a hydroponic set-up. The advantage of this system is that there is a high degree of control (e.g. nutrients supplied). In addition, it allows easy harvesting of both roots and shoots. The set-up of the system is shown in Figure 15.

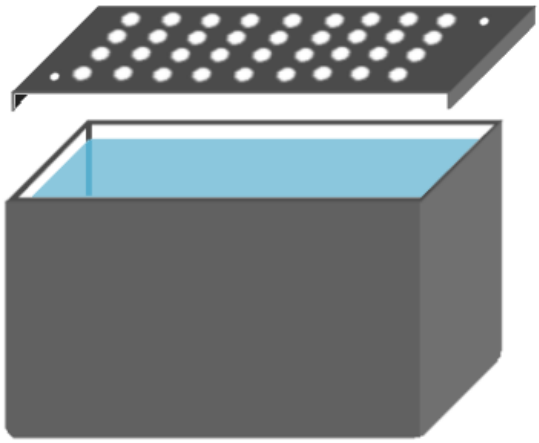

Figure 15: Set-up of the hydroponic culture system [10, p. 3]

The tray on this figure (bottom part) was filled with distilled water or nutrient solution. The upper part shows the lid with multiple holes. Each of the holes was filled with a holder for a plant (see below).

To prepare the plant holders, 1.5 mL microtubes of which the bottom part was cut off were filled with a low phosphate (LP) Hoagland solution (Attachment A) containing 4.5 g/L agar N°2 (Figure

16). The LP solution was used since uranium can form complexes with phosphates, leading to a reduced bioavailability. When the agar solution was solidified, the tubes were placed in the lid, containing 36 plants per lid. The lids were placed in the trays, which were filled with distilled water.

Figure 16: Schematic representation of preparing the plant holders [10, p. 4]

Before sowing *Arabidopsis thaliana* plants, seeds were sterilised in 0.1% NaClO during 1 min. followed by rinsing with sterile distilled water (4 x 5 min). Hereafter, seeds were placed on moist filter paper at 4°C during three days to ensure homogeneous germination. Two seeds were placed in each agar bed and the whole culture was placed in a climate chamber (Microclima 1000E, Snijders Scientific B.V) with 14 h of light (photosynthetic photon flux density of 150-170 µmol $s^{-1}m^{-2}$), day/night temperature of 22 °C/18 °C and a constant humidity of 65% as is shown in Table 3.

Table 3: Climate chamber program [10, p. 5]

Process step				**ChambE**	**HmdtyE**	**illumE**	
ChambS	**HmdtyS**	**illumS**	**Time**				
1. All but 24				22 °C	65 %	65 %	
22 °C	65 %	65 %	05:30				*15:00 → 20:30*
2. All but 24				18 °C	65 %	10 %	
22 °C	65 %	65 %	00:30				*20:30 → 21:00*
3. Dark				18 °C	65 %	10 %	
18 °C	65 %	10 %	09:30				*21:00 → 06:30*
4. All but 24				22 °C	65 %	65 %	
18 °C	65 %	10 %	00:30				*06:30 → 07:00*
5. All but 24				22 °C	65 %	65 %	
22 °C	65 %	65 %	08:00				*07:00 → 15:00*
6. Repeat				0.0	0.0	0.0	
1.0	0.0	99.0	00:00				

After one week in the climate chamber the agar beds were “thinned out” so that only one plant per bed remains. In addition, the distilled water in the trays was replaced with a high phosphate (HP) Hoagland solution (Attachment A), which was replaced twice a week.

After a growing period of 18 days, the *Arabidopsis thaliana* plants were exposed to a zero and 25 µM uranium concentration. For the contamination, uranium ($UO_2(NO_3)_2.6H_2O$ (SPI chemicals, USA) was added to empty trays. Afterwards, 1.35 L HL LP was added and pH was adjusted to pH 5.5 (i.e. pH of Hoagland LP without uranium). After 3 days of exposure, roots and shoots were harvested. During harvest, their weight is recorded once after which the samples were frozen in liquid nitrogen and stored at -80 °C for further analyses. In addition, samples were taken for uranium determination.

3.2. Gene expression

Different genes of the antioxidative defence mechanism were measured to analyse the influence of U exposure. Besides the reference genes, the genes of interest include PCS1, MT2a, GSH1 and 2, different GSTs of the tau class (U1, U2, U4, U7, U19, U25), miR408, SAP12, OX1-5 and CSD1/2. This analysis provides an indication of the production and usage of GSH and the amount of oxidative stress. To analyse the gene expression of root and shoot samples after exposure to uranium, an RNA extraction was performed on the samples, followed by cDNA synthesis and analysis with Real Time qPCR.

3.2.1. Primer development

To be able to analyse the gene expression, there was need for specific primers. Some primers were already available at SCK CEN, but for other genes primers needed to be developed. These genes are shown in Table 4.

Table 4: Genes for primer development

Gene name	AT number
GSTU1	AT2G29490
GSTU2	AT2G29480
GSTU4	AT2G29460
GSTU5	AT2G29450
GSTU7	AT2G29420
GSTU19	AT1G78380
GSTU24	AT1G17170
GSTU25	AT1G17180
miR408	At2g47015
SAP12	At3g28210

To generate the primers, the sequence of each gene was looked up on www.ncbi.nlm.nih.gov using the AT number. Next the website www.ncbi.nlm.nih.gov/tools/primer-blast generated possible primers based on the sequence of the gene. Two settings were adapted on this site. First of all the maximum product size was set to 250 bp. Secondly the organism was set on *Arabidopsis thaliana*. These results were tested on www.eurofinsgenomics.eu/en/ecom/tools/oligo-analysis. The results that passed this test could be further tested as functional primers.

3.2.2. RNA extraction

To extract the RNA, the samples were disrupted by using a mixer mill/cryo mill (Retsch MM 400). Approximately ten Zirconia (Biospec) beads (2.0 mm) were added to each sample after which they were placed in pre-cooled Mixer Mill adaptors and grounded for 3.5 minutes at 30 Hz. After the samples are grounded, RNA was extracted by using the Qiagen RNeasy plant mini kit, following the manufacturer's protocol (as shown in Figure 17) [11]. Once the RNA was extracted, the quantity and purity was checked with the Nanodrop ND-1000 (Thermo Fisher Scientific).

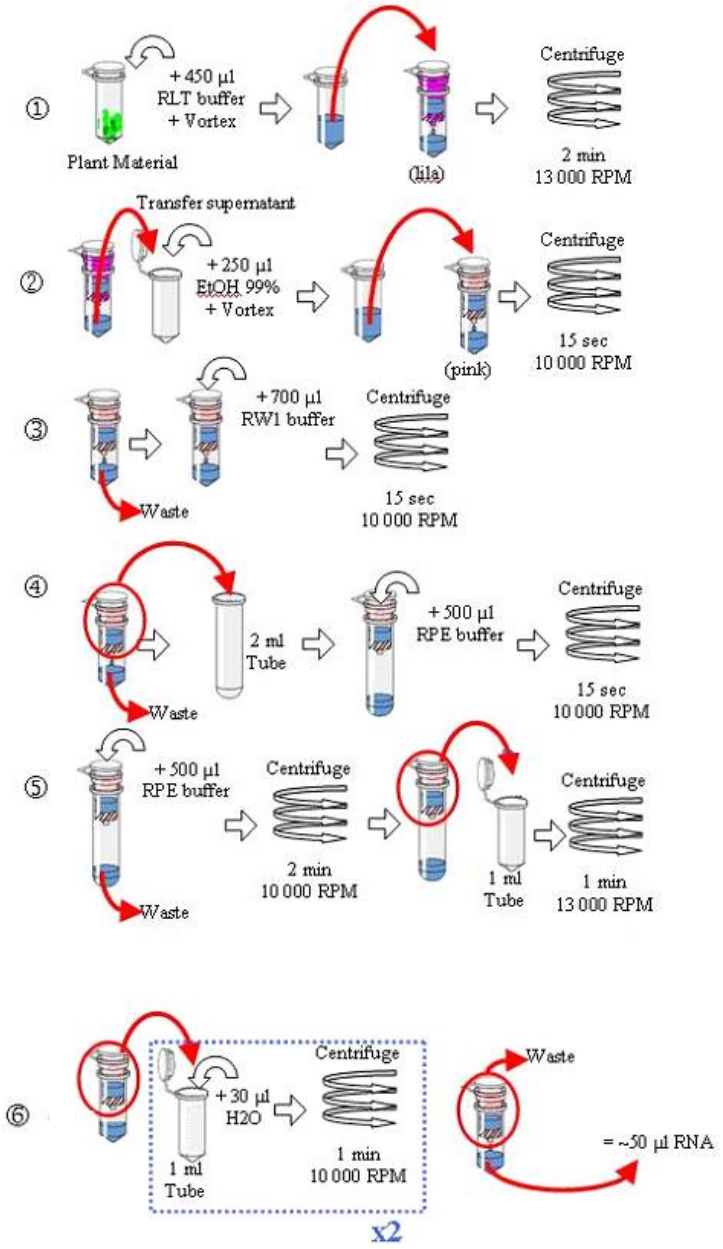

Figure 17: Schematic overview of the Qiagen RNeasy plant mini kit [11, p. 4]

3.2.3. cDNA synthesis

Before synthesis of cDNA, genomic DNA components that were possibly extracted together with the RNA were removed. This was done using the Turbo DNA-free Kit (Invitrogen) as shown in Figure 18.

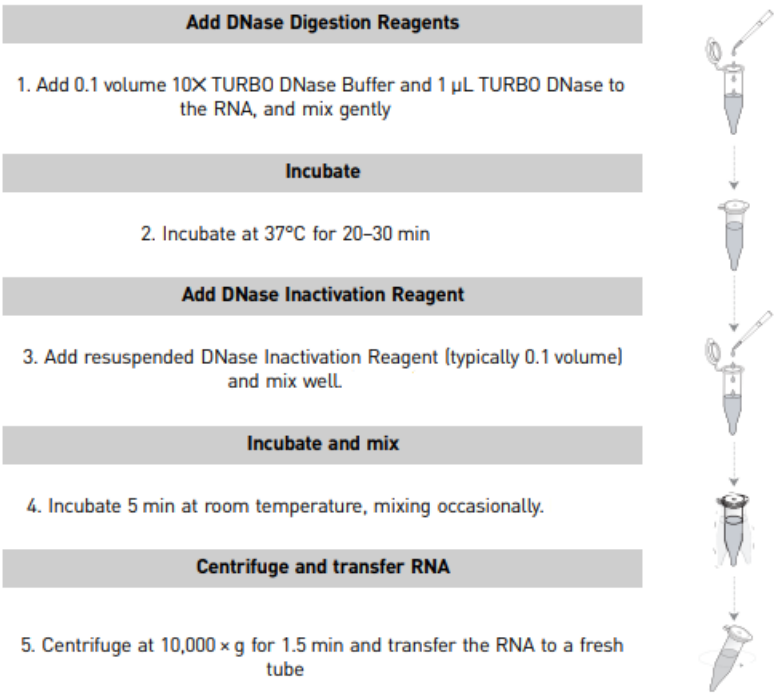

Figure 18: Turbo DNA free Kit protocol [41, p. 4]

After removing the gDNA, cDNA was synthesized according to the standard protocol using the PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa) (Table 5) and the Peqstar Gradient thermocycler (VWR) using the following cycling conditions: 15 min at 37 °C (reverse transcription), 5 s at 84 °C (inactivation of reverse transcriptase) and storage at 4 °C. cDNA samples were diluted with RNase and DNase free water (1:10) for real-time qPCR and were stored at -20 °C.

Table 5: Revers transcription mixture [12, p. 2]

Component	**Volume/reaction**
Master mix	
Buffer	4 µl
RT enzyme Mix I	1 µl
Oligo Dt primers	1 µl
Random 6mers	1 µl
Reaction mixture	
Master mix	7 µl
RNA sample (1 µg/13 µl)	13 µl

For testing the efficiencies of the new developed primers (see 3.2.1), a mixed cDNA sample was prepared by transferring 1 µL cDNA per sample into a single 1.5 ml tube. This sample was used to test the efficiency and specificity of the primers (3.2.4.1).

3.2.4. Real-time qPCR

All cDNA samples and primers were flicked and spinned before making the master mix and stored on ice during usage. Primers were dissolved to a concentration of 100 µM in RNase free water. A working stock of the primers was made by diluting the primers with RNase-free water (1/10 in 500 µl). The amount of reagents needed for each reaction is given in Table 6.

Table 6: Master mix [42, p. 4]

Fast SYBR Green PCR Master Mix	5 µl
Primer Forward (10 µM – 0.3 µM)	0.3 µl
Primer Reverse (10 µM – 0.3 µM)	0.3 µl
RNase free water	1.9 µl

3.2.4.1. Primer efficiencies

The amplification efficiency of each primer was tested with the mixed cDNA sample by diluting it ¼ for 5 times, resulting in following concentration ratio's: 1, 1/4, 1/16, 1/64, 1/256 and 1/1024. From each sample of the dilution series, 2.5 µl was added to 7.5 µL of the master mix. After centrifugation (1 min, 2000 rpm), the real-time qPCR was executed on the ABI Prism 7500 with the program shown in Table 7. Two NTC's were added for each primer, where the sample was replaced with 2.5 µl RNase free water. After the amplification, a melting curve was generated to check the specificity of the primer.

Table 7: Program setting RT-qPCR [42, p. 3]

15 min	95 °C
40 cycles: • 15 s • 30 s • 30 s	 • 94 °C • 50-60 °C (5-8 °C below T_m primers) • 72 °C
Meltingcurve	

After amplification, primer efficiencies were calculated in Microsoft Excel. Treshold values (Ct) were plotted relative to the log of the concentrations of the dilution series. By using the slope of the function of the trendline that was generated, the primer efficiency was calculated using following formula: $(10^{(-1/slope)})-1$. Primers with an efficiency between 90-100% were accepted for further use.

3.2.4.2. Genes of interest

The master mix was mixed and each PCR tube was filled with 7.5 µl master mix. Next 2.5 µl cDNA (< 250 ng) of each sample was added to the plate and the plate was centrifuged for 1 min at 2000 rpm. The real-time qPCR was executed on the ABI Prism 7500 with the program that is shown in Table 7. A no template control (RNase free water instead of cDNA) was used to detect possible contamination of the master mix.

The gene expression was relatively quantified to different reference genes. The stability of the reference genes was tested using Graynorm [42] [43]. Based on the reference genes, a normalisation factor was generated, which was used to normalise the expression values of the samples. Gene expression of the genes of interest was plotted relative to the control conditions by using the $2^{-\Delta Ct}$ method.

3.3. Uranium uptake

The U uptake was analysed to track down if GSH has an influence/function in the uptake and in the root to shoot translocation. The uranium concentration in root and shoots samples were measured using ICP-MS. To remove U from the outside of the roots, root samples were washed twice with 10 mM $Pb(NO_3)_2$ during 10 min. and once with distilled water (10 min.). Root and shoot samples were oven-died during one week at 70 °C. Next, the dried sample were weighted in a 20 ml glass vial and calcinated in a muffle furnace at 550°C. After the samples were cooled down to room temperature, they were dissolved in 1 ml 1 M HCl, heated on the sand bath and diluted with 9 ml demineralized water. A blank sample (1 ml HCl (1 M) and 9 mL demineralized water) was also prepared. Samples were filtered with a 0.45 µm filter (Acrosdisc® Syrige filter) and measured with ICP-MS. The uranium concentration in the samples was calculated with following formula: [16]

$$\text{Concentration U (µg/g)} = \frac{(C-blank)*B}{1000*A}$$

In this formula:
A = netto weight (g) from the oven dried sample
B = netto weight (g) solution +dissolved ash
C = U concentration (µg/l) in the filtrate
1000 = conversion from kg to g (µg/l -> µg/kg, mass density of water)

3.4. GSH concentration

The amount of GSH was analysed to discover the importance of GSH in the antioxidative defence mechanism and to follow the influence of U exposure on the GSH synthesis. This measurement was also a confirmation of the reduced GSH level in the *cad2-1* mutant. The GSH concentration in roots and shoots of wild-type and *cad2-1* plants were measured spectrophotometrically using a multiscan spectrum variable wavelength plate reader (Thermo Labsystems, Cergy Pontoinse, France) and a Corning 96-well UV transparent plate. First, an extraction was done by grinding the frozen samples in liquid nitrogen. Afterwards, 1 ml +0.2 M HCl were added and samples were vortexed. The extract was centrifuged for 4 min at 16,000 g and 0.5 ml supernatant was neutralized with 50 µl NaH_2PO_4 (0.2 M, pH 5.6) and NaOH (0.2 M) until the final pH was between 5 and 6.

The measurement of the GSH concentration started with measuring the total glutathione concentration (GSH + GSSG). This could be done with a GR-dependent reduction of 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB) which is shown in Figure 19. Therefore, 10 µl neutralized extracted, 10 µl GR (20 U ml^{-1}), 0.1 ml NaH_2PO_4 (0.2 mM, pH 7.5), 10 µl NADPH (10 mM), 10 µl DTNB (12 mM), 10 µl EDTA (10 mM) and 60 µl water were added to the wells. The reaction was monitored at 412nm for 5 min. For the measurement of GSSG, 0?2 ml neutralized extract was first incubated for 30 min with 1 µl 2-vinylpyridine (VPD). This step will complex GSH (masking reagent in Figure 19). The remaining VPD was then removed by centrifuge. Thereafter this measurement followed the same principle as described above. Afterwards the concentration GSH was calculated by the total amount of glutathione minus the amount of GSSG [14].

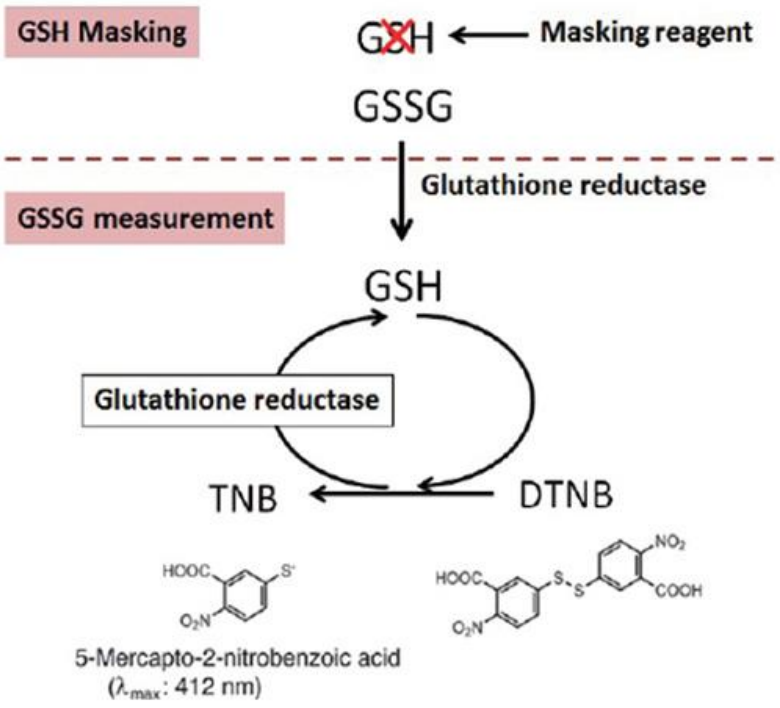

Figure 19: GR-dependent reduction of DTNB [44]

3.5. Enzymatic activity

The activity of different enzymes related to the oxidative defence mechanisms of *Arabidopsis thaliana* plants were measured. These enzymes are catalase (CAT), guaiacol peroxidase (GPOD), syringaldazine peroxidase (SPOD), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione transferase (GST). This analysis reveals the reaction of the antioxidative defence mechanism and could give an indication if the absence of GSH induces a stronger antioxidative defence response or not. In addition, the measurement of GST activity was optimised. This measurement could give an indication of the importance of GST in the detoxification mechanisms under U stress.

To analyse the enzymatic activity, an extraction was done by grinding the samples in 1.5 mL extraction buffer (12,114 g TRIS, 0.3722 g EDTA and 0.1542 g DTT per liter water, pH 7.8) supplied with a spatula tip of polyvinylpyrrolidone (PVP). The extract was centrifuged for 10 min at 13000 rpm.

For the analysis of the enzymes, each one had his own specific solutions, which will be summarised below. Each of these protocols were executed with a plate reader method using a 96-well plate. Unless it is described otherwise the enzyme capacity can be calculated with following formula: [16]

$$\frac{U}{g} = \frac{\Delta A * V_c * V_b}{\Delta t * \varepsilon * d * m * V_e}$$

In this formula:

ε = millimolar extinction coefficient ($\frac{1}{mM*cm}$)

d = light path (0.56 cm)

V_b = volume of extraction buffer (1.5 ml)

V_c = reaction volume of the well (0.2 ml)

V_e = volume of the extract in the well (ml)

m = fresh weight of extracted sample (g)

- **CAT**

The CAT enzyme catalyse the reaction from two hydrogen peroxides to two hydrogen and one oxygen, like shown below [16].

$$2H_2O_2 \rightarrow 2\ H_2O + O_2$$

This reaction was visual due to the formation of air bubbles. The amount of hydrogen peroxides that was removed by CAT was measured at 240 nm. The solutions that needed to be prepared are shown in Table 8.

Table 8: Solutions for CAT measurement [16, p. 2]

Phosphate buffer (pH 7,0)	- 3.403 g KH_2PO_4 - 250 ml H_2O - pH adjustment with KOH
CAT H_2O_2 buffer	- 100 µl H_2O_2 (35%) - 20 ml phosphate buffer (pH 7.0)

Once the buffers were made, the wells were filled with 10 µl of sample extract and 190 µl of the CAT H_2O_2 buffer. The activity was calculated with V_e = 0.010 ml and ε_{H2O2} = 40 $mM^{-1}cm^{-1}$

- **GPX**

GPX is a group of enzymes that uses guaiacol as an electron donor as shown below. [16]

$$4\ \text{Guaiacol} + 2\ H_2O_2 \rightarrow 8\ H_2O + \text{guaiacol}_{ox}$$

In this analyses the oxidized form of guaiacol (tetraguaiacol) was measured at 436 nm. For The GPX enzyme, four solutions needed to be made which are listed in Table 9.

Table 9: Solutions for GPX measurement [16, p. 3]

Phosphate buffer (0,1M; pH 7,0)	- 3.403 g KH_2PO_4 - 250 ml H_2O - pH adjustment with KOH
H_2O_2 (8mM)	- 100 µl H_2O_2 (35%) - 6 ml H_2O
Guaiacol (90mM)	- 50 µl stock solution - 5 ml H_2O
Guajacol-H_2O_2 master mix	- 4 ml each (1:1 ratio)

The wells were filled with 140 µl phosphate buffer, 10 µl sample extract and 50 µl Guaiacol master mix. The enzymatic activity was calculated with $\varepsilon_{oxidized\ guaiacol} = 25.5\ mM^{-1}cm^{-1}$.

- **SPX**

Just like GPX this group uses a common electron donor, namely syringaldazine, like shown in the reaction below [16].

$$Syringaldazine + H_2O_2 \rightarrow 2\ H_2O + syringaldazine_{ox}$$

The oxidized form of the electron donor was measured at 530 nm. For this analysis three solutions had to be prepared as listed in Table 10.

Table 10: Solutions for SPX measurements [16, p. 4]

TRIS buffer (0.1M; ph 7,5)	- 3.0285 g TRIS - 250 ml H_2O - pH adjustment with HCl (5 M)
Syringaldazine (SAZ)	- 3.6 mg SAZ - 1 ml methanol - 2 ml dioxane
H_2O_2 (10 mM)	- 50 µl H_2O_2 - 5 ml H_2O

Once the solutions were made, the wells were filled with: 155 µl TRIS buffer, 20 µl H_2O_2, 20 µl sample extract and 5 µl SAZ. The enzymatic activity of SPX was calculated with $V_e = 0.020$ ml and $\varepsilon_{oxidized\ syringaldazine} = 11.6\ mM^{-1}cm^{-1}$.

- **APX**

This group of enzymes all use AsA as electron donor as described in the following reaction [16]:

$$Ascorbaat + H_2O_2 \rightarrow 2\ H_2O + ascorbaat_{ox}$$

In this analyses the amount of AsA that reacts was measured at 298 nm. The solutions that needed to be made are listed in Table 11.

Table 11: Solutions for APX measurement [16, p. 5]

HEPES-EDTA (0.1 M; 1 mM; pH 7.0)	- 5.958 g HEPES - 0.093 g Na_2EDTA - 250 ml H_2O - pH adjustment with KOH
Na AsA (30 mM)	- 0.297 g Na AsA - 50 ml H_2O
H_2O_2 (20 mM)	- 200 µl H_2O_2 - 10 ml H_2O
AsA-H_2O_2 master mix	- 740 µl H_2O_2 - 2 ml Na AsA

Next the wells were filled with 155 µl HEPES-EDTA, 18 µl sample extract and 27 µl AsA-H_2O_2 master mix. The activity of APX was calculated with V_e = 0.018 ml and ε_{AsA} = 11.6 $mM^{-1}cm^{-1}$.

- **SOD**

The enzyme SOD catalyses the reaction from superoxide to oxygen as shown below [16].

$$2O_2^{\circ-} + 2H^+ \rightarrow O_2 + H_2O_2$$

This analyses was different from the others, the measurement of SOD relied on an inhibition reaction. First the blank samples were measured (no addition of sample). In these samples the reactions of Figure 20 takes place [16].

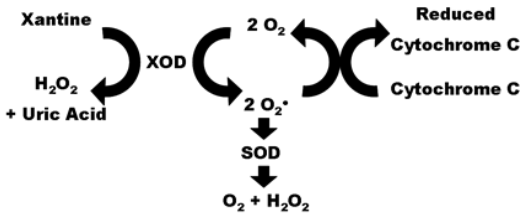

Figure 20: schematic overview of the reactions during SOD measurement [16, p. 7]

The first reaction was catalysed by xanthine oxidase (XOD). In this analyses the amount of reduced Cyt C was measured at 550 nm. By adding sample extract, in which SOD is present, the second reaction was inhibited due the oxidation of superoxide to oxygen. For this measurement six solutions, which are shown in Table 12, needed to be prepared [16].

Table 12: Solutions for SOD measurement [16, p. 6]

KH_2PO_4 buffer (50 mM; pH 7.8)	- 3.402 g KH_2PO_4 - 500 ml H_2O - pH adjustment with KOH
Xanthine (0.5 mM; pH 7.8)	- 1.9 mg xanthine - 25 ml phosphate buffer (boil to dissolve)
XOD (pH 7.8)	- 25 µl XOD - 500 µl phosphate buffer
Na_2EDTA (1 mM; pH 7.8)	- 37.6 mg Na_2EDTA - 100 ml phosphate buffer
Cytochrome C (0.1 mM; pH 7.8)	- 18.576 mg cytochrome C - 15 ml phosphate buffer
SOD master mix	- 3 ml EDTA - 3 ml xanthine - 3 ml cytochrome C

First the four blank samples were measured, the other wells were filled as followed: 5 µl XOD, 5 µl sample extract (not for blank samples), 130 µl phosphate buffer (135 µl for blank samples) and 60 µl SOD master mix. The activity was calculated with the following formulas [16] with V_e = 0.005 ml.

$$\%inhibition = \frac{(\frac{\Delta A}{\Delta t})_{ref} - (\frac{\Delta A}{\Delta t})_{sample}}{(\frac{\Delta A}{\Delta t})_{ref}} * 100\%$$

$$z(U) = \frac{\%\ inhibition}{50\%}$$

$$\frac{U}{g} = z(U) * \frac{V_b}{V_e * m}$$

- **GR**

Glutathione reductase catalyses the NADPH dependent reduction from GSSG to GSH as shown below [16].

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

This analyse measures the amount of GSSG that was reduced at 340 nm. For the GR measurement four solutions needed to be prepared like shown in Table 13.

Table 13: Solutions for GR measurement [16, p. 8]

TRIS-EDTA buffer (0.1 M; 1 mM; pH 8)	- 3.059 g TRIS - 93 mg Na_2EDTA - 250 ml H_2O - pH adjustment with HCl
GSSG (82 mM)	- 25 mg GSSG - 500 µl H_2O
NADPH (6 mM)	- 2.5 mg NADPH - 500 µl H_2O
GR master mix	- 300 µl GSSG - 300 µl NADPH

Once the solutions were made the wells were filled with 165 µl TRIS-EDTA buffer, 7 µl GR master mix and 28 µl sample extract. The enzymatic activity of GR was calculated with V_e = 0.028 ml and ε_{GSSG} = 6.22 $mM^{-1}cm^{-1}$.

- **GST**

In this method GST catalyses the reaction that is illustrated in Figure 21.

Figure 21: Reaction from CDNB with GSH [45]

In this method GSH was measured at 340 nm .The measurement of GST has up to now not be performed in the BIS lab. Therefore, the measurement of this enzyme has to be optimised during this thesis. The protocol used in Kehinde Olajide Erinle et al. *[46]*. was used as starting point. Table 14 lists all the reagents for a 3ml reaction mixture needed for the GST measurement.

Table 14: Reaction mixture for GST measurement [46]

Reaction mixture		
	Volume (ml)	**Concentration in reaction mixture (mM)**
Potassium phosphate buffer (300 mM; pH 6,5)	1	100
GSH (3.16 mM)	0,95	1
1-chloro-2,4-dinitro-benzene (3.16 mM) ➔ 10 mM CDNB + 50% aceton	0,95	1

Since a 200 µl reaction was used, each well contained 7 µl sample extract and 193 µl reaction mixture. The GST activity was calculated with V_e = 0.007 ml and ε_{GSH} = 9.6 $mM^{-1}cm^{-1}$ [46].

3.6. Lipid peroxidation

The lipid peroxidation can be used as an indicator for the membrane damage caused by an increased production of ROS. Products of membrane deterioration such as Malonaldehyde, were measured by a 2-thiobarnituric acid (TBA) method. The method started with the preparation of a 0.1% trichloroacetic acid (TCA) solution, which was made by dissolving 0.1 g TCA in 100 ml demineralized water. The next solution was a 0.5% TBA solution in a 20% TCA solution. This was made by dissolving 20 g TCA in 80 ml demineralized water. Thereafter 0.5 g TBA was added. To dissolve the TBA the beaker can be heated or placed in an ultrasonic bath. To become the desired concentration, the solution was transferred in a 100 ml volumetric flask and adjust with demineralized water.

80-100 mg of the sample was put in liquid nitrogen and were added three zirconia beads. The samples were shredded using the mixer mill for 3.5 min at 30Hz. While working on ice 1 ml of the 0.1% TCA solution was added and homogenized using a vortex. Afterwards the sample was centrifuged at 13000 rpm for 10 min. During this time, small holes were made in the cap of 2 ml microtubes with a syringe needle. After centrifuging, 400 µl of the sample was added in the tubes. For a blank sample TCA was used with the same volume. The samples were incubate for 30 min at 80 °C. Immediately after incubation they were cooled on ice. To pellet TBA precipitates the solutions were centrifuged again for 5 min at 13500 rpm. As the last step 200µl supernatant was transferred to the 96-well plate and the absorption was measured at 532nm and 600nm [18].

The malonaldehyde concentration was calculated with the following formula [18]:

$$\frac{MDA\ (nmol)}{FW\ (g)} = \frac{\Delta A_{corrected} * 3{,}5 * x * 1000}{\varepsilon * b * y}$$

In this formula:

$\Delta A_{corrected} = (A_{532} - A_{600})_{sample} - (A_{532} - A_{600})_{blank}$

b = light path length (0.56 cm for 200 µl)

ε = mM extinction coefficient (155 mM^{-1} cm^{-1})

x = ml TCA 0,1% used for extraction (1 ml)

y = fresh weight used for extraction (g)

1000 = conversion for µmol to nmol

3.5 = dilution factor

Chapter 4

Research paper

OXIDATIVE STRESS RESPONSE IN ARABIDOPSIS THALIANA AFTER EXPOSURE TO URANIUM: THE ROLE OF GLUTATHIONE

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ABSTRACT

Anthropogenic activities have caused pollution of various compartments of our environment with heavy metals, including uranium. Although it is a non-essential element, uranium can easily be taken up by plants where it can cause toxic effects, including oxidative stress. Plants have developed an antioxidative defence mechanism to counteract this stress. Glutathione (GSH) is an important antioxidant in this mechanism with three major function: redox homeostasis, metal homeostasis and detoxification. Although several studies have investigated the uranium-induced stress responses, the role of GSH during uranium stress is not completely known. The present study aimed to further investigate the role of GSH in *Arabidopsis thaliana* during uranium exposure. Therefore seedlings were cultivated for 18 days in a hydroponic setup with Hoagland nutrient solution, followed by three days of exposure to different uranium concentrations, ranging from 0-50 µM. After RNA extraction, the complete transcriptome of the plants was sequenced using the Illumina HiSeq2000 platform. Due to limited root-to-shoot translocation and the limited timeframe, only the data of the roots were processed during this master thesis. Uranium exposure clearly disturbed the sulfur metabolism by causing a sulfur starvation response. No differentially expressed genes related to glutathione synthesis where found, indicating no increased GSH biosynthesis. However, it seems that plants are trying to regulate the sulphur starvation response by providing cysteine for its essential functions as structural role in proteins by breaking down glucosinolates. Based on the differentially expressed genes, the results indicate that GSH probably don't play an important role in detoxifying reactive oxygen species via the AsA-GSH cycle under uranium stress, since genes related to this cycle were not significantly affected. In addition, no indications for the synthesis or presence of phytochelatins were found. However, uranium disturbs the homeostasis of multiple metals among which Fe. Finally, there seems to be an important role for the role for glutathione-S-.transferases in the detoxification of uranium and ROS during uranium exposure.

Keywords: *Arabidopsis thaliana, glutathione, oxidative stress, RNA sequencing, uranium.*

INTRODUCTION

Anthropogenic activities have caused pollution of various compartments of our environment with heavy metals, including uranium. Uranium pollution is mainly caused by the phosphate industry and metal mining and milling [2] [3] [8] [47] [48]. Uranium is a naturally occurring heavy metal and radionuclide which is found in the earth's crust with an average concentration from 2 to 5 mg/kg [47] [48]. Due to its long decay half-life (4.47 * 10^9 years) and low specific activity (1.25 * 10^4 Bq/g) the risk for chemical toxicity is greater than radiological toxicity [2] [3] [8] [49]. The aqueous uranyl ion (UO_2^{2+}) is proposed as the most toxic form since it is able to replace and interact with Ca^{2+}, Mg^{2+}, phosphate and carboxyl groups. Although uranium is a non-essential element, it can be taken up by plants. Once taken up, it can cause effects on macroscopic and cellular level such as a reduced growth, interaction with proteins and nucleic acids and it can affect membrane permeability [2]. In addition, uranium can induce oxidative stress in plants, resulting in a disturbed balance between the production and elimination of reactive oxygen species (ROS). Plant possess enzymatic and non-enzymatic antioxidative defence mechanisms to deal with this disturbance and to control the amount of ROS. The enzymatic antioxidative defence mechanism consists of superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD). In the non-enzymatic defence mechanism, ascorbate (AsA) and glutathione (GSH) are important antioxidants [6] [8] [9]. GSH plays an important role in (1) detoxification, (2) redox homeostasis and (3) metal homeostasis [6].

Earlier studies have shown that GSH plays an important role in plants response to heavy metal induced stress, including uranium [9] [29] [50]. However, the specific role of GSH under U stress has not been fully investigated. Therefore, the main goal of this internship is to unravel the role of GSH in *Arabidopsis thaliana* after exposure to uranium. *Arabidopsis thaliana* is used because it is a well-known plant and serves as a model organisms of flowering plant. Furthermore, this plants has a short life cycle, is easy to grow and needs only a limited amount of space. Finally, its entire genome has been sequenced and annotated and a lot of mutants are available.

Prior to the start of this master thesis, an RNA sequencing was performed on *Arabidopsis thaliana* plants that were grown for 18 days and exposed to different uranium concentrations (3, 6.25, 12.5, 25, 50 µM) during three days.

METHODOLOGY

Cultivation of *Arabidopsis thaliana* plants and treatment

Arabidopsis thaliana seeds were surface-sterilised and rinsed with 0.1% NaClO during 1 min. followed by sterile distilled water (4 x 5 min.). To ensure homogeneous germination the seeds were placed on moist filter paper at 4 °C during three days. Afterwards seeds were sown on plugs from 1.5 ml microtubes filled with 4.5 g/L agar N°2 (Merck) in low phosphate Hoagland solution. 36 plugs were positioned on a lid which was placed on a container filled with distilled water. After one week of growth, water was replaced with 1.35 L modified Hoagland solution with a pH of 5.5 (1 mM KNO_3, 0.3 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM $NH_4H_2PO_4$, 1.62 µM $FeSO_4$, 0.78 µM EDTA, 4.6 µM H_3BO_3, 0.9 µM $MnCl_2$, 32 nM $CuSO_4$, 55.6 nM H_2MoO_4, 76.5 nM $ZnSO_4$) (attachement A). Plants were cultivated in a climate chamber (Microclima 1000E, Snijders Scientific B.V.) with 14 h of light (photosynthetic photon flux density of 150-170 µmol/m2s), day/night temperature of 22°C/18 °C and a constant humidity of 65%. After a growing period of 18 days, plants were exposed to 0, 3, 6.25, 12.5, 25 and 50 µM uranium, which was added as $(UO_2(NO_3)_2.6H_2O$ (SPI chemicals, USA) from a stock solution (100 mM) to the Hoagland solution. Afterwards, the pH of the Hoagland solution was adjusted to pH 5.5. After three days of exposure, plants were harvested. Root fresh weight was recorded and the samples were frozen and stored at -80 °C with liquid nitrogen.

RNA extraction and deep sequencing

Total RNA extraction was performed by manufacturers recommendations using the RNeasy Plant Mini Kit (Qiagen, Venlo, Limburg, Netherlands). The quantity and quality of the RNA was controlled with NanoDrop ND1000 and BioAnalyzer (Agilent Technologies), respectively [51]. At the University of Antwerp, the full transcriptome of the *Arabidopsis thaliana* plants were sequenced by manufacturers recommendations on the Illumina HiSeq2000 platform with the TruseqTM RNA sample prep kit (version 2-single read-50 bp). The RNA-seq data sets were normalized, a False discovery Rate (FDR) (Benjamini & Hochberg correction) and the log fold changes (LFC) were calculated with the EdgeR package for R [51] to search for the differentially expressed genes. The following cut off values were used to determine the differentially expressed genes between treated samples and the control sample: $|Log_2$ Fold Changes (LFC)| > 1 and FDR < 0.05 [51]. These results were processed into Venn diagrams with http://bioinformatics.psb.ugent.be/webtools/Venn/.

GO enrichment

Gene ontology analysis was executed with the web-based tool Metascape [52]. This tool enables us to determine the statistically significant over-represented GO categories after uranium exposure. Analyses were done separately for the up- and downregulated genes for *Arabidopsis thaliana* in each condition. Results were processed in Microsoft Excel. Since the main goal of this project is to study the role of GSH, a visual representation of the GSH-related GO terms was made.

RESULTS AND DISCUSSION

The root-to-shoot translocation of uranium is small [2] [3] [4] [21]. This means that most of the uranium taken up by plants will stay in the roots. Due to this fact and the limited timeframe of this internship, only the data for roots of *Arabidopsis thaliana* are analysed and discussed in this master thesis.

To start the data analyses, a correlation plot (shown in Figure 22) is made to illustrate the variation between the different conditions.

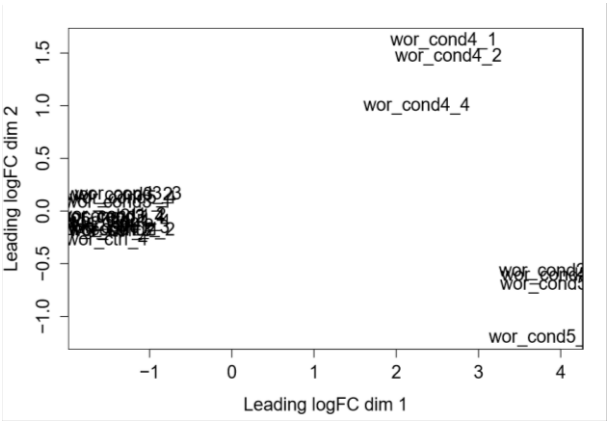

Figure 22: Correlation plot of the RNA sequencing gene expression profile of *Arabidopsis thaliana* plants exposed to different uranium concentrations for three days (cond1: 3 µM, cond2: 6.25 µM, cond3: 12.5 µM, cond4: 25 µM and cond5: 50 µM uranium). The distances correspond to differences in the biological variation between samples.

In this correlation plot three clusters are clearly formed. The first cluster consists of the control samples and the samples exposed to 3 µM, 6.25 µM and 12.5 µM of uranium. This indicates that those conditions are very similar to each other, suggesting that these low concentrations of uranium are not sufficient to induce significant responses in *Arabidopsis thaliana*. The plants exposed to 25 µM and 50 µM uranium are separately clustered from cluster 1 on the first dimension. This indicates that the plants at these conditions are affected differently than in conditions with a low uranium concentration. This difference is also visible in the fresh weight of *Arabidopsis thaliana* roots in various uranium conditions as shown in Figure 23. In the second dimension there is a clear separation between the samples exposed to 25 µM and 50 µM showing a different response in these concentrations.

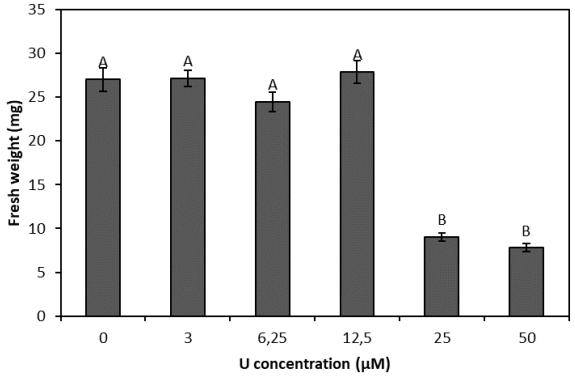

Figure 23: Root fresh weight of *Arabidopis thaliana* that were exposed to different uranium concentration for three days. Data represent the average ± SE of at least 40 biological replicates. Different letters indicate significant differences ($p<0.05$)

Next, the differential expressed genes (DEGs) were identified for *Arabidopsis thaliana* in each condition with the following cut-off values: $|\text{Log}_2$ Fold-Change (LFC)$|$ > 1 and FDR < 0.05. The amount of up-and downregulated DEGs in *Arabidopsis thaliana* per condition are shown in Figure 24 (A).

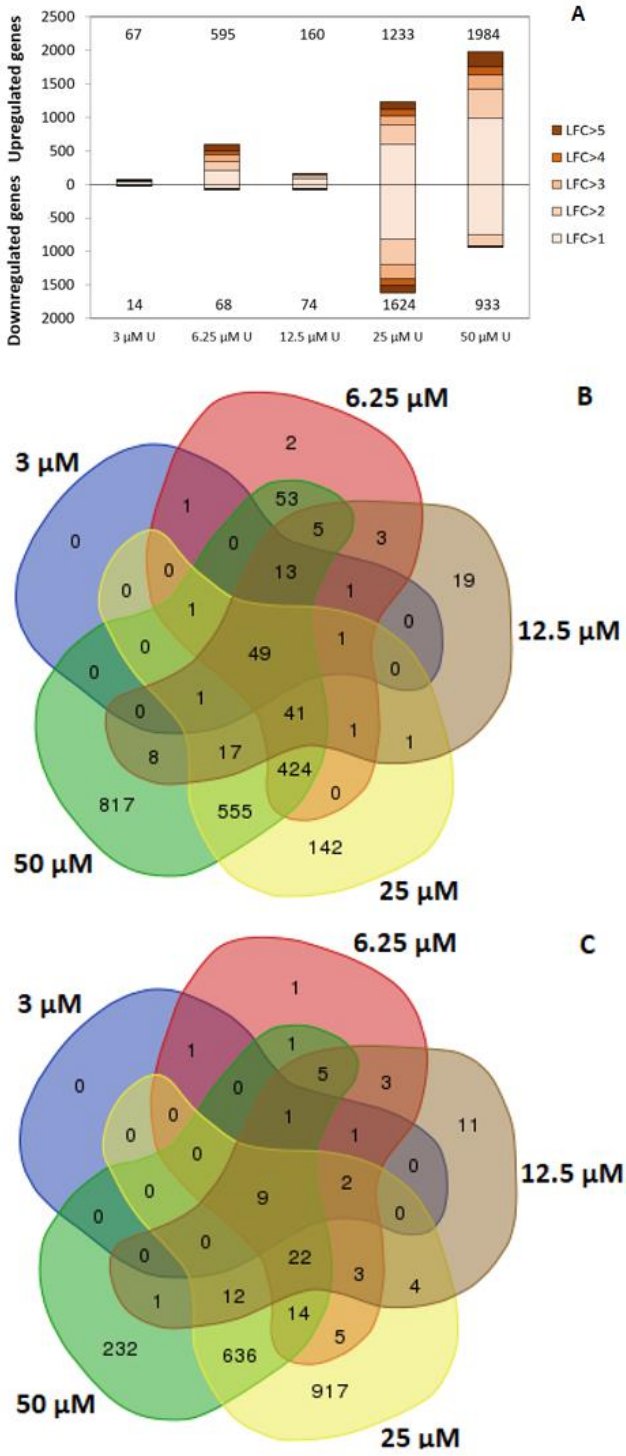

Figure 24: (A) Differential expressed genes in *Arabidopsis thaliana* after exposure to different uranium concentrations for three days; (B) Venn diagram showing the overlap of DEGs that were upregulated in the plant for each exposure condition; (C) Venn diagram showing the overlap of DEGs that were downregulated in the plant for each exposure condition.

There are 67, 595, 160, 1233, 1984 genes upregulated and 14, 68, 74, 1624, 933 genes downregulated in plants exposed to uranium, going from low to high uranium

concentrations respectively. At the lowest uranium concentrations (3-12.5 µM U), there are only a limited amount of genes affected, which possibly explains the clustering of those samples, together with the non-exposed control plants as shown in Figure 22. At the two highest uranium concentrations there are more genes differentially expressed, which might be the consequence of a higher stress response due to the higher uranium concentration.

The Venn diagram of the upregulated gens (Figure 24(B)) reveals that 49 genes are commonly upregulated through all five conditions. The three lowest exposure conditions have respectively 0, 2 and 19 genes which are uniquely upregulated. For the conditions with a higher uranium concentration there are 1088 genes commonly expressed. This may explain why these two conditions are separated from cluster 1 by dimension 1 in the correlation plot (Figure 22). Besides the high amount of commonly expressed genes in the 25 µM and 50 µM exposure condition, there are also respectively 142 and 817 genes uniquely upregulated. This can explain the difference in dimension 2 in the correlation plot between the higher concentrations.

For the downregulated genes (Figure 24 (C)), a similar trend as in the upregulated genes is present, with only 9 genes commonly affected in all the exposure conditions. After exposure to 25 and 50 µM U, there are 917 and 232 genes uniquely downregulated, while 693 genes were in common between both conditions.

To analyse the biological functions of the DEGs, a gene ontology (GO) enrichment analyses was done using the web-based tool Metascape [52]. Metascape provided bar charts displaying the significantly enriched GO terms (Supplemental figures in annex). A general observation is the enrichment of DEGs for GO terms related to hormone metabolism, including abscisic acid, jasmonic acid and salicylic acid. A similar observation is made by Doustaly *et al*. (2014) [47] in 7 weeks old *Arabidopsis thaliana* plants exposed to 50µM uranium. It is known that phytohormones have an important role in plants to counteract stress, including heavy metal stress, since in addition to their controlling function in plant growth and development they have an important role as signalling molecules [53]. Future work could investigate the concentrations of these hormones to further reveal their importance in the response of plants to uranium. In addition, different mutants of *Arabidopsis thaliana* could be used such as aba2-2, dad1 or sid2-2 which are respectively deficient in abscisic acid, jasmonic acid and salicylic acid to obtain a more detailed view of the role of the different phytohormones in the activation of the defence response under uranium stress.

Furthermore, the GO term 'aging' was enriched after exposure to 6.25 µM U or higher U concentrations. This might indicate a deterioration or loss of function of process in the plant. It is known that aging or early senescence can be caused by an imbalance in the cellular redox state [54]. As such, it is often observed in plants after exposure to biotic or abiotic stresses [29] [50] [55] [56]. Early senescence is a recycling process whereby nutrients are translocated from parts that will die to storage parts like seeds/grains. By doing this, the plant optimizes its capacity to reproduce and it ensures a next generation [57] [58].

Since the main goal of this master thesis is to unravel the role of glutathione under U stress, this study will further discuss the GO terms which are relevant to the main functions of glutathione. A visual representation of the related terms is made in Figure 25. The GO terms are divided into four groups, linking them with the GSH metabolism and the three main functions of GSH, i.e. (1) detoxification, (2) redox homeostasis and (3) metal homeostasis. This will enable this master thesis to further explore the role of GSH under U stress.

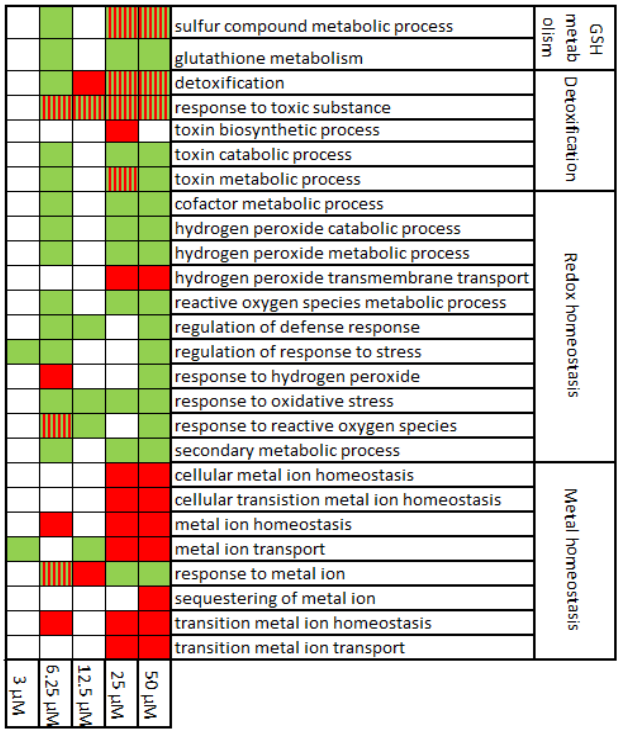

Figure 25: Visualization of significantly affected GO terms obtained after GO enrichment analyses of differentially expressed genes in *Arabidopsis thaliana* after exposure to different uranium concentrations for three days. Green = enriched in upregulated genes, Red = enriched in downregulated genes, Green/red (striped) = enriched in both up-and downregulated genes.

A first view reveals that after exposure to 25 µM U and 50 µM U there are a lot of commonly affected GO terms. Besides this, it is remarkable that there are much more enriched GO terms affected after exposure to 6.25 µM as compared to 12.5 µM U. The concentration 6.25 µM has also more DEGs in common with the higher uranium concentrations, as shown in Figure 24 (A). However, there is no clear explanation for this trend at the moment.

Glutathione metabolism

The genes related to 'sulfur compound metabolic process' are affected in *A. thaliana* plants mainly after exposure to 25 and 50 µM uranium. The upregulated genes in this term are involved in the sulfur compound catabolism while the downregulated ones belongs to the sulfur compound biosynthesis. This indicates that the sulfur metabolism is disturbed after uranium exposure. A decrease of the sulfur concentration after uranium exposure was observed by Mertens *et al.* (unpublished

results), possibly indicating a decreased sulfur uptake. The sulfur compound biosynthetic processes include each pathway that produces sulphur-containing compounds, including cysteine and GSH. The first gene in sulfur assimilation (ATP sulfurylase (APS1)) is downregulated in the plant after uranium exposure. APS1 activates sulphate in an adenoylation reaction [59]. Downregulation of this gene indicates that the sulfur metabolism is clearly disturbed under uranium exposure. The downregulated genes in the GO terms related to GSH metabolism are mainly involved in the glucosinolate biosynthetic process. As reviewed by Czerniawski *et al*. (2018) this pathway uses GSH-conjugates as intermediates to produce glucosinolates [60]. There are also multiple upregulated BGLU genes present. These genes are upregulated during sulfur starvation to recycle sulfur from glucosinolates (secondary metabolism) to the primary sulphur metabolism [61].

Two genes involved in the degradation of GSH were also upregulated after exposure to 25 and 50 µM uranium. These genes, namely AT5G26220 and GGT2 which respectively encode for gamma-glutamyl cyclotransferase and glutamate glyoxylate aminotransferase, are upregulated as response to sulfur starvation as consequence from the disturbed sulfur metabolism [62]. This possibly indicates that the plants are trying to regulate the sulphur starvation response by providing cysteine for its essential functions as structural role in proteins. However, more research is needed to confirm this hypothesis.

The GO term 'GSH metabolism' is enriched in upregulated genes after exposure to 6.25, 25 and 50 µM uranium. This term consist almost fully out of genes encoding for GSTs. They encode for different GSTs from the class phi (GSTF) and tau (GSTU), which are the two biggest GST classes. The upregulation of these GST suggest that they have an important role under uranium stress. Since their role is mainly detoxification of xenobiotics and ROS, their importance will be further discussed below.

Detoxification

Under detoxification there was an enrichment for the terms 'toxin catabolic process' and 'toxin metabolic process' after exposure to 6.25, 25 and 50 µM uranium. Almost all the genes that were upregulated in these terms were GSTs belonging to the phi and tau class. In general GSTs are important in the detoxification mechanism. They are able to catalyse the conjugation between xenobiotics and GSH and are involved in the detoxification of ROS. Overexpression of rice GSTs in transgenic *Arabidopsis thaliana* plants revealed that GSTs can enhance oxidative stress tolerance by ROS scavenging [63]. It is possible that GST also scavenge ROS during uranium exposure, but further research is needed. Previous work of Hossain *et al*. (2012) reviewed that GSTU3/4/12 are overexpressed after cadmium exposure in rice roots and reported that they have a role in the direct quenching of cadmium ions [64]. It is possible that the same mechanism of detoxification occurs by uranium exposure. Multiple LSU genes (response to low sulfur (LSU1, LSU3 and LSU4)) are upregulated in the plants after exposure to 6.25 µM uranium. This observation is in agreement with the disturbed sulfur assimilation which was described in the paragraph on GSH metabolism. The LSU genes are also involved in the detoxification of oxidants. Together with the increased expression of GSTs, this may indicate the importance of the detoxification function of GSH.

An enrichment for the upregulated genes DIN9, HIPP20 and 21 was also observed. These genes are related to cadmium homeostasis and detoxification. "HIPPs are metallochaperons that transport metal ions inside the cell and are involved in heavy metal homeostasis and detoxification." [65, p. 538]. It is possible that they are also involved in the detoxification of uranium, but further research is needed to confirm this hypothesis.

Besides these GSTs, the GO term "detoxification" entails genes related to the detoxification of ROS which indicates a disturbed redox homeostasis. Those gene swill be further discussed in the paragraph 'Redox homeostasis'.

Redox homeostasis

The GO terms related to redox homeostasis are mainly enriched for genes with an upregulated transcription. In contrast to the expectations, there was almost no differential expression of genes related to the AsA-GSH cycle except for (DHAR1). This suggests that the importance of GSH under uranium stress is probably not accomplished via those pathways to detoxify H_2O_2.

The enrichment for the downregulated genes are mainly in the term 'hydrogen peroxide transmembrane transport' after exposure to 25 and 50 µM uranium. Most of the genes in the term 'hydrogen peroxide transmembrane transport' belong to aquaporins. Aquaporins are known to form pores through membranes, which make a quick and reversible water transport possible. Their expression varies with different abiotic stress conditions including heavy metal stress (as reviewed by Afzal *et al.* (2016)) [66]. A similar decrease was observed by Aranjuelo *et al*. (2014). Their study suggested that the decrease transcription for aquaporins is linked to a decreased translocation of uranium to the shoots [9]. Another possible explanation is described, which suggests that the *Arabidopsis* plant downregulates these genes to prevent water loss from their roots [67].

At the lowest uranium concentration, there are almost no signs of heavy metal induced stress. Nevertheless after exposure to 6.25 µM uranium or higher, there are indications of oxidative stress responses based on the enrichment of the GO terms 'hydrogen peroxide metabolic process', 'reactive oxygen species metabolic process' and 'the response to oxidative stress'. It has been shown before that uranium can induce oxidative stress in *Arabidopsis thaliana* plants [3] [8].

As mentioned above, genes related to the detoxification of ROS are affected. These genes include superoxide dismutase (FeSOD and CuZnSOD), catalase (CAT1/3) and multiple peroxidases. Superoxide dismutase forms the first line of defence in the antioxidative defence mechanism. An upregulation of FSD1 was observed under these conditions. FDS1 is a gene encoding for FeSOD which is located in the chloroplast, mitochondria

and cytosol. In contrary to genes encoding for FeSOD, genes encoding for CuZnSOD are frequently downregulated in this function, which can be related to a disturber metal homeostasis (see below). CAT1 and CAT3 are also frequently upregulated. This observation is in agreement with Vanhoudt *et al*. (2011) [19] who stated that FSD1 and CAT1 are upregulated in the roots after exposure to 100 µM uranium for one day. Peroxidases and the peroxidase superfamily proteins are important elements in the antioxidative defence mechanism after exposure to uranium. Genes encoding for peroxidases are mainly upregulated in the term 'response to toxin substance'. All these peroxidase possess heme binding and peroxidase activity. The peroxidase superfamily protein was hereby encoded by different genes. The peroxidases differ in their cellular location, with one common location i.e. the extracellular region. Together with catalase they form the second line of defence in the antioxidative defence mechanism by converting peroxides into water.

Metal homeostasis

Concerning metal homeostasis, this work was mainly interested in the production of phythochelatins (PC). Phythochelatins are important chelators of metals and therefore important in metal homeostasis. PCs are polymers from GSH which synthesis is activated by metal exposure, in theory (and during exposure of other metals (e.g. Ag, Cd, Zn)) [33]. In this work, however, there was no enrichment of genes that could indicate PC synthesis. This observation suggests that PCs do not contribute to the metal homeostasis during uranium exposure. This result is in agreement with Horemans *et al*. (unpublished results) where no increase in PC content was found in *Arabidopsis thaliana* plants after U exposure.

However, we observed an enrichment of downregulated genes related to metal transport, more specific in genes related to Ca, Cu, K, Mg, Mn, Ni, Zn and Fe transport/homeostasis after exposure to 25 and 50 µM uranium. This implicates that uranium exposure disturbs the homeostasis of multiple metals. The most affected metals are Zn and Fe. For Zn the disturbance genes include heavy metal ATPase (HMA3), metal tolerance protein (MTP3) and two zinc-regulated/iron-regulated proteins (ZIP3/9). These genes are involved in respectively the sequestration into the vacuole, zinc transport/tolerance and transmembrane transport/deficiency response [68]. Other Fe-related genes, such as transporter genes IRT1 and IRT2, ferric reductase detective 3 (FRD3), ferric reduction oxidase (FRO2), Fe deficiency induced transcription factor (FRU) and three vacuole iron transporters, were also affected. The majority of these genes were also described in a study by Doustaly *et al.* (2014) [47]. They revealed that uranium can disturb the iron uptake and signalling in roots, for example: " uranyl triggered a root iron-excess response resulting in downregulation of FIT1, FRO2, IRT1, AHA2 and AHA7." [47, p. 818]. A second work by Berthet *et al.* (2017) [48] also studied this phenomenon and revealed that uranyl competes with Fe to form a complex with phosphate and is able to displace Fe in this complex. Uranyl and Fe also compete to bind with pectin or hemicellulose components in the root cell walls [48]. These two phenomena potentially result in an increase in soluble Fe and disturbs the Fe homeostasis.

Besides these specific transporters, the transition metal ion transport was also harmed. This includes genes in Zn uptake (BTSL) and Mn transport (PML4). Additionally, the gene for the copper chaperon of CuZnSOD (CCS) was downregulated. This downregulation can be the consequence of the disturbed Cu homeostasis, which was mentioned before. A study from Saenen *et al.* (2015) described the decrease in the expression of CuZnSOD (suppressed by miR398b/c) as a possible consequence of a disturbed Cu homeostasis [8]. In the present study, an upregulation of the miR398c transcript levels was also observed, which is in agreement of the study by Saenen *et al.* (2015).

A previous study on Cd exposed plants by Zhang *et al* (2019) revealed that "MYB49 affects the expression of genes involved in heavy metal uptake, transport and tolerance (…) MYB49 directly regulates the expression of bHLH38, bHLH101, HIPP22 and HIPP44 by binding to their promotor.", those bHLHs increase Cd accumulation [65, p. 538]. After exposure to 25 µM uranium, the gene encoding for MYB49 is downregulated. This led to a downregulation for the genes encoding for bHLH38/101. These two genes are involved in the regulation of IRT1 expression. This could indicate that the MYB49 gene is important in limiting uranium uptake by *Arabidopsis thaliana* plants. However, more research is needed to further investigate this hypothesis.

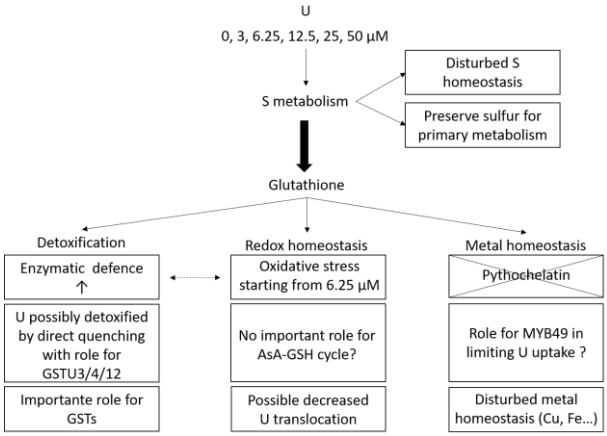

Figure 26: Schematic overview of the results & discussion of Arabidopsis thaliana roots after exposure to different uranium concentrations

CONCLUSION

Using RNA sequencing analysis the potential role of GSH in the response of *Arabidopsis thaliana* plants exposed for three days to 0-50 µM of uranium was studied. Figure 26 provides a schematic overview of the results and discussion described above. This work revealed that the *Arabidopsis thaliana* plant experience oxidative stress starting from 6.25 µM uranium. Starting from 25 µM this stress response was visible in a reduced fresh weight of the roots. Despite the fact that the oxidative stress started from 6.25 µM uranium, there was a clear difference in the response in plants exposed to the lower (3-12.5 µM) and higher (25-50 µM) uranium

concentrations and even between 25 µM and 50 µM. There was a general enrichment of GO terms related to hormone metabolism and 'aging', which emphasizes their importance during the uranium-induced stress response.

This work observed a disturbed sulfur metabolism in *Arabidopsis thaliana* after exposure to 25 or 50 µM uranium. It seems that the plants redirect sulfur from the secondary to the primary metabolism by breaking down glucosinolates. Under the function redox homeostasis no evidence was found for the importance of GSH in the AsA-GSH cycle under uranium stress. Under uranium stress *Arabidopsis* probably scavenge H_2O_2 mainly by CAT and peroxidases, where an increased expression was observed. In addition, GSTs have an important role in both detoxification. The observed downregulation of aquaporins suggest that the plant decreases the translocation of uranium in attempt to protect the shoots against an increasing oxidative stress. The observations under metal homeostasis indicates that there is no involvement of PCs during uranium exposure. But uranium clearly disturbed the homeostasis of multiple metals and especially the homeostasis of Fe. Finally, it seems that MYB49 seems to play an important role in regulating the uranium uptake by *Arabidopsis thaliana* plants.

Further research to investigate the role of GSH during uranium exposure could include the use of *Arabidopsis thaliana* mutant cad2-1 which has a defect in gamma-glutamylcysteine synthetase, the first enzyme of the biosynthesis of GSH. Because of this defect the cad2-1 mutant has only 30% of the wild-type GSH level. It is also interesting to study the enzymatic activity of the enzymes in the antioxidative defence mechanism in this mutant under uranium stress. Other aspects that could be investigated with this mutant are uranium uptake, lipid peroxidation and the effect on growth. During the internship, primers for the GST tau class were designed. Due the covid-19 restrictions present during this master thesis, they still need to be tested for their efficiency, but they can provide more information about GST expression levels in the cad2-1 mutant. Finally, since there is no direct link between the transcript level and protein level, it is interesting to study the enzymatic activity of these GSTs during uranium exposure.

ACKNOWLEDGMENTS

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Attachment A: Instruction for the preparation of Hoagland nutrient solution

"HP" Hoagland

Macro elements without phosphorus ..... 100 ml for 10 L
Phosphorus solution ..... 50 ml for 10 L
Iron Solution ..... 0.6 ml for 10 L
Micro elements ..... 1 ml for 10 L

"LP" Hoagland

Macro elements without phosphorus ..... 100 ml for 10 L
Phosphorus solution ..... 12.5 ml for 10 L
Iron Solution ..... 0.6 ml for 10 L

Table 15: Reagens and concentrations needed for the preparation of Hoagland nutrient solution

Macro elements			1 L /10 L	H 1:1
	g.mol-1	g / 2 L		mM
KNO_3	101,11	20.4		100
$Ca(NO_3)_2 \cdot 4H_2O$	236,15	14.16		30
$MgSO_4 \cdot 7H_2O$	246,48	9.8		20
Phosphorus			1L / 10L	
$NH_4H_2PO_4$	115,03	4.6		20
Iron Solution		g / 250 ml	6ml / 10L	
$FeSO_4 \cdot 7H_2O$	278,02	1,9		27
Na_2-EDTA$\cdot 2H_2O$	372,2	1,25		13
Micro elements		g / 1 L	10 ml /10 L	
H_3BO_3	61,83	2,86		46
$MnCl_2 \cdot 4H_2O$	197,91	1,81		9.1
$CuSO_4 \cdot 5H_2O$	249,68	0,08		0.32
H_2MoO_4	161,97	0,09		0.55
$ZnSO_4 \cdot 7H_2O$	287,54	0,22		0.76

Attachment B: Barcharts provided by GO enrichment

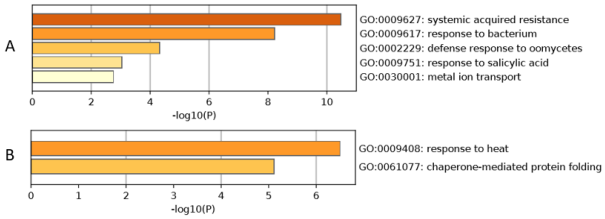

Figure 27: Metascape bar chart for 3 µM uranium with (A) the upregulated DEGs, (B) the downregulated DEGs

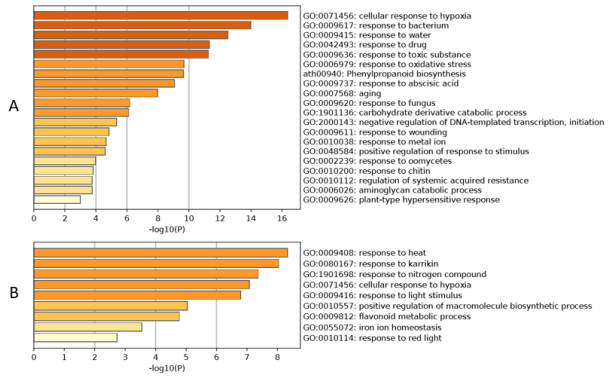

Figure 28: Metascape bar chart for 6.25 µM uranium with (A) the upregulated DEGs, (B) the downregulated DEGs

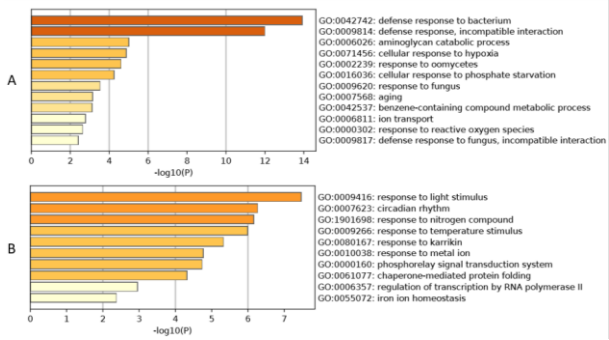

Figure 29: Metascape bar chart for 12.5 µM uranium with (A) the upregulated DEGs, (B) the downregulated DEGs

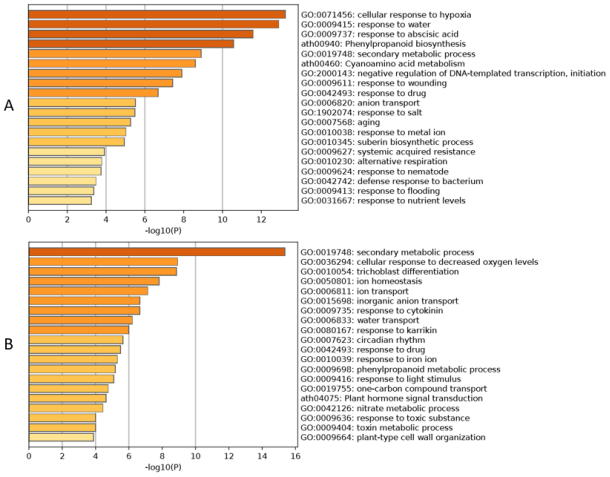

Figure 30: Metascape bar chart for 25 µM uranium with (A) the upregulated DEGs, (B) the downregulated DEGs

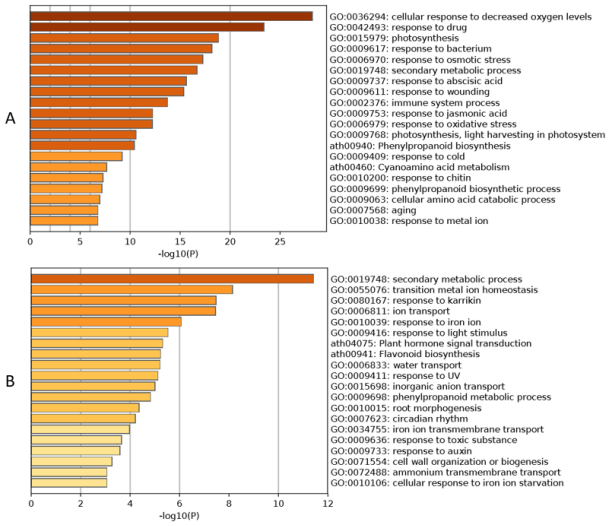

Figure 31: Metascape bar chart for 50 µM uranium with (A) the upregulated DEGs, (B) the downregulated DEGs