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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen: milieu en gezondheid

Masterproef

Oxidative stress and endoreduplication in metal-exposed *Arabidopsis thaliana*: Does alternative oxidase 1a play a role?

Promotor :
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

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8-OHdG	8-hydroxydeoxyguanosine
AOX	Alternative oxidase
APX	Ascorbate peroxidase
AsA	Ascorbate
BER	Base excision repair
Ca	Calcium
CAT	Catalase
Cd	Cadmium
CDK	Cyclin-dependent kinase
Cu	Copper
DSB	Double strand DNA break
DHA	Dehydroascorbate
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
Fe	Iron
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HR	Homologous recombination
IARC	International Agency for Research on Cancer
ICP-MS	Inductively coupled plasma mass spectrometry
MMR	Mismatch repair
NHEJ	Non-homologous end joining
O_2^{o-}	Superoxide
^{o}OH	Hydroxyl radical
Pb	Lead
PCD	Programmed cell death
PI	Propidium iodide
PVP	Polyvinylpyrrolidone
Ra	Radium
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription real-time polymerase chain reaction
SAZ	Syringaldazine
SOD	Superoxide dismutase
SPX	Syringaldazine peroxidase
Th	Thorium
U	Uranium
UO_2^+	Uranyl ion
XOD	Xanthine oxidase
Zn	Zinc

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Time flies! I can still clearly remember my first day at Hasselt University, almost five years ago. Now, I am writing the last pieces of my thesis and I am almost graduating. Writing this thesis would not have been possible without the help and support of many people.

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SAMENVATTING

Vervuiling van de omgeving met metalen is een probleem dat wereldwijd vele regio's treft en negatieve gevolgen heeft voor de gezondheid van verscheidene organismen. Omdat de meeste metalen in de omgeving niet voorkomen als afzonderlijke polluenten, is het belangrijk de gemengde aard van metaalvervuiling in rekening te brengen bij het bestuderen van de effecten van metaalblootstelling. Twee metalen die vaak samen voorkomen in de omgeving zijn cadmium (Cd) en uranium (U). Ze kunnen opgenomen worden door planten en plantengroei en -ontwikkeling verstoren. Dit kan verklaard worden op basis van twee belangrijke eigenschappen van deze metalen: (1) hun genotoxiciteit en (2) hun vermogen om oxidatieve stress te induceren, gekenmerkt door een verhoogde productie van reactieve zuurstofvormen (ROS), die mogelijk ook DNA-schade induceren. Organellen die een belangrijke rol spelen in de oxidatieve stressrespons, zijn mitochondriën. Vooral de mitochondriale alternatieve oxidasen (AOXen) zijn stressgevoelige enzymen. Ze reduceren mitochondriale ROS-productie en worden vaak geïnduceerd in planten onder stresscondities op het niveau van transcriptie, translatie en capaciteit. Voorgaand onderzoek heeft een inductie aangetoond van AOX1a, de meest dominante AOX isovorm in *Arabidopsis thaliana*, op transcriptioneel en translationeel niveau. Daarom stellen we de hypothese dat AOX1a een rol speelt in de oxidatieve stressrespons en DNA-schade en -herstel geïnduceerd door metaalblootstelling in *A. thaliana*. Bovendien worden ook de effecten van metaalblootstelling op endoreduplicatie – een alternatieve vorm van de celcyclus waarin DNA-replicatie plaatsvindt zonder mitose – onderzocht, aangezien onderzoek heeft aangetoond dat dit proces geïnduceerd wordt door mitochondriale oxidatieve stress in humane HK-1 cellen. Ook de rol van AOX1a in dit proces wordt nagegaan, omdat dit enzym mitochondriale ROS productie reduceert.

Om de hypotheses van deze studie te onderzoeken, worden wildtype en *aox1a*-knockout *A. thaliana* planten blootgesteld aan 5 of 10 µM Cd, 25 µM U of een combinatie van 2.5 µM Cd en 12.5 µM gedurende 24 u (en 72 u voor de flowcytometrische analyse van de nucleaire DNA inhoud). De bepaling van de transcriptie van pro- en antioxidatieve genen, antioxidatieve enzymcapaciteiten en concentraties van de antioxidatieve enzymen ascorbaat (AsA) en glutathion (GSH) en hun redoxstatus toont aan dat AOX1a waarschijnlijk niet betrokken is in de metaalgeïnduceerde oxidatieve stressrespons, aangezien de geobserveerde responsen zeer gelijkaardig zijn tussen beide genotypes. Een vergelijking van de responsen geïnduceerd door Cd en U toont echter aan dat de mechanismen die aan de basis liggen van de oxidatieve stressrespons geïnduceerd door beide metalen waarschijnlijk verschillen. Verder verminderen zowel Cd als U de mate van oxidatieve DNA schade, bepaald als de hoeveelheid 8-deoxyhydroxyguanosine (8-OHdG), en de expressie van de meeste genen die gemeten werden via "reverse transcription real-time polymerase chain reaction" (RT-qPCR). Deze respons wordt niet geobserveerd in respons op blootstelling aan zowel Cd als U, wat mogelijk wijst op een antagonistische werking tussen beide metalen. Verder verhoogt blootstelling aan alle vier metaalcondities de mate van endoreduplicatie, gemeten via flowcytometrie, in *A. thaliana* blaadjes. De start van deze metaalgeïnduceerde toename in endoreduplicatie blijkt uitgesteld te zijn in *aox1a*-mutante blaadjes, wat mogelijk wijst op een rol voor AOX1a in de regulatie van dit proces.

ABSTRACT

Metal contamination is a problem affecting many regions worldwide, causing negative health effects in several organisms. As in the environment most metals do not occur as single pollutants, it is important to take into account the mixed nature of metal contamination when studying the effects of metal exposure. Two metals frequently occurring together in the environment, are cadmium (Cd) and uranium (U). They can be taken up by plants and cause disturbances of plant growth and development. This observation can be explained by two important characteristics of these metals: (1) their genotoxicity and (2) their ability to induce oxidative stress, characterized by an increased production of reactive oxygen species (ROS), possibly also leading to DNA damage. Organelles playing an important role in the oxidative stress response are mitochondria. In particular, the mitochondrial alternative oxidases (AOXs) are stress-sensitive enzymes, which reduce mitochondrial ROS production and are often induced in plants under stress conditions at the level of transcription, translation and capacity. Previous research has shown an induction of AOX1a, the most dominant AOX isoform in *Arabidopsis thaliana*, at transcriptional and translational levels after Cd exposure. Therefore, we hypothesized a role for this enzyme in the oxidative stress response and DNA damage and repair induced by metal exposure in *A. thaliana*. In addition, the effects of metal exposure on endoreduplication – an alternative form of the cell cycle consisting of DNA replication without mitosis – are investigated, as this process has been shown to be induced by mitochondrial oxidative stress in human HK-1 cells. Therefore, the role of AOX1a in this process is investigated as well, as this enzyme is known to reduce mitochondrial ROS production.

To reach the objectives of this study, wild-type and *aox1a*-knockout *A. thaliana* seedlings are grown in hydroponics and exposed to either 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h (and 72 h for the flow cytometric measurements of nuclear DNA content). An investigation of the transcription of selected pro- and antioxidative genes, antioxidative enzyme capacities and concentrations of the antioxidative metabolites ascorbate (AsA) and glutathione (GSH) and their redox status, shows that AOX1a is probably not involved in the metal-induced oxidative stress response, as responses are very similar between both genotypes. A comparison of the responses induced by Cd and U, however, indicates that the mechanisms underlying the oxidative stress response induced by both metals are probably different. In addition, both Cd and U decrease the extent of oxidative DNA damage, determined as the amount of 8-deoxyhydroxyguanosine (8-OHdG), and the expression of most DNA repair genes measured using reverse transcription real-time polymerase chain reaction (RT-qPCR). The latter response is not observed after combined exposure to Cd and U, possibly indicating an antagonistic action between both metals. Furthermore, exposure to all four metal conditions increases the extent of endoreduplication, measured by flow cytometry, in *A. thaliana* leaves. The onset of this metal-induced increase in endoreduplication seems to be delayed in *aox1a*-mutant leaves, possibly pointing towards a role for AOX1a in the regulation of this process.

1. INTRODUCTION

Contamination of the environment, caused by metals, is a problem affecting many regions worldwide. Although certain metals are **naturally present** in the environment, they are also often released by **anthropogenic sources**. After their introduction in the environment, metals can be taken up by plants, negatively affecting plant growth and development, and enter the food chain, possibly causing health effects in humans [1-3].

In the environment, most metals do not occur as single pollutants. Research has shown that the response of plants to a combination of two different abiotic stresses is unique and cannot directly be extrapolated from the response to each of the stressors applied individually. Therefore, it is important to take into account the **mixed nature of metal contamination** when studying the effects of metal exposure in plants [4].

An example of a metal never occurring as a single pollutant is uranium (U), always present together with other radioactive elements, such as thorium (Th) and radium (Ra), and non-radioactive contaminants, such as cadmium (Cd), zinc (Zn) and copper (Cu) [5].

In this project, the effects of **exposure to Cd and U in *Arabidopsis thaliana* plants** are studied. Since both elements are known to negatively affect human health and plant growth and development, it is important to get a better insight into the mechanisms underlying the toxicity of these metals. As previous research has shown that the effects induced by combined exposure to both metals differ markedly from those induced by exposure to only one of them, the effects of **combined exposure to both metals** are investigated as well [5].

1.1. Cadmium

1.1.1. Characteristics and presence in the environment

Cadmium is a metal **naturally present** in all environmental matrices. It occurs in association with the sulfide ores of Zn, lead (Pb) and Cu and as an impurity in phosphate minerals. In addition, it can be introduced in the environment by **anthropogenic sources**, such as smelting and refining of non-ferrous metals, fossil fuel combustion and municipal waste incineration. The use of commercial fertilizers derived from rock phosphate and sewage sludge also contributes to environmental Cd contamination [6]. Several Belgian areas such as the Meuse valley near Liège and the northern parts of the Kempen region are polluted by Cd, which is mainly due to historical emissions from non-ferrous industries [7].

1.1.2. Effects of cadmium in humans

Cadmium can be taken up from contaminated soils by plants, introducing it into the **food chain** as it also accumulates in vegetables and crops grown for human consumption [1, 8]. Therefore, food is the most important source of human Cd exposure. Other important sources are cigarette smoke, house dust and contaminated air and water [8].

Although dietary uptake is a more important route of Cd uptake than inhalation, absorption via the lungs is higher than uptake via the gastrointestinal tract. After absorption, Cd enters the bloodstream and is transported to several organs. It is mainly stored in the renal

cortex, although high exposure doses can also lead to increased Cd concentrations in the liver. Excretion of Cd is generally slow, resulting in a very long biological half-life of several decades [6].

The most important target organ for chronic dietary Cd exposure is the **kidney**. Renal damage caused by Cd is characterized by proximal tubular reabsorptive dysfunction, leading to severe disease or even death, in case of prolonged exposure to high concentrations [8]. Cadmium can also induce effects on **bone** by decreasing calcium absorption and bone mineralization and enhancing bone resorption. In addition, it has been classified as a group 1 human **carcinogen** by the International Agency for Research on Cancer (IARC) [6].

1.1.3. Effects of cadmium in plants

Although Cd is a non-essential element, it is readily **taken up by plants** via transmembrane carriers for essential nutrients such as calcium (Ca), iron (Fe) and Zn. The degree of Cd uptake by plants depends on its concentration in the soil and its bioavailability, modulated by the presence of organic matter, pH, redox potential, temperature and concentrations of other elements. It first enters the roots and is then transported to the above-ground parts of the plant, although most of the Cd ions are retained in the roots [2].

Cadmium causes **several effects in plants on morphological and physiological levels**. Examples are leaf roll, chlorosis, reduced growth, disturbances of the water balance, damage to the photosynthetic apparatus, inhibition of mitochondrial oxidative phosphorylation and inhibition of stomatal opening. It has also been shown to interfere with the uptake, transport and use of several elements and water by plants [9]. Although it is not redox-active, Cd is able to induce **oxidative stress**. It increases the production of reactive oxygen species (ROS) by replacing redox-active elements, such as Fe, and by inhibiting the activity of several antioxidative enzymes and metabolites. Furthermore, it increases the enzymatic production of ROS by inducing NADPH oxidase activity. It also increases subcellular ROS production in mitochondria, chloroplasts and peroxisomes [10]. In addition, Cd is able to induce **DNA damage**, as discussed in section 1.5.

1.2. Uranium

1.2.1. Characteristics and presence in the environment

Uranium is a primordial radionuclide and metal **naturally present** in all environmental matrices [3, 11]. It occurs in hydrothermal veins, sedimentary rocks and pyritic conglomerate beds. The most prevalent form in which U occurs in watery solutions in the environment, is the **uranyl ion** (UO_2^+) which forms soluble complexes with carbonate, phosphate and sulfate ions that can readily be transported in the environment [12]. Currently, 22 U isotopes are recognized, of which ^{234}U, ^{235}U and ^{238}U are naturally occurring [3]. In this project, ^{238}U is used, as it is the most abundant naturally occurring isotope [11].

Uranium can also be introduced in the environment by **anthropogenic sources**, such as U mining, milling and processing, phosphate mining, coal use and inappropriate waste

disposal. One of the most important industrial applications of U is its use as a fuel in nuclear power plants [3].

1.2.2. Effects of uranium in humans

As U is naturally present in the environment in limited concentrations, humans are always exposed to low U concentrations via food, air, water and soil. Uranium can be taken up by ingestion, aerosol inhalation and wounds. More than 95 % of the U taken up by ingestion is excreted in the feces within days. Of the small portion that enters the bloodstream, 50% is filtered by the kidneys and excreted, 22% is accumulated in the **bones** and 21% in the **kidneys**, possibly causing renal dysfunction. Inhaled U can either enter the bloodstream or be retained in the **lungs**, depending on its particle size. When retained in the lungs, it can decay to other radionuclides, which can cause a radiological or chemical hazard. In addition, stochastic effects such as **cancer** can manifest at a later stage [3, 13].

Because of its low specific activity (12.4 kBq g^{-1}), U is only **weakly radiotoxic**. It emits alpha particles that are unable to penetrate the superficial layer of the skin, but have a high ionizing capacity over a short pathway when they travel through tissue. Therefore, internal U contamination and accumulation in specific organs poses a high risk for irreversible cellular damage. Ionizing radiation induces molecular and cellular effects directly through energy transfers to macromolecules or indirectly through a water radiolysis reaction and subsequent production of ROS [13].

The **chemical toxicity** of U is higher than its radiotoxicity and also highly dependent on the form in which it is present. The soluble forms of U are more toxic as compared to the non-soluble forms. The major cause of chemical toxicity is UO_2^+. Macromolecules such as nucleic acids, proteins and lipids can be attacked, leading to DNA damage, enzyme inactivation and membrane damage [12, 13].

1.2.3. Effects of uranium in plants

Although U and its decay products are not essential for plant growth, they are **taken up by plants** [14]. Soil characteristics such as pH influence the form in which U is present, thereby influencing its uptake and transfer in plants [15]. The form of U that is most readily taken up and transferred to the shoots, is UO_2^+. However, U mainly accumulates in the roots with **very limited transfer to the shoots** [14].

To date, **information on U toxicity effects in plants is limited**. Moreover, the results of the available studies are often contradictory. Several endpoints and toxicity effects were reported, such as stunted lateral root growth, chlorosis and yellow turning roots [16]. In addition, certain studies have shown a transient hormesis response in which low U concentrations tend to increase plant growth [16, 17]. Uranium can also interfere with the uptake and distribution of several plant nutrients, thereby disturbing their function in plant cells [16]. It has also been shown to induce **oxidative stress**, as demonstrated by Vanhoudt *et al*. (2011) [18, 19]. In this study, exposure to U concentrations ranging from 0.1 to 100 µM for 24 to 72 h significantly affected the expression and activity of several antioxidative enzymes in roots and leaves of *A. thaliana* plants. The expression of certain lipoxygenases

and the concentrations of the antioxidative metabolites ascorbate (AsA) and glutathione (GSH) were affected as well. In addition, U is also able to induce **DNA damage** in animals [20, 21] and plants [17], as discussed in section 1.3.

1.3. DNA damage and repair, cell cycle arrest and endoreduplication

As mentioned before, both Cd and U can be taken up from contaminated soils by plants and cause disturbances of plant growth and development. These disturbances can be explained by two important characteristics of both metals: their **genotoxicity** and their ability to induce **oxidative stress**.

1.3.1. DNA damage and repair

Both U and Cd are **genotoxic**. Studies have demonstrated the induction of DNA damage by U in several animal species such as zebrafish and rats [20, 21]. Also in plants, U seems to be able to induce DNA damage, as shown by Vandenhove *et al.*(2006) [17]. In the latter study, exposure of *Phaseolus vulgaris* roots to 100 µM U significantly increased the percentage tail DNA in the comet assay, indicating an increased number of double strand DNA breaks (DSBs), while exposure to 1000 µM U completely dispersed the DNA [17]. Uranium can react with DNA either directly through DNA adducts or hydrolysis of the phosphate groups or indirectly through oxidative stress. Furthermore, it can induce mutations [20]. Cadmium is also able to induce DNA damage in plants, as demonstrated by Liu *et al.* (2012) [22]. It is capable of inducing point mutations, small inserts and deletions, rearrangements, ploidy changes, single and double strand breaks, base substitutions and oxidized bases [22].

DNA damage causes reduced protein synthesis, cell membrane destruction and damage to photosynthetic proteins, negatively affecting plant growth and development [23]. Plant cells can respond to DNA damage by undergoing **programmed cell death (PCD)** or by going into **cell cycle arrest** to enable DNA repair mechanisms to restore the damage [24].

1.3.2. Endoreduplication

Although cells stop dividing during cell cycle arrest, they can still replicate their nuclear genome in a process called **endoreduplication**. Endoreduplication is an alternative form of the cell cycle, in which DNA replication (S-phase) proceeds without mitosis (M-phase), resulting in **polyploidy**. It occurs in several organisms and is very common in plants [25]. The main challenge in establishing an endocycle is to keep the cyclin-dependent kinase (CDK) activity high enough to enable DNA replication, but below the level triggering mitosis [26]. Several important regulators of CDK activity and their interactions are presented in Fig. 1.

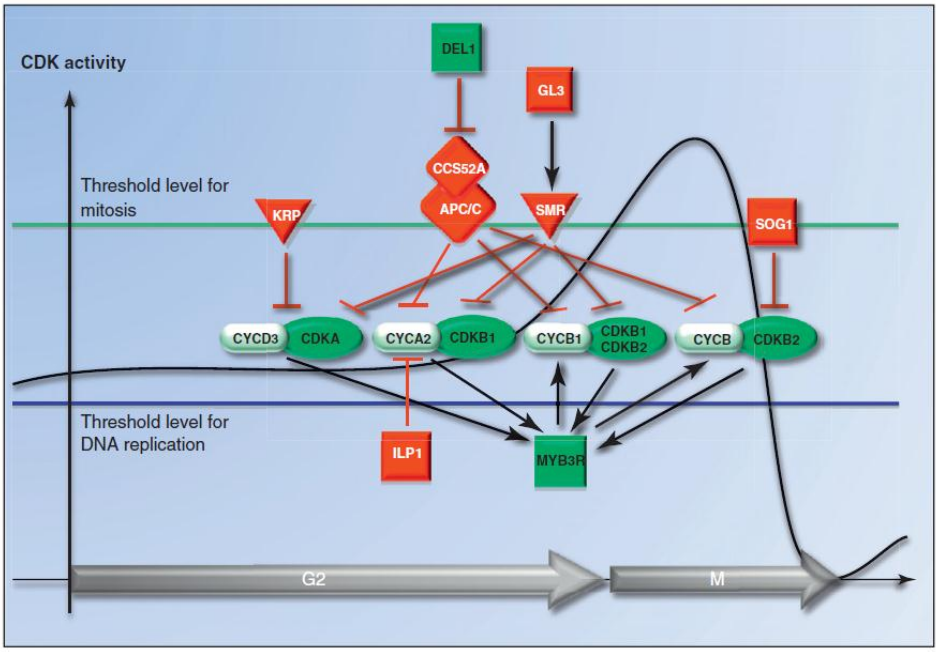

Figure 1. Regulatory wiring of the G2-M control machinery in mitosis and endoreduplication. Positive interactions are indicated with a black arrow, negative with a red T-line. Positive regulators of mitosis (negative regulators of endoreduplication) in green with black font color, positive regulators of endoreduplication (negative regulators of mitosis) in red with white font color. Transcriptional regulators are drawn in squares, CDK inhibitors in triangles, protein degradation machinery with a diamond. The long horizontal blue and green lines indicate hypothetical threshold levels for DNA replication and mitosis, respectively. CYC: cyclin; KRP: KIP-related protein; DEL1: DP-E2F-like 1; CCS52A: cell cycle switch protein 52A; APC/C: anaphase-promoting complex/cyclosome; GL3: GLABRA 3; SMR: SIAMESE-related; SOG1: suppressor of gamma radiation 1; ILP1: increased level of polyploidy 1; MYB3R: 3-repeat MYB protein (Adapted from De Veylder *et al.*, 2011 [26]).

The physiological significance of endoreduplication, however, is poorly understood. It might provide a mechanism to increase gene expression as more DNA template is available. Also, it might maintain an optimal ratio between cell and nuclear size [27]. Since the extent of endoreduplication in plants strongly depends on age, older cells often have a higher nuclear DNA content [25]. In addition, research has shown that endoreduplication in plants can be **affected by several stress factors**, such as DNA damage, Cd exposure, UV-B irradiation and exposure to 2-methoxyestradiol [28-31]. A likely benefit of entry into endoreduplication is that it prevents cells with damaged DNA from proliferating and passing on the damaged DNA to their daughter cells as well as from dying [30]. However, the exact mechanisms underlying the induction of endoreduplication under stress conditions are not fully elucidated yet.

1.4. Oxidative stress

A second mechanism by which Cd and U can disturb plant growth and development, is the induction of **oxidative stress**. This is defined as a disturbance of the balance between cellular pro- and antioxidants in favor of the former, leading to an increased production of ROS, such as superoxide (O_2^{o-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical (^oOH). **Increased production of ROS** can lead to degradation of proteins, cross-linking of DNA and peroxidation of membrane lipids. However, ROS are also important **signal transduction components**, as H_2O_2 can initiate signaling responses leading to enzyme activation, gene

expression and PCD. Therefore, it is important that ROS levels are kept within a certain range to enable signal transduction without causing damage to cellular macromolecules [10, 32, 33].

To prevent oxidative damage, organisms possess an extensive **antioxidative defense system** consisting of both enzymes and metabolites. Superoxide dismutase (SOD) catalyzes the dismutation of O_2^{o-} to H_2O_2. Subsequently, H_2O_2 can be eliminated by catalases (CATs) or peroxidases. Catalases directly convert H_2O_2 to H_2O and O_2. In contrast, the detoxification of H_2O_2 by peroxidases requires the oxidation of a co-substrate. Plants possess ascorbate peroxidases (APXs) and glutathione peroxidases, which use ascorbate (AsA) and glutathione (GSH), respectively, as a co-substrate to neutralize H_2O_2 [34]. Also peroxiredoxins, reducing H_2O_2 to H_2O via the oxidation of their own thiol groups, are part of the antioxidative systems. They are converted back to their reduced form by electron donors such as thioredoxins, glutaredoxins and GSH [34, 35]. Examples of antioxidative metabolites are the water soluble metabolites AsA and GSH, which are kept in their reduced form by the action of the AsA-GSH cycle. Finally, the lipid soluble vitamin E protects membranes against lipid peroxidation [36].

In physiological conditions, ROS are a by-product of mitochondrial metabolism in the respiratory **electron transport chain (ETC)** (Fig. 3a) [37]. In plants under (a)biotic stress, the electron carriers in the ETC often show an over-reduction, which can lead to an increased electron leakage. The leaked electrons can subsequently reduce O_2, thereby increasing ROS production [38]. Indeed, it is known from literature that Cd induces mitochondrial ROS production in animals [39] as well as in plants [40]. Also U seems to enhance mitochondrial ROS production at least in rat kidneys [41].

To limit mitochondrial ROS production, these organelles possess an extensive antioxidative defense system. In addition, plants possess several alternative components of the ETC, such as the alternative oxidases (AOXs). These stress-sensitive enzymes, located at the matrix side of the inner mitochondrial membrane, directly reduce O_2 to H_2O_2, thereby bypassing complexes III and IV and **reducing ROS production** [42]. Moreover, AOXs also play an important role in the avoidance of cell death under stress conditions [43]. Indeed, research has shown that cells with reduced AOX levels are more prone to the induction of PCD by H_2O_2, salicylic acid and the protein phosphatase inhibitor cantharidin [44]. Furthermore, AOX is an important target of **retrograde signaling** from the mitochondria to the nucleus in plants [43].

Higher plants possess two AOX gene families, *AOX1* and *AOX2*, each consisting of several isoforms. In *A. thaliana*, the model organism used in this study, *AOX1a* is the most dominant isoform [45]. Literature shows that AOXs are often induced under stress conditions in different plant species at the level of transcription, translation and enzyme activity [46-48]. Although no data are available to date that indicate the importance of AOX in U exposed plants, research has shown that exposure of *A. thaliana* to 5 and 10 µM Cd causes an increased AOX1a transcription and translation [49]. This leads to the **hypothesis that AOX1a plays a role in the oxidative stress response in metal-exposed *A. thaliana*.**

As research has shown that mitochondrial oxidative stress, caused by 2-methoxyestradiol, is able to induce endoreduplication in human HK-1 cells [31] and Cd and U are also known to induce oxidative stress, we **hypothesize that both metals induce endoreduplication** in *A. thaliana* leaves. We will also investigate the **involvement of**

AOX1a in this process, as it is induced by Cd exposure and is known to reduce mitochondrial ROS production, thereby possibly affecting endoreduplication.

1.5. Objectives

The main goal of this project is to gain more insight into **the role of AOX1a in oxidative stress, DNA damage and repair and endoreduplication in plants exposed to Cd and/or U**. The plant species used in this project, *A. thaliana*, is one of the most widely used model organisms in plant research. Its genetic information is widely available, simplifying the use of molecular techniques. In addition, it is relatively easy to grow and transform and multiple mutants are available [50].

In a first part of the project, the role of AOX1a in the oxidative stress response in Cd- and U-exposed *A. thaliana* plants is determined. Both wild-type and *aox1a*-knockout plants are cultivated in a hydroponic system. After 18 days, they are exposed to Cd, U or a combination of both metals for **24 h**, as several oxidative stress-related parameters significantly differed between metal-exposed and control plants at this time point in previous experiments [19, 51].

Subsequently, roots and leaves are harvested and their **metal uptake** is determined using inductively coupled plasma mass spectrometry (ICP-MS). Furthermore, we also keep track of the **growth** of control and metal-exposed seedlings.

Subsequently, the effects on several oxidative stress-related parameters are determined. Firstly, the expression of several pro- and antioxidative genes is determined using reverse transcription real-time polymerase chain reaction (RT-qPCR). Secondly, the capacity of six antioxidative enzymes is spectrophotometrically determined. Also the concentrations of GSH and AsA and their redox state are assessed using a plate reader method.

The next objective of the project is to determine the influence of AOX1a on the **amount of DNA damage and the extent of DNA repair** in plants exposed to Cd and/or U. The amount of 8-hydroxydeoxyguanosine (8-OHdG) is determined using ELISA, while the extent of DNA repair is assessed by the measurement of the expression of genes involved in several DNA repair pathways.

The third and last objective of the project is the assessment of the extent of **endoreduplication** in Cd- and U-exposed plants and the role of AOX1a in this process. Therefore, the nuclear DNA content is determined in the leaves of plants exposed to Cd, U or a combination of both metals for 24 or 72 h using flow cytometry. Furthermore, the expression of several genes possibly involved in the regulation of endoreduplication is determined after 24 h of exposure using RT-qPCR.

Finally, the effects of Cd and U exposure are compared between the two genotypes to get a better understanding of the role of AOX1a in metal-induced oxidative stress, DNA damage and repair and endoreduplication. In addition, the effects are compared between the Cd- and U-exposed plants and the plants exposed to a combination of both metals, to increase our insight into the interaction between both metals.

2. MATERIALS AND METHODS

In this project, wild-type and *aox1a*-knockout *A. thaliana* plants (ecotype Columbia) were exposed to Cd (5 or 10 µM), U (25 µM) or a combination of both metals (2.5 µM Cd and 12.5 µM U) for 24 h. Subsequently, the effects on several oxidative stress-related parameters, DNA damage and repair and endoreduplication were determined.

2.1. Plant culture and metal exposure

Wild-type and *aox1a*-knockout *A. thaliana* seeds were surface-sterilized and spread on moist filter paper at 4°C for 3 days to synchronize germination. They were sown on plugs from 1.5 mL polyethylene centrifuge tubes cut in half, filled with 0.6 % agar in a Hoagland solution with a low phosphate concentration. The plugs were put in a PVC cover on top of a container filled with 1.35 L of a modified Hoagland solution (consisting of 1 mM KNO_3, 0.3 mM $Ca(NO_3)_2$ x 4 H_2O, 0.2 mM $MgSO_4$ x 7 H_2O, 0.1 mM $NH_4H_2PO_4$, 1.62 mM $FeSO_4$ x 7 H_2O, 0.81 mM Na_2-EDTA x 2 H_2O, 4.6 µM H_3BO_3, 0.9 µM $MnCl_2$ x 4 H_2O, 32 nM $CuSO_4$ x 5 H_2O, 55.6 nM H_2MoO_4 and 76.5 nM $ZnSO_4$ x H_2O) , with each cover containing 36 plugs. The plants were grown in a growth chamber with a 14 h photoperiod (photosynthetic photon flux density of 160 to 170 mol $m^{-2}s^{-1}$ at the leaf level) , day/night temperatures of 22°C/18°C and a relative humidity of 65 %.

Subsequently, 18-day old seedlings were exposed to 5 µM $CdSO_4$, 10 µM $CdSO_4$, 25 µM $UO_2(NO_3)_2$ or a combination of 2.5 µM $CdSO_4$ and 12.5 µM $UO_2(NO_3)_2$. Since phosphate has been shown to limit the U availability to plants [52], Hoagland with a lower phosphate concentration (0.025 mM) was used after contamination of the plants with Cd and U. The pH of the Hoagland solution in the metal-contaminated containers was adjusted to the same value of that in the containers with control plants (approximately 5.5). After 24 or 72 h of exposure, roots and leaves were harvested, weighed, snap frozen in liquid nitrogen and stored at -80°C for further analyses except for endoreduplication and element analysis.

2.2. Cadmium and uranium analysis

Samples for Cd and U analysis were dried at 70°C for at least 1 week. To remove surface-bound Cd and U, roots were first washed for 10 minutes in 1 mM $Pb(NO_3)_2$ and twice for 10 minutes in dH_2O. After determination of their dry weight, root and leaf samples were dry-ashed in a muffle furnace at 550°C and digested in a 0.1 M HCl solution. Subsequently, the Cd and U content of the samples was determined using ICP-MS at the Chemistry Department of the SCK•CEN (Dr. Peter Van Bree).

2.3. Determination of gene expression using reverse transcription real-time PCR

Frozen root and leaf tissue was grounded using the MM 400 (Retsch) under frozen conditions. Then, RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Both the concentration and the purity of the RNA were determined using the Nanodrop® ND-1000

Spectrophotometer (Nanodrop Technologies, Inc). The RNA integrity was checked on the 2100 Bioanalyzer (Agilent Technologies). Subsequently, RNA was stored at -80°C.

Starting from the extracted RNA, cDNA was synthesised using the QuantiTect Reverse Transcription Kit (Qiagen). In order to obtain comparable results in real-time PCR, the same amount of starting RNA material (1 µg) was used for each sample. Subsequently, the cDNA was diluted 1/10 in RNase-free H_2O and stored at -20°C.

Real-time PCR reactions were performed according to the Fast SYBR® Green Master Mix Protocol (Applied Biosystems). Reactions were performed in 96-well plates in the 7900HT Fast Real-Time PCR System (Applied Biosystems). In each well, 8 µL of mastermix was added to 2 µL of 1/10 diluted cDNA. The mastermix consisted of 5 µL 2x Fast SYBR ® Green mastermix, 0.3 µL forward primer, 0.3 µL reverse primer and 2.4 µL RNase-free H_2O for each sample.

The data acquired from this experiment were normalized against the expression of three reference genes (roots: *TIP41* (AT4G34270), *SAND* (AT2G28390) and *F-BOX* (AT5G15710); leaves: *UBC* (AT5G25760), *SAND* and *F-BOX*) according to the $2^{-\Delta Ct}$ method [53]. The sequences of the forward and reverse primers used for the gene expression measurements, are presented in Table 1.

2.4. Analysis of enzyme capacities

Frozen root and leaf samples were grounded using the MM 400 (Retsch) and extracted in a buffer (pH 7.8) containing 0.1 M TRIS, 1 mM Na_2-EDTA and 1 mM DTT. Before shredding, a spatula tip of polyvinylpyrrolidone (PVP) was added, to adsorb polyphenols that could interfere with the enzymatic reactions. For the determination of APX capacities, the same extraction buffer was used after addition of 10 mM AsA. After shredding the samples, they were centrifuged (10 minutes, 13000 rpm, 4°C) and the supernatant was diluted 1/2 in the extraction buffer described above. The capacities of several antioxidative enzymes were determined spectrophotometrically in the diluted supernatant at 25°C.

The capacity of CAT was assessed in 96-well UV-plates using the PowerWave XS plate reader (BioTek). In each well, 10 µL of sample extract was added to 190 µL of a 49 mM H_2O_2 solution. The absorbance at 240 nm was monitored kinetically.

To determine the guaiacol peroxidase (GPX) capacity, 150 µL 0.1 M phosphate buffer, 10 µL sample extract and 40 µL guajacol mastermix, consisting of 90 mM guaiacol and 163 mM H_2O_2 mixed on a 1:1 ratio were added in each well of a plastic 96-well plate. Subsequently, the appearance of tetraguajacol was monitored kinetically at 436 nm.

Syringaldazine peroxidase (SPX) capacity was assessed in 96-well UV-plates. In each well, 155 µL 0.1 TRIS (pH 7.5), 20 µL 98 mM H_2O_2, 20 µL plant extract and 5 µL syringaldazine (SAZ) were added. The appearance of oxidized SAZ was monitored kinetically at 530 nm.

For the determination of the glutathione reductase (GR) capacity, 165 µL TRIS-EDTA buffer (0.1 M TRIS; 1 mM Na_2-EDTA) (pH 8), 7 µL GR mastermix (1:1 mix of 82 mM GSSG and 6 mM NADPH) and 28 µL were added in each well of a 96-well UV-plate. The decrease of NADPH, used for the reduction of GSSG, was followed kinetically at 340 nm.

The APX capacity was determined in UV-cuvettes using the Ultrospec 2000 UV/VIS Spectrophotometer (Pharmacia Biotech). In each cuvette, 665 µL HEPES-EDTA (0.1 M HEPES; 1mM EDTA) buffer, 100 µL 30 mM Na-ascorbate, 35 µL 196 mM H_2O_2 and 200 µL sample extract were added. Subsequently, the appearance of dehydroascorbate (DHA) was measured spectrophotometrically at 298 nm.

The activity of SOD was measured on the Ultrospec 2000 UV/VIS Spectrophotometer (Pharmacia Biotech) in plastic cuvettes. In each cuvette, 580 µL KH_2PO_4 buffer (pH 7.8), 100 µL1 mM EDTA, 100 µL 0.5 mM xanthine, 100 µL 0.1 mM cytochrome C, 100 µL extract and 20 µL xanthine oxidase (XOD) was added. The extent of cytochrome C reduction (measured at 550 nm) was compared to this in a blank, not containing any sample extract. Then, the relative inhibition of cytochrome C reduction by the sample extract was calculated as a measure of SOD capacity.

2.5. Metabolite analysis

Reduced, oxidized and total AsA and GSH concentrations were determined using a plate reader method for measuring redox couples adapted from Queval and Noctor [54]. As AsA levels in roots are often below the detection limit, AsA concentrations were only determined in leaf samples. Glutathione levels were assessed in both root and leaf samples.

Frozen root and leaf samples were grounded using the MM 400 (Retsch) and extracted into 600 µL of 200 mM HCl. After centrifugation (15 minutes, 13000 rpm, 4°C), 30 µL 200 mM NaH_2PO_4 (pH 5.6) was added to 300 µL of the supernatant. Subsequently, the pH of all samples was adjusted to 4.5 using 200 mM NaOH. For the measurement of total AsA, samples were incubated with 25 µM DTT and 120 mM NaH_2PO_4 (pH 7.5) for 15 minutes at 20°C to fully reduce the AsA pool. Subsequently, the pH of the samples was adjusted to pH 5.5, the optimal pH for ascorbate oxidase, using 200 mM HCl. Further, all measurements were performed as described by Queval and Noctor [54].

Oxidized AsA and reduced GSH were calculated as the difference between total and reduced AsA and total and oxidized GSH, respectively.

2.6. Detection of oxidative DNA damage

Frozen root and leaf tissue was grounded using the MM 400 (Retsch) under frozen conditions. Then, DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). The concentration and purity of the DNA were determined spectrophotometrically using the Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Inc).

To digest the DNA samples, 38 µL of DNA was incubated at 100°C for 2 minutes and treated overnight with 3 µL 250 mM potassium acetate buffer (pH 5.4), 3 µL 10 mM zinc sulphate and 2 µL nuclease P1 (6.25 U/µL; Sigma-Aldrich) at 37°C. Then, it was treated for 2 h with 6 µL 0.5 M Tris-HCl (pH 8.3) and 2 µL alkaline phosphatase (0.31U/µL; Sigma-Aldrich) at 37°C. Subsequently, the concentration of 8-OHdG was determined using the New 8-OHdG Check Kit (Japan Institute for the Control of Aging), which provides a competitive enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of 8-OHdG.

The assay was performed according to the manufacturer's instructions. Finally, the absorbance at 415 nm was measured using the PowerWave XS plate reader (BioTek).

2.7. Flow cytometric analysis of nuclear DNA content

The determination of the extent of endoreduplication was performed on fresh leaves, immediately after harvesting, using the CyStain PI Absolute P kit (Partec). For each condition, four to six fresh rosettes were analyzed. The three youngest leaves with a size of at least 1 mm were separately chopped with a fresh razor blade in a petri dish containing 500 µL extraction buffer. After incubation in the buffer for approximately 1 minute, the solution was filtered through a 50 µm nylon filter (Celltrics) and 2 mL staining solution, consisting of 2 mL staining buffer, 120 µL propidium iodide (PI) solution and 6 µL RNase solution per sample, was added to the flow-through. The samples were then incubated in the dark for at least one hour and their nuclear DNA content was analyzed on the BD Accuri C6 Flow Cytometer (BD Biosciences) with a FL2 585/40 nm filter.

2.8. Statistical analysis

The statistical analysis of the data obtained in the experiments described above, was performed for each genotype separately using a one-way ANOVA. Normality and homoscedasticity of the data were verified using the Shapiro-Wilk and Fligner-Killeen test, respectively. When necessary, data were transformed (logarithm, exponent, inverse or square root). Gene expression data were standardly log transformed. As a post hoc test, the Tukey multiple comparison of means was used. In case the assumptions for ANOVA were not met, statistical analysis of the data was performed using a non-parametric Kruskal-Wallis test, followed by the post hoc Wilcoxon test. The False Discovery Rate (FDR) control was used to correct for multiple comparisons. All statistical analyses were performed in R (version 2.15.1, The R Foundation for Statistical Computing).

Table 1. Overview of the primer sequences (5'-3') used to determine the expression of the reference genes and genes of interest using RT-qPCR. *TIP41*: TIP41-like; *UBC*: ubiquitin conjugating enzyme; *SAND*: SAND-family; *F-BOX*: F-BOX protein; *AOX1a*: alternative oxidase 1a; *LOX*: lipoxygenase; *RBOH*: respiratory burst oxidase homolog; *CSD*: Cu/Zn superoxide dismutase; *FSD*: Fe superoxide dismutase; *MSD1*: Mn superoxide dismutase 1; *CAT*: catalase; *APX1*: ascorbate peroxidase 1; *GR*: glutathione reductase; *ATM*: ataxia-telangiectasia mutated; *ATR*: ATM- and RAD3-related; *PARP*: poly(ADP-ribose) polymerase; *XRCC1*: homolog of X-ray repair cross complementing 1; *LIG4*: ligase 4; *MMH*: formamidopyrimidine DNA glycosylase; *OGG1*: 8-oxoguanine DNA glycosylase 1; *PCNA*: proliferating cellular nuclear antigen; *MSH*: MUTS homolog; *CCS52A*: cell cycle switch protein 52A; *DEL1*: DP-E2F-like 1; *GL3*: GLABRA3; *ILP1*: increased level of polyploidy 1; *KRP*: KIP-related protein; *SMR*: SIAMESE-related.

GENE	FORWARD PRIMER	REVERSE PRIMER
REFERENCE GENES		
TIP41	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
UBC	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
SAND	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
F-BOX	TTTCGGCTGAGAGGTTCGAGT	GATTCCAAGACGTAAAGCAGATCAA
ALTERNATIVE RESPIRATION		
AOX1a	CTCTTCGTTGGCCTACCGATT	AACCATTCCAGGTACTGCTGCTAC
PRO-OXIDATIVE GENES		
LOX1	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC
LOX2	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC
RBOHC	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
RBOHD	AACTCTCCGCTGATTCCAACG	TGGTCAGCGAAGTCTTTAGATTCCT
ANTIOXIDATIVE GENES		
CSD1	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
CSD2	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
FSD1	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
FSD2	TTGGAAAGGTTCAAGTCGGCT	CATTTGCAACGTCAAGTCTATTCG
FSD3	AACGGGAATCCTTTACCCGA	TGTCTCCACCACCAGGTTGC
MSD1	ATGTTTGGGAGCACGCCTAC	AACCTCGCTTGCATATTTCCA
CAT1	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
CAT2	AACTCCTCCATGACCGTTGGA	TCCGTTCCCTGTCGAAATTG
CAT3	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA
APX1	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
GR1	CTCAAGTGTGGAGCAACCAAAG	ATGCGTCTGGTCACACTGC
GR2	GCCCAGATGGATGGAACAGAT	TAGGGTTGGAGAATGTTGGCG
DNA REPAIR		
ATM	TGATGTGCGCTGTTTCAGCTAA	GATGGGTAATGGAGTTGTGCTGATA
ATR	GCCGATGAAGCCGAGATACTT	GTAAGCGGTTTATCATCGCAGTAAA

PARP1	TGCATTGGGAGAAATACATGAGC	CCGAGCCCTTTGGTCGAG
PARP2	ATCGGAGGTGATTGATCGGTATG	AAATCATGAGGTATCACTGTGTAGAACTCT
XRCC1	TGGGCCAGGGATGACCTAAG	CCGCAGCTATTCGCTTGATTT
KU80	CTTCTTCCAGCACAACTCCTCAA	CTACGCATCGCAGGACCTACAT
LIG4	TGATGTATCGG ATATCAAGGGCA	GAATGGGACCGAGGCACG
RAD51	GTCCAACAACAAGACGATGAAGAA	AACAGAAGCAATACCTGCTGCC
MMH	GGTGCTATTTATATCAAAGGCGTTG	ACTTTGAATACTTAGATGGCCACTCC
OGG1	AAGAGTTTAGAAAGGCTGGTTTTGG	TCATTACCACCACCTGGCTTTG
PCNA1	GTGCTCAGGCAGAACACAACTG	ACCTCAGGGCAAATGAGAGAGAC
PCNA2	GGACAGCAAACATTGTGCTCAG	GAAAGCGAAACAGGCTCGTTC
MSH2	GACTGCATCTTTGCCCGTGT	ATATCGATGCGGTTTCAAGCA
MSH7	ATCAACTCTTCTTCGTGCAACATGT	CACGAGGGAGATTTCGCAAG
ENDOREDUPLICATION		
CCS52A1	AGTGGGACTAGGGAACTGTGTGTATT	GCAAACACTATCCTCAGCTCCG
CCS52A2	AATTGCGTAGATACCAACAGCCA	GGTTCTGGGAATACCCGTGTGT
DEL1	CTCCCTTGATGACGCTGCAA	TGCTATATCATAAAGCCGCCTCAC
GL3	CCGAGTGGTACTATTTGGTTTGTATGT	TTGCACAACCATATCGGTTCAC
ILP1	TCACACGAACCCACAGTCG	TGAGCCAAGCCTTTTCTCGA
KRP1	AATTGATGACGGAGATGCCAAC	CTAATGGCTTCTCCTTCTCGAAATC
KRP2	GGAATAAGTTGTTGGAATGTTCTATGAAGT	AACCCACTCGTATCTTCCTCCAC
SIAMESE	ATCGAGCGGTTCTTCTCCTCTG	GGAAACTTCTTCGCCGCTTG
SMR1	CAAGATCCGATCCAAAACCTCA	TTCTTGGGATGTGGGTGTGC
SMR4	TGATGGTGGTGAGAAAACGAGA	TCTCTTCGAGGCTGTGCGTAG
SMR5	CAGCATATCCGCCTTGTCCA	CTGCTACCACCGAGAAGAACAAGT
SMR7	ACATCGATTCGGGCTTCACTAA	CCGTGGGAGTGATACAAATTCC

3. RESULTS

To investigate the role of AOX1a in oxidative stress, DNA damage and repair and endoreduplication in metal-exposed *A. thaliana*, both wild-type and *aox1a*-knockout plants were exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h, after which the effects on several parameters involved in these processes were determined and compared between the two genotypes. The *aox1a*-knockout plants contain a T-DNA insertion in both alleles of the *AOX1a* gene, causing them to have no functional AOX1a enzymes.

3.1. Growth responses

After exposure to Cd, U or a combination of both metals for 24 h, the fresh weight of roots and leaves was determined (Fig. 2). The results indicate that metal exposure caused a significantly decreased root growth of both wild-type and *aox1a*-knockout plants after 24 h already (Fig. 2A). In contrast to exposure to a single stressor, combined exposure to Cd and U did not significantly affect root growth of wild-type plants at this time point. In *aox1a*-knockout mutants, however, combined Cd and U exposure did lead to decreased root growth, although to a lesser extent than exposure to only one these metals (Fig. 2A).

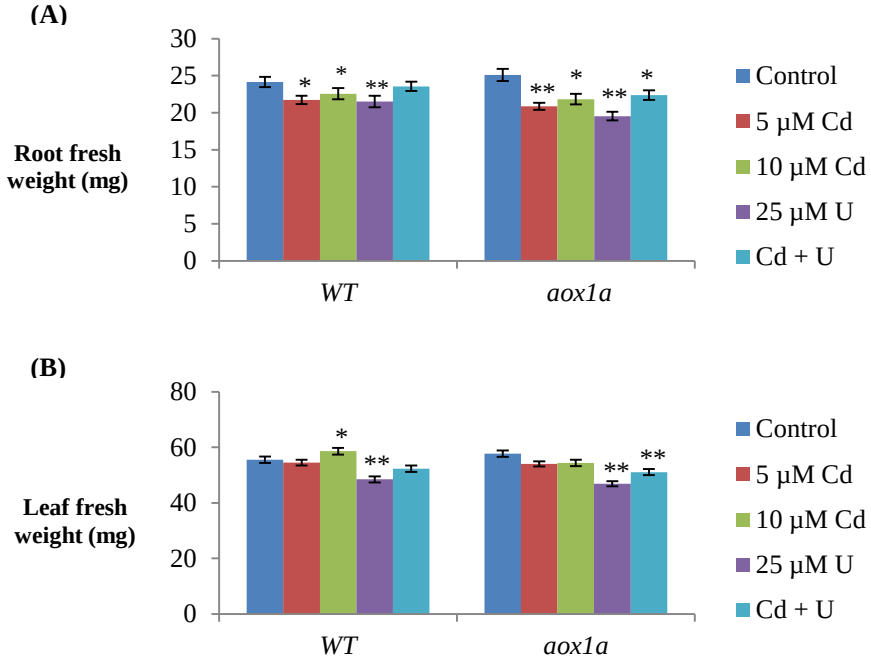

Figure 2. Fresh weight (mg) of roots (A) and leaves (B) of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of at least 130 biological independent replicates. Significance levels compared to its own genotype under control conditions (Non-parametric test): *: $p < 0.05$; **: $p < 0.01$.

In leaves, slightly different growth responses were observed as compared to roots, as leaf growth was not affected by exposure to 5 µM Cd (Fig. 2B). Exposure to 10 µM Cd, however, significantly increased leaf growth of wild-type plants, which was not observed for mutant leaves. Exposure to 25 µM U significantly reduced leaf growth in both genotypes, while the

decrease in leaf fresh weight after combined exposure to Cd and U was again only significant in *aox1a*-knockout seedlings (Fig. 2B).

3.2. Cadmium and uranium content

In order to determine the amount of Cd and U taken up by the plants from the metal-contaminated Hoagland solution, Cd and U concentrations in roots and leaves were determined using ICP-MS.

The Cd and U contents in roots are presented in Table 2. The uptake of both metals in roots was highly similar between wild-type and *aox1a*-knockout seedlings (Table 2). Although under control conditions a small amount of Cd (close to the detection limit) was present in roots, this amount greatly increased after exposure to 5 or 10 µM Cd and a combination of Cd and U. The uptake of Cd after exposure to 10 µM Cd was about three times the amount taken up from a Hoagland solution containing 5 µM Cd. The amount of Cd taken up from the Hoagland solution containing 2.5 µM Cd and 12.5 µM U, was slightly higher than half of the concentration taken up after exposure to 5 µM Cd. In U-exposed roots, a slight increase in Cd uptake compared to the control could be observed as well. However, this increase was negligible as compared to the increases observed after exposure to Cd or a combination of Cd and U (Table 2).

The same response was observed for U uptake in roots exposed to 10 µM Cd (Table 2). Although a slight increase in U uptake was visible under these exposure conditions, it was negligible compared to the increase in U uptake after exposure to U or a combination of Cd and U. The amount of U taken up by the roots of plants exposed to a combination of 2.5 µM Cd and 12.5 µM U was about half of the amount taken up by the roots of seedlings exposed to 25 µM U (Table 2).

Table 2. Metal concentrations (µg/g DW) in **roots** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of at least 3 biological independent replicates. Significance levels compared to its own genotype under control conditions (One-way ANOVA): : $p < 0.05$; : $p < 0.01$. N.D.: not detectable.

WT					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
Cd	2.29 ± 0.76	614.33 ± 25.25	1866 ± 85.16	8.62 ± 1.14	369.22 ± 5.88
U	0.93 ± 0.13	N.D.	12.07 ± 2.93	6787.71 ± 245.24	3249.51 ± 124.52
aox1a					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
Cd	1.57 ± 0.14	616.13 ± 23.80	1806.72 ± 30.15	9.81 ± 0.79	388.67 ± 24.58
U	1.52 ± 0.17	2.09 ± 0.51	11.48 ± 2.34	7167.08 ± 282.83	3944.02 ± 345.92

In Table 3, Cd and U contents in leaves of metal-exposed seedlings are presented. As in roots, metal uptake in leaves was highly similar between the two genotypes (Table 3). While exposure to 5 µM Cd caused a significant increase in Cd concentrations in leaves, this increase was almost doubled after exposure to 10 µM Cd. Exposure to a combination of 2.5 µM Cd and 12.5 µM U caused an increase in leaf Cd concentrations, which was about half the size of the increase induced by exposure to 5 µM Cd (Table 3).

Exposure to 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U significantly increased U concentrations in the leaves of *A. thaliana* seedlings. The increase caused by U exposure was about three times the increase caused by combined exposure to Cd and U (Table 3). However, the U concentrations in leaves were very low as compared to those observed in roots.

Table 3. Metal concentrations (µg/g DW) present in **leaves** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of at least 3 biological independent replicates. Significance levels compared to its own genotype under control conditions (One-way ANOVA): : $p < 0.05$; : $p < 0.01$. N.D.: not detectable.

WT					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
Cd	1.14 ± 0.75	453.81 ± 19.18	875.90 ± 73.79	0.96 ± 0.28	192.74 ± 16.73
U	0.08 ± 0.01	0.10 ± 0.02	0.08 ± 0.01	1.80 ± 0.09	0.64 ± 0.09
aox1a					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
Cd	0.30 ± 0.09	437.30 ± 8.80	896.25 ± 70.55	0.81 ± 0.20	203.02 ± 13.92
U	0.08 ± 0.01	N.D.	0.10 ± 0.02	2.22 ± 0.69	0.68 ± 0.15

3.3. Oxidative stress-related responses in an 'omics' approach

In order to get more insight in the oxidative stress response induced in metal-exposed *A. thaliana*, three parameters were determined in roots and leaves of plants exposed to Cd and/or U at different biological organization levels: (1) expression of selected pro- and antioxidative genes, (2) antioxidative enzyme capacities and (3) AsA and GSH concentrations and their redox state. To investigate the role of AOX1a in the metal-induced oxidative stress response, the effects on these parameters were determined in both wild-type and *aox1a*-knockout seedlings and compared between both genotypes.

3.3.1. Expression of selected pro- and antioxidative genes

The expression of several pro- and antioxidative genes was determined in wild-type and *aox1a*-knockout plants exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. The expression of *AOX1a* was assessed as well to verify earlier results and check its response to U exposure. Results of the measurements are presented in Table 4 for the roots and Table 5 for the leaves.

Under control conditions, the expression of most of the genes measured did not significantly differ between the two genotypes in roots (Table 4). Moreover, in general changes in gene expression caused by Cd, U and combined exposure to both metals were very similar between roots of wild-type and *aox1a*-knockout mutants, although significance levels sometimes differed between both genotypes (Table 4).

In roots, ***AOX1a* expression** significantly increased by exposure to any of the four metal exposures, with the strongest induction observed in U-exposed roots. As expected, *AOX1a* was not reliably detected in mutant roots (Table 4).

In Table 4, it is shown that the expression of several **pro-oxidative genes** was significantly affected by metal exposure in the roots of wild-type and *aox1a*-knockout plants. The

expression levels of lipoxygenase 1 (*LOX1*) and respiratory burst oxidase homolog D (*RBOHD*) significantly increased by exposure to both 5 and 10 µM Cd. A similar, though much weaker, increasing trend in *LOX1* and *RBOHD* gene expression could be observed after exposure to U and a combination of Cd and U in the mutant roots. In contrast to *RBOHD*, *RBOHC* levels were decreased by exposure to 10 µM Cd and 25 µM U in both genotypes.

Exposure to Cd, U or a combination of both metals also significantly altered the expression of several **antioxidative genes** in leaves of both genotypes. The expression of Cu/Zn superoxide dismutase (*CSD1*) decreased after exposure to 5 µM Cd, 25 µM U and a combination of both metals, with U having a stronger effect as compared to Cd. The effect of Cd on *CSD1* expression could not be observed when a concentration of 10 µM was applied (Table 4). While the expression of *CSD2* was decreased by all four metal exposure conditions in both genotypes, exposure to 25 µM U and a combination of Cd and U caused the largest decrease (Table 4). The three different Fe superoxide dismutase (*FSD*) isoforms were differently affected by metal exposure. While Cd and U exposure both strongly increased *FSD1* expression, both metals downregulated *FSD2* and *FSD3* expression. Combined exposure to Cd and U upregulated *FSD1*, while *FSD2* and *FSD3* expression stayed largely unaffected (Table 4). In general, the effect of the four metal exposures on the expression of manganese superoxide dismutase 1 (*MSD1*), the only mitochondrial SOD isoform, was very small, although some differences were statistically significant. Regarding H_2O_2 scavenging enzymes, the three *CAT* isoforms all were upregulated or unaffected by exposure to Cd. In contrast, exposure to U and a combination of Cd and U caused a decreasing trend in *CAT* expression. Expression of *APX1* increased in both genotypes after exposure to 25 µM U, while the other three exposure conditions had little effect. Expression levels of *GR1*, the cytosolic *GR* isoform, in contrast, were significantly elevated by all metal exposures, while expression of the *GR2*, the *GR* isoform located in chloroplasts and mitochondria was only increased after exposure to 5 µM in the mutant roots (Table 4).

Table 4. Expression levels of *AOX1a* and oxidative stress-related genes in **roots** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* plants exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. In the first column ('UNEXPOSED') the gene expression under control conditions is compared for both genotypes. The columns 'WT' and '*aox1a'* give an overview of the responses of wild-type and *aox1a*-knockout plants, respectively. The expression in metal-exposed roots is represented relative to their own control (= 1.00 ± S.E.). Values are the mean ± S.E. of at least 3 biological independent replicates. Significance levels (One-way ANOVA): = $p < 0.05$; = $p < 0.01$; = $p < 0.05$; = $p < 0.01$ for an increased and decreased expression, respectively. *AOX*: alternative oxidase; *CSD:* copper/zinc superoxide dismutase; *FSD:* iron superoxide dismutase; *MSD:* manganese superoxide dismutase; *CAT*: catalase; *APX:* ascorbate peroxidase; *GR*: glutathione reductase; *LOX*: lipoxygenase; *RBOH*: respiratory burst oxidase; N.D.: not detectable.

ROOTS	**UNEXPOSED**		**WT**				***aox1a***			
	WT	*aox1a*	5 µM Cd	10 µM Cd	25 µM U	Cd + U	5 µM Cd	10 µM Cd	25 µM U	Cd + U
ALTERNATIVE RESPIRATION										
AOX1a	1.00 ± 0.01	N.D.	2.46 ± 0.29	6.12 ± 0.07	6.80 ± 0.59	3.17 ± 0.70	N.D.	N.D.	N.D.	N.D.
PRO-OXIDATIVE GENES										
LOX1	1.00 ± 0.10	0.75 ± 0.06	6.00 ± 1.06	50.00 ± 5.84	1.46 ± 0.07	1.18 ± 0.33	10.87 ± 2.18	85.18 ± 14.07	2.17 ± 0.32	1.35 ± 0.13
RBOHC	1.00 ± 0.08	0.86 ± 0.08	0.89 ± 0.14	0.74 ± 0.06	0.42 ± 0.02	0.87 ± 0.07	1.49 ± 0.05	0.74 ± 0.12	0.51 ± 0.07	0.86 ± 0.09
RBOHD	1.00 ± 0.15	0.83 ± 0.06	2.66 ± 0.51	5.71 ± 0.57	1.08 ± 0.17	1.37 ± 0.08	4.90 ± 0.19	5.85 ± 1.02	1.73 ± 0.32	1.32 ± 0.01
ANTIOXIDATIVE GENES										
CSD1	1.00 ± 0.03	1.06 ± 0.04	0.55 ± 0.14	0.96 ± 0.07	0.08 ± 0.00	0.18 ± 0.01	0.53 ± 0.05	1.07 ± 0.07	0.07 ± 0.00	0.17 ± 0.01
CSD2	1.00 ± 0.03	1.00 ± 0.03	0.64 ± 0.02	0.70 ± 0.05	0.08 ± 0.00	0.33 ± 0.02	0.85 ± 0.20	0.72 ± 0.05	0.08 ± 0.01	0.34 ± 0.01
FSD1	1.00 ± 0.03	0.87 ± 0.05	4.80 ± 0.24	3.47 ± 0.22	11.28 ± 0.57	7.67 ± 0.25	5.25 ± 0.22	5.09 ± 0.59	12.41 ± 1.26	9.89 ± 0.83
FSD2	1.00 ± 0.06	0.99 ± 0.05	0.77 ± 0.03	0.47 ± 0.05	0.72 ± 0.04	1.13 ± 0.06	0.80 ± 0.03	0.43 ± 0.04	0.69 ± 0.06	1.07 ± 0.13
FSD3	1.00 ± 0.06	0.84 ± 0.06	0.67 ± 0.04	0.38 ± 0.05	0.76 ± 0.04	0.81 ± 0.06	0.90 ± 0.08	0.47 ± 0.03	0.83 ± 0.01	0.86 ± 0.08
MSD1	1.00 ± 0.01	1.08 ± 0.03	1.02 ± 0.00	1.20 ± 0.05	1.17 ± 0.02	1.18 ± 0.06	0.91 ± 0.03	1.16 ± 0.06	1.08 ± 0.09	1.32 ± 0.07
CAT1	1.00 ± 0.04	0.96 ± 0.10	2.04 ± 0.17	5.79 ± 0.38	0.90 ± 0.03	0.63 ± 0.05	1.74 ± 0.10	8.10 ± 0.31	0.84 ± 0.03	0.63 ± 0.05
CAT2	1.00 ± 0.12	0.75 ± 0.07	1.17 ± 0.13	1.12 ± 0.13	0.40 ± 0.04	0.59 ± 0.05	1.67 ± 0.17	1.19 ± 0.20	0.65 ± 0.12	0.72 ± 0.05
CAT3	1.00 ± 0.07	0.87 ± 0.09	1.03 ± 0.20	2.10 ± 0.31	0.42 ± 0.06	0.46 ± 0.04	1.64 ± 0.06	3.10 ± 0.55	0.59 ± 0.12	0.56 ± 0.05
APX1	1.00 ± 0.09	0.89 ± 0.08	0.88 ± 0.07	1.01 ± 0.05	2.12 ± 0.13	1.20 ± 0.03	1.20 ± 0.15	1.22 ± 0.08	2.47 ± 0.30	1.33 ± 0.12
GR1	1.00 ± 0.02	0.98 ± 0.03	1.58 ± 0.05	2.20 ± 0.19	2.85 ± 0.11	1.59 ± 0.07	1.46 ± 0.05	3.02 ± 0.40	2.90 ± 0.08	1.59 ± 0.07
GR2	1.00 ± 0.05	0.84 ± 0.05	1.07 ± 0.10	1.05 ± 0.07	0.80 ± 0.03	0.84 ± 0.02	1.53 ± 0.16	1.12 ± 0.08	1.00 ± 0.08	0.96 ± 0.09

In leaves, generally all genes showed a slightly lower expression in the mutant under control conditions as compared to the wildtype, possibly pointing to a lower basal gene expression level (Table 5). As was the case for roots, changes in gene expression levels measured in metal-exposed leaves were similar for both genotypes (Table 5).

In wild-type leaves, ***AOX1a* expression** increased after exposure to 5 and 10 µM Cd. Exposure to 25 µM U and a combination of Cd and U did not significantly affect leaf *AOX1a* levels. As expected, *AOX1a* levels could not be measured in mutant leaves (Table 5).

As in roots, expression of several **pro-oxidative genes** was significantly altered in the leaves. The expression of all pro-oxidative genes measured either remained unaltered or was upregulated in response to Cd and/or U exposure (Table 5). While *LOX2* levels significantly increased in mutant leaves after exposure to all four metal exposures, no significant changes were observed in leaves of wild-type seedlings. On the other hand, *LOX1* expression was significantly upregulated in both genotypes after exposure to 10 µM Cd. While *RBOHC* expression levels strongly increased by exposure to 5 and 10 µM Cd and combined exposure to Cd and U, *RBOHD* expression remained unaltered after exposure to any of these metals (Table 5).

As in roots, expression of several **antioxidative genes** was significantly altered in the leaves (Table 5). In general, responses in *aox1a*-knockout leaves were highly similar to those observed in wild-type leaves. While *CSD2* expression strongly decreased after exposure to all four metal exposures, *CSD1* expression was only lowered by U and combined exposure to Cd and U. Leaf *FSD1* expression levels significantly increased by exposure to 25 µM U in both genotypes. The combination of Cd and U also elevated *FSD1* expression, although this was only significant in mutant leaves. Leaf *FSD2* and *FSD3* expression were downregulated by all four metal exposure conditions., while *MSD1* expression was not affected by any of the metals (Table 5). While *CAT3* expression was downregulated by all four conditions, this response was only observed after exposure to 5 µM Cd and combined exposure to Cd and U for *CAT1*. Expression levels of *CAT2* showed a decreasing trend in the wild-type leaves after exposure to most of the metals, while the opposite was observed in mutant leaves (Table 5). In both genotypes, *APX1* expression was upregulated by Cd exposure. However, it was significantly downregulated in wild-type leaves exposed to a combination of Cd and U, a response that was not observed in mutant leaves (Table 5). After Cd exposure, a strong upregulation of *GR1* expression was observed in the leaves of both genotypes, while *GR2* expression decreased (Table 5).

Table 5. Expression levels of *AOX1a* and oxidative stress-related genes in **leaves** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* plants exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. In the first column ('UNEXPOSED') the gene expression under control conditions is compared for both genotypes. The columns 'WT' and '*aox1a'* give an overview of the responses of wild-type and *aox1a*-knockout plants, respectively. The expression in metal-exposed leaves is represented relative to their own control (= 1.00 ± S.E.). Values are the average ± S.E. of at least 3 biological independent replicates. Significance levels (One-way ANOVA): = $p < 0.05$; = $p < 0.01$; = $p < 0.05$; = $p < 0.01$ for an increased and decreased expression, respectively. *AOX*: alternative oxidase; *CSD:* copper/zinc superoxide dismutase; *FSD:* iron superoxide dismutase; *MSD:* manganese superoxide dismutase; *CAT*: catalase; *APX:* ascorbate peroxidase; *GR*: glutathione reductase; *LOX*: lipoxygenase; *RBOH*: respiratory burst oxidase; N.D.: not detectable.

LEAVES	UNEXPOSED		WT				*aox1a*			
	WT	*aox1a*	5 µM Cd	10 µM Cd	25 µM U	Cd + U	5 µM Cd	10 µM Cd	25 µM U	Cd + U
ALTERNATIVE RESPIRATION										
AOX1a	1.00 ± 0.09	N.D.	2.77 ± 0.22	2.92 ± 0.36	1.24 ± 0.07	0.88 ± 0.24	N.D.	N.D.	N.D.	N.D.
PRO-OXIDATIVE GENES										
LOX1	1.00 ± 0.19	0.72 ± 0.05	1.17 ± 0.11	2.31 ± 0.22	0.86 ± 0.02	1.02 ± 0.24	1.91 ± 0.37	3.70 ± 0.58	1.18 ± 0.08	1.08 ± 0.17
LOX2	1.00 ± 0.20	0.57 ± 0.02	0.67 ± 0.03	1.30 ± 0.32	0.99 ± 0.07	0.54 ± 0.19	1.84 ± 0.34	1.68 ± 0.06	1.80 ± 0.04	1.44 ± 0.12
RBOHC	1.00 ± 0.28	0.93 ± 0.30	117.78 ± 3.74	128.87 ± 22.23	1.56 ± 0.62	39.46 ± 9.82	206.90 ± 60.14	107.02 ± 30.20	1.83 ± 0.47	28.38 ± 18.45
RBOHD	1.00 ± 0.10	0.83 ± 0.25	0.87 ± 0.11	1.57 ± 0.18	1.22 ± 0.29	1.74 ± 0.55	1.61 ± 0.06	1.72 ± 0.21	1.28 ± 0.13	1.39 ± 0.16
ANTIOXIDATIVE GENES										
CSD1	1.00 ± 0.07	0.76 ± 0.04	0.97 ± 0.07	0.72 ± 0.08	0.13 ± 0.01	0.17 ± 0.01	0.94 ± 0.12	1.02 ± 0.11	0.18 ± 0.03	0.24 ± 0.02
CSD2	1.00 ± 0.12	0.98 ± 0.03	0.19 ± 0.00	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.13 ± 0.02	0.12 ± 0.02	0.14 ± 0.05	0.11 ± 0.03
FSD1	1.00 ± 0.19	0.77 ± 0.13	0.63 ± 0.04	0.66 ± 0.01	2.72 ± 0.17	1.61 ± 0.29	0.65 ± 0.04	0.97 ± 0.17	3.55 ± 0.51	2.24 ± 0.33
FSD2	1.00 ± 0.09	0.92 ± 0.05	0.25 ± 0.01	0.19 ± 0.02	0.55 ± 0.04	0.59 ± 0.16	0.27 ± 0.02	0.19 ± 0.02	0.60 ± 0.03	0.86 ± 0.05
FSD3	1.00 ± 0.13	0.90 ± 0.01	0.16 ± 0.02	0.14 ± 0.02	0.48 ± 0.03	0.52 ± 0.14	0.25 ± 0.01	0.20 ± 0.02	0.47 ± 0.02	0.67 ± 0.05
MSD1	1.00 ± 0.07	0.90 ± 0.01	0.94 ± 0.02	0.94 ± 0.05	0.81 ± 0.04	0.88 ± 0.04	0.80 ± 0.08	0.93 ± 0.04	1.02 ± 0.04	1.02 ± 0.03
CAT1	1.00 ± 0.09	0.88 ± 0.10	0.61 ± 0.04	1.01 ± 0.15	0.68 ± 0.01	0.40 ± 0.09	0.48 ± 0.06	1.44 ± 0.16	0.71 ± 0.01	0.59 ± 0.08
CAT2	1.00 ± 0.13	0.66 ± 0.12	0.49 ± 0.04	0.60 ± 0.05	1.08 ± 0.22	0.68 ± 0.18	1.34 ± 0.10	1.12 ± 0.13	1.74 ± 0.28	1.26 ± 0.07
CAT3	1.00 ± 0.02	0.84 ± 0.06	0.17 ± 0.02	0.37 ± 0.05	0.50 ± 0.13	0.38 ± 0.13	0.36 ± 0.03	0.54 ± 0.07	0.57 ± 0.07	0.52 ± 0.05
APX1	1.00 ± 0.07	0.67 ± 0.05	1.22 ± 0.07	1.39 ± 0.14	0.83 ± 0.05	0.64 ± 0.02	1.59 ± 0.11	2.55 ± 0.23	1.08 ± 0.02	1.03 ± 0.13
GR1	1.00 ± 0.05	0.76 ± 0.03	4.21 ± 0.12	3.07 ± 0.32	1.24 ± 0.07	1.08 ± 0.09	3.09 ± 0.50	4.71 ± 0.36	1.60 ± 0.13	1.48 ± 0.10
GR2	1.00 ± 0.10	0.84 ± 0.10	0.39 ± 0.03	0.50 ± 0.01	0.95 ± 0.07	0.67 ± 0.17	0.55 ± 0.05	0.61 ± 0.02	1.17 ± 0.15	1.01 ± 0.02

3.3.2. Antioxidative enzyme capacities

The **capacities of the antioxidative enzymes** SPX, GPX, CAT, APX and GR were spectrophotometrically determined in both roots and leaves. In leaf samples, the SOD capacity was determined as well. The results of these measurements in roots and leaves are presented in Table 6 and Table 7, respectively. In both organs, the response of enzyme capacities to metal exposure was highly similar between the two genotypes.

Table 6. Capacities (mU/g FW) of antioxidative enzymes in **roots** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of at least 3 biological independent replicates. SPX: syringaldazine peroxidase; GPX: guaiacol peroxidase; CAT: catalase; APX: ascorbate peroxidase; GR: glutathione reductase. Significance levels compared to its own genotype under control conditions (One-way ANOVA): : $p < 0.05$; : $p < 0.01$.

WT					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
SPX	65.73 ± 0.80	60.70 ± 3.19	52.02 ± 4.73	53.12 ± 2.63	50.80 ± 2.66
GPX	86.18 ± 7.60	83.49 ± 3.68	83.44 ± 4.50	85.10 ± 6.11	71.17 ± 3.35
CAT	0.26 ± 0.02	0.28 ± 0.03	0.26 ± 0.00	0.20 ± 0.02	0.26 ± 0.02
APX	29.59 ± 2.05	33.28 ± 0.57	35.35 ± 2.76	45.46 ± 0.66	31.57 ± 1.03
GR	0.61 ± 0.09	0.46 ± 0.11	0.45 ± 0.10	0.40 ± 0.07	0.49 ± 0.01
aox1a					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
SPX	64.41 ± 4.36	60.37 ± 4.28	57.48 ± 5.11	53.41 ± 3.50	51.94 ± 1.43
GPX	81.82 ± 3.48	87.48 ± 3.76	87.05 ± 3.19	94.00 ± 6.52	78.17 ± 3.26
CAT	0.26 ± 0.02	0.27 ± 0.02	0.31 ± 0.02	0.22 ± 0.02	0.29 ± 0.01
APX	33.25 ± 0.82	37.51 ± 1.32	35.27 ± 0.83	38.82 ± 0.56	32.30 ± 0.90
GR	0.57 ± 0.07	0.49 ± 0.12	0.43 ± 0.10	0.53 ± 0.16	0.42 ± 0.10

In roots, exposure to Cd, U or a combination of both for 24 h did not cause any significant alterations in the capacities of the measured enzymes, except for APX. Its capacity was significantly increased by exposure to 25 µM U. This response could be observed in the roots of both wild-type and *aox1a*-knockout plants, although more pronounced in wild-type plants (Table 6).

While in roots, APX capacity was mainly affected by U exposure, it was most strongly influenced by exposure to 10 µM Cd in the leaves (Table 7). The capacities of CAT, GR and SOD in the leaves of both genotypes were not significantly altered by any of the exposure conditions. In contrast, SPX and GPX capacities were increased in both genotypes by exposure to 5 µM Cd, a response that was even more intense after exposure to the highest Cd concentration (Table 7).

Table 7. Capacities (mU/g FW) of antioxidative enzymes in **leaves** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of at least 3 biological independent replicates. SPX; syringaldazine peroxidase; GPX: guaiacol peroxidase; CAT: catalase; APX: ascorbate peroxidase; GR: glutathione reductase; SOD: superoxide dismutase;. Significance levels compared to its own genotype under control conditions (One-way ANOVA): : $p < 0.05$; : $p < 0.01$.

WT					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
SPX	3.98 ± 0.21	7.77 ± 0.89	10.04 ± 0.82	3.64 ± 0.39	3.78 ± 0.50
GPX	2.51 ± 0.07	4.36 ± 0.33	6.56 ± 0.27	4.04 ± 0.49	2.49 ± 0.07
CAT	1.13 ± 0.07	0.99 ± 0.20	1.06 ± 0.24	1.72 ± 0.18	1.11 ± 0.12
APX	22.84 ± 2.22	29.94 ± 2.50	30.75 ± 3.86	22.82 ± 4.49	23.71 ± 1.19
GR	0.60 ± 0.21	0.66 ± 0.13	0.53 ± 0.11	0.59 ± 0.17	0.45 ± 0.11
SOD	133.64 ± 30.89	156.60 ± 14.58	155.13 ± 26.05	157.34 ± 15.87	142.66 ± 29.11
aox1a					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
SPX	3.65 ± 0.26	6.71 ± 0.64	9.54 ± 0.94	4.20 ± 0.34	4.33 ± 0.41
GPX	2.41 ± 0.15	3.66 ± 0.15	6.93 ± 0.35	3.25 ± 0.26	3.19 ± 0.31
CAT	1.55 ± 0.17	1.14 ± 0.15	1.22 ± 0.08	1.62 ± 0.20	1.28 ± 0.09
APX	20.61 ± 1.87	32.49 ± 1.81	39.69 ± 3.11	26.24 ± 5.91	19.79 ± 1.53
GR	0.64 ± 0.15	0.60 ± 0.12	0.53 ± 0.16	0.66 ± 0.12	0.47 ± 0.13
SOD	150.58 ± 33.56	142.57 ± 15.61	135.01 ± 29.73	143.05 ± 14.21	124.52 ± 29.80

3.3.3. Antioxidative metabolite concentrations

The concentrations of the oxidized and reduced forms of the **antioxidative metabolites** AsA and GSH, as well as their total concentrations, were spectrophotometrically determined in both metal-exposed roots (Table 8) and leaves (Table 9). In both organs, antioxidative metabolite concentrations were very similar between the two genotypes studied.

Table 8. Glutathione concentrations (nmol/g FW) in **roots** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of 3 biological independent replicates. GSH: glutathione (reduced form); GSSG: glutathione disulfide (oxidized form). Significance levels compared to its own genotype under control conditions (One-way ANOVA): : $p < 0.05$.

WT					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
GSH + GSSG	123.23 ± 16.36	121.16 ± 30.07	107.76 ± 21.14	162.51 ± 12.62	81.56 ± 24.23
GSH	101.23 ± 22.65	117.03 ± 30.58	98.99 ± 22.54	133.91 ± 13.03	78.36 ± 25.99
GSSG	22.00 ± 7.00	4.13 ± 0.58	8.77 ± 1.46	28.59 ± 3.41	3.20 ± 1.84
aox1a					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
GSH + GSSG	131.22 ± 18.90	117.13 ± 21.06	111.76 ± 17.65	168.98 ± 15.29	83.67 ± 20.35
GSH	100.06 ± 30.74	113.13 ± 21.67	104.18 ± 18.96	150.37 ± 15.92	79.95 ± 21.32
GSSG	31.16 ± 12.76	4.01 ± 0.75	7.58 ± 1.63	18.62 ± 2.89	3.72 ± 1.55

In roots, AsA concentrations were not determined, as they often fall below the detection limit. Total and reduced root GSH concentrations were not significantly affected by any of the exposure conditions. Oxidized glutathione concentrations, however, significantly decreased in both genotypes after exposure to 5 µM Cd and to a combination of Cd and U. A decreasing trend in GSSG levels was also observed after exposure to 10 µM Cd (Table 8).

In leaves of both genotypes, total, reduced and oxidized leaf AsA levels did not significantly differ from the control levels in all conditions studied (Table 9).

Similar to the results observed in roots, total and reduced leaf GSH concentrations were not significantly altered in both genotypes. Oxidized glutathione concentrations, in contrast, were significantly lower after exposure to 5 and 10 µM Cd in wild-types leaves. Exposure to 25 µM U also caused a decreasing trend in GSSG levels in wild-type leaves. In *aox1a*-knockout mutants, similar trends were observed, although GSSG did not decrease in response to 25 µM U. Oxidized glutathione levels were below detection limit in wild-type and *aox1a*-knockout leaves exposed to a combination of Cd and U, indicating they were lower than the GSSG concentrations measured in leaves exposed to the other metal conditions (Table 9).

Table 9. Ascorbate and glutathione concentrations (nmol/g FW) in **leaves** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of at least 3 biological independent replicates. AsA: ascorbate (reduced form); DHA: dehydroascorbate (oxidized form); GSH: glutathione (reduced form); GSSG: glutathione disulfide (oxidized form). Significance levels compared to its own genotype under control conditions (One-way ANOVA): : $p < 0.05$; = $p < 0.01$. N.D.: not detectable.

WT					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
AsA + DHA	4323 ± 56	3866 ± 271	3350 ± 291	4400 ± 326	4068 ± 286
AsA	3762 ± 198	3515 ± 293	2793 ± 343	3784 ± 168	3554 ± 0.22
DHA	561 ± 187	352 ± 28	557 ± 216	616 ± 220	514 ± 69
GSH + GSSG	370.05 ± 9.51	294.56 ± 48.99	327.52 ± 18.94	274.06 ± 4.38	319.47 ± 40.63
GSH	341.16 ± 14.32	293.24 ± 48.60	322.79 ± 19.00	260.79 ± 4.50	319.34 ± 40.47
GSSG	28.89 ± 6.36	1.32 ± 0.52	4.73 ± 0.35	13.27 ± 1.77	N.D.
aox1a					
	Control	**5 µM Cd**	**10 µM Cd**	**25 µM U**	**Cd + U**
AsA + DHA	4363 ± 128	3812 ± 140	3522 ± 190	4573 ± 314	3829 ± 299
AsA	3520 ± 86	3482 ± 166	2980 ± 170	3632 ± 297	3077 ± 290
DHA	844 ± 231	330 ± 26	542 ± 74	941 ± 189	763 ± 41
GSH + GSSG	367.16 ± 8.16	300.91 ± 23.35	367.55 ± 36.61	279.76 ± 7.24	320.50 ± 38.24
GSH	347.73 ± 11.97	299.42 ± 23.04	363.13 ± 36.41	258.91 ± 7.20	321.16 ± 38.53
GSSG	19.43 ± 5.81	1.49 ± 0.46	4.43 ± 0.45	20.85 ± 3.96	N.D.

3.4. Metal-induced DNA damage and repair

In order to get more insight into the extent of DNA damage and repair induced in *A. thaliana* seedlings by metal exposure, the amount of oxidative DNA damage and the expression of selected genes involved in different DNA repair pathways were determined. In analogy with the measurements of nuclear DNA content (see section 3.5), measurements of DNA damage and repair were only performed in leaves. To investigate the role of AOX1a in these processes, results were compared between wild-type and *aox1a*-knockout leaves.

3.4.1. Amount of oxidative DNA damage in metal-exposed leaves

To investigate the induction of **oxidative DNA damage** by exposure to Cd, U and a combination of both metals, the 8-OHdG content in leaves of plants exposed to 5 µM or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h, was assessed using ELISA. To investigate the role of AOX1a, the effects were compared between wild-type and *aox1a*-knockout leaves (Fig. 3).

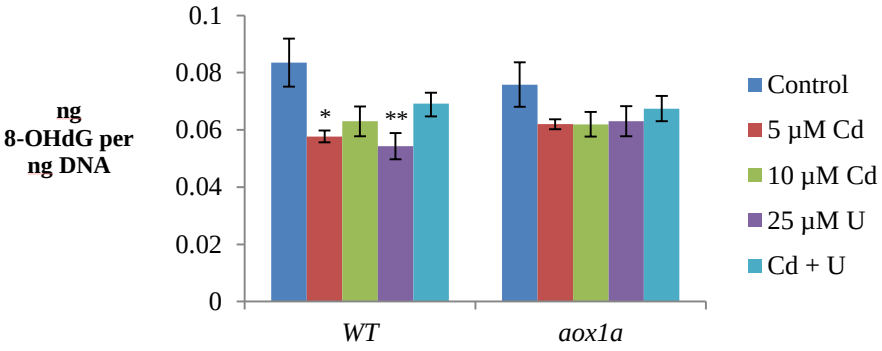

Figure 3. Amount of oxidative DNA damage as ng 8-OHdG per ng DNA in wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* **leaves** exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Data represent the mean ± S.E. of 4 biological independent replicates. Significance levels compared to its own genotype under control conditions (One-way ANOVA): *: $p < 0.05$; **: $p < 0.01$. 8-OHdG: 8-hydroxydeoxyguanosine.

A significant decrease in oxidative DNA damage was observed in wild-type *A. thaliana* leaves after exposure to 5 µM Cd and 25 µM U. This decrease in the amount of DNA damage was also visible in wild-type leaves exposed to 10 µM Cd and a combination of Cd and U, although these responses were weaker (Fig. 3).

In contrast to the results observed in wild-type leaves, the amount of oxidative DNA damage in *aox1a*-knockout leaves was not significantly altered by any of the four metal exposures, although a decreasing trend could be observed as well (Fig. 3).

3.4.2. Expression of genes involved in DNA repair mechanisms

To gain further insight into the molecular mechanisms underlying DNA damage and repair in plants exposed to Cd, U or a combination of both, the **expression of genes involved in several DNA repair pathways** was determined at 24 h after the start of exposure. To assess the role of AOX1a in metal-induced DNA damage and repair, responses were compared between wild-type and *aox1a*-knockout leaves.

Under control conditions, the expression of selected DNA repair genes was generally lower in mutant as compared to wild-type leaves (Table 10). This could also be observed for the expression of oxidative stress-related genes (Table 5), possibly pointing to a lower basal gene expression level in *aox1a*-mutant leaves.

The expression levels of ataxia-telangiectasia mutated (*ATM*) and ATM- and RAD3-related (*ATR*), two genes involved in **signaling of DNA damage**, were differentially influenced in the leaves of both genotypes (Table 10). In wild-type leaves, both *ATM* and *ATR* expression levels were mainly decreased by exposure to 5 µM Cd and 25 µM U, while 10 µM

Cd and a combination of both metals did not cause significant changes in *ATM* and *ATR* gene expression. However, a decreasing trend in *ATM* expression could be observed after combined exposure to Cd and U. In contrast to the response in wild-type leaves, *ATR* expression was not altered by any of the metal exposure conditions in mutant leaves. A significant decrease in *ATM* expression in this genotype could be observed for all metal treatments, except 5 µM Cd (Table 10).

Expression levels of formamidopyrimidine DNA glycosylase (*MMH*), a gene involved in the excision repair of oxidized bases, decreased in wild-type leaves in response to all four metal exposure conditions, while in the leaves of *aox1a*-knockout plants, only exposure to 25 µM U caused a significantly decreased *MMH* expression. The expression of 8-oxoguanine DNA glycosylase 1 (*OGG1*), another gene involved in excision repair of oxidative DNA damage, decreased in response to both 5 and 10 µM Cd in both genotypes, while 25 µM U and combined exposure to Cd and U caused no significant alterations in its expression. While the same response was observed for poly(ADP-ribose) polymerase 1 (*PARP1*), the expression of *PARP2* increased in response to both Cd concentrations. Uranium exposure, in contrast, lowered *PARP2* expression. Combined exposure to both Cd and U seemed to cause a response similar to that of Cd exposure alone (Table 10). Expression levels of homolog of X-ray repair cross complementing 1 (*XRCC1*), a gene involved in **base excision repair** (BER), increased in response to exposure to both Cd concentrations and combined exposure to Cd and U in both genotypes. Exposure to 25 µM U did not affect *XRCC1* expression (Table 10).

The expression of all measured **mismatch repair** (MMR) genes decreased in response to all exposure conditions relative to the control. However, combined exposure to Cd and U seemed to induce no alterations in the expression of MMR genes (Table 10).

In *aox1a*-knockout leaves, a significant upregulation of *KU80*, a gene involved in the **non-homologous end joining** (NHEJ) pathway of DSB repair, was observed in mutant leaves after exposure to 5 µM Cd, a response that was the opposite from the response in wild-type leaves, where *KU80* levels showed a decreasing trend (Table 10). The expression of *KU80* significantly decreased by exposure to 25 µM U in both genotypes and after combined exposure to Cd and U in the mutant (Table 10). While in mutant leaves the expression of ligase 4 (*LIG4*), another gene involved in NHEJ, was clearly upregulated in response to both Cd concentrations, this response was much weaker or absent in the wildtype (Table 10).

The expression of *RAD51*, a gene involved in the **homologous recombination** (HR) pathway of DSB repair, was downregulated by exposure to 5 and 10 µM Cd and 25 µM U. In both genotypes, this response was mostly pronounced after exposure to both Cd concentrations (Table 10).

Table 10. Expression levels of DNA repair genes in **leaves** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* plants exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. In the first column ('UNEXPOSED') the gene expression under control conditions is compared for both genotypes. The columns 'WT' and '*aox1a'* give an overview of the responses of wild-type and *aox1a*-knockout plants, respectively. The expression in metal-exposed leaves is represented relative to their own control. Values are the average ± SE of at least 3 biologically independent replicates. Significance levels (One-way ANOVA): = $p < 0.05$; = $p < 0.01$; = $p < 0.05$; = $p < 0.01$ for an increased and decreased expression, respectively. *PARP*: poly(ADP-ribose) polymerase; *KU80*: KU80 homolog; *LIG4*: ligase 4; *ATM*: ataxia-telangiectasia mutated; *ATR*: ATM- and RAD3-related; *MMH*: formamidopyrimidine DNA glycosylase; *OGG1*: 8-oxoguanine DNA glycosylase 1; *PCNA*: proliferating cellular nuclear antigen; *MSH*: MUTS homolog; *XRCC1*: homolog of X-ray repair cross complementing 1.

LEAVES	UNEXPOSED		WT				*aox1a*			
	WT	*aox1a*	5 µM Cd	10 µM Cd	25 µM U	Cd + U	5 µM Cd	10 µM Cd	25 µM U	Cd + U
DNA damage signalling										
ATM	1.00 ± 0.17	0.79 ± 0.12	0.27 ± 0.01	0.72 ± 0.14	0.33 ± 0.09	0.45 ± 0.06	0.89 ± 0.11	0.50 ± 0.04	0.48 ± 0.02	0.52 ± 0.10
ATR	1.00 ± 0.13	0.71 ± 0.07	0.46 ± 0.04	0.75 ± 0.11	0.56 ± 0.07	1.00 ± 0.02	0.89 ± 0.11	0.94 ± 0.08	0.77 ± 0.07	1.03 ± 0.12
Base excision repair (BER)										
MMH	1.00 ± 0.06	0.78 ± 0.05	0.65 ± 0.07	0.68 ± 0.01	0.51 ± 0.03	0.68 ± 0.01	1.00 ± 0.07	0.78 ± 0.01	0.67 ± 0.02	0.87 ± 0.06
OGG1	1.00 ± 0.08	0.92 ± 0.04	0.65 ± 0.05	0.67 ± 0.03	0.78 ± 0.04	0.89 ± 0.03	0.74 ± 0.07	0.67 ± 0.02	0.86 ± 0.03	0.94 ± 0.03
PARP1	1.00 ± 0.09	0.91 ± 0.07	0.53 ± 0.02	0.51 ± 0.02	0.73 ± 0.10	0.82 ± 0.06	0.59 ± 0.09	0.47 ± 0.04	0.75 ± 0.05	0.87 ± 0.05
PARP2	1.00 ± 0.10	0.92 ± 0.06	1.34 ± 0.10	1.52 ± 0.10	0.48 ± 0.04	1.30 ± 0.03	1.52 ± 0.12	1.36 ± 0.13	0.53 ± 0.03	1.27 ± 0.09
XRCC1	1.00 ± 0.08	1.01 ± 0.04	2.18 ± 0.22	2.65 ± 0.08	1.08 ± 0.15	1.32 ± 0.25	1.86 ± 0.23	2.42 ± 0.05	1.05 ± 0.03	1.37 ± 0.28
Mismatch repair (MMR)										
PCNA1	1.00 ± 0.09	0.87 ± 0.04	0.34 ± 0.04	0.18 ± 0.02	0.47 ± 0.03	0.74 ± 0.13	0.28 ± 0.02	0.17 ± 0.02	0.55 ± 0.03	0.89 ± 0.10
PCNA2	1.00 ± 0.08	0.82 ± 0.05	0.48 ± 0.07	0.34 ± 0.03	0.60 ± 0.03	0.84 ± 0.08	0.44 ± 0.03	0.33 ± 0.01	0.64 ± 0.04	0.87 ± 0.07
MSH2	1.00 ± 0.10	0.89 ± 0.07	0.32 ± 0.04	0.31 ± 0.02	0.65 ± 0.02	0.73 ± 0.04	0.49 ± 0.02	0.27 ± 0.01	0.68 ± 0.03	0.81 ± 0.04
MSH7	1.00 ± 0.11	0.77 ± 0.06	0.37 ± 0.03	0.40 ± 0.03	0.51 ± 0.02	0.70 ± 0.02	0.53 ± 0.03	0.36 ± 0.02	0.62 ± 0.04	0.88 ± 0.01
Non-homologous end joining (NHEJ)										
KU80	1.00 ± 0.12	0.74 ± 0.09	0.65 ± 0.07	1.12 ± 0.08	0.54 ± 0.10	0.59 ± 0.06	1.56 ± 0.10	1.24 ± 0.09	0.66 ± 0.04	0.84 ± 0.03
LIG4	1.00 ± 0.10	0.75 ± 0.01	1.05 ± 0.11	1.52 ± 0.14	0.70 ± 0.10	1.03 ± 0.04	2.31 ± 0.14	1.98 ± 0.11	0.86 ± 0.08	1.19 ± 0.12
Homologous recombination (HR)										
RAD51	1.00 ± 0.15	0.75 ± 0.11	0.22 ± 0.02	0.26 ± 0.04	0.52 ± 0.07	0.72 ± 0.05	0.48 ± 0.02	0.31 ± 0.00	0.63 ± 0.06	0.78 ± 0.05

3.5. Metal-induced endoreduplication

Finally, the effects of metal exposure on endoreduplication were assessed by determining both the nuclear DNA content and the expression of selected genes involved in the regulation of endoreduplication (Fig. 1). As the analysis of nuclear DNA content using flow cytometry is very difficult to perform in roots, all measurements were only performed in leaves.

3.5.1. Nuclear DNA content

In order to get more insight into the effects of metal exposure on the extent of endoreduplication, the **nuclear DNA content** was determined in separate leaves of *A. thaliana* seedlings exposed to Cd, U or a combination of both. Measurements were performed in the three youngest leaves, as the cell division rate is highest in these leaves, making it easier to detect any changes in the extent of endoreduplication after short term exposure. While all other measurements were only performed after 24 h of metal exposure, the analysis of nuclear DNA content was performed after 72 h of exposure as well. This was due to the fact that plant cells generally only divide once every 24 h, possibly making it difficult to detect large changes in nuclear DNA content after 24 h of exposure. In addition, responses were compared between wild-type and *aox1a*-knockout seedlings to investigate the potential role of AOX1a in the endoreduplication process.

Results of the measurements of nuclear DNA content in the youngest leaf after 24 h and 72 h of exposure are presented in Figs. 4 and 5, respectively.

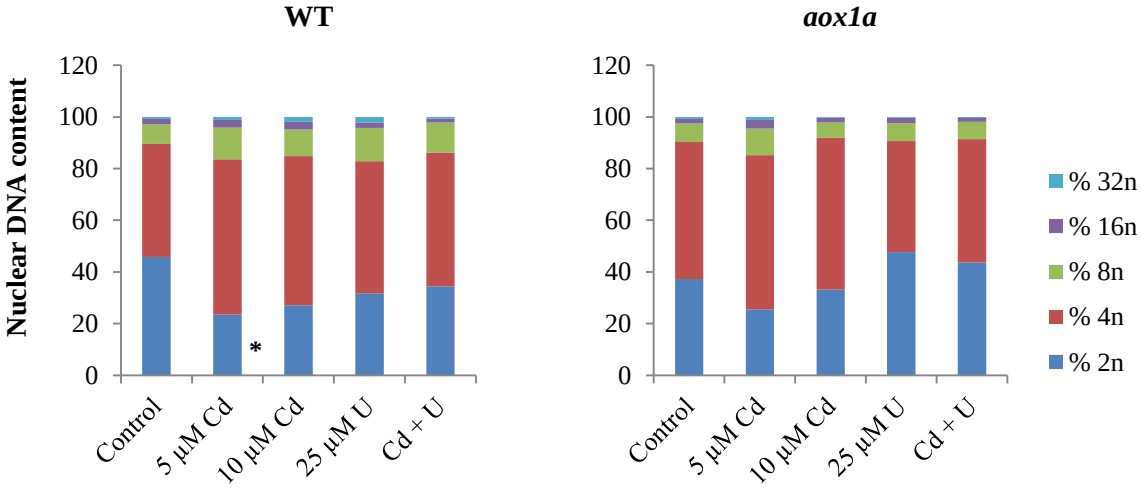

Figure 4. Nuclear DNA content in the **youngest leaf** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for **24 h**. All percentages of a certain n-value are compared to the control of the same genotype. Data represent the mean ± S.E. of 6 biological independent replicates. Significance levels compared to its own genotype under control conditions (indicated on the right side of each bar) (One-way ANOVA): *: $p < 0.05$.

As shown in Fig. 4, nuclear DNA content in the youngest leaf was differently affected by metal exposure between the two genotypes. While metal exposure induced an increasing trend in DNA ploidy in wild-type leaves, as shown by a decrease in the proportion of 2n and an increase in the proportion of nuclei with higher ploidy levels (8n, 16n and 32n), this trend was much weaker, or even absent, in *aox1a*-knockout leaves (Fig. 4). While the proportion of 2n

nuclei showed a decreasing trend in response to all metal exposure conditions in wild-type leaves, it remained unaltered or even showed an increasing trend in mutant leaves. The only exception was the response observed in *aox1a*-knockout leaves exposed to 5 µM Cd, which also showed a slightly decreasing trend in the proportion of 2n nuclei. However, this response was weaker as compared to the response observed in wild-type leaves (Fig. 4). Similar results were observed in the second and third youngest leaves (Supplementary Figs. 1 and 3).

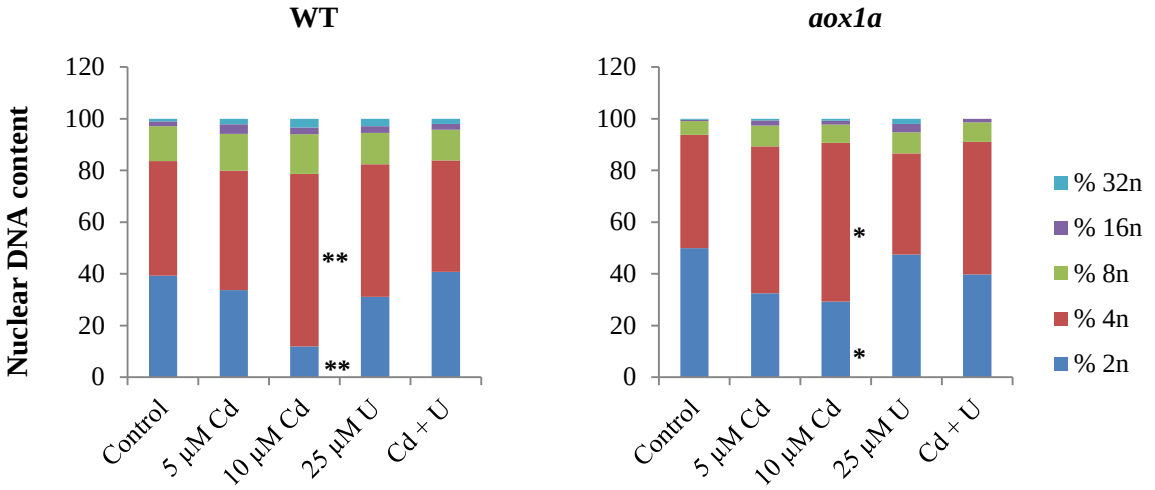

Figure 5. Nuclear DNA content in the **youngest leaf** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for **72 h**. All percentages of a certain n-value are compared to the control of the same genotype. Data represent the mean ± S.E. of 4 biological independent replicates. Significance levels compared to its own genotype under control conditions (indicated on the right side of each bar) (One-way ANOVA): *: $p < 0.05$; **: $p < 0.01$.

After 72 h, a metal-induced increasing trend in nuclear DNA content could be observed in the youngest leaves of wild-type seedlings as well, with the strongest response after exposure to 10 µM Cd, showing a significant increase in the proportion of 4n nuclei and a concomitant decrease in the proportion of 2n nuclei (Fig. 5),. However, a similar, though weaker, response was induced in *aox1a*-knockout leaves as well (Fig. 5), possibly indicating a delayed response in mutant as compared to wild-type leaves. In addition, the increase in endoreduplication was stronger after Cd exposure as compared to U exposure and combined exposure to both metals (Fig. 5). Again, similar responses were observed in the second and third youngest leaves (Supplementary Fig. 2 and 4).

3.5.2. Expression of genes involved in endoreduplication

To investigate the molecular mechanisms underlying the differences in endoreduplication responses observed between the different metal exposure conditions and genotypes, the **expression of several genes involved in endoreduplication** was determined after 24 h of exposure (Table 11).

The expression of cell cycle switch protein 52A 1 (*CCS52A1)*, an activator of the Anaphase-Promoting Complex/Cyclosome that targets cyclins for degradation, significantly decreased in response to exposure to 25 µM U. In *aox1a*-knockout leaves, a significant increase in *CCS52A1* expression was observed after exposure to 5 µM Cd, a response that was absent in wild-type leaves. Also the expression of the second isoform, *CCS52A2*, decreased in

response to 25 µM U. Moreover, exposure to 5 µM Cd significantly lowered *CCS52A2* expression levels in leaves of mutant seedlings (Table 11). In wild-type leaves, the expression of GLABRA 3 (*GL3*), a transcriptional activator of SIAMESE-related (*SMR*) genes, showed a decreasing trend induced by all four metal exposure conditions. In mutant leaves, however, the strongest decrease in *GL3* expression was seen after exposure to 10 µM Cd, while U exposure did not significantly affect *GL3* levels (Table 11). The expression of increased level of polyploidy 1 (*ILP1*), involved in the transcriptional repression of A2-type cyclins, showed a decreasing trend after exposure to all metal conditions in both genotypes. Also expression levels of KIP-related protein 1 (*KRP1*) and *KRP2*, which target cyclin-dependent kinase A for inhibition, and *SIAMESE*, a plant-specific CDK inhibitor, were lower in response to metal exposure, a response which was strongest after Cd exposure. The response of the expression of these genes to combined exposure to Cd and U tended to follow that of exposure to only U (Table 11). The expression of the different *SMR* genes was differently affected by exposure to Cd, U or a combination of both. While *SMR1* levels decreased in response to all four metal exposure conditions, the opposite response was observed for *SMR5* levels, with 25 µM U causing the strongest *SMR5* upregulation. The expression of *SMR4* increased in response to both Cd concentrations and a combination of Cd and U, but decreased in response to U exposure. While *SMR7* expression was not affected by Cd exposure in wild-type leaves, it significantly decreased in response to Cd in *aox1a*-knockout leaves. Exposure to 25 µM U and a combination of both Cd and U induced a decreasing trend in *SMR7* expression levels in leaves of both genotypes (Table 11).

The expression of DP-E2F-like 1 (*DEL1)*, a transcriptional repressor of endoreduplication, significantly decreased after exposure to both Cd concentrations and 25 µM U in leaves of both genotypes. Combined exposure to Cd and U did not significantly affect *DEL1* expression levels (Table 11).

Table 11. Gene expression of positive and negative regulators of endoreduplication in **leaves** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* plants exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. In the first column ('UNEXPOSED') the gene expression under control conditions is compared for both genotypes. The columns 'WT' and '*aox1a*' give an overview of the responses of wild-type and *aox1a*-knockout plants, respectively. The expression in metal-exposed leaves is represented relative to their own control. Values are the average ± SE of at least three biologically independent replicates. Significance levels (One-way ANOVA): = $p < 0.05$; = $p < 0.01$; = $p < 0.05$; = $p < 0.01$ for an increased and decreased expression, respectively. *CCS52A*: cell cycle switch protein 52A; *GL3*: GLABRA3; *ILP1*: increased level of polyploidy 1; *KRP*: KIP-related protein; *SMR*: SIAMESE-related; *DEL1*: DP-E2F-like 1.

LEAVES	**UNEXPOSED**		**WT**				***aox1a***			
	WT	*aox1a*	5 µM Cd	10 µM Cd	25 µM U	Cd + U	5 µM Cd	10 µM Cd	25 µM U	Cd + U
Positive regulators										
CCS52A1	1.00 ± 0.14	0.76 ± 0.09	0.85 ± 0.06	1.05 ± 0.08	0.46 ± 0.03	0.84 ± 0.18	1.70 ± 0.11	1.10 ± 0.05	0.59 ± 0.04	0.91 ± 0.06
CCS52A2	1.00 ± 0.08	0.89 ± 0.06	0.86 ± 0.07	0.98 ± 0.05	0.77 ± 0.01	0.84 ± 0.03	0.67 ± 0.02	1.01 ± 0.06	0.79 ± 0.02	0.97 ± 0.03
GL3	1.00 ± 0.21	0.57 ± 0.09	0.36 ± 0.09	0.45 ± 0.17	0.61 ± 0.10	0.54 ± 0.08	0.60 ± 0.18	0.28 ± 0.06	1.31 ± 0.21	1.02 ± 0.14
ILP1	1.00 ± 0.10	0.83 ± 0.06	0.73 ± 0.03	0.82 ± 0.03	0.67 ± 0.05	0.68 ± 0.05	0.75 ± 0.07	0.88 ± 0.04	0.80 ± 0.04	0.84 ± 0.04
KRP1	1.00 ± 0.11	0.91 ± 0.07	0.72 ± 0.05	0.65 ± 0.03	0.99 ± 0.01	0.52 ± 0.14	0.63 ± 0.06	0.79 ± 0.09	1.18 ± 0.03	0.68 ± 0.09
KRP2	1.00 ± 0.09	0.99 ± 0.08	0.47 ± 0.03	0.34 ± 0.02	0.71 ± 0.01	0.79 ± 0.04	0.43 ± 0.06	0.25 ± 0.05	0.90 ± 0.11	0.85 ± 0.07
SIAMESE	1.00 ± 0.09	0.91 ± 0.03	0.57 ± 0.02	0.52 ± 0.03	0.72 ± 0.03	0.84 ± 0.05	0.45 ± 0.03	0.55 ± 0.02	0.80 ± 0.02	0.98 ± 0.03
SMR1	1.00 ± 0.10	0.82 ± 0.09	0.54 ± 0.05	0.62 ± 0.07	0.35 ± 0.03	0.44 ± 0.08	0.56 ± 0.05	0.85 ± 0.12	0.51 ± 0.07	0.69 ± 0.05
SMR4	1.00 ± 0.19	0.97 ± 0.05	1.98 ± 0.24	1.14 ± 0.12	0.60 ± 0.05	1.42 ± 0.23	1.35 ± 0.09	1.12 ± 0.10	0.67 ± 0.02	1.47 ± 0.23
SMR5	1.00 ± 0.24	1.04 ± 0.35	1.71 ± 0.35	1.54 ± 0.22	7.80 ± 0.83	2.97 ± 0.51	1.33 ± 0.28	1.55 ± 0.25	5.14 ± 0.84	1.81 ± 0.89
SMR7	1.00 ± 0.09	1.07 ± 0.04	0.91 ± 0.11	1.43 ± 0.35	0.71 ± 0.12	0.75 ± 0.06	0.50 ± 0.07	0.64 ± 0.08	0.59 ± 0.04	0.74 ± 0.06
Negative regulators										
DEL1	1.00 ± 0.19	0.69 ± 0.08	0.31 ± 0.04	0.25 ± 0.04	0.44 ± 0.02	0.72 ± 0.12	0.49 ± 0.04	0.30 ± 0.02	0.57 ± 0.05	0.88 ± 0.09

4. DISCUSSION

Metal contamination is a problem affecting many regions worldwide. Therefore, it is important to gain more insight into the effects of metal toxicity on different organisms. In this study, the effects of metal contamination were determined in plants, as they take up metals from contaminated soils, thereby introducing them into the food chain, possibly leading to negative health effects in other organisms as well.

As metals never occur in the environment as single pollutants, it is critical to additionally investigate the effects of combined exposure to multiple stressors [4]. In this study, the short term effects of exposure to Cd, U and a combination of both metals on oxidative stress, DNA damage and repair and endoreduplication were determined in *A. thaliana* seedlings. Furthermore the role of AOX1a, a stress-sensitive mitochondrial enzyme, in these processes was assessed, as this enzyme is known to be induced by Cd exposure in *A.* thaliana and reduces mitochondrial ROS production, possibly affecting the metal-induced oxidative stress response [42]. As ROS are able to induce DNA damage in plants [10] and have been shown to induce endoreduplication in human HK-1 cells [31], AOX1a might play a role in these processes as well.

4.1. Exposure to Cd and/or U affects *A. thaliana* root and leaf growth

In a first part of this study, root and leaf growth of plants exposed to Cd, U or a combination of both for 24 h was determined as a general estimate of the toxicity of the metal concentrations used. In general, a decrease in root growth was observed after exposure to any of the four metal exposure conditions (Fig. 2A). This decrease, however, was stronger in mutant as compared to wild-type roots. This could be related to a higher mitochondrial electron leakage and ROS production in mutant plants under stress conditions [55]. Nonetheless, under control conditions, root growth was slightly higher in mutant seedlings (Fig. 2A), as was the case for the leaves (Fig. 2B). This could possibly be explained by the non-phosphorylating nature of AOX1a. Contrary to electron transport through the classical ETC, electron transport through the alternative ETC does not cause a proton driving force leading to ATP production [43]. Millar *et al.* (1998) [56] have demonstrated in soybean seedlings under control conditions that an increase in respiration via AOX from 5 % on day 4 of development to 55 % on day 17 takes place. This increase is accompanied by a decrease in ATP yield and a decreased growth rate. As AOX1a is the most dominant isoform in *A. thaliana* and *aox1a*-knockout mutants do not contain any functional AOX1a enzymes, we hypothesize that, under control conditions, ATP production is slightly higher in *aox1a*-knockout mutants compared to wildtype seedlings, causing a slightly higher growth rate of these mutants.

While root growth was affected by all metal exposure conditions, leaf growth was mainly decreased by U or by combined exposure to Cd and U (Fig. 2B). As the root-to-shoot transfer for U is very low [14], the observed decrease in leaf growth was probably due to signaling from the roots rather than to direct toxicity effects of U in the leaves. Cadmium exposure did not significantly decrease leaf growth. In wild-type seedlings, exposure to 10 µM Cd even caused a small increase in leaf fresh weight (Fig. 2B). Although this might be considered a

hormesis effect, this is rather unlikely, as no such effect was seen after exposure to 5 µM Cd (Fig. 2B).

4.2. Exposure to Cd does not influence U uptake or vice versa

To identify possible links between the effects of Cd and U and their uptake by *A. thaliana* seedlings, Cd and U concentrations in roots and leaves of metal-exposed plants were determined. In addition, this enabled us to identify possible effects of the metals on each other's uptake or root-to-shoot translocation.

In general, Cd and U uptake in roots and leaves were very similar between wild-type and *aox1a*-knockout plants (Tables 2 and 3), indicating that AOX1a does not influence metal uptake or root-to-shoot translocation.

Both Cd and U were readily taken up from the contaminated nutrient solutions, as shown by the significant increases in root metal content after exposure to Cd, U or a combination of both as compared to the control. Furthermore, Cd and U uptake in the roots was proportional to the Cd and U concentrations present in the Hoagland solution, with higher concentrations in the nutrient solution leading to higher metal concentrations in the roots (Table 2). These results are in agreement with the results of Cuypers *et al.* (2011) [51], who also reported concentration-dependent increases in root and leaf Cd concentrations in *A. thaliana* seedlings exposed to 5 µM and 10 µM for 24 h. Vanhoudt *et al.* (2011) [16] also observed similar results after exposure to U concentrations ranging from 0.1 to 100 µM U for 1, 3 or 7 days.

The presence of very low amounts of Cd and U in roots and leaves of control plants was unexpected, as no metals were added to the nutrient solution of these plants. Therefore, this observation was possibly due to the presence of very small amounts of Cd and U in the climate chambers and containers used for the cultivation of the plants or could originate during the many steps needed to prepare the samples for ICP-MS analysis. However, Cd and U concentrations present in control plants were negligible as compared to those in metal-exposed seedlings (Tables 2 and 3).

While U concentrations in roots of U-exposed plants were very high (Table 2), almost no U was present in leaves (Table 3), indicating a very low root-to-shoot transfer factor for U, as also shown by Vandenhove *et al.* (2006) [17], who exposed *P. vulgaris* to different U concentration ranging from 0.1 µM to 1000 µM for 1 to 7 days, and Vanhoudt *et al.* (2010) [5], who exposed *A. thaliana* seedlings to 5 µM Cd, 10 µM U or a combination of both for 3 days. Cadmium, on the contrary, showed a higher root-to-shoot translocation than U, although the largest amount of Cd ions was retained in the roots (Tables 2 and 3). This observation is also in accordance with results from Vanhoudt *et al.* (2010) [5] and Cuypers *et al.* (2011) [51].

While Vanhoudt *et al.* (2010) [5] found that simultaneous exposure to 5 µM Cd and 10 µM U for 3 days caused an almost two-fold increase in U uptake in roots and leaves of *A. thaliana* plants, no such effect was observed in this study. However, this might be due to the difference in Cd and U concentrations and exposure duration. To fully investigate the effects of Cd on U uptake and vice versa, a larger combined exposure experiment should be performed, including many different Cd and U concentrations and several combinations of these concentrations.

4.3. Although AOX1a is transcriptionally induced by Cd and/or U, it does not seem to be involved in the oxidative stress response

As both Cd and U are known to induce oxidative stress, several oxidative stress-related parameters were determined in *A. thaliana* seedlings exposed to Cd, U and a combination of both for 24 h, to identify and compare the molecular mechanisms underlying the oxidative stress response induced by both metals. In addition, we hypothesized a role for the mitochondrial AOX1a in the metal-induced oxidative stress response, as this enzyme is known to reduce mitochondrial ROS production under stress conditions [42]. To investigate this possible role for AOX1a, results were compared between wild-type and *aox1a*-knockout plants.

As expected, *AOX1a* transcript levels could not be determined in roots and leaves of *aox1a*-knockout mutants (Tables 4 and 5), as they contain a T-DNA insertion in the gene coding for AOX1a. In roots and leaves of wild-type plants, an increase in *AOX1a* expression was observed after exposure to 5 and 10 µM Cd (Tables 4 and 5). This observation is in agreement with the results of Keunen *et al.* (2013) [49], who also reported increased *AOX1a* expression levels in a similar set-up. While the upregulation of *AOX1a* in roots was similar to the upregulation reported by Keunen *et al.* (2013), the increase in *AOX1a* expression in leaves was clearly weaker in our study, although the same Cd concentrations and exposure duration were used. This could be related to the different conditions in which the plants were cultivated. In our study, a Hoagland solution with a lower phosphate concentration was used after the start of metal exposure to prevent high phosphate concentrations from limiting plant U uptake. In addition, light was provided by different lamps, plants were grown under a different photoperiod and plants were grown on agar-containing tubes instead of sand.

To date, no data are available on the effects of U exposure on *AOX1a* expression. In this study, a significant upregulation of *AOX1a* transcript levels was observed in wild-type roots exposed to 25 µM U or a combination of Cd and U (Table 4). This upregulation could be caused by a U-induced impairment of the mitochondrial ETC. Although this has not been shown in plants, Shaki *et al.* (2012) [41] have shown in rat kidneys that U causes disruptions of the ETC at complexes II and III, leading to the induction of ROS formation. In leaves, however, no significant increases in *AOX1a* expression levels were observed (Table 5). This could possibly be explained by the fact that almost no U was translocated to the leaves (Table 3).

In general, the effects of Cd and U exposure on the expression of selected pro- and antioxidative genes (Tables 4 and 5), the capacities of antioxidative enzymes (Tables 6 and 7) and AsA and GSH concentrations and their redox state (Tables 8 and 9), were very similar between wild-type and *aox1a*-knockout seedlings, probably indicating that AOX1a does not play an important role in the modulation of the Cd- and U-induced oxidative stress response.

4.4. Different mechanisms lie at the basis of the oxidative stress response induced by Cd and U

Although no differences could be observed between wild-type and *aox1a*-knockout seedlings, clear differences were present between the effects on oxidative stress-related parameters induced by Cd and U. Therefore, the mechanisms underlying the oxidative stress response induced by these metals are, at least partially, different.

In roots, *LOX1* and *RBOHD* gene expression was strongly upregulated by both Cd concentrations (Table 4), while in leaves this response was seen for *LOX1* and *RBOHC* (Table 5). These results are very similar to those observed by Cuypers *et al.* (2011) [51] after exposure to the same Cd concentrations for 24 h. Uranium exposure also caused small increases in the expression of some of these pro-oxidative genes (Tables 4 and 5), which are comparable to the increases observed in *A. thaliana* roots and leaves exposed to U concentrations ranging from 0.1 to 100 µM for 24 h by Vanhoudt *et al.* (2011) [18, 19]. However, these increases were negligible as compared to those induced by Cd (Tables 4 and 5). Therefore, we can conclude that, while Cd can induce oxidative stress by enhancing the transcription of pro-oxidative genes, probably leading to an increase in O_2^{o-} production and lipid peroxidation, it is unlikely that this is also an underlying mechanism of U-induced oxidative stress.

Overall, the effects on antioxidative gene expression observed after exposure to 5 and 10 µM Cd are highly similar to those reported by Cuypers *et al.* (2011) [51] and Smeets *et al.* (2008) [57], who also exposed *A. thaliana* seedlings to both Cd concentrations for 24 h, indicating that Cd influences the antioxidative defense system. The only exceptions are *CAT1* and *CAT3* expression levels in leaves. While Cuypers *et al.* (2011) [51] reported significant increases in the expression of these genes after Cd exposure, significant decreases were observed in our study (Table 5). However, Cd-induced decreases in *CAT1* and *CAT3* expression levels were also observed by Vanhoudt *et al.* (2010) [5]. In contrast to other studies mentioned, the latter study also investigated the effects of combined exposure to Cd and U. In this study, similar to our set-up, a Hoagland solution with a low phosphate concentration was used after the start of metal exposure to inhibit high phosphate concentrations from limiting U uptake. Therefore, the differential effects observed for *CAT1* and *CAT3* expression levels after Cd exposure in different studies could be related to the phosphate content of the Hoagland solution. This is in accordance to the results of another study performed by Vanhoudt *et al.* (2008) [52], in which *CAT1* expression levels in leaves of seedlings grown in a low phosphate Hoagland solution showed a decreasing trend as compared to those in leaves of plants grown in a high phosphate Hoagland solution under control conditions.

Generally, the effects on antioxidative gene expression induced by exposure to 25 µM U in roots and leaves of *A. thaliana* seedlings (Tables 4 and 5) are comparable to those reported by Vanhoudt *et al.* (2011) [18, 19] in a study investigating the effects of exposure to U concentrations ranging from 0.1 to 100 µM on the oxidative stress response in *A. thaliana*.

Exposure to all metal conditions decreased *CSD* expression in both roots and leaves (Tables 4 and 5). This is likely to be the result of an induction of miR398, a micro-RNA known to regulate *CSD* expression levels [58]. The downregulation of *CSD* expression was

generally stronger after U than after Cd exposure. On the other hand, the upregulation of *FSD1* expression was much stronger after U exposure, possibly compensating for the stronger decrease in *CSD2* expression, as both enzymes are localized in chloroplasts. Despite significant changes in transcription of the different SOD isoforms in leaves (Tables 4 and 5), no significant effect of metal exposure on SOD capacity was observed (Table 7). This is not unusual as there are multiple steps between transcription of the gene and the activity of the corresponding enzyme, causing a difference in timing between gene expression and enzyme activity.

In roots, gene expression of *CAT*, an enzyme involved in H_2O_2 detoxification, was also differently influenced by Cd and U (Table 4). While Cd exposure increased expression levels of all *CAT* isoforms, the opposite response was induced by U. However, no significant changes in the capacity of CAT were observed in either plant organ (Tables 6 and 7). Expression levels of *APX1*, another H_2O_2-detoxifying enzyme, were significantly increased after U exposure, possibly compensating for the decreased *CAT* expression (Table 4). The increased gene expression levels of *APX1* in roots exposed to U were also reflected by an increased capacity of this enzyme under these conditions (Table 7), a trend that was also observed by Vanhoudt *et al.* (2011) [18], after exposure of *A. thaliana* seedlings to 100 µM for 24 h. This increase in APX transcription and activity could possibly function as a defense mechanism against a U-induced increase in H_2O_2 levels. However, H_2O_2 concentrations were not measured in this study. The APX enzyme capacity in roots was not significantly affected by Cd alone or a combined exposure to Cd and U. This is in agreement with the transcript levels of *APX1* in roots, which were also significantly upregulated in response to U only (Table 4). In contrast to the expression of most other antioxidative genes, the response of *APX* expression to combined Cd and U exposure in roots mostly followed the Cd-induced response (Table 4). Despite clear changes in APX transcription and activity, no significant increases in root levels of DHA – the oxidized form to which AsA is converted during the detoxification of H_2O_2 by APX – were observed (Table 8). This could possibly be explained by changes in the capacity of other enzymes involved in the AsA-GSH cycle, such as DHA reductase. However, capacities of these enzyme were not determined in this study. Therefore, it could be interesting to also determine their capacity in future studies.

While in roots Cd exposure did not significantly affect the capacities of the measured antioxidative enzymes (Table 6), it significantly increased the capacities of SPX and GPX in a concentration-dependent fashion in the leaves (Table 7). These observations are in correspondence with the results of Cuypers *et al.* (2011) [51] and Smeets *et al.* (2008) [57]. As peroxidases are key components in the scavenging of H_2O_2, these results indicate that H_2O_2 is an important component of the Cd-induced oxidative stress response in leaves. As SPX is also involved in cell wall lignification, an increase in its capacity might indicate an attempt to limit the cellular uptake of toxic compounds such as Cd and U. Uranium exposure and combined exposure to Cd and U, however, did not cause significant changes in leaf antioxidative enzyme capacities. This might also be related to the very low root-to-shoot translocation levels of U (Table 3). After combined exposure, however, Cd was present in leaves as well (Table 3), but at a level that was possibly below that able to evoke changes in antioxidative enzyme capacities.

Expression levels of *GR1*, an enzyme responsible for the reconversion of GSSG to GSH, increased in both roots and leaves in response to all metal exposure conditions. However, this response was strongest after Cd exposure in the leaves (Table 5). The increase in *GR1* expression levels could indicate an attempt of the cell to keep GSH in its reduced form, as it is possibly needed to bind and sequester metal ions via phytochelatins [59] and could possibly lie at the basis of the decreases in GSSG observed in both roots and leaves exposed to Cd or a combination of Cd and U (Tables 8 and 9). However, this is in disagreement with the enzyme capacity of GR, which remained unaltered after exposure to all metal conditions (Tables 6 and 7).

In both plant organs, effects on pro- and antioxidative gene expression induced by combined exposure to Cd and U tended to coincide with the responses induced by U as a single stressor (Tables 4 and 5). In roots, this observation can be explained by the fact that U concentrations present were much higher as compared to Cd concentrations (Table 2). In leaves, however, U concentrations were very small as compared to Cd (Table 3). Therefore, the effects of mixed exposure on pro- and antioxidative gene expression in the leaves are probably the result of U-induced root-to-shoot signaling. However, our results seem to be in contrast to the results of the mixed exposure experiment of Vanhoudt *et al.* [5], who found that toxicity effects of combined exposure to 5 µM Cd and 10 µM U for 72 h on transcriptional level were mostly influenced by Cd. Nevertheless, metal concentrations and exposure durations differed from those in our study, where plants were exposed to a combination of 2.5 µM Cd and 12.5 µM U for 24 h.

No significant changes in the levels of AsA, GSH and their redox state were observed, except for the changes in GSSG levels as discussed before. This indicates that the plants were able to cope with exposure to the metal concentrations used and that a metabolic equilibrium could be reached.

In general, it can be concluded that although Cd and U both induce oxidative stress, the mechanisms underlying this process are rather different between both metals, as they induce different effects on the transcription of pro- and antioxidative genes, the capacity of antioxidative enzymes and the concentrations of GSSG. As almost no U is translocated to the leaves, the effects of U exposure on the oxidative stress-related parameters in leaves are probably the result of root-to-shoot signaling, as also proposed by Vanhoudt *et al.* (2011) [19]. Furthermore, the effects induced by combined exposure to both Cd and U are generally not equally influenced by Cd and U. Therefore, it is very important to account for mixed exposure conditions when investigating the effects of metal contamination in future experiments.

4.5. Exposure to Cd and/or U does not induce oxidative DNA damage

Both Cd and U are genotoxic. In addition they are both able to induce oxidative stress (as discussed in the previous section), which can also possibly induce DNA damage. Therefore, the effects of Cd and U exposure on DNA damage and repair were investigated in this study.

First, the extent of oxidative DNA damage induced by Cd and/or U exposure was determined in leaves of *A. thaliana* seedlings. In analogy with the measurements of endoreduplication (see section 4.7), measurements of the extent of oxidative DNA damage

were only performed in leaf samples. To identify the possible influence of AOX1a on metal-induced oxidative DNA damage, results were compared between wild-type and *aox1a*-knockout leaves.

As shown in Fig. 3, exposure to Cd or U alone or a combination of both metals did not induce a significant increase in oxidative DNA damage in the leaves of *A. thaliana* seedlings, although both metals are known to induce oxidative stress. Instead, a decreasing trend in the ratio of 8-OHdG to total DNA could be observed after metal exposure. This trend was stronger in wild-type as compared to *aox1a*-knockout leaves (Fig. 3).

To date, no information is available on the effects of Cd and U exposure on oxidative DNA damage in plants yet. The results we observed after Cd exposure in *A. thaliana* leaves, however, are similar to those reported in other organisms. In a study of Calevro *et al.* (1998) [60], the ability of Cd to induce oxidative DNA base modifications in brain cells from *Pleurodeles* larvae was investigated. Although Cd showed the ability to induce DNA strand breaks, no Cd-induced increases in the amount of oxidative DNA base modifications were observed. However, Cd did seem to inhibit the repair of oxidative DNA damage [60]. The latter response was also supported by our study, as the expression of *OGG1*, a gene involved in the BER pathway for the excision of oxidatively damaged nucleotides, was significantly decreased after Cd exposure (Table 10). Similar responses were observed by Emmanouil *et al.* (2007) [61], who reported Cd-induced elevations in DNA breakage, but no elevations in oxidative DNA damage in mussel gills. In addition, a general negative impact of Cd exposure on the repair capacity for oxidative DNA damage was observed in the study of Emmanouil *et al.* (2007) as well [61].

Although no data are available to date on the effects of U on oxidative DNA damage *in planta*, our study shows that U exposure decreases oxidative DNA damage in the leaves to a similar extent as Cd does (Fig. 3). The decrease in oxidative DNA damage induced by combined exposure to Cd and U, however, was smaller as compared to the decreases induced by exposure to only one of the stressors (Fig. 3).

A possible explanation for the lower level in oxidative DNA damage observed after exposure to Cd and U, is the induction of endoreduplication caused by these metals (Fig. 4). As endoreduplication increases the amount of DNA present in a cell, the ratio between the amount of metal and the amount of DNA decreases, thereby possibly decreasing the proportion of DNA bases that can be oxidatively modified by the stressor. As the induction of endoreduplication by Cd and U exposure was clearly weaker or even absent in the leaves of *aox1a*-knockout mutants as compared to wild-type leaves (Fig. 4), this might explain the smaller decrease in oxidative DNA damage in mutant versus wild-type leaves (Fig. 3).

4.6. Exposure to Cd and/or U inhibits DNA repair at the transcript level

In the next part of this study, the effects of exposure to 5 or 10 µM Cd, 25 µM U and a combination of 2.5 µM and 12.5 µM U on DNA repair were investigated at the transcriptional level. As information on the effects of Cd and U on DNA damage and repair is rather scarce, there was no indication in which repair pathways changes on the transcriptional level could be expected in our set-up. Therefore, we chose to determine the expression of genes involved in several important DNA repair pathways (Table 10). In analogy with the measurement of the

extent of oxidative DNA damage (Fig. 3) and endoreduplication (Figs. 4 and 5), measurements were only performed in leaves. Again, the possible role of AOX1a was assessed by comparing the results between wild-type and *aox1a*-knockout leaves. Although to date no information is available on the role of AOX1a in DNA damage and repair in plants, we hypothesize a role for this enzyme, as it reduces production of ROS that could otherwise damage DNA [10, 42].

The measured DNA repair genes, can be subdivided into five classes based on the pathway they are involved in. Both ATM and ATR are master controllers of the DNA damage response, coordinating cell cycle progression and DNA repair pathways. While ATM is mainly activated by DSBs, the response of ATR is stronger in case of replication defects in both plants and animals as review by Waterworth *et al.* (2011) [62]. As both genes were differently affected by metal exposure between wild-type and mutant leaves (Table 10), this might indicate that AOX1a is involved in the regulation of the DNA damage response induced by metal stress, at least at the transcript level.

The second class of measured genes encode enzymes involved in BER. In this pathway, a damaged base is replaced by an undamaged base in several steps, including recognition and removal of the damaged base, incision, gap filling and sealing [23]. In contrast to *ATM* and *ATR*, the expression of the genes involved in BER was similarly affected by metal exposure between the two genotypes, possibly ruling out the involvement of AOX1a in the transcriptional regulation of the BER pathway under metal stress. However, several differences could be observed between the effects of Cd and U on the expression of BER genes. Combined exposure to Cd and U, in contrast, did not significantly affect the expression of genes involved in BER (Table 10).

Another DNA repair pathway, MMR, serves to recognize and repair mismatched bases, often produced by errors during replication or homologous recombination. The MMR mechanism discriminates between correct and incorrect bases and finally corrects the error after DNA synthesis [23]. Both Cd and U exposure were shown to decrease the expression of genes involved in this pathway (Table 10). The effects observed after Cd exposure were in agreement with those reported by Liu *et al.* (2008) [63] after exposure of *A. thaliana* seedlings to comparable Cd concentrations for 18 days. Although to date no data are available on the effects of U on the expression of MMR genes, our results indicate effects comparable to those of Cd. As observed for the BER genes, combined exposure to Cd and U did not significantly affect the expression of MMR genes in the leaves (Table 10).

In plants, DSBs are repaired either by HR, using a highly similar sequence in the genome as a template for repair, or NHEJ, which randomly rejoins DNA ends with little sequence dependence [62]. Although both DSB repair mechanisms are present in plants, most DSBs are repaired by NHEJ [62]. While a clear upregulation of NHEJ genes could be observed in mutant leaves exposed to 5 µM Cd, such response was not present in wild-type leaves. Although exposure to 10 µM Cd increased the expression of NHEJ genes in leaves of both genotypes, this response was stronger in the mutant (Table 10). A possible explanation could be that Cd has a higher ability to induce DSBs in the mutant as compared to the wild-type leaves. However, to be able to draw firm conclusions, the amount of DSBs should be determined in metal-exposed leaves of both genotypes, for example using a western blotting

assay with antibodies directed against gamma-H2AX [64]. Exposure to U, in contrast, induced a decreasing trend in the expression of NHEJ genes (Table 10).

In conclusion, it can be summarized from Table 10 that 24 h exposure to Cd generally resulted in a decrease in the expression of genes involved in DNA repair, although a few exceptions were present. Indeed, inhibition of DNA repair has been proposed as a major factor underlying Cd genotoxicity as reviewed by Bertin and Averbeck (2006) [65]. A similar response was observed after U exposure (Table 10), although no data are available on the effects of U exposure on DNA repair in plants yet. Therefore, additional studies are needed to verify our results. Interestingly, combined exposure to Cd and U generally did not influence the expression of genes involved in DNA repair (Table 10), possibly indicating an antagonistic action between both metals.

When interpreting these results, however, it should be kept in mind that changes in gene expression are not always reflected by changes in protein abundance and enzyme capacity. Therefore, the effects of Cd and U exposure on DNA repair should be investigated on these levels as well, for example by western blotting with antibodies directed against DNA repair enzymes. In addition, mutants defective in certain aspects of DNA repair could be used.

4.7. Exposure to Cd and/or U induces endoreduplication in *A. thaliana* leaves

Research has shown that endoreduplication can be induced by several stress factors in plants, as demonstrated by Yamasaki *et al.* (2010) [28] in cucumber exposed to UV-B irradiation and by Fusconi *et al.* (2006) [29] in *Pisum sativum* exposed to Cd. Therefore, the effects of Cd, U and combined exposure on this process were investigated in this study as well. As it is very difficult to determine the nuclear DNA content in roots with the current procedure, ploidy levels were only determined in leaves. Measurements were performed in the three youngest leaves, as cells in these leaves have the highest division rating, making is easier to detect any changes in the extent of endoreduplication after short term metal exposure.

From the results obtained in this experiment, it can be concluded that exposure to Cd, U and combined exposure to both metals for 24 and 72 h induce endoreduplication in leaves of wild-type *A. thaliana* seedlings (Figs. 4 and 5). These results are in accordance with the results of Fusconi *et al.* (2006) [29], who observed a decrease in the proportion of 2n nuclei and an increase in the proportion of 4n nuclei in primary roots of *P. sativum* seedlings exposed to 25 and 250 µM Cd. Similar results were reported by Repetto *et al.* (2007) [66] in roots of two pea genotypes exposed to the same Cd concentrations. To date, no data are available on the effects of U on nuclear DNA content. Our results suggest, however, that U exposure causes similar, though weaker, effects on endoreduplication as compared to Cd (Fig. 4 and 5). As both metals are known to induce oxidative stress in *A. thaliana* seedlings, we hypothesize a role for ROS in the induction of endoreduplication caused by Cd and U. This hypothesis is supported by the study of Ting *et al.* (2010) [31], who reported an increase in the extent of endoreduplication in human HK-1 cells exposed to 2-methoxyestradiol, related to the ability of this compound to induce mitochondrial oxidative stress.

4.8. Metal-induced endoreduplication is delayed in leaves of *aox1a*-knockout seedlings

While 24 h of Cd and U exposure clearly induced endoreduplication in the leaves of *A. thaliana* seedlings, this response was much weaker, or even absent, in *aox1a*-knockout leaves (Fig. 4). After 72 h of exposure, however, a metal-induced increase in ploidy levels could also be observed in mutant leaves. Nevertheless, this increase was weaker as compared to that observed in wild-type leaves (Fig. 5). Therefore, we conclude that AOX1a defiency probably has a delaying effect on the induction of endoreduplication caused by Cd and U exposure. As AOXs are known to reduce mitochondrial ROS production under stress conditions [42] and mitochondrial ROS production was shown to induce endoreduplication in human HK-1 cells [31], the delay in the onset of metal-induced endoreduplication is a rather unexpected response. However, the results of this study indicate that AOX1a does not play a crucial role in the metal-induced oxidative stress response, which might imply that ROS production is not elevated in *aox1a*-knockout mutants. In conclusion, additional research is needed to gain more insight in the mechanisms underlying the metal-induced increase in endoreduplication and the delayed onset of this response in *aox1a*-knockout leaves.

4.9. A possible role for *SMR* genes in metal-induced endoreduplication

To investigate the molecular mechanisms underlying the metal-induced increase in ploidy levels of *A. thaliana* leaves and the different responses observed between the two genotypes, the expression of several genes involved in endoreduplication was determined and compared between the different metal exposure conditions and genotypes (Table 11). As changes at the transcriptional level are generally fast, gene expression was only determined after 24 h of exposure.

From the results shown in Table 11, it can be concluded that the gene expression levels of most of the measured positive regulators of endoreduplication decreased or remained unaltered in response to metal exposure. This appears to be in disagreement with the results obtained from the flow cytometric measurements of nuclear DNA content, where an increase in endoreduplication after 24 h of metal exposure was observed (Fig. 4). However, as mentioned before, changes in gene expression levels are not always reflected in changes in enzyme capacities. As described by De Veylder *et al.* (2011) [26], the activity of many endoreduplication regulators can also be regulated by post-translational modifications such as phosphorylation, or degradation in proteasomes. The increase in *SMR4* and *SMR5* expression levels (Table 11) however does support the increased level of endoreduplication observed after exposure to Cd and U. The only exception is the decrease in *SMR4* expression after exposure to 25 µM U, which does not coincide with the observed increased endoreduplication. This decrease however seems to be compensated for by a large increase in *SMR5* expression in U-exposed leaves (Table 11). As this increase was stronger in wild-type leaves as compared to the increase observed in *aox1a*-knockout leaves, this supports the lower extent of endoreduplication in the latter genotype. The same is true for the decrease in *SMR7* expression in mutant leaves, which was smaller or even absent in wild-type leaves (Table 11). Finally, the decrease in the expression of *DEL1*, a negative regulator of endoreduplication,

observed after exposure to Cd and U (Table 11), supports the increase in endoreduplication in wild-type leaves as well.

As nuclear ploidy was measured in separate leaves of a different age, it would in future be interesting to also measure gene expression levels in separate leaves instead of complete rosettes.

5. CONCLUSION AND SYNTHESIS

In this study, the effects of exposure to Cd and U or a combination of both metals on the oxidative stress response, DNA damage and repair and endoreduplication – including possible relations – were determined in *A. thaliana* seedlings. The combination of Cd and U was chosen for its realism, as both metals frequently co-occur in polluted environments. In addition, the role of AOX1a, a stress-sensitive component of the mitochondrial alternative ETC, was determined in these plant stress processes, as previous research demonstrated an increased transcription, translation and capacity of this enzyme under stress conditions. In order to achieve this, both wild-type *A. thaliana* seedlings and *aox1a*-knockout mutants, lacking functional AOX1a enzymes, were grown hydroponically for 18 days, after which they were exposed to either 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for 24 h. Next, the induction of oxidative stress, DNA damage and repair and endoreduplication by the metal exposures was investigated. Oxidative stress responses investigated included the effects of metal exposure on the expression of selected pro- and antioxidative genes, the capacity of antioxidative enzymes and the concentrations of the antioxidative metabolites AsA and GSH and their redox state. To investigate the induction of DNA damage, the amount of oxidative DNA damage and the expression of genes involved in several DNA repair pathways were assessed. Finally, the extent of endoreduplication and the gene expression of several positive and negative regulators of this process were investigated. To gain insight in the role of AOX1a in the processes studied, all responses were compared between wild-type and *aox1a*-knockout plants.

It was shown here that Cd exposure did not influence the amount of U taken up into the plants or vice versa, which is in contrast with results of earlier studies. However, this might be due to the fact that different metal concentrations and exposure durations were applied in earlier experiments. To gain more insight into the interactions between Cd and U, an extensive combined exposure study should be set up, using different Cd and U concentrations in several combinations.

Interestingly, an increase of *AOX1a* gene expression was observed in roots of *A. thaliana* seedlings exposed to Cd, U or a combination of both. Although increases in *AOX1a* transcription had already been reported in the past, to date, no data are available on the effects of U and combined exposure to Cd and U on *AOX1a* expression. In leaves, however, no significant increases in *AOX1a* expression could be observed after exposure to U alone or in combination with Cd, which is probably related to the very low root-to-shoot transfer of U.

Despite the significant increases in *AOX1a* expression levels induced by Cd (in both roots and leaves), U and combined exposure to both (in roots only), the effects of metal exposure on the different oxidative stress-related parameters were very similar between wild-type *aox1a*-knockout seedlings, potentially ruling out a crucial role for AOX1a in the metal-induced oxidative stress response. However, several differences could be observed between the effects induced by Cd or U. This indicates that, although Cd and U are both known to induce oxidative stress, the mechanisms underlying the oxidative stress response probably differ between both. In general, the effects of combined exposure to Cd and U on the

expression of pro- and antioxidative genes tend to coincide with the effects induced by exposure to U only.

It was shown that all four metal exposure conditions decrease the amount of oxidative DNA damage to a higher extent in wild-type as compared to *aox1a*-knockout leaves. This could possibly be explained by the metal-induced increase in endoreduplication observed in the final part of this study. As endoreduplication increases the amount of nuclear DNA, the ratio between the amount of metal and DNA decreases, thereby possibly lowering the proportion of DNA bases that can be oxidatively modified by the stressor. In addition, exposure to Cd and U decreased the expression of most DNA repair genes measured, with a few exceptions. This is in agreement with previous studies, which reported the inhibition of DNA repair as one of the major mechanisms underlying Cd genotoxicity. However, no data are available on the effects of U exposure on DNA repair in plants yet, implying the need for additional studies to verify our results. Combined exposure to Cd and U generally only had very little effect on the expression of the measured DNA repair genes, possibly pointing towards an antagonistic action of both metals.

Exposure to Cd and U and combined exposure to both for 24 h was shown to induce endoreduplication in wild-type *A. thaliana* leaves, a response that was much weaker or even absent in *aox1a*-knockout leaves. After 72 h of exposure however, an increase in nuclear DNA content was observed in mutant leaves as well, although still weaker than in wild-type leaves. This led to the hypothesis that AOX1a is involved in the regulation of endoreduplication, as a lack of functional AOX1a enzymes seems to delay the onset of metal-induced endoreduplication. Neither the increase in endoreduplication after metal exposure nor the clear difference between both genotypes could be explained by changes in the transcription of endoreduplication regulators, as the expression of most positive regulators was negatively influenced after 24 h of metal exposure. The decrease in expression levels of *DEL1*, a negative regulator of endoreduplication, and the increases in *SMR4* and *SMR5*, however, did support the increase in nuclear DNA content observed after metal exposure. To further identify the mechanisms underlying metal-induced endoreduplication, positive and negative regulators of this process should also be studied at the levels of protein abundance and enzyme capacity, as many of them are known to be regulated by post-translational modifications and degradation in proteasomes.

In general, it can be concluded that the mechanisms underlying the oxidative stress response induced by Cd and U are different. As combined exposure to Cd and U generally induces effects that are rather unpredictable based on the effects induced by exposure to only one of both metals, the combined nature of metal exposure should be accounted for in future studies. While AOX1a does not seem to play an important role in the oxidative stress response induced by Cd and U, it is possibly involved in the regulation of DNA repair and endoreduplication in metal-exposed *A. thaliana* seedlings. Its role in these processes should be further investigated, for instance by using different AOX1a-overexpressor lines.

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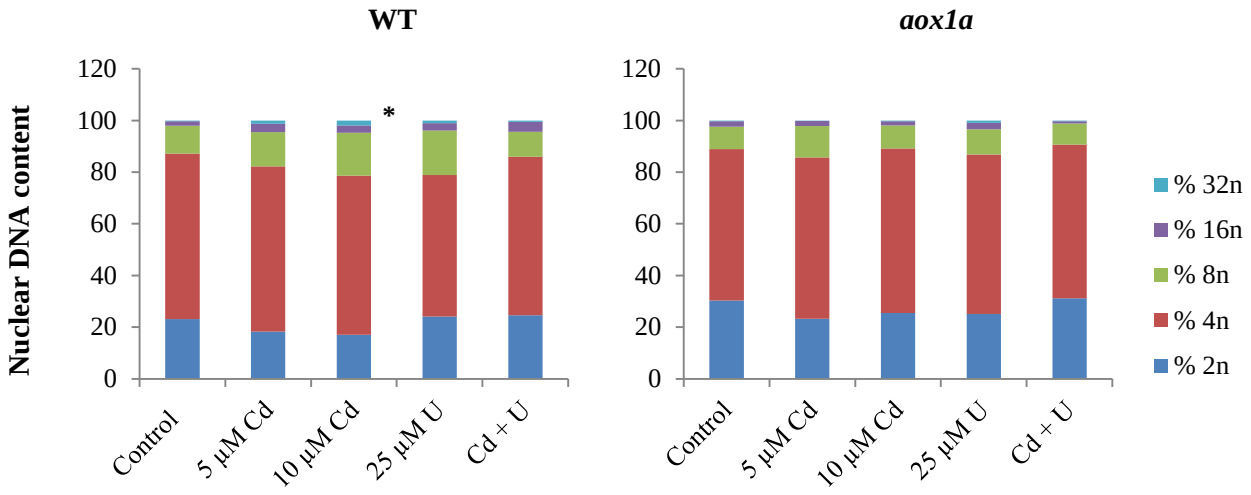
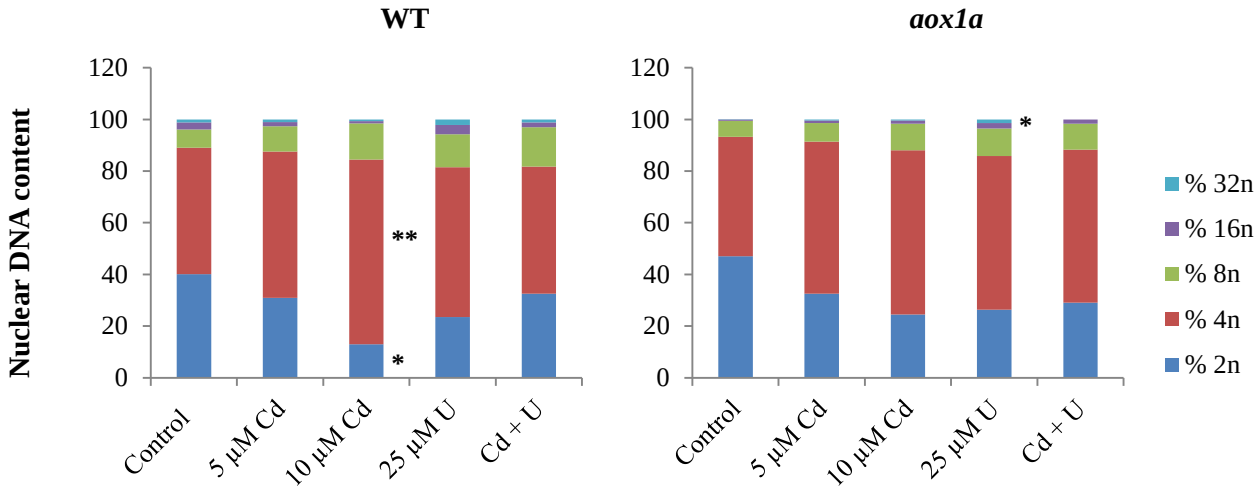
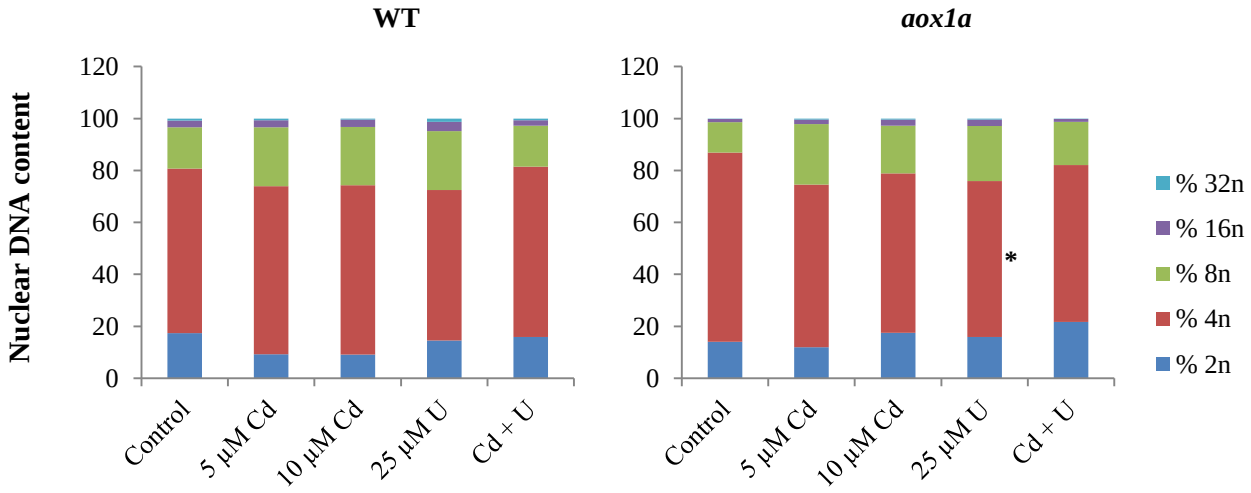
SUPPLEMENTARY DATA



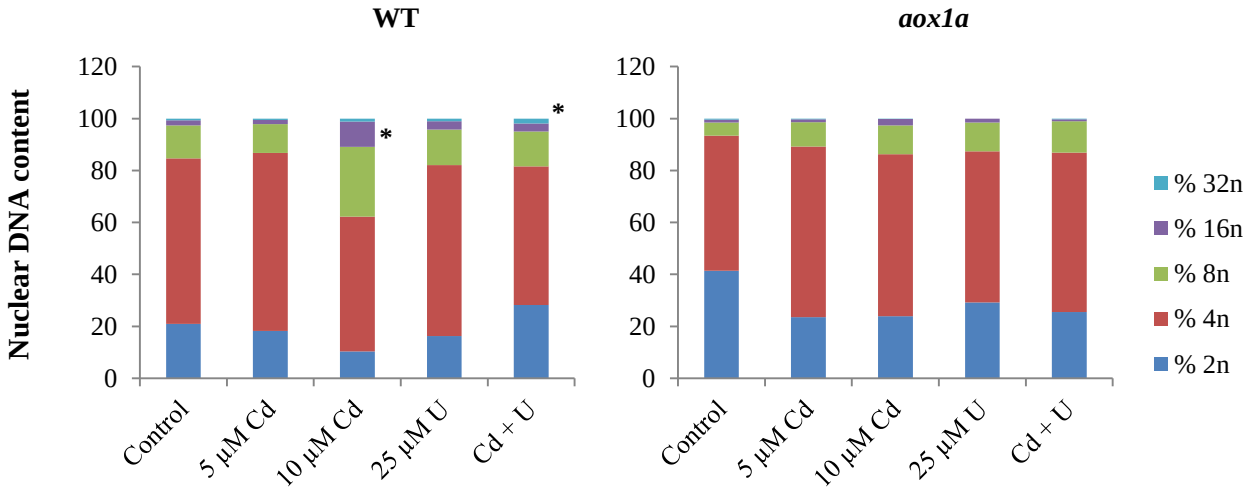
Supplementary Figure 1. Nuclear DNA content in the **second youngest leaf** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for **24 h**. All percentages of a certain n-value are compared to the control of the same genotype. Data represent the mean ± S.E. of 6 biological independent replicates. Significance levels compared to its own genotype under control conditions (indicated on the right side of each bar) (One-way ANOVA): *: $p < 0.05$



Supplementary Figure 2. Nuclear DNA content in the **second youngest leaf** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for **72 h**. All percentages of a certain n-value are compared to the control of the same genotype. Data represent the mean ± S.E. of 4 biological independent replicates. Significance levels compared to its own genotype under control conditions (indicated on the right side of each bar) (One-way ANOVA): *: $p < 0.05$; **: $p < 0.01$.



Supplementary Figure 3. Nuclear DNA content in the **third youngest leaf** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for **24 h**. All percentages of a certain n-value are compared to the control of the same genotype. Data represent the mean ± S.E. of 6 biological independent replicates. Significance levels compared to its own genotype under control conditions (indicated on the right side of each bar) (One-way ANOVA): *: $p < 0.05$.



Supplementary Figure 4. Nuclear DNA content in the **third youngest leaf** of wild-type (WT) and *aox1a*-knockout (*aox1a*) *A. thaliana* seedlings exposed to 5 or 10 µM Cd, 25 µM U or a combination of 2.5 µM Cd and 12.5 µM U for **72 h**. All percentages of a certain n-value are compared to the control of the same genotype. Data represent the mean ± S.E. of 4 biological independent replicates. Significance levels compared to its own genotype under control conditions (indicated on the right side of each bar) (One-way ANOVA): *: $p < 0.05$.

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