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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef
Cadmium-induced effects on endoreduplication in *Arabidopsis thaliana*

Promotor :
Prof. dr. Ann CUYPERS

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

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.



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LIST OF ABBREVIATIONS

•OH	Hydroxyl radical
¹O₂	Singlet oxygen
³O₂	Ground state oxygen
APC	Anaphase-promoting complex
APx	Ascorbate peroxidase
AsA	Ascorbate
Ca	Calcium
CAT	Catalase
Cd	Cadmium
CDK	Cyclin-dependent kinases
Cu	Copper
Cys	Cysteine
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
Fe	Iron
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRx	Glutaredoxin
GSH	Glutathione
GSH1	γ-glutamylcysteine synthetase
GSH2	Glutathione synthetase
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
H₂O₂	Hydrogen peroxide
IARC	International Agency for Research on Cancer
ICP-OES	Inductivity-coupled plasma optical emission spectrometer
Mg	Magnesium
MT	Metallothioneins
NADPH	Nicotinamide adenine dinucleotide phosphate
O₂^{•-}	Superoxide radical ion
O₂²⁻	Peroxide ion
Pb	Lead
PCs	Phytochelatin
PCS	Phytochelatin synthase
PI	Propidium iodide
PRx	Peroxiredoxins
Rb	Retinoblastoma tumor suppressor protein
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription real-time polymerase chain reaction
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TRx	Thioredoxin
Vit E	Vitamin E
Zn	Zinc

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SAMENVATTING

Vervuiling van het milieu met metalen door menselijke activiteiten beïnvloedt vele regio's wereldwijd. Cadmium (Cd) is een toxisch metaal dat makkelijk wordt opgenomen door planten en daardoor in de voedselketen terecht komt, dit leidt tot negatieve gevolgen voor de gezondheid van verscheidene organismen. Eén van de belangrijkste effecten van Cd in planten is de inductie van oxidatieve stress door een verhoogde productie van reactieve zuurstofvormen (ROS). Om de cellulaire redox balans te behouden, bezitten organismen een antioxidatief verdedigingssysteem bestaande uit enzymen en metabolieten zoals ascorbaat (AsA) en glutathion (GSH). Daarnaast blijken AsA en GSH betrokken te zijn in de regulatie van de celcyclus. In een alternatieve celcyclus, endoreduplicatie genaamd, wordt chromosomaal DNA gerepliceerd zonder dat de cel mitose ondergaat. Dit resulteert in plantencellen die meerdere kopieën van het diploïde genoom bezitten, of endopolyploidie. Daarnaast blijkt endoreduplicatie geïnduceerd te worden door externe stressfactoren zoals bijvoorbeeld UV-B straling. Daarom stellen we de hypothese dat Cd endoreduplicatie beïnvloedt in blaadjes van *Arabidopsis thaliana* en hierbij oxidatieve stress gebruikt wordt als een onderliggend mechanisme.

Om deze hypothese te onderzoeken werden in deze studie de effecten van Cd op endoreduplicatie en de mogelijke link met de Cd-geïnduceerde oxidatieve stress onderzocht in blaadjes van *Arabidopsis thaliana*. De eerste onderzoeksfase van deze studie had daarom als doel de mate van endoreduplicatie te bepalen in aparte blaadjes van *A. thaliana* gegroeid onder **controle omstandigheden**, van dag 11 tot dag 21 na het zaaien. Metingen door middel van flowcytometrie hebben aangetoond dat de **mate van endoreduplicatie** stijgt naarmate de blaadjes ouder worden door de opeenvolging van endocycli. Daarnaast werd er aangetoond dat endoreduplicatie een belangrijke rol speelt in de ontwikkeling van het blad, voornamelijk tijdens de expansiefase van bladgroei. Omdat endoreduplicatie leeftijdsafhankelijk blijkt te zijn en mogelijk gereguleerd wordt door redox signalisatie, werden ook **oxidatieve stressgerelateerde parameters** en leeftijdsafhankelijke verschillen bestudeerd onder controle omstandigheden. De concentratie GSH was in het algemeen lager in oude blaadjes ten opzichte van jonge blaadjes. Expressieniveaus van oxidatieve stress markeringen waren in het algemeen gestegen in oude blaadjes vergeleken met jonge blaadjes. Daarom stellen we dat deze effecten te wijten zijn aan verhoogde gevoeligheid van jonge blaadjes voor stressfactoren, resulterend in een grotere behoefte aan metabolieten. Terwijl oude blaadjes, door naderende senescentie, verlaagde antioxidatieve capaciteiten en verhoogde niveaus van oxidatieve stress vertonen.

In de tweede fase van deze studie werden **Cd-geïnduceerde effecten op endoreduplicatie** en de mogelijke link met de **Cd-geïnduceerde oxidatieve stress** onderzocht in *A. thaliana* blootgesteld aan 0, 1, 2.5, 5, 7.5 en 10 μM Cd, vanaf dag 11 na het zaaien. Ondanks het feit dat onderzoek heeft aangetoond dat verscheidene externe stressfactoren endoreduplicatie induceren, tonen onze resultaten dat Cd blootstelling de mate van endoreduplicatie in blaadjes van *A. thaliana* verlaagt. Tegen onze verwachtingen in werden de expressieniveaus van *SMR* genen, positieve regulatoren van endoreduplicatie, verhoogd na blootstelling aan Cd. Expressieniveaus van *DEL1*, een negatieve regulator, werden daarentegen niet beïnvloedt door blootstelling aan Cd. Aangezien deze *SMR* genen ook een rol blijken te spelen in de ROS-geïnduceerde DNA schade respons, stellen we de hypothese dat ROS-geïnduceerde DNA schade *ATM* activeert en deze op zijn beurt de expressie van *SMR* genen induceert. Aangezien belangrijke regulatoren van endoreduplicatie dus een rol spelen in de Cd-geïnduceerde oxidatieve DNA schade respons, en concentraties van antioxidatieve metabolieten, net zoals endoreduplicatie, sterk leeftijdsafhankelijk blijken te zijn, is Cd-geïnduceerde oxidatieve stress mogelijk betrokken in de Cd-geïnduceerde effecten op endoreduplicatie.

ABSTRACT

Environmental metal pollution caused by anthropogenic activities affects many regions worldwide. **Cadmium (Cd)** is a toxic metal which is easily taken up by plants and thereby introduced into the food chain, causing negative health effects in many organisms. One of the most important effects of Cd in plants is the induction of **oxidative stress** by an increased production of reactive oxygen species (ROS). In order to maintain the cellular redox balance, organisms possess an antioxidative defence system consisting of enzymes and metabolites. In addition to their role as antioxidative metabolites, ascorbate (AsA) and glutathione (GSH) are involved in the regulation of the cell cycle as well. In an alternative cell cycle mode called **endoreduplication**, chromosomal DNA is replicated without intervening mitoses. This results in plant cells containing multiples of the diploid genome and thus endopolyploidy. In addition, endoreduplication seems to be affected by external stress factors such as high UV-B irradiation. Therefore, we hypothesized that Cd affects endoreduplication in leaves of *Arabidopsis thaliana* using oxidative stress as an underlying mechanism.

In this study, Cd-induced effects on endoreduplication and the possible link with the Cd-induced oxidative challenge were investigated in leaves of *A. thaliana* seedlings. The first objective of the study was to investigate the **extent of endoreduplication** in separate leaves of *A. thaliana* grown under **control conditions** from day 11 to day 21 after sowing. Flow cytometric measurements showed the extent of endoreduplication to increase with increasing leaf age by successive endocycling. In addition, endoreduplication was shown to play an important role in leaf development, especially during the expansion phase of growth. Since endoreduplication seems leaf age-dependent and may possibly be affected by redox signalling, **oxidative stress-related parameters** and age-related differences herein were measured under control conditions as well. Concentrations of GSH were generally lower in old leaves as compared to young leaves and expression levels of oxidative stress markers were increased in old leaves as compared to young leaves. We hypothesize that these effects might be due to a combination of increased vulnerability of younger leaves to external stressors and therefore higher antioxidative metabolite concentrations, and decreased oxidative capacities of old leaves approaching senescence which results in higher levels of oxidative stress.

In the second part of this study, **Cd-induced effects on endoreduplication** and the possible link with the **Cd-induced oxidative challenge** were investigated in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5, and 7.5 or 10 μM Cd, starting from day 11 after sowing. Although research has shown several external stress factors to induce endoreduplication, our results showed Cd exposure to decrease the extent of endoreduplication in leaves of *A. thaliana*. In contrast to our expectations, *SMR* genes, positive regulators of endoreduplication, were increased in response to Cd exposure while expression levels of *DEL1*, a negative regulator of endoreduplication, remained unaltered. Since the measured *SMR* genes are also known to be involved in the ROS-induced DNA damage response, we hypothesize that ROS-induced DNA damage activates *ATM* and subsequently the *SMR* genes. This was supported by increased gene expression levels of *ATM* and oxidative stress markers. Since important regulators of endoreduplication seem to be involved in the Cd-induced oxidative DNA damage response and antioxidative metabolite concentrations seem to be leaf age-dependent, similar to endoreduplication, Cd-induced oxidative stress may be involved in the Cd-induced effects on endoreduplication.

1 INTRODUCTION

Extensive mining and industrial activities during the late 19th and early 20th century in addition to diverse anthropogenic activities nowadays, have led to a widespread environmental pollution by metals including **cadmium** (Cd)^(1, 2). Their occurrence in the atmosphere, soil and water can cause problems to all organisms. After being released into the environment, these metals can be taken up by plants and introduced into the food chain, causing risk for human health⁽³⁾.

1.1 Cadmium

1.1.1 Cadmium in the environment

Cadmium is a metal present in soils, water and the atmosphere and occurs as complex oxides, sulphides and carbonates in zinc (Zn), lead (Pb) and copper (Cu) ores. It is released from **natural sources** such as the earth's crust and mantle by volcanic activity and weathering of rocks. However, historic anthropogenic Cd releases deposited in soils, landfills and waste piles have led to Cd concentrations exceeding those from natural sources. Up to now, **anthropogenic activities** such as the mobilisation of Cd impurities in raw materials (e.g. phosphate minerals, fossil fuels, Zn and Cu) and its intentional use in products and processes (e.g. non-ferrous metal production, power plants, cement production, nickel-Cd battery industry, phosphate fertilizers) still contribute to environmental Cd pollution^(4, 5). For example, in Belgium, the rural northern part of the Kempen is polluted by Cd, primarily due to past emissions by zinc smelters from non-ferrous industries⁽⁶⁾.

1.1.2 Cadmium in humans

During the last decades, Cd has raised great concern and has been recognised not only as an occupational health hazard but also as a serious risk to environmentally exposed populations^(6, 7). Since Cd can be easily taken up from contaminated soils by plants, it is accumulated in crops and vegetables and introduced into the **food chain**. Therefore, human exposure to Cd is mainly due to the ingestion of contaminated food or exposure to tobacco smoke^(7, 8). Other possible sources of exposure are house dust, ambient air and drinking water⁽⁴⁾. Although dietary uptake of Cd is the primary route of exposure, absorption of Cd via the lungs is higher than via the gastrointestinal tract. Cadmium is inhaled as particulate matter and its absorption is strongly dependent upon particle size and solubility⁽⁹⁾.

Once absorbed into the bloodstream, Cd is mainly stored in internal organs such as the liver and kidneys. With increasing exposure intensity, an increasing proportion of the absorbed Cd is stored in the liver, although the highest concentrations are generally found in the renal cortex⁽⁴⁾.

The **kidney** is considered the critical target organ for chronic dietary Cd exposure. Effects of Cd-induced renal damage include proximal tubular reabsorptive dysfunction, which results in increased protein excretion in urine. Furthermore, prolonged exposure to high levels of Cd may lead to decreases in calcium absorption, **bone** mineralization and bone formation in addition to an increase in bone resorption, causing the so called *itai-itai* disease⁽⁷⁾. The very long biological half-life of Cd, ranging from 10 to 30 years in the human body due to a generally slow excretion, is an important cause for human Cd toxicity⁽¹⁰⁾. In addition, the International Agency for Research on Cancer (IARC) has classified Cd as a group I human **carcinogen**⁽⁴⁾.

1.1.3 Cadmium in plants

Although Cd is a non-essential element and toxic metal for plants, it is readily taken up by their roots due to its high mobility and water solubility. Furthermore, transport of Cd ions via transmembrane

carriers involved in the uptake of Ca^{2+} , Fe^{2+} , Mg^{2+} , Cu^{2+} and Zn^{2+} is facilitated due to their chemical similarity with these essential elements. The degree of **Cd uptake** by plants is strongly dependent on its concentration in the soil and its bioavailability, modulated by the soil pH, redox potential, temperature, presence of organic matter and concentrations of other elements^(8, 11).

Once taken up by the roots, Cd-ligand complexes can reach the xylem through an apoplastic and/or symplastic pathway, and are transported to the aerial parts of the plant. However, most are retained in the roots⁽³⁾.

Chlorosis, leaf roll and stunted growth of both roots and stems are visible symptoms caused by **Cd toxicity**. On a physiological level, Cd affects enzyme activities, damages the photosynthetic apparatus, disturbs the water balance and inhibits stomatal opening and mitochondrial oxidative phosphorylation. In this way, it affects important physiological processes such as photosynthesis, transpiration and respiration. Furthermore, Cd has been reported to interfere with uptake, transport and use of several elements and water by plants^(2, 3). On a cellular level, the most important effect caused by Cd is oxidative stress⁽²⁾.

1.2 Cd-induced oxidative stress

Although Cd is a non-redox-active element and thus unable to generate free radicals directly, it has been shown to induce the production of **reactive oxygen species (ROS)**. These are partially reduced forms of ground state oxygen ($^3\text{O}_2$) such as singlet oxygen ($^1\text{O}_2$), superoxide radical ion ($\text{O}_2^{\bullet-}$), peroxide ion (O_2^{2-}), hydrogen peroxide (H_2O_2). Furthermore, hydroxyl radicals ($^{\bullet}\text{OH}$) are produced through the Fenton reaction in which a free redox-active metal such as Fe^{2+} is oxidized to Fe^{3+} , using H_2O_2 . Afterwards, Fe^{3+} is reduced back to Fe^{2+} using $\text{O}_2^{\bullet-}$ as a substrate. The overall reaction, called the Haber-Weiss reaction, uses H_2O_2 and $\text{O}_2^{\bullet-}$ to produce O_2 , OH^- and the highly reactive $^{\bullet}\text{OH}$ ⁽¹²⁾. The increased ROS production causes **oxidative stress**, which is defined as a disruption of the cellular balance between pro- and antioxidants in favour of the former.

Cadmium is shown to induce oxidative stress via many different pathways. For example, studies have reported **mitochondrial ROS production**, caused by physiological activity of the respiratory chain, to be increased by Cd. Furthermore, Cd has been shown to induce **NADPH oxidase activity**, increasing the production of $\text{O}_2^{\bullet-}$. Moreover, by its replacement of iron (Fe) due to interference with thiol groups in proteins, Cd increases the **free Fe concentration** and therefore the cellular amount of free redox-active metals, which can directly produce hydroxyl radicals ($^{\bullet}\text{OH}$) through the Fenton reaction. In addition, if interactions take place between Cd and **antioxidative enzymes**, their catalytic function may be inhibited. Finally, in order to prevent all of these interactions, Cd is scavenged by **glutathione (GSH)**. Due to the high affinity of Cd for thiols, GSH is a primary target for free Cd-ions. However, the Cd-induced depletion of reduced GSH will result in a disturbed redox balance, leading to an oxidative challenge⁽¹³⁾.

High levels of ROS can cause **oxidative damage**, which is the disruption of cellular macromolecules such as degradation of proteins, cross-linking of DNA and peroxidation of membrane fatty acids⁽²⁾.

However, other than this damaging property, ROS have been shown to fulfil important **signalling functions**. By this means, oxidative stress can cause cellular damage, although, controlled levels of ROS play an important role in modulating signalling networks that control both physiological processes and stress responses⁽¹⁴⁾. It is therefore of great importance to keep these levels of ROS within a certain range, a function which is performed by the **antioxidative defence system**. For this reason, in certain cases the term **oxidative challenge** is more appropriate to use.

1.2.1 Antioxidative defence system

In order to maintain the cellular redox balance, organisms possess an **antioxidative defence system** consisting of both enzymes and metabolites⁽¹⁵⁾.

1.2.1.1 Antioxidative enzymes

An interacting network of antioxidative enzymes provides protection against oxidative stress. **Superoxide dismutases** (SODs) catalyse the dismutation of $O_2^{\cdot-}$ to H_2O_2 and are classified according to their metal cofactor. Plant cells possess three different SODs: CuZnSOD, FeSOD and MnSOD. Subsequently, in order to detoxify H_2O_2 , **peroxidases** such as ascorbate peroxidase (APx) and glutathione peroxidase (GPx) couple the reduction of H_2O_2 to H_2O to the oxidation of ascorbate (AsA) and glutathione, respectively. In addition, peroxiredoxins (PRxs) are important H_2O_2 -removal systems due to cysteine (Cys) at the active sites of which the sulfhydryl- or thiol-groups (-SH) are oxidized by H_2O_2 ⁽¹⁶⁾. After oxidation, they are converted back to their reduced form by thiols from thioredoxin (TRx), glutaredoxin (GRx) and GSH⁽¹⁷⁾. In contrast, **catalases** (CATs) break down H_2O_2 molecules to H_2O and O_2 without the use of any cellular reducing equivalents⁽¹³⁾.

1.2.1.2 Antioxidative metabolites

Besides enzymes, certain metabolites also play an important role in antioxidative defence. Two groups of antioxidative metabolites are distinguished: lipid-soluble or hydrophobic metabolites such as vitamin E (vit E) and water-soluble or hydrophilic metabolites such as GSH and AsA⁽¹³⁾.

Vitamin E or tocotrienol effectively inhibits the peroxidation of lipids by its incorporation into lipid environments such as cellular membranes. It is hypothesized that Vit E is oxidized by quenching lipid radicals. The generated free radical (chromanoxyl) form is then reduced back by interactions between water- and lipid-soluble substances⁽¹⁸⁾.

Glutathione is a thiol-containing tripeptide (γ -glutamyl-cysteinyl-glycine) and essential hydrophilic metabolite with thiol-disulphide interactions, making it a key antioxidant. The thiol group of cysteine enables a reversible oxidation of reduced glutathione by donating an electron directly to unstable molecules such as ROS. In this way, GSH is able to scavenge ROS non-enzymatically. After donating an electron, GSH itself becomes reactive and reacts with another reactive GSH to form glutathione disulphide (GSSG), which is recycled back to GSH by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutathione reductase (GR). In addition to this continuous reduction of GSSG by GR, GSH is also synthesized *de novo* via a pathway involving two enzymes, γ -glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2)^(17, 19). Besides its role in antioxidative defence, GSH is also involved in the detoxification of free metal ions, thereby regulating metal homeostasis. Glutathione S-transferase (GST) catalyses the conjugation forming a GSH-metal complex⁽¹⁷⁾. In addition, GSH is the precursor of **phytochelatins** (PCs), which are metal-binding peptides synthesized by phytochelatin synthase (PCS) in response to Cd and other metals. Phytochelatins are able to sequester the metal as well, reducing the circulation of free Cd^{2+} ions. After chelation, these complexes are transported to the vacuole, leaving cytoplasmic enzymes undamaged⁽³⁾. In the same way, **metallothioneins** (MTs) are proteins rich in cysteine clusters which detoxify Cd by complexation and vacuolar sequestration⁽¹³⁾.

Ascorbate or vitamin C is another abundant water-soluble antioxidative metabolite which plays essential roles in multiple physiological processes. Together, GSH and AsA participate in the **AsA-GSH cycle** which is located in various plant subcellular compartments and is responsible for detoxification of H_2O_2 (Figure 1). In this cycle, AsA is used by the plant-specific APx to reduce H_2O_2 to H_2O . The oxidized form of AsA, dehydroascorbate (DHA), is then recycled by dehydroascorbate

reductase (DHAR) using GSH as an electron donor. Finally, the oxidized dithiol GSSG is reduced again to GSH using electrons from NADPH in a reaction catalysed by GR^(17, 20). In this way, GSH transfers its reducing equivalents to enzymes or components in order to maintain the cellular redox balance⁽¹³⁾.

In addition to their role in the antioxidative defence system, both GSH and AsA are involved in the regulation of **the cell cycle** and thereby affect plant growth and development.

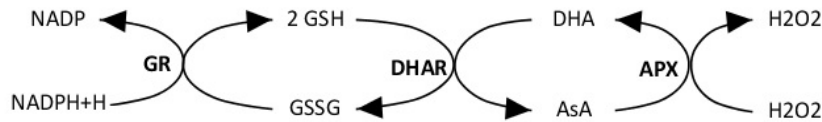


Figure 1: The glutathione-ascorbate cycle. GR: glutathione reductase; GSH: glutathione; GSSG: glutathione disulphide; DHA: dehydroascorbate; DHAR: DHA reductase; AsA: ascorbate; APX: ascorbate peroxidase (adapted from Meyer *et al.* (2008)⁽²⁰⁾).

1.3 The cell cycle

The cell cycle is an ordered sequence of events leading to cell growth and finally cell division into two separate daughter cells containing identical genetic material. This cell-division cycle typically consists of four phases: a gap 1 (G₁) phase, a synthesis (S) phase, a gap 2 (G₂) phase and finally mitosis (M).

During the **G₁ phase** the cell is metabolically active and grows continuously. This phase is followed by the **S phase**, during which DNA replication takes place, doubling the nuclear DNA content from 2C to 4C (C-value refers to the degree of ploidy and is relative to the replication state of the cell⁽²¹⁾). After DNA synthesis is fully completed, the cell will enter the **G₂ phase**, in which it will continue to grow and synthesize proteins prior to mitosis. During the **M phase**, mitosis takes place. This will separate the chromosomes and is followed by cytokinesis. Cells can also exit the G₁ phase to enter the **G₀**, a quiescent stage of the cycle in which the cell remains metabolically active but no longer proliferates, unless it is stimulated by extracellular signals to do so⁽²²⁾.

In plants as well as other eukaryotes, **cyclin-dependent kinases** (CDK) are of great importance in the regulation of cell cycle progression. Their regulatory subunits, **cyclins**, fluctuate in phase with the cell cycle (Figure 2A). Which target proteins are phosphorylated by the CDK-cyclin complex is crucial to cell cycle progression and is determined by the associated cyclin, as each catalytic subunit can associate with diverse cyclins. Three classes of CDK-cyclin complexes regulate progression through the cell cycle: G₁, S and M CDK-complexes.

During **early G₁**, CDK activity is kept low in order to form pre-replication complexes at replication origins in preparation for the next S phase. In **late G₁ phase**, G₁ CDK activity rises due to the increasing level of G₁ cyclins until it is sufficient to inactivate the S phase inhibitor Rb, releasing the S CDK complexes' activity and triggering the **S phase**. During the S and G₂ phase, mitotic CDK complexes are already synthesized, although their activity is kept low. In **late G₂ phase**, transcription of M cyclins increases until a high level of M CDK-cyclin complexes is achieved and the cell enters the **M phase**. The anaphase-promoting complex (APC) will now become active and degrades anaphase inhibitors, thus commencing anaphase. Later in M phase, M cyclins are degraded by the APC, lowering the CDK activity back to early G₁ levels⁽²³⁾.

As mentioned before, GSH and AsA play an important role in the regulation of the cell cycle as well. *Arabidopsis thaliana* mutants containing lowered endogenous **AsA** concentrations, showed retarded cell division and slow plant growth. It is hypothesized that the AsA-enhanced transition from G₁ to S is due to the use of AsA for the biosynthesis of hydroxyproline-rich proteins required for this progression. In contrast, only a critical concentration of **GSH** is required for cell cycling⁽²⁵⁾. In G₁ phase,

GSH is recruited into the nucleus so that the total cellular GSH pool exceeds the level present at early G₁, which is considered essential for G₁ to S phase progression⁽²⁶⁾.

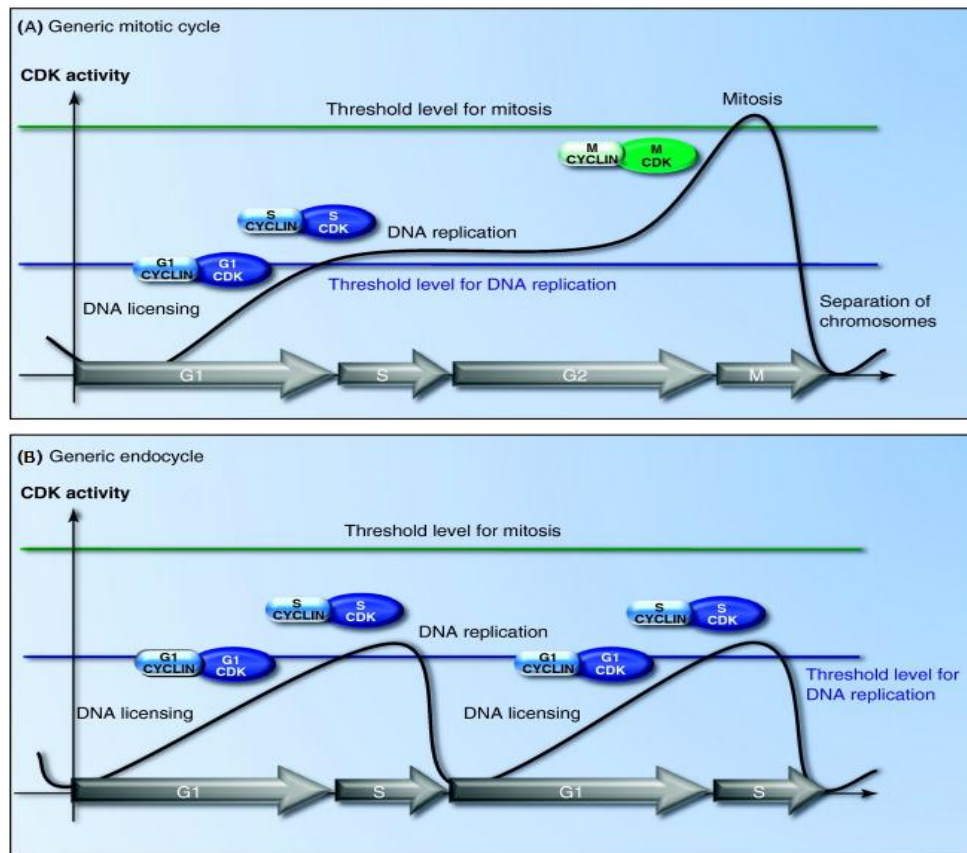


Figure 2: Roles of CDK-cyclin complexes in mitotic and endoreduplication cycles. **(A)** A typical mitotic cycle. During early G₁, CDK activity must be low to allow licensing of replication origins. Late in G₁, CDK levels rise due to increasing levels of G₁ cyclins, until the level of CDK activity is sufficient to inactivate Rb (or its homologs) and trigger S phase, including firing of replication origins and S-phase transcription. Maintenance of S phase requires specific CDK–cyclin complexes. During G₂, transcription of M-phase cyclins rises, until a high level of active CDK–cyclin complexes is reached and M phase begins. In mid-M phase, mitotic cyclins are destroyed by the APC/C and CDK levels once again drop to early G₁ levels. **(B)** Typical endocycle. CDK levels of G₁ and S continue to cycle between low levels in a G₁-like phase for licensing of the replication origins and the increased levels are needed to trigger S. Mitotic CDK complexes either never form or their activity is suppressed, with CDK activity never reaching the level necessary to trigger M as a consequence. CDK: cyclin-dependent kinases (adapted from De Veylder *et al.* (2011)⁽²⁴⁾)

1.3.1 Endoreduplication

Besides the regular cell division cycle, plant cells can enter an alternative cell cycle mode called **endoreduplication**. During endoreduplication, chromosomal DNA is replicated (S-phase) without intervening mitoses (M-phase). This will give rise to polyploid plant cells which contain multiples of the diploid genome equivalent.

Besides higher plants also algal and fern cells as well as other eukaryotes undergo endoreduplication. Furthermore, many cell types show polyploidy, although it is mostly found in those undergoing differentiation and expansion. Between different species, ploidy levels vary greatly, ranging from 4C to 32C in *A. thaliana* and up to 24576C in extreme cases such as *Arum maculatum*⁽²⁷⁾. Moreover, within the same plant, older tissues often display higher levels of ploidy than younger ones.

Endoreduplication is considered essential for **growth** and **developmental processes**, although its physiological purpose still remains unclear⁽²⁴⁾. Because of its high occurrence in tissues showing high metabolic activity, it is often hypothesized that endoreduplication provides a mechanism for increasing

the availability of DNA template in order to increase gene expression levels. Correspondingly, endoreduplication is suggested to be linked to increased nuclear volume and/or cell size. It has therefore been considered as a mechanism to provide sufficient DNA prior to massive increases in tissue mass, determining a potential range of cell volumes rather than a strict cell size⁽²⁸⁾. Besides its role in developmental processes, endoreduplication can be affected by **external stress factors**. For example, Gegas *et al.* (2014) has recently shown high UV-B irradiation to stimulate endoreduplication in leaves of *A. thaliana*⁽²⁹⁾. Similarly, water-deficit stress has been shown to increase endoreduplication in *Arabidopsis* leaf mesophyll cells⁽³⁰⁾. These findings suggest that endoreduplication may provide a mechanism to mitigate effects of exogenous stress.

The endoreduplication cycle is regulated by most of the same key regulators of the classical cell division cycle (Figure 2B). In order to establish an endocycle, the main challenge is to keep CDK-activity below a level that triggers mitosis⁽²⁴⁾. This lowered CDK-activity is achieved either at the transcriptional or the post-translational level. At the transcriptional level, the down-regulation of genes encoding M cyclins was shown during the transition from mitosis to endoreduplication. At the post-translational level, activators of the APC promote proteolysis of M cyclins and therefore the inactivation of M-phase CDK kinase activity. Furthermore, two inhibitory proteins (ICK1 and ICK2) were found to inactivate CDK activity in *Arabidopsis*. However, their actual involvement in the impairment of M-CDK activity still needs to be confirmed. Altogether, the differential regulation of these key regulators determines whether a cell follows the cell division cycle or enters the endocycle, which is regulated at the transcriptional or post-translational level⁽²⁸⁾. Several important regulators and their interactions are presented in Figure 3.

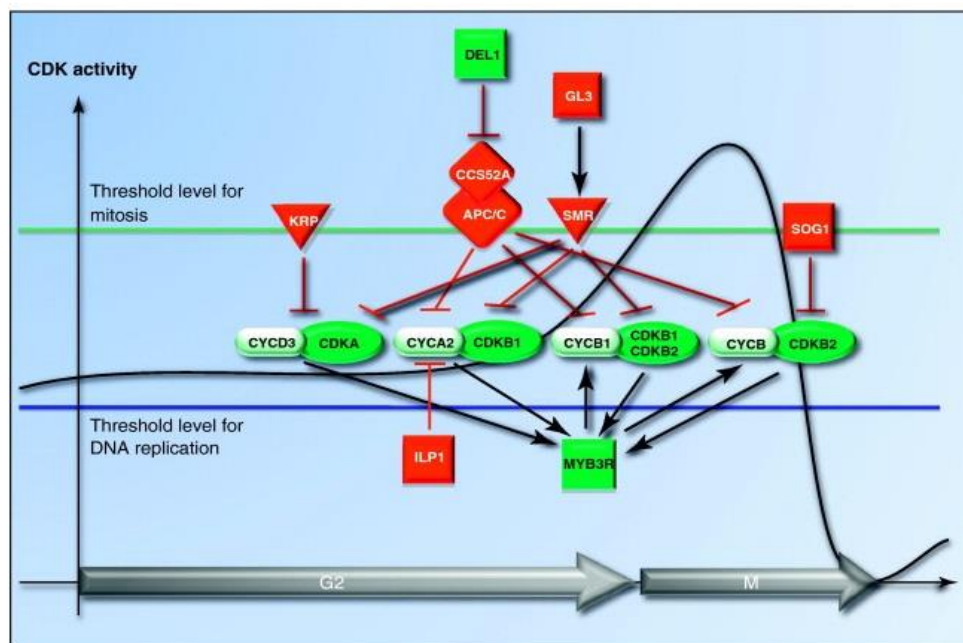


Figure 3: Regulatory wiring of the G2-M control machinery in mitosis and endoreduplication. Positive interactions are indicated with a black arrow, negative with a red T-line. Positive regulators of mitosis in green, positive regulators of endoreduplication in red with. Transcriptional regulators are drawn in squares, CKIs in triangles, protein degradation machinery with a diamond. The horizontal blue and green lines indicate hypothetical thresholds levels for DNA replication and mitosis, respectively. Please note that the biologically relevant composition of most of CDK–cyclin complexes is not clear at the moment and the presented data is largely derived from interaction studies. The transcriptional activation of the SMR SIM by GL3 limited to trichomes. However, a similar activation of SMRs in other cell types and tissues by developmentally acting transcription factors can be hypothesized. 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)⁽²⁴⁾).

1.4 Objectives

The goal of this project is to increase our knowledge on **endoreduplication in plants**. In addition to studying this alternative cell cycle under **control** conditions, the effects of **Cd exposure** and the possible link with the Cd-induced **oxidative challenge** are investigated. For this project *Arabidopsis thaliana* was used. Its small genome and the availability of its genetic information facilitate molecular research. Furthermore, its rapid life cycle and relatively easy cultivation make this species a suitable model plant.

1.4.1 Endoreduplication and oxidative stress related parameters under control conditions

The first objective of this project is to study endoreduplication in *A. thaliana* leaves during normal growth and development. For this purpose, *A. thaliana* wild-type plants are grown under **control conditions** in a hydroponic cultivation system. Starting from day 11 after sowing, when the first two rosette leaves have fully emerged, the plants are analysed phenotypically during 10 days of normal development. For this, rosette diameter, leaf surface area, and fresh weight are measured daily.

Furthermore, nuclear ploidy levels of the first leaf pair are measured daily using flow cytometry. The nuclear DNA content of younger leaves is measured as well, (if these have already fully emerged) at day 12, 14, 18 and 21 after sowing. As preliminary experiments have shown that the level of nuclear ploidy is strongly dependent on the leaf age, measurements are performed on separate leaves.

Moreover, two oxidative stress-related parameters are studied under control conditions and compared between the oldest and younger leaves of the plant. Firstly, the concentration of GSH as well as the redox state are determined using a plate reader method. Secondly, expression levels of oxidative stress marker genes are measured using reverse transcription real-time polymerase chain reaction (RT-qPCR).

1.4.2 Cd-induced effects on endoreduplication and the link with the Cd-induced oxidative challenge

The next objective is to determine **Cd-induced effects on endoreduplication** in leaves of *A. thaliana*. Preliminary experiments have shown only mild effects on nuclear ploidy levels after short-term exposure of 19 day-old plants to Cd. However, since endoreduplication is most often found in metabolically active cells including those undergoing differentiation and expansion, plants exposed to Cd starting from an earlier time point during development may show more pronounced effects. Therefore, 11 day-old *A. thaliana* plants are exposed to a range of Cd concentrations.

The uptake and accumulation of Cd and essential nutrients in the leaves is determined after 10 days of exposure using inductively-coupled plasma optical emission spectrometry (ICP-OES).

Nuclear ploidy levels of leaves are measured 24 hours, 72 hours, 7 days and 10 days after start of the exposure. Expression levels of important positive and negative regulators of endoreduplication as well as DNA damage signalling genes are measured in response to 11 days of Cd exposure.

A possible link with the **Cd-induced oxidative challenge** is investigated via the analysis of several oxidative stress-related parameters 10 days post-exposure. Leaf age-related differences in GSH and AsA concentrations and redox states are measured in addition to expression levels of oxidative stress marker genes. Furthermore, the extent of lipid peroxidation, a frequently used oxidative stress marker, is assessed using the TBARS assay.

2 MATERIALS AND METHODS

In this study Cd-induced effects on endoreduplication and the possible link with the Cd-induced oxidative challenge were investigated in *A. thaliana* leaves (ecotype Columbia). To this end, nuclear ploidy levels and several oxidative stress-related parameters were determined in leaves of wild-type plants exposed to a range of environmentally relevant Cd concentrations (1, 2.5, 5, 7.5 or 10 μM CdSO₄).

2.1 Plant cultivation and Cd exposure

Seeds were surface-sterilized using a sodium hypochlorite solution and Tween 80. In order to synchronize germination, the seeds were spread on moist filter paper and incubated for 3 days at 4 °C in the dark. Next, they were grown in a hydroponic cultivation system as described by Keunen *et al.* (2011)⁽³¹⁾. Therefore, they were first sown on sand-filled 15 mL polypropylene centrifuge tubes cut in half. These tubes were placed in a PVC frame on top of a container filled with a modified Hoagland solution (0.505 mM KNO₃, 0.15 mM Ca(NO₃)₂ × 4 H₂O, 0.1 mM NH₄H₂PO₄, 0.1 mM MgSO₄ × 7 H₂O, 4.63 μM H₃BO₃, 0.91 μM MnCl₂ × 4 H₂O, 0.03 μM CuSO₄ × 5 H₂O, 0.06 μM H₂MoO₄ × H₂O, 0.08 μM ZnSO₄ × 7H₂O, 1.64 μM FeSO₄ × 7 H₂O and 0.81 μM Na₂-EDTA). Each frame contained 90 tubes and one oxygenator, continuously adding O₂ to the solution. The plants were grown in a growth chamber at 65% relative humidity with day/night temperatures of 22 °C/18 °C and a 12 hours photoperiod. A combination of blue, red and far-red LED lights was used (170 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at the rosette level) to obtain a spectrum simulating the photosynthetic active radiation (PAR) in sunlight. The modified Hoagland nutrient solution was refreshed twice a week to maintain nutrient availability and pH.

Subsequently, 11-day old seedlings were exposed to 0, 1, 2.5, 5, 7.5 or 10 μM Cd by addition of CdSO₄ to the Hoagland solution. Leaves were harvested 24 hours, 72 hours, 7 days or 10 days after exposure to Cd. Phenotypic measurements were performed immediately after harvesting of the plants, as described in section 1.2. Consequently, leaves were snap frozen in liquid nitrogen and stored at -70 °C for further analyses. Samples for element analysis were dried in an oven at 80 °C.

2.2 Kinematic analysis

Phenotypic analyses were performed by weighing and scanning (Epson Perfection V330 Photo at 300 dpi) plant rosettes and measuring root length. Next, all rosette leaves were detached and scanned separately. The rosette diameter and leaf surface area were analysed using ImageJ software (U. S. National Institutes of Health). In this way, rosette growth and leaf development were monitored.

2.3 Element analysis

Leaf samples were analysed for Cd, phosphorous (P), sulphur (S), Zn, Fe and Cu content using inductively-coupled plasma optical emission spectrometry (ICP-OES) (Agilent Technologies 700 Series). Prior to measurement, leaf samples were dried for 2 weeks at 80 °C and digested using 70-71% HNO₃ and 37% HCl. Blank and reference (Trace elements in spinach, 1570a, Standard Reference Material) samples were used as a control. Finally, the samples were dissolved in 2% HCl and measured.

2.4 Analysis of nuclear DNA content

The nuclear DNA content, and thus the extent of endoreduplication, was determined using flow cytometry. Since nuclear ploidy levels have been shown to correlate with leaf age, all flow cytometric measurements were performed on separate leaves.

For the extraction and staining of nuclei, the CyStain PI Absolute P kit (Partec) was used. First, the leaf was chopped with a fresh razor blade in a petri dish containing 250 μ L extraction buffer. After incubation for approximately 2 minutes, the extract was filtered through a 50 μ m nylon filter (Partec Celltrics®). Next, 1 mL staining solution was added, containing 1000 μ L staining buffer, 6 μ L propidium iodide (PI) and 3 μ L RNase. According to the size of the leaves, the volumes of extraction buffer and staining solution were halved if necessary. After at least 1 hour of incubation in the dark at 4 °C, the nuclear DNA content of 5000 gated nuclei was analysed using the BD FACSCalibur™ Flow Cytometer (BD Biosciences). For this purpose, the red/orange emission (FL-2, 585/42 nm) was measured after excitation by a blue argon-ion laser (488 nm) and plotted against the forward scatter (FSC). Analysis of the data was performed using FCS Express 4 Image Cytometry software (De Novo Software).

2.5 Gene expression analysis

First, frozen leaf tissue was grounded using two stainless steel beads using the MM400 Mixer Mill (Retsch). Next, the RNA was isolated using the RNAqueous® Kit (Ambion Inc.). Both the concentration and purity of the RNA extracts were determined using the Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Inc). Subsequently, the RNA samples were stored at -70 °C.

During the next step, the extracted RNA was converted to cDNA using the Primescript™ RT reagent Kit (TaKaRa Bio). For each sample, the same amount of RNA starting material (1 μ g) was used in order to obtain comparable results after real-time PCR. Prior to the reverse transcription reaction, genomic DNA was removed using the TURBO DNA-free™ Kit (Ambion). Afterwards, the obtained cDNA samples were diluted 1/10 using 1/10 TE buffer 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 at universal cycling conditions (20 s 95 °C, 40 cycles of 3 s at 95 °C and 30 s at 60 °C) in the 7500 Fast Real-Time PCR System (Applied Biosystems). Each well contained 2 μ L of 1/10 diluted cDNA and 8 μ L of mastermix, consisting of 5 μ L Fast SYBR® Green Master Mix, 0.3 μ L forward primer, 0.3 μ L reverse primer and 2.4 μ L RNase-free water. Melting curves were used to check PCR product specificity and were set 15 s at 95 °C, 60 s at 60 °C, 15 s at 95 °C and 15 s at 60 °C.

Using the $2^{-\Delta Ct}$ method, expression levels of three reference genes (*SAND*, *UBC* and *EF-1 α*) were used to normalize the expression of the genes of interest. Reference genes were selected using the GrayNorm algorithm⁽³²⁾. Forward and reverse primer sequences are listed in Table 1.

2.6 Metabolite analysis

Reduced, oxidized and total concentrations of AsA and GSH were measured using a plate reader method adapted from Queval and Noctor⁽³³⁾. In order to compare AsA and GSH concentrations between leaves of a different age, rosettes were subdivided in samples consisting of leaf 1 to 4 and leaf 6 to 9 (Figure 4).

First, frozen leaves were extracted in 200 mM HCl using a cooled mortar and pestle. After centrifugation for 10 minutes at 13200 rpm and 4 °C, 30 μ L 200 mM NaH₂PO₄ (pH 5.6) was added to 300 μ L of supernatant. Next, the pH of the samples was adjusted to 4.5 using 200 mM NaOH.



Figure 4: Rosette of a 21-day old *A. thaliana* plant. Leaves are numbered from the oldest leaf (leaf 1) to the youngest leaf (leaf 9)

Reduced AsA was measured at 265 nm. Total AsA was measured at 265 nm after incubation of the samples with 25 μ M dithiothreitol and 120 mM NaH_2PO_4 (pH 7.5) for 15 minutes at 20 $^\circ\text{C}$, in order to fully reduce the AsA pool. Subsequently, the pH of the samples was adjusted to pH 5.5 using 200 mM HCl. This way, AsA oxidation was measured for 15 minutes after addition of 10 μ L ascorbate oxidase. By subtracting the amount of reduced AsA from total AsA, DHA was determined.

In order to determine total GSH, the GR-dependent reduction of 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB) was measured during 5 min at 412 nm. Glutathione disulphide was measured after incubation of the samples with 2-vinylpyridine for 30 minutes at 20 $^\circ\text{C}$ to precipitate reduced GSH. The difference between total GSH and GSSG was used to calculate reduced GSH. All measurements were performed using the FLUOstar Omega plate reader (BMG Labtech).

2.7 Analysis of lipid peroxidation

In order to measure the extent of lipid peroxidation, levels of thiobarbituric acid (TBA)-reactive substances (TBARS) such as malondialdehyde were measured spectrophotometrically. First, leaves were homogenised in 1 mL 0.1% trichloroacetic acid (TCA) using a cooled mortar and pestle. Next, the samples were centrifuged for 10 minutes at 13200 rpm and 4 $^\circ\text{C}$. Subsequently, 400 μ L of supernatant was added to 1 mL of 0.5% TBA in 20% TCA in duplo. For blank measurements, 0.1% TCA was used instead of supernatant. Then, samples were incubated for 30 minutes at 95 $^\circ\text{C}$. After incubation, the samples were cooled down immediately and centrifuged for 10 minutes at 13200 rpm and 4 $^\circ\text{C}$. Finally, the absorbance was measured at 532 nm (total absorbance) and 600 nm (aspecific absorbance) using the UV-1602 spectrophotometer (Shimadzu).

2.8 Statistical analysis

The data obtained from the experiments described above were statistically analysed using ANOVA. First, the normality of the data was verified using the Shapiro-Wilk test. Homoscedasticity of the data was tested using a residual plot and the Bartlett's test. In case of a non-normal distribution or heteroscedasticity of data, transformations (logarithmic, exponential, inverse or square root) were performed. Gene expression data were standardly log transformed. Post-hoc pairwise comparisons were performed using the Tukey-Kramer multiple comparisons of means. In case the assumptions for ANOVA were not met, a non-parametric Kruskal-Wallis test was used. In this case, the Wilcoxon Signed-Rank test was used for pairwise comparisons of the data. All these statistical analyses were performed in R (The R Foundation for Statistical Computing; version 3.1.2).

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. *SAND*: SAND-family; *UBC*: ubiquitin conjugating enzyme; *EF1 α* : elongation factor 1 α ; *UPOX*: upregulated under oxidative stress; *TIR*: toll-interleukin-resistance; *SMR*: SIAMESE-related; *DEL1*: DP-E2F-like 1; *ATM*: ataxia-telangiectasia mutated; *ATR*: ATM- and RAD3-related.

Gene	Locus	Forward primer	Reverse primer
Reference genes			
<i>SAND</i>	AT2G28390	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
<i>UBC</i>	AT5G25760	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
<i>EF1α</i>	AT2G18720	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA
Oxidative stress marker genes			
<i>UPOX</i>	AT2G21640	GACTTGTTTCAAAAACACCATGGAC	CACTTCCTTAGCCTCAATTTGCTTC
<i>Defensin-like protein</i>	AT2G43510	ATGGCAAAGGCTATCGTTTCC	CGTTACCTTGCGCTTCTATCTCC
<i>Unknown protein</i>	AT1G19020	GAAAATGGGACAAGGGTTAGACAAA	CCCAACGAAAACCAATAGCAGA
<i>Unknown protein</i>	AT1G05340	TCGGTAGCTCAGGGTAAAGTGG	CCAGGGCACAACAGCAACA
<i>TIR</i>	AT1G57630	ACTCAAACAGGCGATCAAAGGA	CACCAATTCGTCAAGACAACACC
Endoreduplication genes			
<i>SMR4</i>	AT5G02220	GATGGTGGTGAGAAAACGAGA	TCTCTTCGAGGCTGTGCGTAG
<i>SMR1</i>	AT3G10525	CAAGATCCGATCCAAAACCTCA	TTCTGGGATGTGGGTGTGC
<i>SMR5</i>	AT1G07500	CAGCATATCCGCTTGTCCA	CTGCTACCACCGAGAAGAACAAGT
<i>SMR7</i>	AT3G27630	ACATCGATTGCGGCTTCACTAA	CCGTGGGAGTGATACAAATTCC
<i>DEL1</i>	AT3G48160	CTCCCTTGATGACGCTGCAA	TGCTATATCATAAAGCCGCCTCAC
DNA Repair genes			
<i>ATM</i>	AT3G48190	TGATGTGCGCTGTTTCAGCTAA	GATGGGTAATGGAGTTGTGCTGATA
<i>ATR</i>	AT5G40820	GCCGATGAAGCCGAGATACTT	GTAAGCGGTTTATCATCGCAGTAAA

3 RESULTS

3.1 Endoreduplication and oxidative stress-related parameters under control conditions

In the first part of the study, endoreduplication was investigated in *A. thaliana* leaves during 10 days of growth and development under control conditions. Furthermore, two oxidative stress-related parameters, GSH concentrations and gene expression of oxidative stress markers, were studied under control conditions.

3.1.1 Nuclear ploidy levels in unexposed leaves

Endoreduplication was first studied in leaves of *A. thaliana* grown under control conditions. Starting from day 11 after sowing, when the first two rosette leaves had fully emerged, nuclear ploidy levels were measured using flow cytometry. The nuclear DNA content of the first leaf pair (leaves 1 and 2) was determined daily. Ploidy levels of younger leaves, if these had already fully emerged, were defined at day 12, 14, 18 and 21 after sowing.

The distribution of nuclear ploidy levels in the first leaf pair clearly shifted from major proportions of cells containing 2C and 4C at day 11, to cells containing a nuclear DNA content of 2C, 4C, 8C, 16C and 32C at day 21 after sowing (Figure 5A). More specifically, the fraction of cells of leaf 1 and 2 containing a 2C nuclear DNA content immediately started decreasing, until day 18 after sowing, where it seemed to have reached a minimum (Figure 5B). The proportion of cells containing a nuclear DNA content of 4C first raised, although soon began decreasing and eventually dropped as well (Figure 5C). On the other hand, the proportion of 8C increased strongly until day 16 after sowing, and then decreased (Figure 5D). In contrast, the fraction of 16C increased slowly until day 15, at which point it started increasing more steeply (Figure 5E). The proportion of 32C was negligible until day 16 after sowing, at which it increased rapidly (Figure 5F). In general, as the fraction of cells of a certain nuclear ploidy level (e.g. 4C) started increasing, the fraction of cells of the preceding ploidy level (in this case 2C) started decreasing. Furthermore, this transition to higher nuclear ploidy levels was confirmed by the endoreduplication factor (EF) – the number of endocycles per 100 cells – as it increased almost linearly from day 11 to 21 (Figure 5G). The EF was determined as follows: $0 \times 2C (\%) + 1 \times 4C (\%) + 2 \times 8C (\%) + 3 \times 16C (\%) + 4 \times 32C (\%)$.

A similar pattern was observed when comparing the distribution of nuclear ploidy levels and the EF between different leaves of a plant within one point in time. *Arabidopsis thaliana* seedlings grown for 21 days under control conditions showed increasing levels of nuclear ploidy from the youngest leaf (leaf 8) to the oldest leaves (leaf 1-2) (Figure 6A). The proportion of cells containing a 2C (Figure 6B) and 4C (Figure 6C) nuclear DNA content was higher in young leaves (e.g. leaf 8-5) as compared to the older leaves (leaf 4-1). The fraction of 8C cells though was highest in leaf 5, 4 and 3 as compared to the other leaves (Figure 6D). In comparison to the oldest leaves, the proportion of 16C cells was lower in younger leaves and even absent in the youngest (Figure 6E). Although some cells of leaf 1-2 had already reached a 32C nuclear DNA content, proportions of 32C were negligible or absent in younger leaves (Figure 6F). Correspondingly, the EF of older leaves was higher than that of younger leaves (Figure 6G).

Furthermore, the EF of leaves 1-2 and leaves 3-8 was plotted against the surface area of these leaves (Figure 7). In both leaves 1-2 and leaves 3-8 a strong positive correlation between the EF and leaf surface area of $R^2 = 0.8526$ ($p < 0.01$) and $R^2 = 0.8748$ ($p < 0.01$), respectively, was observed, indicating a clear relationship between the extent of endoreduplication and the surface area of the

leaves. In addition, the EF of the first leaf pair increased to a greater extent with increasing leaf surface area as compared to that of younger leaves (leaves 3-8). Furthermore, this could also be observed when comparing leaf 3 to leaf 4, leaf 4 to leaf 5 and so on (Supplementary Figure 1).

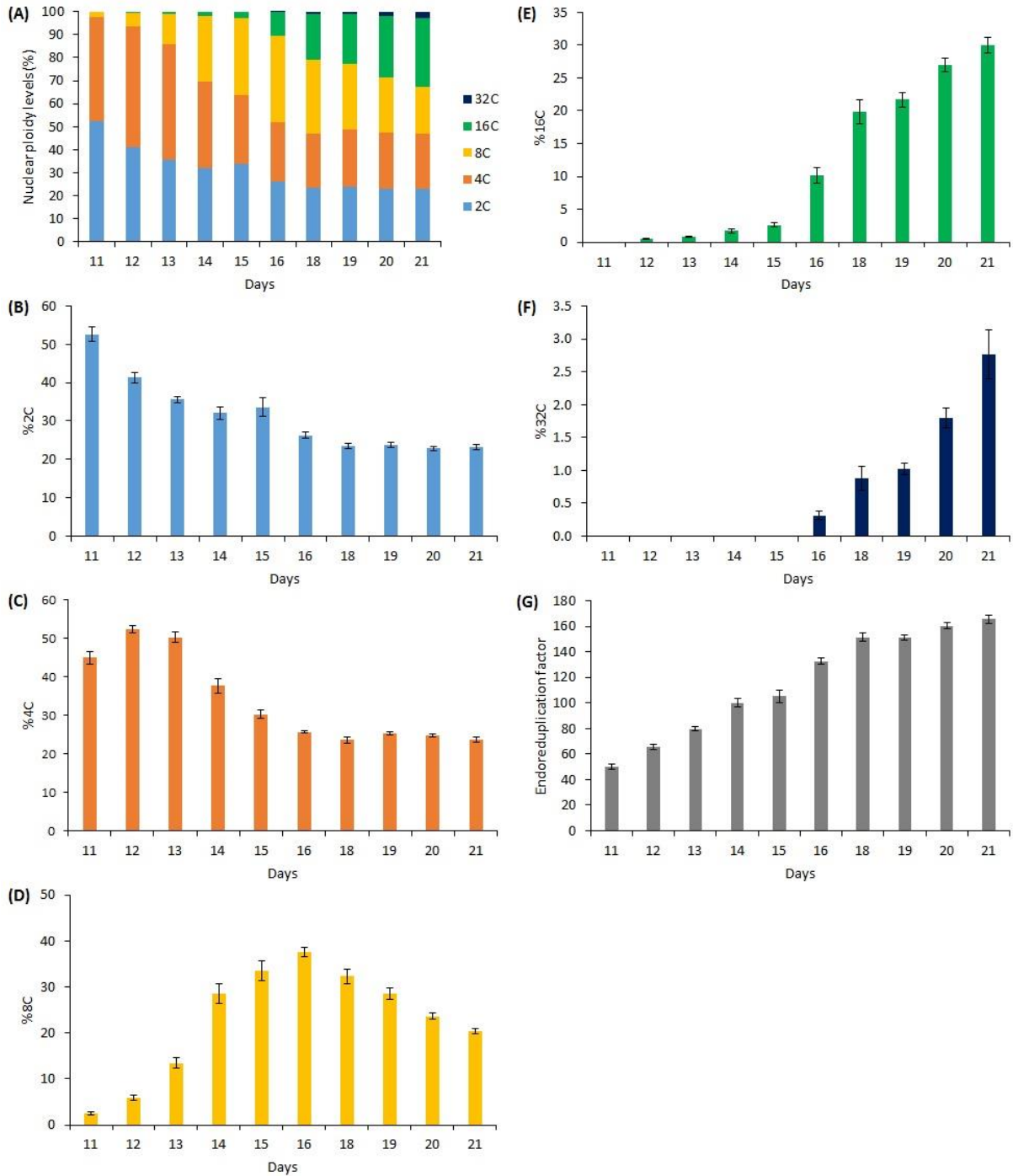


Figure 5: Nuclear ploidy levels (%) (A), the fraction of cells (%) containing a nuclear DNA content of 2C (B), 4C (C), 8C (D), 16C (E) or 32C (F) and the endoreduplication factor (G) in the first leaf pair of *A. thaliana* seedlings grown under control conditions from day 11 until day 21 after sowing. Data represent the mean (\pm S.E.) of 4 biological independent replicates.

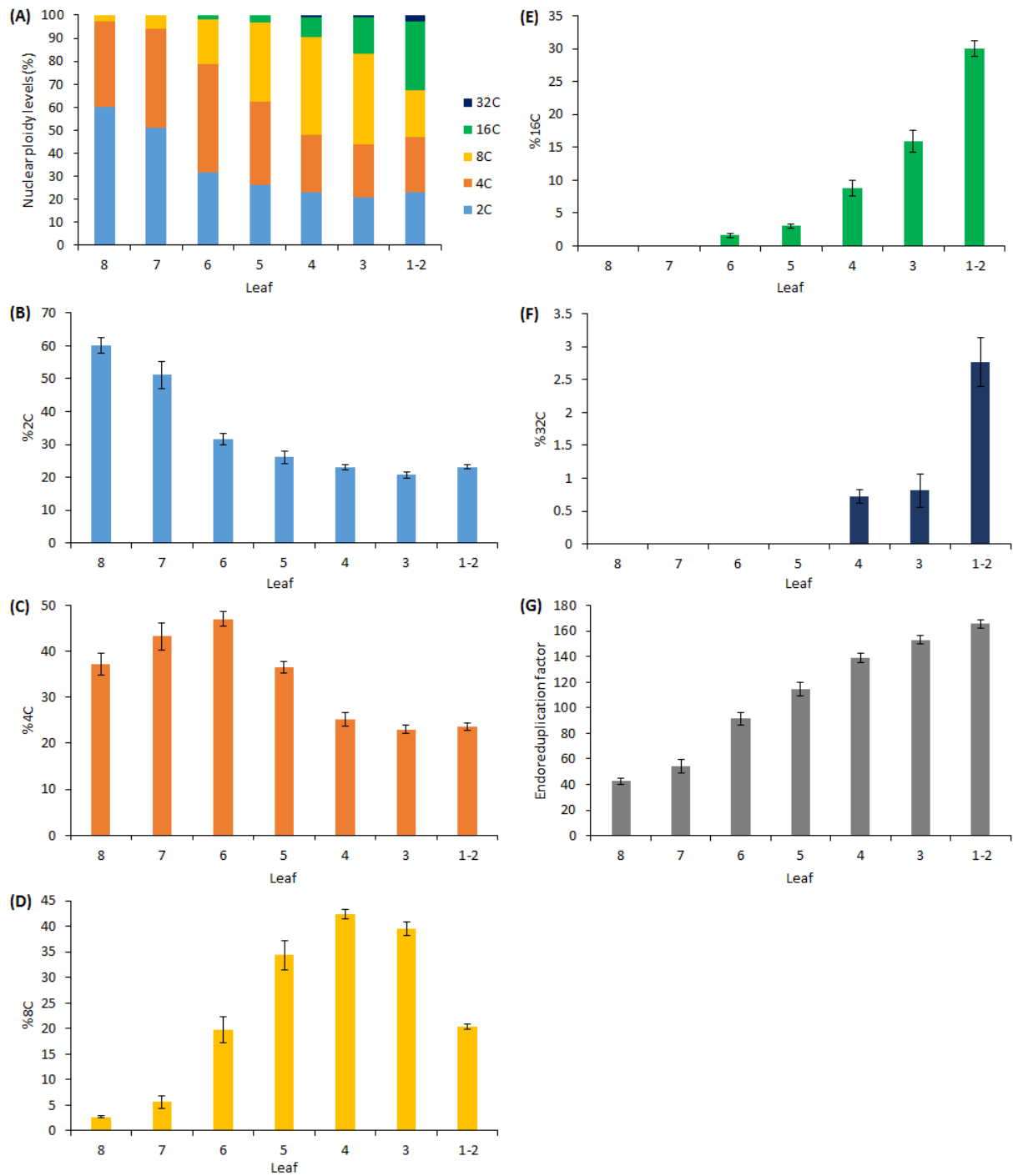


Figure 6: Nuclear ploidy levels (%) (A), the fraction of cells (%) containing a nuclear DNA content of 2C (B), 4C (C), 8C (D), 16C (E) or 32C (F) and the endoreduplication factor (G) in leaves of *A. thaliana* seedlings grown for 21 days under control conditions. Data represent the mean (\pm S.E) of 4 biological independent replicates.

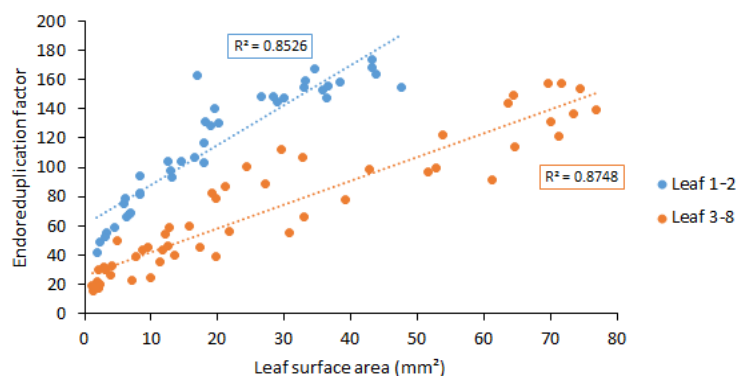


Figure 7: Correlation plot of the endoreduplication factor versus leaf surface area (mm²) of leaves 1-2 and leaves 3-8 of *A. thaliana* seedlings grown for 21 days under control conditions. Measurements were performed at several time points from day 11 to day 21 after sowing.

3.1.2 Oxidative stress-related parameters in unexposed leaves

In order to acquire more insight into the oxidative challenge under control conditions and age-related differences herein, two oxidative stress-related parameters were compared between young (leaves 6 to 9) and old (leaves 1 to 4) leaves of *A. thaliana* seedlings grown for 21 days under control conditions.

Firstly, leaf age-related differences in concentrations and redox state of GSH were determined in leaves of *A. thaliana* seedlings using spectrophotometry. Both the concentrations of GSH and GSSG as well as the total concentration of GSH were significantly lower in old leaves as compared to young leaves (Table 2).

Table 2: Glutathione concentrations (nmol/g FW) in young (leaves 6-9) and old leaves (leaves 1-4) of 21-day old *A. thaliana* seedlings. Data represent the mean \pm S.E. of 4 biological independent replicates. Significance levels compared to young leaves (one-way ANOVA): ■: p-value < 0.01. FW: fresh weight; GSH: glutathione (reduced form); GSSG: glutathione disulphide (oxidized form).

Leaves	GSH + GSSG	GSH	GSSG
Young	360.14 \pm 25.72	329.05 \pm 23.39	31.08 \pm 3.21
Old	219.74 \pm 6.67	207.27 \pm 6.92	12.47 \pm 1.07

Secondly, the expression of five oxidative stress marker genes was determined via RT-qPCR and compared between young and old leaves of *A. thaliana* grown for 21 days under control conditions (Table 3). Gene expression levels of *AT1G57630* were significantly higher in old leaves as compared to young leaves. A similar trend was observed for oxidative stress markers *AT2G43510*, *AT1G19020* and *AT1G05340*.

Table 3: Expression levels of oxidative stress marker genes in young (leaves 6-9) and old leaves (leaves 1-4) of 21-day old *A. thaliana* seedlings. The expression levels in old leaves are represented relative to those in young leaves (= 1.00 \pm S.E.). Data represent the mean \pm S.E. of at least 3 biological independent replicates. Significance levels (one-way ANOVA): ■: p-value < 0.01 for an increased expression compared to young leaves. Significant differences are indicated only in case both normalized and non-normalized data (not-shown) are statistically significant.

Leaves	<i>AT2G21640</i>	<i>AT2G43510</i>	<i>AT1G19020</i>	<i>AT1G05340</i>	<i>AT1G57630</i>
Young	1.00 \pm 0.14	1.00 \pm 0.08	1.00 \pm 0.45	1.00 \pm 0.29	1.00 \pm 0.19
Old	0.71 \pm 0.12	4.64 \pm 0.79	3.63 \pm 1.68	3.31 \pm 1.29	16.24 \pm 3.66

3.2 Cadmium-induced effects on endoreduplication and the possible link with the Cd-induced oxidative challenge

In the second part of this study, Cd-induced effects on endoreduplication were studied. Concentrations of Cd and essential elements were determined in leaves of *A. thaliana*. In addition, the length of the roots as well as rosette weight were measured in order to evaluate the effect of Cd on growth and development. Nuclear ploidy levels and expression levels of regulators of endoreduplication and DNA damage signalling genes were measured in response to Cd exposure. Furthermore, the possible link with the Cd-induced oxidative challenge was investigated by analysis of GSH and AsA concentrations, expression levels of oxidative stress marker genes as well as measurements of TBARS concentrations.

3.2.1 Uptake of Cd and nutrients

To determine the amount of Cd which was taken up by the plants and accumulated in the leaves, Cd concentrations were measured in leaves of plants exposed to 0, 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing, using ICP-OES (Figure 8). Furthermore, the leaf concentrations of essential macroelements (i.e. P and S) and microelements (i.e. Zn and Fe) was measured.

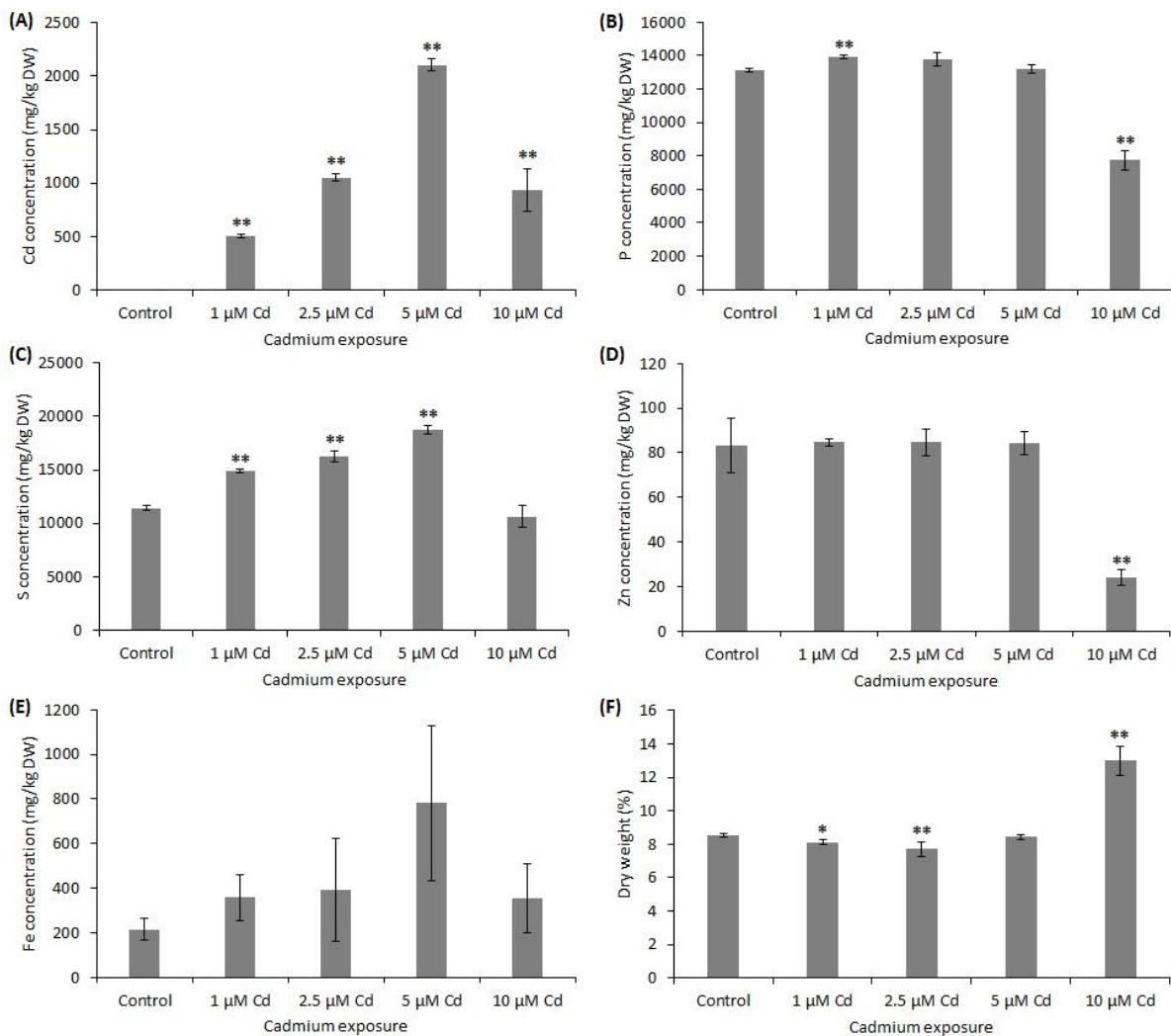


Figure 8: Concentrations (mg/kg DW) of cadmium (A), phosphorous (B), sulphur (C), zinc (D) and iron (E) and percentage dry weight (F) in leaves of *A. thaliana* seedlings exposed to 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing. Data represent the mean \pm S.E. of at least 5 biological independent replicates. Significance levels compared to control conditions (one-way ANOVA): *: p-value < 0.05; **: p-value < 0.01. DW: dry weight; Cd: cadmium; P: phosphorous; S: sulphur; Zn: zinc; Fe: iron.

The Cd concentration present in leaves of *A. thaliana* seedlings was significantly increased after 10 days of exposure to 1, 2.5, 5 and 10 μM Cd, starting from day 11 after sowing (Figure 8A). Cadmium was hereby accumulated by plant leaves in a dose-dependent manner as the concentration of Cd doubled from 1 μM Cd to 2.5 μM Cd and consequently from 2.5 μM Cd to 5 μM Cd. However, leaves of plants exposed to 10 μM Cd accumulated less than half the concentration of Cd as compared to leaves of plants exposed to 5 μM Cd. The concentration of P present in the leaves was significantly increased in plants exposed to 1 μM Cd but decreased in those exposed to 10 μM Cd (Figure 8B). Furthermore, S concentrations were significantly higher in Cd-exposed plants as compared to the control, except for plants exposed to 10 μM Cd (Figure 8C). Accumulation of Zn in leaves of plants exposed to 1, 2.5 and 5 μM Cd was not affected, although it was significantly decreased in response to 10 μM Cd (Figure 8D). No significant difference was found for the concentrations of Fe (Figure 8E). The dry weight of leaves of plants exposed to 1 and 2.5 μM Cd was significantly lower as compared to the control, though it was significantly higher in those exposed to 10 μM Cd (Figure 8F).

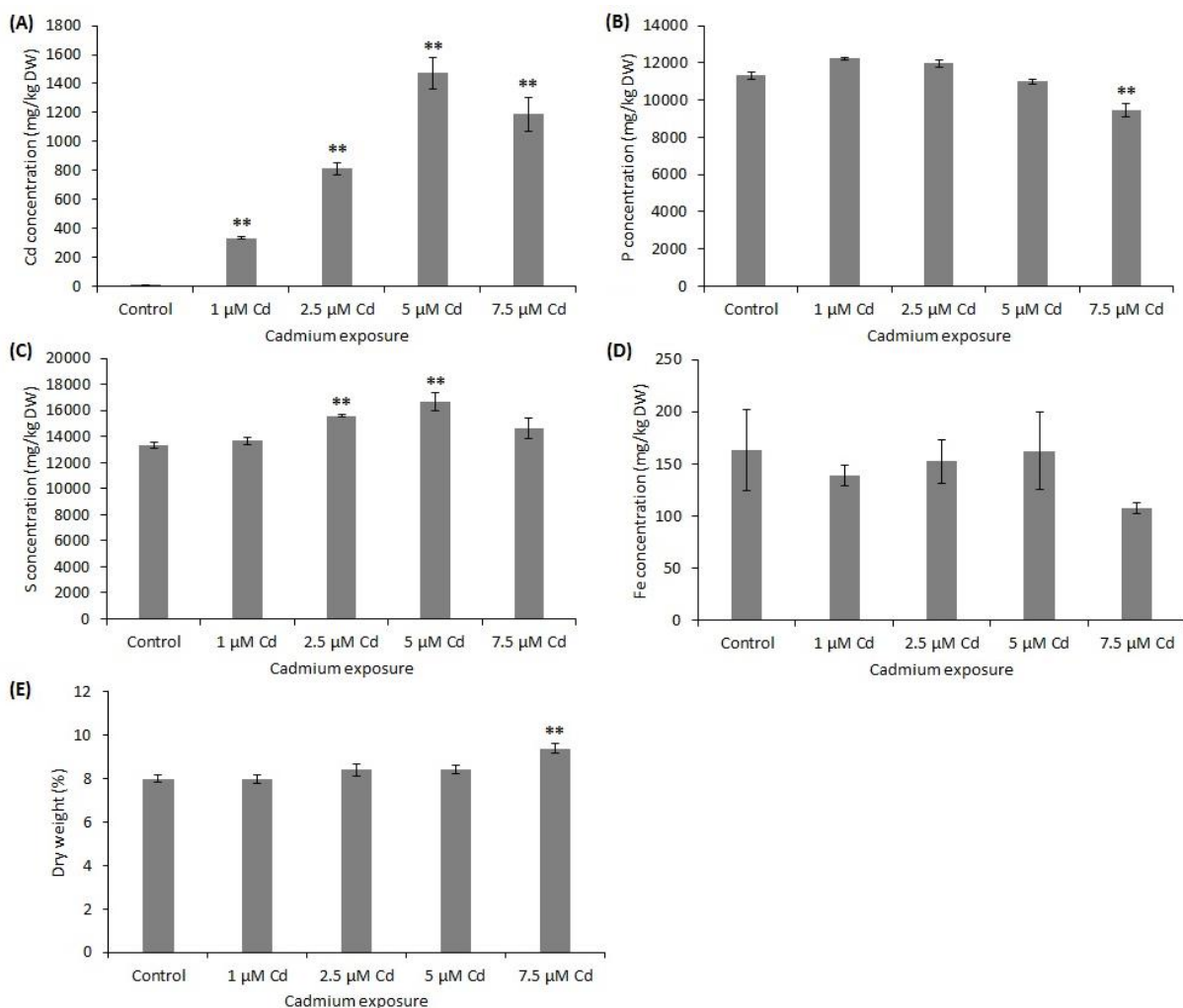


Figure 9: Concentrations (mg/kg DW) of cadmium (A), phosphorous (B), sulphur (C), and iron (D) and percentage dry weight (E) in leaves of *A. thaliana* seedlings exposed to 1, 2.5, 5 or 7.5 μM Cd for 10 days, starting from day 11 after sowing. Data represent the mean \pm S.E. of at least 4 biological independent replicates. Significance levels compared to control conditions (one-way ANOVA): **: p-value < 0.01. DW: dry weight; Cd: cadmium; P: phosphorous; S: sulphur; Fe: iron.

In a similar experiment, Cd, P, S and Fe concentrations were measured in leaves of *A. thaliana* seedlings exposed to 1, 2.5, 5 and 7.5 μM Cd for 10 days, starting from day 11 after sowing. The Cd concentration

present in the leaves was increased significantly in response to exposure to 1, 2.5, 5 and 7.5 μM Cd (Figure 9A). Furthermore, the concentration of P was significantly lower in leaves exposed to 7.5 μM Cd (Figure 9B). Leaf concentrations of S were increased in plants exposed to 2.5 and 5 μM Cd (Figure 9C). No significant difference was found for the concentrations of Fe (Figure 9D). The percentage dry weight of leaves of plants exposed to 7.5 μM Cd was significantly increased as compared to the control (Figure 9E). Because of technical issues, the concentration of Zn could not be measured.

3.2.1.1 Growth responses

In order to evaluate whether growth and development of the plants were disturbed by exposure to 1, 2.5, 5 or 10 μM Cd, root length and rosette weight were determined.

Compared to the control, the root length of plants exposed to 10 μM Cd was significantly reduced (Figure 10A). Exposure of plants to lower Cd concentrations did not significantly inhibit root growth. However, rosette weight was significantly lower for plants exposed to 2.5, 5 and 10 μM Cd as compared to the control (Figure 10B).

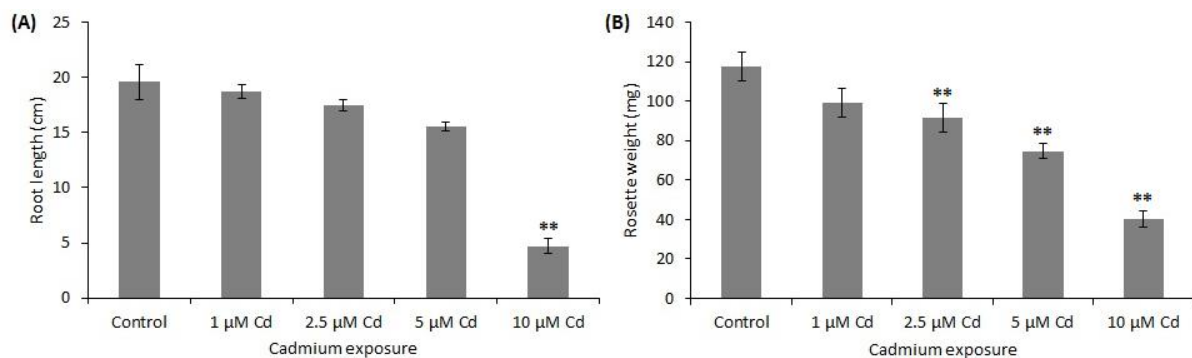


Figure 10: Root length (cm) (A) and rosette weight (mg) (B) of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing. Data represent the mean \pm S.E. of 6 biological independent replicates. Significance levels compared to control conditions (one-way ANOVA): **: p-value < 0.01.

3.2.2 Nuclear ploidy and expression levels of regulators of endoreduplication in Cd-exposed leaves

To determine the effects of Cd on endoreduplication, *A. thaliana* seedlings were exposed to 0, 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing. Using flow cytometry, nuclear ploidy levels of leaves 1 and 3 were analysed 24 hours, 72 hours, 7 days and 10 days after exposure. Furthermore, expression levels of negative and positive regulators of endoreduplication were measured in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 and 7.5 μM Cd for 11 days, starting from day 11 after sowing.

After 24 hours of Cd exposure, nuclear ploidy levels were not significantly affected in leaf 1 (Figure 11A). However, the proportion of cells of leaf 1 containing 16C was significantly decreased in response to exposure to 5 μM Cd for 72 hours (Figure 11C). Furthermore, the fraction of cells containing a 4C nuclear DNA content was significantly increased in response to exposure to 10 μM Cd at the same time point. This corresponded with significantly decreased proportions of 8C and 16C cells in leaf 1 of these plants as compared to the control. Correspondingly, the EF was decreased in leaf 1 of plants exposed to 5 and 10 μM Cd for 72 hours. After 7 days of exposure, the fraction of cells of leaf 1 containing a nuclear DNA content of 16C was significantly decreased in response to 10 μM Cd as compared to the control (Figure 11E). At the same time, the EF was significantly reduced in leaf 1 of plants exposed to 5 μM Cd. No significant effects were observed in plants exposed for 10 days (Figure

11G). Similarly, the EF was not significantly affected in response to Cd exposure (Figure 11H).

After 72 hours of exposure, no significant effects concerning nuclear ploidy levels were found in leaf 3 as compared to the control (Figure 12A). The proportion of cells containing a 16C nuclear DNA content was significantly decreased in leaf 3 of plants exposed to 10 μM Cd for 7 days (Figure 12C). Though, the EF was not significantly affected in response to Cd exposure for 7 days (Figure 12D).

However, after 10 days of exposure to Cd nuclear ploidy levels of leaf 3 were significantly decreased (Figure 12E and F). After 10 days of exposure, the fraction of cells in leaf 3 containing a nuclear DNA content of 2C was not significantly affected in response to Cd exposure (Figure 12E and Supplementary Figure 2A). The proportion 4C was significantly increased in leaf 3 of plants exposed to 10 μM Cd (Figure 12E and Supplementary Figure 2B). However, fractions of cells containing a 8C nuclear DNA content were significantly higher in leaf 3 of plants exposed to 1, 2.5 and 5 μM Cd as compared to the control (Figure 12E and Supplementary Figure 2C). Proportions of 16C cells were significantly lower in plants exposed to all Cd concentrations tested as compared to the control (Figure 12E and Supplementary Figure 2D). The fraction of cells containing a 32C nuclear DNA content was significantly lower in leaf 3 of plants exposed to 5 and 10 μM Cd (Figure 12E and Supplementary Figure 2E). On average, the extent of endoreduplication in leaf 3 of Cd-exposed plants was reduced. This was confirmed by the EF, which was significantly lower in leaf 3 of plants exposed to 1, 5 and 10 μM Cd as compared to the control (Figure 12F).

Furthermore, expression levels of regulator genes of endoreduplication and DNA damage signalling genes were determined in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 and 7.5 μM Cd for 11 days, starting from day 11 after sowing (Table 4). In general, the expression of positive regulators of endoreduplication such as SIAMESE-related (*SMR*) genes *SMR4*, *SMR5* and *SMR7* was significantly increased after exposure to 5 and 7.5 μM Cd for 11 days. Additionally, expression levels of *SMR4* increased after exposure to 1 μM Cd while levels of *SMR5* increased after exposure to 1 and 2.5 μM Cd as well. The transcriptional repressor of endoreduplication DP-E2F-like (*DEL1*) was not significantly affected in response to Cd exposure. The expression of ataxia-telangiectasia mutated (*ATM*), involved in DNA damage signalling, was significantly increased in plants exposed to 7.5 μM Cd. In contrast, the expression of ATM- and RAD3-related (*ATR*) was not significantly affected by any of the Cd exposure conditions.

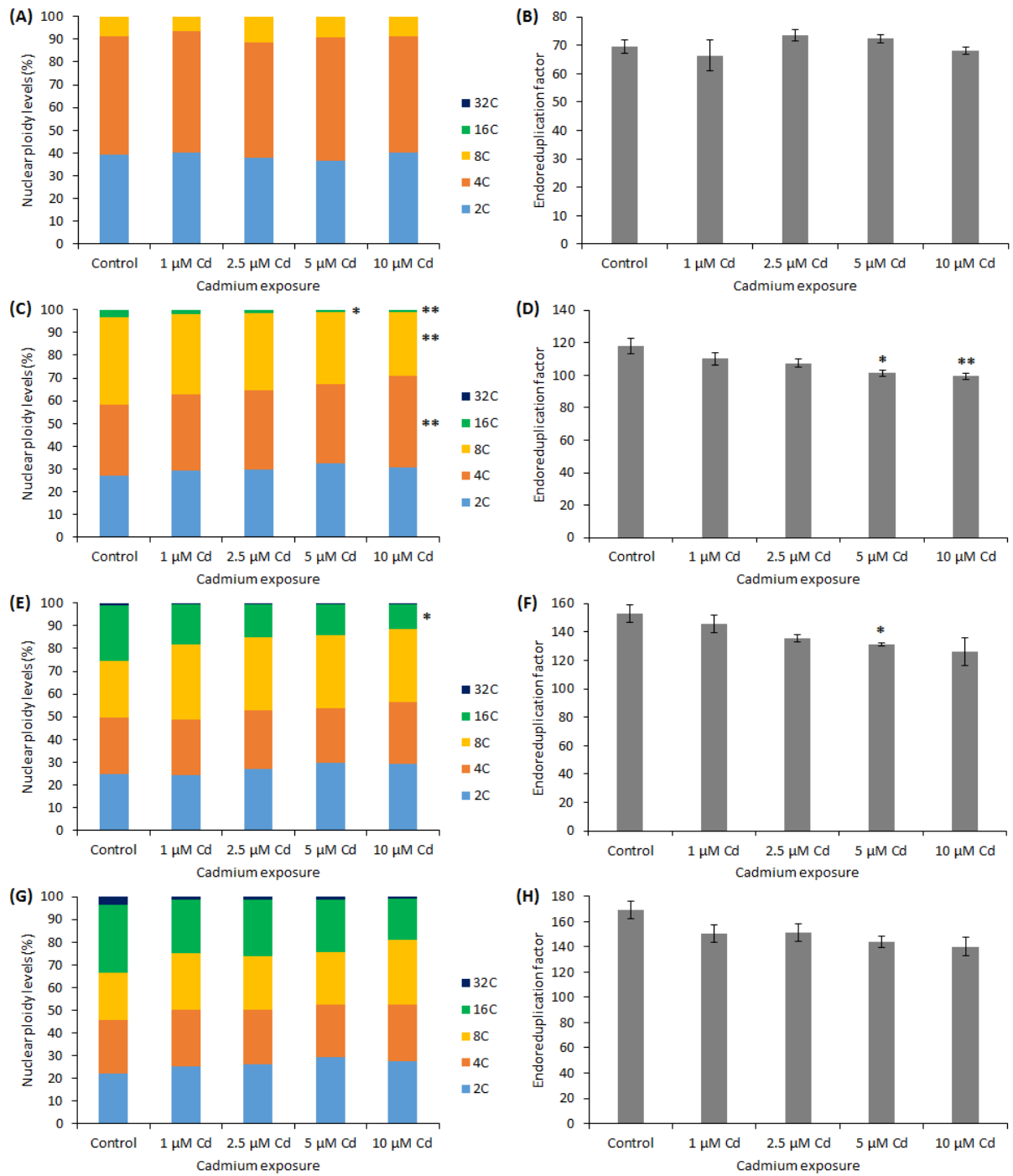


Figure 11: Nuclear ploidy levels (%) and the endoreduplication factor in leaf 1 of *A. thaliana* seedlings after 24 hours (**A, B**), 72 hours (**C, D**) 7 days (**E, F**) and 10 days (**G, H**) of exposure to 0, 1, 2.5, 5 or 10 μM Cd, starting from day 11 after sowing. Data represent the mean (\pm S.E.) of 4 biological independent replicates. Significance levels compared to control conditions (one-way ANOVA): *: p-value < 0.05; **: p-value < 0.01.

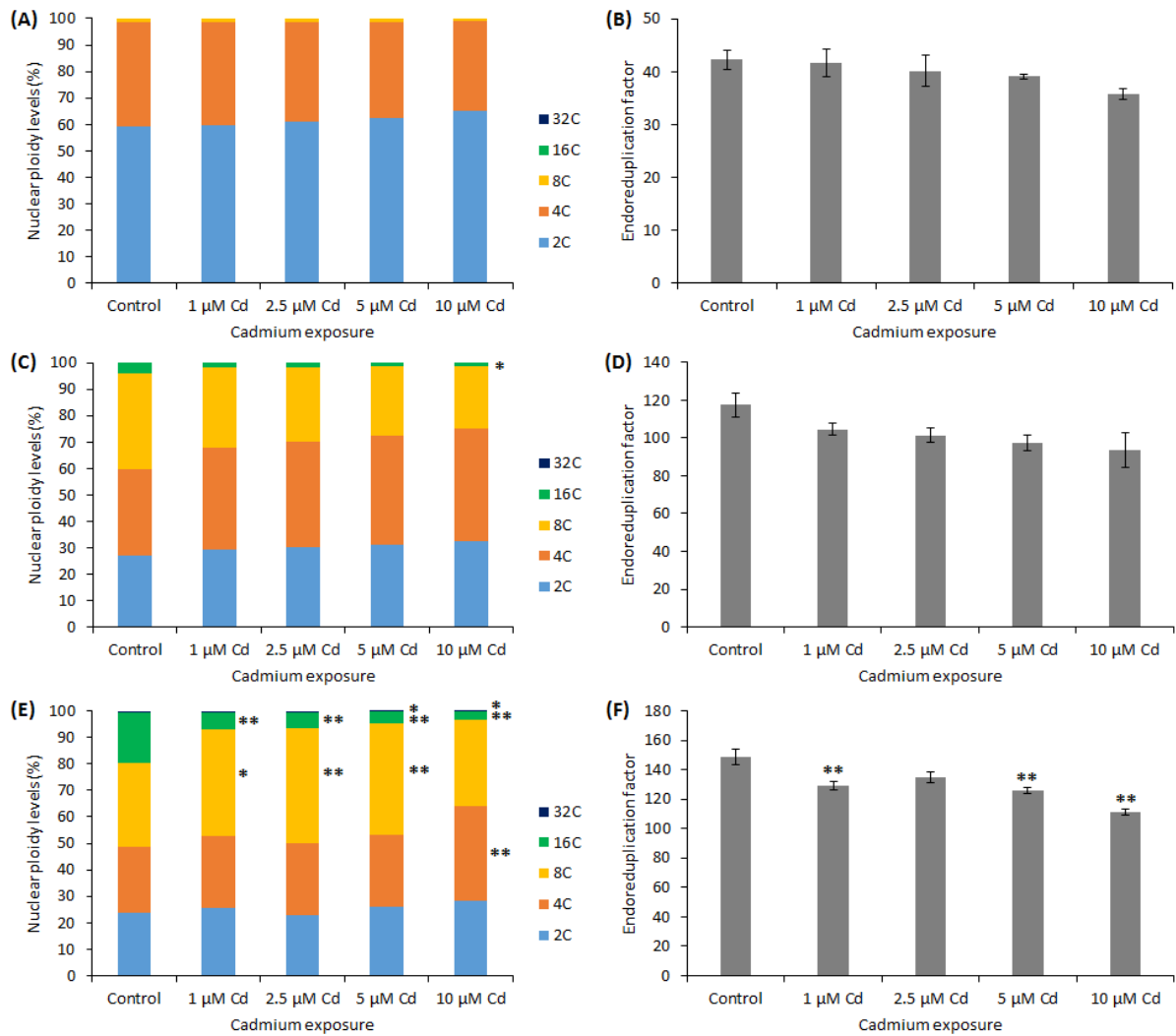


Figure 12: Nuclear ploidy levels (%) and the endoreduplication factor in leaf 3 of *A. thaliana* seedlings after 72 hours (A, B), 7 days (C, D) and 10 days (E, F) of exposure to 0, 1, 2.5, 5 or 10 μM Cd, starting from day 11 after sowing. Data represent the mean (\pm S.E.) of 4 biological independent replicates. Significance levels compared to control conditions (one-way ANOVA): *: p-value < 0.05; **: p-value < 0.01.

Table 4: Expression levels of positive and negative endoreduplication regulator genes and DNA damage signalling genes in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 or 7.5 μM Cd for 11 days, starting from day 11 after sowing. Gene expression levels of Cd-exposed plants are represented relative to control conditions ($= 1.00 \pm \text{S.E.}$). Data represent the mean \pm S.E. of at least 5 biological independent replicates. Significance levels (one-way ANOVA): ■: p-value < 0.05; ■: p-value < 0.01 for an increased expression compared to the control. Significant differences are indicated only in case both normalized and non-normalized data (not-shown) are statistically significant. *SMR*: SIAMESE-related; *DEL1*: DP-E2F-like 1; *ATM*: ataxia-telangiectasia mutated; *ATR*: ATM- and RAD3-related.

Gene	Control	1 μM Cd	2.5 μM Cd	5 μM Cd	7.5 μM Cd
Positive regulators					
<i>SMR1</i>	1.00 \pm 0.09	0.86 \pm 0.03	0.88 \pm 0.06	0.86 \pm 0.07	0.82 \pm 0.10
<i>SMR4</i>	1.00 \pm 0.12	1.46 \pm 0.13	1.37 \pm 0.23	3.26 \pm 0.11	3.11 \pm 0.52
<i>SMR5</i>	1.00 \pm 0.20	5.00 \pm 1.05	2.66 \pm 0.50	6.16 \pm 1.06	3.03 \pm 0.61
<i>SMR7</i>	1.00 \pm 0.13	1.13 \pm 0.12	3.55 \pm 0.94	11.81 \pm 3.35	10.76 \pm 3.22
Negative regulators					
<i>DEL1</i>	1.00 \pm 0.10	1.02 \pm 0.13	0.99 \pm 0.08	1.02 \pm 0.04	1.10 \pm 0.08
DNA damage signalling					
<i>ATM</i>	1.00 \pm 0.08	0.88 \pm 0.08	1.23 \pm 0.16	1.19 \pm 0.03	1.45 \pm 0.05
<i>ATR</i>	1.00 \pm 0.15	1.26 \pm 0.06	1.16 \pm 0.21	1.28 \pm 0.03	1.21 \pm 0.18

3.2.3 The effect of Cd exposure on oxidative stress-related parameters

The possible link between Cd-induced effects on endoreduplication and the Cd-induced oxidative challenge was investigated by the measurement of three oxidative stress-related parameters: AsA and GSH concentrations and redox state, lipid peroxidation and expression of oxidative stress marker genes. Measurements of AsA and GSH and lipid peroxidation were performed on leaves harvested from *A. thaliana* seedlings exposed to 0, 1, 2.5, 5, 7.5 or 10 μM Cd for 10 days starting from day 11 after sowing. Gene expression levels were determined in leaves of *A. thaliana* exposed to 0, 1, 2.5, 5 or 7.5 μM Cd for 11 days, starting from day 11 after sowing.

3.2.3.1 Antioxidative metabolite concentrations

The concentrations and redox state of the antioxidative metabolites AsA and GSH were determined using spectrophotometry, in leaves of *A. thaliana* seedlings exposed to 1, 2.5, 5 and 10 μM Cd for 10 days, starting from day 11 after sowing. In order to gain insight into leaf age-related differences in antioxidative metabolite concentrations in Cd-exposed plants, measurements were compared between young (leaves 6-9) and old leaves (leaves 1-4).

In general, concentrations of total, reduced and oxidized AsA as well as GSH were lower in older leaves in comparison to younger leaves (Table 5). However, in both old and young leaves of plants exposed to 10 μM Cd, total and reduced GSH concentrations were significantly increased as compared to the control. At the same time, decreased concentrations of GSSG were found in old leaves of plants exposed to 2.5 μM Cd and young leaves of plants exposed to 2.5 and 5 μM Cd. In addition, total and reduced AsA concentrations were significantly increased in both old and young leaves of plants exposed to 10 μM Cd.

Table 5: Ascorbate and glutathione concentrations (nmol/g FW) in young (leaves 6-9) and old leaves (leaves 1-4) of *A. thaliana* seedlings exposed to 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing. Data represent the mean \pm S.E. of at least 5 biological independent replicates. Significance levels compared to young leaves of plants exposed to the same Cd concentration: **: $p < 0.01$ and significance levels compared to control conditions of the same leaf age (two-way ANOVA): increase ■: p -value < 0.05 ; ■: p -value < 0.01 decrease ■: p -value < 0.05 ; ■: p -value < 0.01 . FW: fresh weight; GSH: glutathione (reduced form); GSSG: glutathione disulphide (oxidized form); AsA: ascorbate (reduced form); DHA: dehydroascorbate (oxidized form).

Leaves	Control	1 μM Cd	2.5 μM Cd	5 μM Cd	10 μM Cd
Young					
GSH+GSSG	309.02 \pm 16.52	337.77 \pm 30.10	335.36 \pm 15.76	351.70 \pm 17.36	484.09 \pm 29.82
GSH	266.78 \pm 13.14	317.45 \pm 33.61	320.63 \pm 14.78	337.68 \pm 20.09	440.13 \pm 36.02
GSSG	21.12 \pm 2.51	10.16 \pm 4.68	7.37 \pm 3.03	5.96 \pm 0.98	12.78 \pm 4.77
AsA+DHA	3.49 \pm 0.23	3.59 \pm 0.40	3.64 \pm 0.38	3.94 \pm 0.20	6.91 \pm 0.57
AsA	2.70 \pm 0.46	2.73 \pm 0.32	2.52 \pm 0.24	2.68 \pm 0.37	4.80 \pm 0.54
DHA	0.79 \pm 0.42	0.86 \pm 0.19	1.13 \pm 0.23	1.26 \pm 0.24	1.92 \pm 0.52
Old					
GSH+GSSG	196.96 \pm 7.02**	202.19 \pm 9.27**	214.93 \pm 6.39**	212.87 \pm 10.81**	345.16 \pm 11.75**
GSH	180.14 \pm 5.44**	177.81 \pm 7.99**	206.47 \pm 5.90**	199.95 \pm 9.39**	330.64 \pm 11.15
GSSG	8.41 \pm 1.05**	12.19 \pm 6.65	4.23 \pm 0.72	6.46 \pm 0.86	7.26 \pm 1.05
AsA+DHA	1.82 \pm 0.12**	1.72 \pm 0.08**	1.71 \pm 0.13**	1.42 \pm 0.13**	4.60 \pm 0.55**
AsA	1.26 \pm 0.07**	1.15 \pm 0.08**	1.12 \pm 0.13**	1.14 \pm 0.11**	3.64 \pm 0.53
DHA	0.56 \pm 0.09	0.57 \pm 0.06	0.59 \pm 0.08	0.28 \pm 0.12*	0.96 \pm 0.25

3.2.3.2 Lipid peroxidation

In order to determine the extent of lipid peroxidation in Cd-exposed plants, TBARS levels were spectrophotometrically determined in leaves of plants exposed to 0, 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing (Figure 13). Leaves of plants exposed to 10 μM Cd showed a strongly significant increase of TBARS levels as compared to leaves of control plants. No significant effects were found after exposure to lower Cd concentrations.

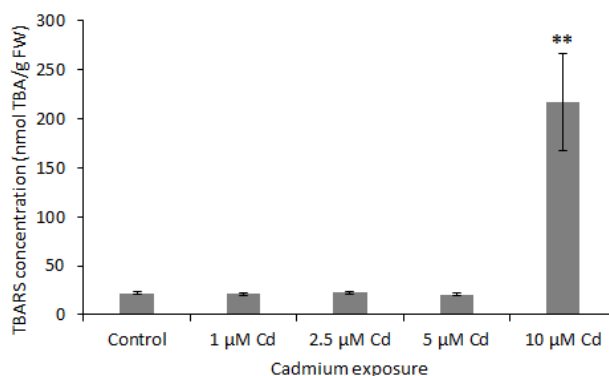


Figure 13: Thiobarbituric acid-reactive substances (TBARS) ($\mu\text{mol/g}$ FW) in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing. Data represent the mean \pm S.E. of at least 5 biological independent replicates. Significance levels compared to control conditions (one-way ANOVA): **: p-value < 0.01. FW: fresh weight.

In a similar experiment, levels of TBARS were determined in leaves of plants exposed to 0, 1, 2.5, 5 or 7.5 μM Cd for 10 days, starting from day 11 after sowing (Figure 14). No significant differences were found.

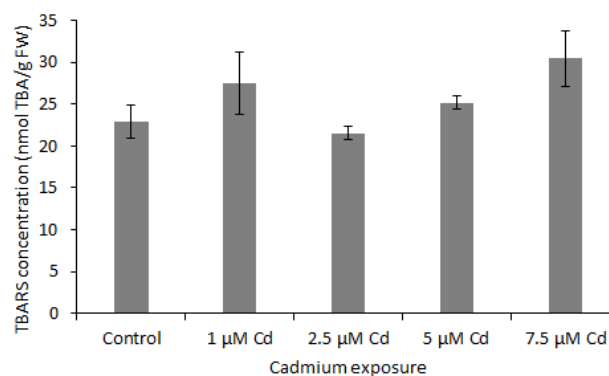


Figure 14: Thiobarbituric acid-reactive substances (TBARS) ($\mu\text{mol/g}$ FW) in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 or 7.5 μM Cd for 10 days, starting from day 11 after sowing. Data represent the mean \pm S.E. of at least 5 biological independent replicates. FW: fresh weight

3.2.3.3 Expression of genes involved in oxidative stress

To further investigate the molecular mechanisms underlying Cd-induced effects on endoreduplication and their link to Cd-induced oxidative stress, expression levels of oxidative stress marker genes were determined via RT-qPCR. The expression of these genes was measured in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 and 7.5 μM Cd for 11 days, starting from day 11 after sowing (Table 6). In general, the expression of the oxidative stress marker genes was significantly increased in response to exposure to 1, 2.5, 5 and 7.5 μM Cd except for *AT2G21640*, which only responded to 5 and 7.5 μM Cd.

Table 6: Expression levels of oxidative stress marker genes in leaves of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 or 7.5 μM Cd for 11 days, starting from day 11 after sowing. Gene expression levels of Cd-exposed plants are represented relative to control conditions ($= 1.00 \pm \text{S.E.}$). Data represent the mean \pm S.E. of at least 5 biological independent replicates. Significance levels (one-way ANOVA): ■: p-value < 0.01 for an increased expression compared to the control. Significant differences are indicated only in case both normalized and non-normalized data (not-shown) are statistically significant.

Gene	Control	1 μM Cd	2.5 μM Cd	5 μM Cd	7.5 μM Cd
Oxidative stress marker genes					
<i>AT2G21640</i>	1.00 \pm 0.09	1.44 \pm 0.13	1.59 \pm 0.11	3.08 \pm 0.25	2.91 \pm 0.82
<i>AT2G43510</i>	1.00 \pm 0.26	3.29 \pm 0.61	3.52 \pm 0.84	4.20 \pm 0.67	4.51 \pm 0.52
<i>AT1G19020</i>	1.00 \pm 0.18	5.93 \pm 0.99	4.15 \pm 0.93	7.31 \pm 1.20	4.79 \pm 0.91
<i>AT1G05340</i>	1.00 \pm 0.29	18.40 \pm 3.06	12.80 \pm 3.44	24.83 \pm 2.93	16.23 \pm 3.08
<i>AT1G57630</i>	1.00 \pm 0.17	4.99 \pm 1.05	3.74 \pm 0.89	7.07 \pm 1.50	5.04 \pm 0.99

4 DISCUSSION

Environmental pollution by metals is a problem affecting many regions worldwide. Cadmium, a toxic metal and group I carcinogen is considered a worldwide concern in terms of its influence on environmental quality and health. Furthermore, Cd is easily taken up by plants and thereby introduced into the food chain, causing negative health effects in many organisms.

In this study, we investigated Cd-induced effects on endoreduplication in *A. thaliana* leaves and the possible link with the Cd-induced oxidative challenge. In order to evaluate the effect of Cd exposure on endoreduplication, this alternative cell cycle mode was first studied in leaves of *A. thaliana* during 10 days of growth under control conditions. Since endoreduplication seems to be age-dependent and possibly affected by redox signalling, it was furthermore interesting to determine age-related differences in oxidative stress related parameters under control conditions as well. Besides its function in the normal development and growth of the plant, endoreduplication can be induced by external stress factors such as UV-B irradiation⁽²⁹⁾. Therefore, we measured the effects of Cd-exposure on nuclear ploidy levels and expression levels of endoreduplication regulator genes in plants exposed for 10 days, starting from day 11 after sowing. Additionally, several oxidative stress-related parameters were studied in order to investigate the possible link of Cd-induced effects on endoreduplication with the Cd-induced oxidative challenge.

4.1 Endoreduplication and oxidative stress-related parameters under control conditions

In the first part of this study we investigated the extent of endoreduplication in leaves of *A. thaliana* grown under control conditions. Therefore, we determined the extent of endoreduplication during 10 days of growth and development, starting from day 11 after sowing. Furthermore, oxidative stress-related parameters were studied in leaves of *A. thaliana* grown under control conditions. This way, both endoreduplication and oxidative stress-related parameters were investigated under control conditions, in order to compare these with the Cd-induced response later on.

4.1.1 Endoreduplication in unexposed leaves of *A. thaliana*

First we determined the extent of endoreduplication in leaves of *A. thaliana* seedlings during growth and development under control conditions. Different environmental factors affect the level of endoreduplication. For instance, light intensity and soil water content were shown to influence nuclear ploidy levels in leaves of *A. thaliana*⁽³⁰⁾. The specific growth conditions (e.g. relative humidity, temperature, photoperiod, growth medium etc.) used for cultivation of the plants will therefore most likely exert control on the extent of endoreduplication. Beemster *et al.* (2005)⁽³⁴⁾ has already shown an increasing nuclear ploidy levels in leaves 1 and 2 of *A. thaliana* from day 10 until day 22 after sowing. However, in order to evaluate the effect of Cd exposure on endoreduplication in the next part of this study, it is of great importance to define the extent of endoreduplication under control conditions, using our specific growth conditions. Therefore, nuclear ploidy levels of the first leaf pair (leaves 1-2) were measured daily using flow cytometry starting from day 11 after sowing, when the first two rosette leaves had fully emerged. Furthermore, the nuclear DNA content of younger leaves, if these had already fully emerged, was determined 24 hours, 72 hours, 7 days and 10 days after sowing.

From the results obtained in this experiment, it can be concluded that nuclear ploidy levels in the first leaf pair increase with increasing leaf age by successive endocycling (Figure 5). These results were in accordance with the results of Galbraith *et al.* (1991)⁽³⁵⁾, who showed an increasing trend of nuclear ploidy in the first, second and third leaf pair (leaves 1-2, 3-4 and 5-6 respectively) of *A. thaliana*

from day 9 to day 21 after germination. Gegas *et al.* (2014)⁽²⁹⁾ showed increasing levels of endopolyploidy in leaf 5 of Col-0 *A. thaliana* plants from 5 to 15 days post initiation. Moreover, Beemster *et al.* (2005)⁽³⁴⁾ showed an increasing extent of endoreduplication in the first leaf pair of *A. thaliana* from day 14 until day 22 after sowing. In addition, the authors reported that from day 14 onwards, 8C cells were observed in combination with a reduced cell division rate, therefore indicating endoreduplication had started in the first leaf pair. They therefore suggested the leaf tissue to have shifted from proliferation (cell division rate matches the expansion rate) to expansion (cells continue to expand but stop dividing). These are the first two developmental phases through which growth is achieved. When cells no longer expand, the third developmental phase of growth, that is maturity, has been reached⁽³⁴⁾. Endoreduplication therefore seems most important during expansion. Since our results show the proportion of 8C cells to increase strongly at day 13-14 after sowing as well, we suggest endoreduplication in the first leaf pair to start at this time point, indicating the onset of the expansion phase.

Furthermore, older leaves of *A. thaliana* showed higher nuclear ploidy levels as compared to younger leaves of the same plant grown for 21 days under control conditions (Figure 6). This is confirmed by the EF as it increased gradually from leaf 8 to leaves 1-2. These results are in agreement with those reported by Galbraith *et al.* (1991)⁽³⁵⁾, who showed a higher extent of endoreduplication in older leaf pairs as compared to younger leaf pairs within the same plant, 21 days after germination. These findings suggest that endoreduplication is tissue dependent and that growth of the leaves and thus its developmental phase strongly determines the extent of endoreduplication.

Moreover, a positive correlation was found when plotting the EF against the leaf surface area (Figure 7). This indicated a positive relationship between nuclear DNA content and leaf surface area. These results were confirmed by Massonnet *et al.* (2011)⁽³⁶⁾ who reported a positive correlation between the surface area of leaf 6 and the EF. These findings support our previous suggestion that endoreduplication is important during expansion, and thereby correlated with leaf surface area.

Together, these findings provide the general picture of endoreduplication in leaves of *A. thaliana* grown under control conditions. Furthermore, they indicate that endoreduplication plays an important role in normal growth and development of *A. thaliana* leaves under control conditions. Moreover, the extent of endoreduplication seems to be strongly dependent on leaf age, which is coupled to a developmental phase. To the best of our knowledge, no data have been published concerning daily evaluations of nuclear ploidy levels in different leaves of *A. thaliana*. In future studies, it would be interesting to determine the cell number, size and division rate parallel to the leaf area and nuclear DNA content. Additionally, *A. thaliana* plants mutated in *DEL1* or *FZR2/CCS52A1*, having an enhanced or reduced extent of endoreduplication, respectively, can be used to improve our understanding regarding the role of endoreduplication in these growth and developmental phases.

4.1.2 Oxidative stress-related parameters under control conditions

In order to gain more knowledge on the oxidative challenge under control conditions, antioxidative metabolite concentrations and gene expression levels of oxidative stress markers were determined in leaves of *A. thaliana*. Furthermore, as endoreduplication seems strongly dependent on leaf age, these oxidative stress-related parameters were compared between young (leaves 6 to 9) and old (leaves 1-4) leaves of 21-day old *A. thaliana* seedlings grown under control conditions.

Overall, concentrations of total, reduced and oxidized GSH were lower in old leaves as compared to young leaves (Table 2). We hypothesize that younger leaves most likely suffer a greater vulnerability to environmental stress factors as compared to older leaves. Therefore, they require an

improved antioxidant defence system which is governed by the pool sizes of antioxidants and the ability to keep these pools in their reduced forms. To date, no data have been published on the difference in glutathione concentrations between old and young leaves of *A. thaliana*. In addition to their role in the antioxidative defence system, both GSH and AsA are supposed to be involved in the regulation of the cell cycle. On the other hand, merely the presence of a critical concentration of GSH is required for cell division to occur while any rise above this level is redundant. However in most cases, in terms of the cell cycle their role in redox signalling seems more important rather than the compound itself⁽²⁵⁾. Nevertheless, since younger leaves most likely contain more tissue undergoing cell division, this could explain the increased concentrations of GSH in comparison to older leaves. To further investigate these hypotheses it would be interesting to perform similar experiments using the *cad2-1* *A. thaliana* mutant, containing only 15-30% of GSH compared to wild-type levels, in combination with measurements of the cell division rate and occurrence.

Secondly, gene expression levels of several oxidative stress markers were determined in young and old leaves of *A. thaliana* plants grown for 21 days under control conditions (Table 3). Although no significant age-related differences were found except for one oxidative stress marker, *AT1G57630*, an increasing trend was observed when comparing the expression levels of old leaves to young leaves. Gadjev *et al.* (2006)⁽³⁷⁾ reported these genes to be hallmarks for the general oxidative stress response because of their consistent up-regulation in response to different oxidative stress-causing agents. Therefore, our findings indicate that the old leaves suffer increased levels of oxidative stress as compared to young leaves. Furthermore, *AT1G57630* seems to be expressed during the final stage of leaf development, when the leaf ceases metabolic activity and reaches senescence⁽³⁸⁾. Moreover, leaf senescence seems to be closely related to oxidative stress tolerance⁽³⁹⁾.

In combination with the decreased concentrations of GSH in old leaves as compared to young leaves, these findings strongly suggest that the old leaves were most likely suffering increased levels of oxidative stress caused by decreased antioxidative capacities. The observed effect is most likely due to these leaves coming closer to leaf senescence, as was indicated by increased expression levels of *AT1G57630*, in combination with increased GSH concentrations in young leaves due to a higher vulnerability to environmental stressors.

4.2 Cd-induced effects on endoreduplication and the possible link with the Cd-induced oxidative challenge

In the second part of the study, the main objective was to investigate the possible link between Cd-induced effects on the oxidative challenge and endoreduplication. Therefore, the uptake of Cd and acquisition of nutrients was defined in response to Cd exposure. Furthermore, nuclear ploidy levels and expression levels of important regulators of endoreduplication and DNA damage signalling genes were determined in leaves of plants exposed to Cd. Finally, we investigated leaf age-related differences in antioxidative metabolite concentrations, expression levels of oxidative stress marker genes and the extent of lipid peroxidation in response to Cd exposure.

4.2.1 Cadmium uptake and nutrient acquisition

Concentrations of Cd, macro- (P and S) and microelements (Zn and Fe) were determined in leaves of 21-day old Cd-exposed *A. thaliana* plants in order to evaluate their uptake and accumulation in the leaves in response to Cd exposure.

Cadmium was readily taken up from the Cd-contaminated Hoagland solution and accumulated in the leaves. This was shown by increased Cd concentrations in leaves of plants exposed for 10 days

to all Cd concentrations tested (Figure 8A). Furthermore, concentration-dependent increases in leaf Cd content were observed for plants exposed to 1, 2.5 and 5 μM Cd. However, leaves of plants exposed to 10 μM Cd accumulated only half the concentration of Cd in comparison to those exposed to 5 μM Cd. We hypothesize that this is due to disturbed growth of the roots which might reduce Cd uptake and subsequent translocation to the aerial parts of the plant. This was confirmed by a significant decrease in root length of plants exposed to 10 μM Cd as compared to the control (Figure 10A). However, these results are not in accordance to the results of Cuypers *et al.* (2011)⁽¹⁴⁾, who reported a concentration-dependent increase in leaf Cd concentrations of *A. thaliana* seedlings exposed to 5 and 10 μM Cd for 24 hours starting 3 weeks after sowing. However, in the current study, *A. thaliana* seedlings were exposed to Cd for 10 days starting from day 11 after sowing. Since these plants were exposed for a longer period starting from a younger age, they will most likely experience an increased vulnerability to the Cd-induced toxicity. In addition, acquisition of P (Figure 8B) and Zn (Figure 8D) further supports our hypothesis, as the accumulation of both essential elements was significantly decreased in leaves of plants exposed to 10 μM Cd as compared to the control, most likely due to disturbed root growth. In addition, Jozefczak *et al.* (2014)⁽⁴⁰⁾ reported the relative translocation of Cd from roots to leaves to be lower in plants exposed to 10 μM Cd as compared to those exposed to 5 μM Cd. A similar result was found in a study by Nocito *et al.* (2011)⁽⁴¹⁾ who reported an increasing Cd retention in the roots with increasing Cd concentrations. These findings could further contribute to the limited accumulation of Cd observed in leaves of plants exposed to 10 μM Cd.

The leaf S concentration of plants exposed to 1, 2.5 and 5 μM Cd was furthermore significantly increased in a dose-dependent manner (Figure 8C). This could be explained by an increased sulphate uptake and assimilation for improving the synthesis of GSH and PCs in order to mitigate the Cd-induced oxidative stress and reduce the circulation of free Cd^{2+} ions⁽¹⁹⁾. In contrast to the plants exposed to 1, 2.5 and 5 μM Cd, the S concentration of leaves of plants exposed to 10 μM Cd was not significantly increased. These latter most likely will not be able to take up sufficient amounts of S due to disturbed root growth, as was suggested for the other nutrients. In addition, as the roots were in direct contact with the Cd containing nutrient solution, these organs will possibly suffer excessive oxidative stress. In this way, most of the S which was taken up, might be consumed in order to protect the roots from oxidative damage. Another possible explanation may be the fact that the uptake of inorganic sulphate is mediated via energy-dependent proton/sulphate co-transport systems located in the surface cell layers of roots⁽⁴²⁾. Depletions of ATP induced by oxidative damage in the roots may therefore inhibit uptake of S. Finally, ATP sulphurylase is shown to be easily affected by Cd⁽³⁾. As this enzyme catalyses the first reaction – which is ATP-dependent as well – in S assimilation⁽⁴³⁾, its interaction with Cd may cause a reduced uptake of inorganic sulphate. Therefore, less S may be accumulated in the leaves of plants exposed to 10 μM Cd compared to those exposed to lower Cd concentrations.

However, in comparison to the control, this limited uptake of S in plants exposed to 10 μM Cd was not significantly decreased either. As this was the case for most of the other nutrients, it further confirms the increased demand of S uptake and assimilation in response to Cd exposure.

Because of an impaired accumulation of Cd and essential elements together with disturbances of root growth in plants exposed to 10 μM Cd, a similar experiment was performed exposing plants to 7.5 μM Cd instead of 10 μM Cd (Figure 9). In general, the results of this experiment are in line with these described above. However, the uptake of essential nutrients was not as strongly affected by exposure to 7.5 μM Cd as compared to 10 μM Cd.

In order to fully investigate the uptake and translocation to the leaves of Cd as well as essential elements after Cd exposure, their concentrations should be measured in both roots and leaves.

Moreover, it would be interesting to determine the concentrations and synthesis rate of PCs in both organs in order to further clarify the results concerning S accumulation.

4.2.2 Cadmium-induced effects on endoreduplication and its regulators in leaves of *A. thaliana*

Besides its role in growth and developmental processes, research has shown that endoreduplication can be induced by external stress factors. Thereby it may provide a mechanism to mitigate the effects of exogenous stress. For instance, the extent of endoreduplication was increased in response to high UV-B irradiation in leaves of *A. thaliana*, as was recently shown by Gegas *et al.* (2014)⁽²⁹⁾. Moreover, nuclear ploidy levels were increased by water-deficit stress in *Arabidopsis* leaf mesophyll cells. In this part of the study, Cd-induced effects on endoreduplication and its regulators in *A. thaliana* leaves were investigated. Therefore, nuclear ploidy levels were determined in leaves 1 and 3 of plants exposed to 0, 1, 2.5, 5 and 10 μM Cd for 24 hours, 72 hours, 7 days and 10 days. Expression levels of important regulators of endoreduplication were determined in leaves of plants exposed to 0, 1, 2.5, 5 and 7.5 μM Cd for 11 days, starting from day 11 after sowing.

After 24 hours of exposure to Cd, no significant effect was observed on the extent of endoreduplication in the oldest leaf (leaf 1) and nuclear ploidy levels appeared to be equally distributed in exposed and non-exposed plants (Figure 11A). After 72 hours of exposure, leaf 1 of plants exposed to 10 μM Cd showed significantly increased proportions of 4C and decreased proportions of 8C and 16C (Figure 11C). However, only the proportion of 16C cells in leaf 1 was decreased after 7 days of exposure to 10 μM Cd (Figure 11E). Although a similar trend was observed in leaf 1 as compared to leaf 3 of *A. thaliana* plants exposed to Cd for 10 days, endoreduplication being significantly reduced in leaf 3, the extent of endoreduplication in leaf 1 was no longer significantly reduced as compared to the control at this time point (Figure 11G).

Leaf 3 showed no significant differences in nuclear ploidy levels in response to Cd exposure of plants for 72 hours (Figure 12A). However, plants exposed to 10 μM Cd for 7 days showed a significant decreased proportion of 16C cells (Figure 12C). Additionally, after 10 days of exposure to Cd, the extent of endoreduplication in leaf 3 of *A. thaliana* seedlings was significantly reduced (Figure 12E and Supplementary Figure 2). This effect was shown by significantly decreased proportions of cells containing nuclear ploidy levels of 16C (1, 2.5, 5 and 10 μM Cd) and 32C (5 and 10 μM Cd) after 10 days of exposure. Additionally, it was confirmed by a significantly reduced EF in leaves of plants exposed to 1, 5 and 10 μM Cd as compared to the control (Figure 12F).

In the control experiment in which we daily determined nuclear ploidy levels in leaf 1-2 of *A. thaliana* seedlings during growth, we have shown endoreduplication, and therefore expansion, of these leaves to start around day 14 after sowing, when they were approximately 4 days old (Figure 5). This further explains our findings, as nuclear ploidy levels of leaf 1 were affected 72 hours after exposure, which is at day 14 after sowing. At this time point during growth, the extent of endoreduplication in leaf 1 starts to increase and is therefore most likely affected in response to Cd exposure. At this time, leaf 3 will be roughly one day old. However, after 7 days of exposure, when decreased proportions of 16C cells were observed, these leaves will be approximately 5 days old. Furthermore, after 10 days of exposure to Cd, the strongest reduction of nuclear ploidy levels was observed in leaf 3. Hence, we suggest that the delayed effect on endoreduplication in response to Cd in leaf 3 compared to leaf 1 is due to a delay in the expansion phase, in which endoreduplication seems to play an important role.

Although leaf 1 showed a similar trend compared to leaf 3 after 10 days of exposure to Cd, the extent of endoreduplication was no longer significantly reduced in leaf 1 at this time point. Therefore,

we suggest that the expansion phase of leaf 1, in which Cd exposure affected endoreduplication, was probably coming to an end after 10 days of exposure and the leaf approached the maturity phase of development in which cells no longer expand. These results are in accordance with increased expression levels of the oxidative stress marker *AT1G57630*, which is expressed during the final stage of leaf development, when the leaf ceases metabolic activity and reaches senescence. Nevertheless, although these findings suggest that the effect of Cd on endoreduplication of leaf 1 has weakened after 10 days of exposure, the EF clearly showed a decreasing trend in an almost dose-dependent manner. In order to further investigate this, nuclear ploidy levels of younger leaves such as leaf 3 should be measured throughout the whole process of growth as well, from proliferation until senescence is reached.

Interestingly, the proportion of cells containing a nuclear DNA content of 8C was significantly increased in leaf 3 of plants exposed to 1, 2.5 and 5 μM Cd for 10 days (Figure 12E and Supplementary Figure 2C). This indicates that while cells of leaves of unexposed plants undergo successive endocycles reaching 16C nuclear DNA content proportions of nearly 20% (Supplementary Figure 2D), most of the cells in the Cd-exposed leaf cells were stalled at 8C. Sugimoto-Shirasu *et al.* (2002)⁽⁴⁴⁾ reported that DNA topoisomerase VI (topo VI; a subclass of type II DNA topoisomerases, type IIB) is required for successive endocycling beyond the 8C level in *Arabidopsis*, as it resolves DNA entanglements during DNA replication. Therefore, we hypothesize that Cd inhibits topo VI and thereby endocycling beyond 8C nuclear ploidy levels. In addition, topo VI requires the divalent metal ion Mg^{2+} for both structural and catalytic functions, such as relaxation of supercoiled DNA. Another divalent metal ion such as Ca^{2+} was furthermore shown to modulate topo VI activity by replacing Mg^{2+} , making the re-ligation process less effective⁽⁴⁵⁾. These findings support our hypothesis, as Cd^{2+} , another divalent metal ion, might replace Mg^{2+} , thereby affecting the activity of topo VI and consequently the process of endoreduplication. In order to further investigate this effect, it would be interesting to use *A. thaliana* mutants defective in topo VI. Since several enzymes of the DNA replication machinery (e.g. DNA polymerase) contain a Mg^{2+} cofactor, the effect of Cd exposure on these enzymes should be investigated as well.

To date, no data have been published on the effects of Cd exposure on endoreduplication in leaves of *A. thaliana*. In contrast to our results, Fusconi *et al.* (2006)⁽⁴⁶⁾ have shown Cd exposure of *Pisum sativum* to increase the proportion of primary root cells containing a 4C nuclear DNA content while decreasing the proportion of 2C after 48 hours of exposure. However, 4C nuclear ploidy levels do not necessarily imply endoreduplication, as the nuclear DNA content raises from 2C to 4C during the mitotic cycle as well. Furthermore, the proportion of cells containing a nuclear DNA content of 8C was not affected. However, this dissimilarity in Cd response may be due to the fact that these nuclear ploidy levels were measured in a different organ of a different organism which was cultivated using different growth conditions. Nevertheless, in a future experiment it would be interesting to determine Cd-induced effects on endoreduplication in roots of *A. thaliana* using the same growth conditions used in the current study on *A. thaliana* leaves.

In order to investigate the mechanisms underlying the Cd-induced decrease in endoreduplication, leaf expression levels of important regulators of endoreduplication were measured in response to 0, 1, 2.5, 5 and 7.5 μM Cd exposure for 11 days, starting from day 11 after sowing (Table 4). In spite of these reduced nuclear ploidy levels in response to Cd exposure, expression levels of *DEL1*, a transcriptional repressor of endoreduplication, were not significantly affected in response to Cd.

Neither the expression levels of the measured positive regulators of endoreduplication explained the reduced extent of endoreduplication, as these were generally increased in response to

Cd exposure. These positive regulators of endoreduplication were all *SMR* genes, which encode a plant-specific class of CDK inhibitors (CKIs) and are therefore suggested to play a role in cell cycle regulation. However, these *SMR* genes were shown to be involved in the DNA damage response as well⁽⁴⁷⁾. Therefore, the increased expression levels of *SMR* genes may indicate increased DNA damage in our samples. Given the increased expression levels of the measured oxidative stress marker genes in response to Cd exposure, this is highly possible. Yi *et al.* (2014)⁽⁴⁷⁾ have shown *SMR5* and *SMR7* to be part of a signalling cascade inducing cell cycle checkpoints in response to ROS-induced DNA damage in leaf cells. Therefore, their expression depends on *ATM*, which is activated by DNA double-strand breaks (DSBs), rather than the expected *ATR* kinase, which is activated by DNA single-strand breaks or stalled replication forks. For this reason, the DNA damage signalling genes *ATM* and *ATR* were measured as well. Our results confirm the findings of Yi *et al.* (2014)⁽⁴⁷⁾ as the positive regulators *SMR4*, *SMR5* and *SMR7* were all significantly increased after exposure to 5 and 7.5 μM Cd and *ATM* was increased in leaves of plants exposed to 7.5 μM Cd. On the contrary, no significant differences were found for *ATR* expression levels.

From these findings we hypothesize that the increased expression levels of *SMR* genes are due to stimulation by ROS-induced DNA damage and the consequent activation of *ATM* (in 7.5 μM Cd exposed plants). This was furthermore supported by the increased expression of oxidative stress marker genes in response Cd exposure. However, no such relation between *SMR*, *ATM* and the oxidative stress markers was found for lower Cd concentrations. This may be caused by excessive amounts of DSBs in the highest Cd concentration. Changes at the transcriptional level are generally fast and most likely of short duration. A possible explanation may be the fact that we measured expression levels at 11 days after exposure, starting from 11 days after sowing. Therefore, Cd-induced effects on transcriptional level may have completely diminished by that time.

To fully investigate this, the amount of DNA damage in response to Cd exposure should be measured in addition to the expression of oxidative stress markers, endoreduplication regulators and DNA damage signalling genes. Furthermore, expression levels should be determined on different time points between 0 and 11 days after exposure to gain more insight on both short- and long-term Cd-induced changes on transcriptional level. In addition to the genes measured in this study, we should measure expression levels of *WEE1*, a gene which is induced quickly upon DNA stress and is involved in cell cycle progression and the regulation of CDK activity⁽⁴⁷⁾. Furthermore, it would be interesting to determine CDK activity itself, parallel to these measurements.

4.2.3 Cadmium-induced oxidative challenge

In this next part of the study, we investigated the possible link between Cd-induced effects on the oxidative challenge and endoreduplication. Our results suggest that endoreduplication is strongly dependent on leaf age and is furthermore reduced in leaves of plants exposed to Cd. Therefore, we investigated leaf age-related differences in antioxidative metabolite concentrations of Cd-exposed plants as well. Furthermore, expression levels of oxidative stress marker genes were determined in response to Cd exposure. Finally, the concentration of TBARS was measured in order to evaluate the Cd-induced oxidative damage.

Antioxidative metabolite concentrations were measured in young (leaves 1-4) and old (leaves 6-9) leaves of *A. thaliana* exposed to 0, 1, 2.5, 5 and 10 μM Cd for 10 days, starting at day 11 after sowing. As also shown in the previous experiment, investigating leaf age-related differences in GSH concentrations and redox state under control conditions, concentrations of total, reduced and oxidized GSH were lower in old leaves as compared to young leaves (Table 5). This trend was observed in leaves

of both control plants and Cd-exposed plants. Moreover, the same tendency was found for concentrations of total, reduced and oxidized AsA, as these were all higher in young leaves as compared to old leaves. These results further support our hypothesis that old leaves possess lower antioxidative capacities as compared to younger leaves. However, due to a higher vulnerability of young leaves to environmental stressors as compared to old leaves they may require increased antioxidative capacities. The correct explanation might be somewhere in-between.

Furthermore, significantly decreased concentrations of GSSG were observed in young (2.5 and 5 μM Cd) and old leaves (2.5 μM Cd) in response to Cd exposure. These results are in accordance to the results of Jozefczak *et al.* (2014)⁽⁴⁰⁾, who reported significantly decreased leaf GSSG concentrations in combination with increased PCs levels and a dose-dependent induction of *GR1* expression in leaves of *A. thaliana* seedlings exposed to 5 and 10 μM Cd for 24 hours, starting from day 19 after sowing. This was accompanied by a dose- and time-dependent increase in reduced and total GSH levels. Therefore, we hypothesize that the decreased GSSG concentration in the leaves of Cd-exposed plants is due to an increased activity of GR, which catalyses the reduction of GSSG to GSH. However, in contrast to Jozefczak *et al.* (2014)⁽⁴⁰⁾, decreased GSSG concentrations in leaves of plants exposed to 2.5 and 5 μM Cd for 10 days did not coincide with increased concentrations of reduced or total GSH. Therefore, in addition to our previous hypothesis, we suggest that the increased GSH pool which was observed by Jozefczak *et al.* (2014)⁽⁴⁰⁾ up to 72 hours of exposure, has diminished after 10 days of exposure as the plant increases the synthesis of PCs for chelation and subsequent sequestration of Cd to less sensitive cell compartments such as the vacuole⁽⁴⁸⁾. To further investigate this hypothesis, gene expression levels of *GR1*, *GSH1*, *GSH2*, *PCS1* and *PCS2* and enzyme activities of GR, GSH1 and GSH2 should be measured. Since PCS is post-transcriptionally activated at the enzyme level by Cd, we should also measure PCS activity.

Moreover, reduced and total GSH levels were significantly increased in both young and old leaves of plants exposed to 10 μM Cd for 10 days starting from day 11 after sowing. This might indicate that chelation by GSH and PCs in this case is insufficient, allowing free Cd^{2+} ions and thereby causing chronic Cd-induced oxidative stress. As the plants try to cope with this increased oxidative stress, the ROS scavenging pathway using AsA might be stimulated. This was supported by increased concentrations of both reduced and total AsA. Furthermore Jozefczak *et al.* (2014)⁽⁴⁰⁾ reported increased levels of AsA in leaves after 48 hours of exposure to 10 μM Cd as well.

Gene expression levels of oxidative stress markers were determined in leaves of *A. thaliana* plants exposed to 0, 1, 2.5, 5 or 7.5 μM Cd for 11 days, starting at day 11 after sowing. Overall, the expression of the oxidative stress marker genes was significantly increased in leaves of all Cd-exposed plants. These results indicate that the plants exposed to Cd suffer increased levels of oxidative stress as compared to the control.

Additionally, the extent of lipid peroxidation, which was measured as the concentration of TBARS, was determined in leaves of *A. thaliana* exposed to 0, 1, 2.5, 5 or 10 μM Cd for 10 days, starting at day 11 after sowing. Significantly increased TBARS levels were found in leaves of plants exposed to 10 μM Cd (Figure 13). Similar results were found by Jozefczak *et al.* (2014)⁽⁴⁰⁾, who observed significant increased leaf TBARS levels after exposure of 19-day old *A. thaliana* plants to 10 μM Cd for 48 and 72 hours. No significant differences were found in a similar experiment which we performed by exposing *A. thaliana* seedlings for 10 days to 7.5 μM Cd instead of 10 μM Cd (Figure 14). Hence, we conclude that these plants were able to cope with the Cd-induced oxidative stress in response to 7.5 μM Cd.

Furthermore, GSSG concentrations were reduced in response to Cd exposure, most likely due to an increased GR activity, recycling GSSG back to GSH and subsequent synthesis of PCs in order to

decrease the amount of free Cd²⁺ ions. However, plants exposed to 10 µM Cd showed increased concentrations reduced and total GSH and AsA in both young and old leaves. In these plants, GSH and PCs chelation of Cd²⁺ might not be sufficient, causing oxidative stress. This latter was confirmed by increased expression levels of oxidative stress markers in response to Cd exposure and subsequent DNA damage. In addition, measurements of TBARS concentrations have shown plants exposed to 10 µM Cd to suffer oxidative damage, though plants were able to cope with concentrations up to 7.5 µM Cd. Furthermore, the accumulation of Cd and essential nutrients measurements as well as root growth were shown to be disturbed in plants exposed to 10 µM Cd for 10 days. Somewhere in-between 7.5 and 10 µM Cd there most likely is a turning point at which the antioxidative defence system and Cd detoxification mechanism fail to deal with the amount of Cd. For this reason, in future studies exposing *A. thaliana* during 10 days starting from day 11, it may be better to use 7.5 µM Cd as the highest Cd concentration.

To conclude, reduced, oxidized and total concentrations of GSH and AsA were lower in old leaves compared to young leaves, in both control plants as Cd-exposed plants. We hypothesized earlier that this may be due to an increased vulnerability of young leaves in combination with a decreased antioxidative capacity of old leaves. However, this may also indicate that these young leaves were still fully in the expansion phase of growth after 10 days of exposure and therefore endocycling was still taking place at high rates. Hence, these leaves may contain increased concentrations of antioxidative metabolites compared to old leaves, in order to protect themselves from oxidative damage during this important phase of growth and to exert control on the endoreduplication process by redox signalling. This may explain why these age-related differences were seen in both control and Cd-exposed plants. However, additional research is needed to further investigate this hypothesis. In future experiments, it would be interesting to measure antioxidative metabolite concentrations in separate leaves and in combination with measurements of nuclear ploidy levels, cell growth parameters and the expression of endoreduplication regulators and oxidative stress markers. Furthermore, using the *cad2-1 A. thaliana* mutant, containing only 15-30% of GSH compared to wild-type levels, and *vtc1-1* mutant, containing only 25-30% of wild-type AsA levels, the importance of these antioxidative metabolites in the process of endoreduplication and Cd-induced effects hereon could be evaluated.

5 CONCLUSION

In this study, Cd-induced effects on endoreduplication and the possible link with the Cd-induced oxidative challenge were investigated in leaves of *A. thaliana* seedlings. Endoreduplication and oxidative stress-related parameters were first studied in plants grown hydroponically under control conditions in order to evaluate the effects of Cd exposure on this alternative cell cycle later in this study. During 10 days of growth and development starting from day 11 after sowing, when the first two rosette leaves had fully emerged, daily flow cytometric measurements of nuclear ploidy levels were performed on separate leaves. It was shown in this study that under control conditions the extent of endoreduplication increased with increasing leaf age by successive endocycling. In addition, as older leaves showed higher ploidy levels as compared to young leaves of the same rosette within one point in time, endoreduplication is strongly dependent on the developmental phase of the leaf. More specifically, this alternative cell cycle seems most important during the expansion phase of growth. Since endoreduplication seems leaf age-dependent, oxidative stress-related parameters were studied in young and old leaves of plants grown for 21 days under control conditions. Concentrations of GSH were generally lower in old leaves as compared to young leaves. We hypothesized that young leaves require an improved antioxidative defence system because of an increased vulnerability to environmental stress factors compared to old leaves. However, increased expression levels of *AT1G57630* in old leaves of plants grown under control conditions indicated these leaves to approach senescence, as this oxidative stress marker gene is expressed during the final stage of leaf development. Since leaf senescence is furthermore strongly related to oxidative stress tolerance, this indicated that the lower concentrations of GSH found in old leaves are due to the leaves coming closer to senescence. Therefore, we hypothesized that the observed effect may be due to a combination of both young leaves suffering an increased vulnerability and old leaves reaching senescence.

In the second part of this study, Cd-induced effects on endoreduplication and the possible link with the Cd-induced oxidative challenge were investigated in leaves of *A. thaliana* seedlings. Preliminary experiments within our research group have shown only mild effects on nuclear ploidy levels of 19-day old plants in response to short-term Cd exposure. However, research has shown endoreduplication to play an important role during growth and development of plants. For this reason, 11-day old *A. thaliana* plants were exposed to 0, 1, 2.5, 5 and 7.5 or 10 μM Cd for 10 days during their growth and development. First, the accumulation of Cd and essential macro- and microelements in the leaves was evaluated. Plants exposed to 1, 2.5 and 5 μM Cd showed a concentration-dependent increase in leaf Cd concentration. Remarkably, leaves of plants exposed to 10 μM Cd accumulated only half the concentration of Cd as compared to plants exposed to 5 μM Cd. Since plants exposed to 10 μM Cd showed disturbed root growth and decreased concentrations of P and Zn were accumulated in leaves of these plants, we hypothesize that this limited accumulation of Cd and essential elements in plants exposed to 10 μM Cd is due to disturbed root growth caused by excessive oxidative damage. In accordance to results of earlier studies, S uptake and translocation was dose-dependently increased in response to Cd exposure. This indicated an improved synthesis of GSH and PCs in order to mitigate Cd-induced oxidative stress and reduce the amount of free Cd^{2+} ions. However, plants exposed to 10 μM Cd did not show significantly increased nor decreased S accumulation in comparison to the control. Even though there is an increased demand for S assimilation in response to Cd exposure, plants exposed to 10 μM Cd were not able to accumulate increased amounts of S due to disturbed root growth. Subsequently, nuclear ploidy levels were measured 24 hours, 72 hours, 7 days and 10 days after the start of exposure. Research has shown external stress factors such as UV-B irradiation to induce endoreduplication in plants. However, the results obtained in this study have shown Cd

exposure to decrease the extent of endoreduplication in leaves of *A. thaliana* exposed to Cd for 10 days, starting from day 11 after sowing. Furthermore, the delayed effect on endoreduplication in response to Cd in leaf 3 compared to leaf 1 is most likely due to a later emergence of leaf 3 and subsequently undergoing the expansion phase. In this phase, endoreduplication plays an important role and is therefore most likely affected. To date, no data have been published on the effects of Cd exposure on endoreduplication in leaves of *A. thaliana*. Interestingly, the proportion of 8C was significantly increased in leaf 3 of plants exposed to 1, 2.5 and 5 μM Cd for 10 days which coincided with significantly decreased proportions of 16C. Research has shown Topo VI to be required for endocycling beyond the 8C level in *Arabidopsis*. Therefore, we hypothesized that Cd affects Topo VI activity by replacement of its Mg^{2+} cofactor, and thereby inhibits endoreduplication beyond 8C nuclear ploidy levels. In addition, expression levels of important regulators of endoreduplication and DNA damage signalling genes were measured. In contrast to our expectations, expression of the negative regulator *DEL1* was not altered after 11 days of Cd exposure. Furthermore, expression levels of the *SMR* genes were significantly increased in response to Cd. Besides their role as positive regulators of endoreduplication, *SMR* genes are shown to be involved in the ROS-induced DNA damage response and are activated by *ATM*, which is activated by DSBs and was significantly increased in response to Cd exposure as well. This led to the hypothesis that the expression of *SMR* was increased in response to ROS-induced DNA damage and consequently the activation of *ATM*, which was supported by increased expression levels of oxidative stress markers. For this reason, it would be interesting to measure the amount of DNA damage in future experiments. In order to investigate the possible link with the Cd-induced oxidative challenge, leaf age-related differences in GSH and AsA concentrations, expression levels of oxidative stress marker genes and lipid peroxidation were determined in response to Cd exposure as well. Reduced and total GSH and AsA levels were increased in both young and old leaves of plants exposed to 10 μM Cd, which might indicate increased oxidative stress. Total, reduced and oxidized GSH and AsA concentrations were all higher in young leaves as compared to old leaves in both non-exposed and Cd-exposed plants. As stated previously, this may be due to a combination of increased vulnerability of young leaves and the old leaves reaching senescence. However, since the young leaves are still in the expansion phase we hypothesize that high concentrations of antioxidative metabolites may be required in order to protect the leaves from oxidative damage during this important developmental phase and possibly to exert control on endoreduplication by redox signalling. More research is needed to further investigate this hypothesis. In combination with the use of *cad2-1* and *vtc1-1* *A. thaliana* mutants, antioxidative metabolite concentrations should be studied in separate leaves together with measurements of nuclear ploidy levels, cell growth parameters and expression of endoreduplication regulators and oxidative stress markers. In addition, increased levels of TBARS, disturbed accumulation of Cd and essential nutrients by disturbed root growth as well as increased expression of oxidative stress markers and DNA damage signalling genes were observed in plants exposed to 10 μM Cd for 10 days, starting from day 11 after sowing. In future studies exposing *A. thaliana* during 10 days of development, 7.5 μM Cd might be used instead of 10 μM Cd.

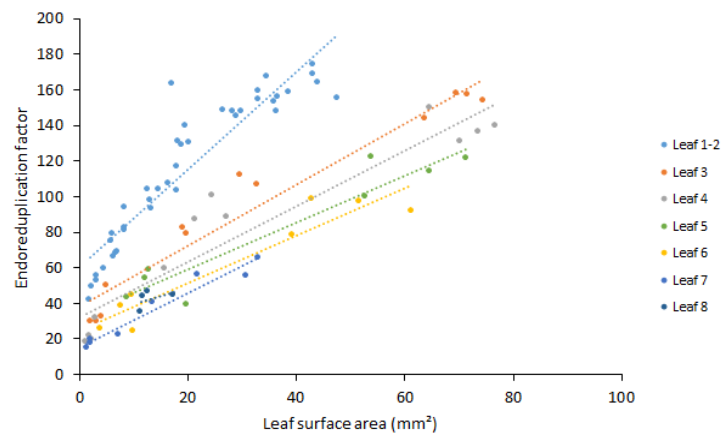
In general, it can be concluded that Cd reduces the extent of endoreduplication in leaves of *A. thaliana*. Furthermore, since endoreduplication plays an important role in growth and developmental processes, more specifically the expansion phase of growth, Cd-induced effects on endoreduplication are most prevalent during this period. In addition, since important regulators of endoreduplication seem to be involved in the Cd-induced oxidative DNA damage response, and antioxidative metabolite concentrations seem to be dependent on leaf age, Cd-induced oxidative stress may be involved in the Cd-induced effects on endoreduplication. Though, this hypothesis should be further investigated.

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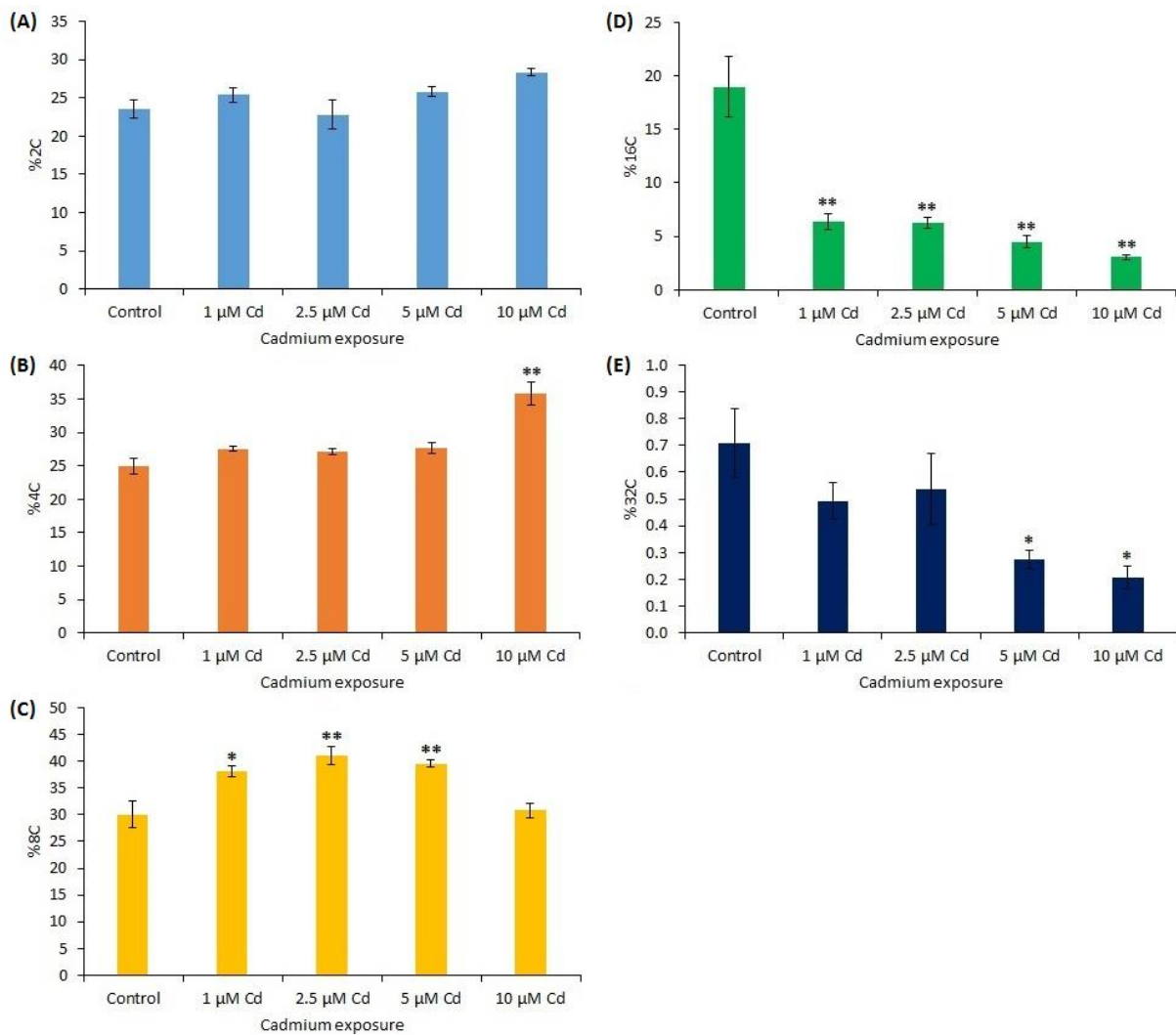
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SUPPLEMENTARY DATA



Supplementary Figure 1: Correlation plot of the endoreduplication factor versus leaf surface area (mm^2) of leaves 1-2, 3, 4, 5, 6, 7 and 8 of *A. thaliana* seedlings grown for 21 days under control conditions. Measurements were performed at several time points from day 11 to day 21 after sowing.



Supplementary Figure 2: The fraction of cells (%) containing a nuclear DNA content of 2C (A), 4C (B), 8C (C), 16C (D) and 32C (E) in leaf 3 of *A. thaliana* seedlings exposed to 0, 1, 2.5, 5 or 10 μM Cd for 10 days, starting from day 11 after sowing. Data represent the mean \pm S.E. of 4 biological independent replicates. Significance levels compared to control conditions (one-way ANOVA): *: p-value < 0.05; **: p-value < 0.01.

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

Jaar: **2015**

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