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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Masterthesis

The role of glutathione in cell cycle regulation and the DNA damage response in cadmium-exposed Arabidopsis thaliana plants

Sofie Achten

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :
Prof. dr. Ann CUYPERS

MENTOR :
Mevrouw Sophie HENDRIX

2017
2018

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
APX	Ascorbic acid peroxidase
AsA	Ascorbic acid
Ca	Calcium
cad2-1	*Cadmium-sensitive 2-1*
CAT	Catalase
Cd	Cadmium
CDKs	Cyclin-dependent kinase
CdS04	Cadmium sulfate
Cys	Cysteine
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
EF	Endoreduplication factor
FCS	Flow cytometry
Fe	Iron
G1	Gap 1
G2	Gap 2
Glu	Glutamate
Gly	Glycine
GR	Glutathione reductase
GRx	Glutaredoxin
GSH	Glutathione
GSH1	γ-glutamylcysteine synthetase
GSH2	Glutathione synthetase
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HNO3	Nitric acid
ICP-OES	Inductively coupled plasma - optical emission spectrometry
M	Mitosis
MS	Murashige and Skoog
NADPH	Nicotinamide adenine dinucleotide phosphate
NTCs	No template controls
O_2^-•	Superoxide
pad2-1	P*hytoalexin-deficient 2-1*
PAR	Photosynthetic active radiation
PbNO3	Lead nitrate
PC	Phytochelatin
PCS	Phytochelatin synthase
PI	Propidium iodide
PRx	Peroxiredoxin
PSB	Pellet solubilization buffer
rax1-1	R*egulator of APX2 1-1*
rml1	*Root meristemless 1*
ROS	Reactive oxygen species
S	Synthesis
SMR	SIAMESE-RELATED
SOD	Superoxide dismutase
TRx	Thioredoxin
WT	Wildtype/wild-type
Zn	Zinc
γ-GC	γ-glutamylcysteine

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ABSTRACT

Many regions worldwide are affected by cadmium (Cd) contamination of the soil. Plants can take up this metal and in that way cadmium can enter the food chain. Thereby it poses a serious threat to human health and is it important to investigate Cd toxicity in plants. Cadmium can cause an increased production of ROS leading to oxidative stress. Elevated ROS levels can cause damage to macromolecules like DNA. Plants poses an extensive antioxidative defense system in order to protect themselves against oxidative damage. An example of such an antioxidative metabolite is glutathione (GSH). Besides it antioxidative defense capacities, it is also involved in cell cycle regulation. In plants, there is an alternative version of the classical cell cycle. This alternative is called endoreduplication.

Preliminary data show that upon external stress conditions such as Cd exposure, both cell division and endoreduplication in *A. thaliana* leaves is inhibited. Glutathione is known for its involvement in both cell cycle regulation and plant responses to Cd, therefore the hypothesis of this project is that GSH is involved in cell cycle regulation in response to Cd exposure in *A. thaliana* leaves. To test this hypothesis, plant responses are compared between WT plants and the GSH-deficient *cadmium-sensitive 2-1* (*cad2-1*) mutant. Several parameters related to cell cycle regulation, oxidative stress and DNA damage are compared between WT and *cad2-1* mutant *A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM Cd. Additionally, a screening method to identify Cd-sensitive *A. thaliana* mutants using 96-well plates was optimized.

Cell cycle-related parameters and gene expression levels related to oxidative stress, DNA damage and cell cycle regulation were significantly affected upon Cd exposure in the mutant as compared to the WT, emphasizing the involvement of GSH in the Cd-induced DNA damage response and cell cycle regulation. Similar responses where observed in other GSH-deficient mutants, meaning that the observed effects were due to decreased GSH levels. Furthermore, the 96-well setup is suitable as a screening method. Different Cd-sensitive parameters responded strongly upon Cd exposure. Because of the differences in the Cd-induced responses between the WT and the GSH-deficient *cad2-1* mutant, it is possible to identify Cd-sensitive mutants via this system.

SAMENVATTING

Vele regio's wereldwijd worden getroffen door cadmium (Cd) vervuiling van de bodem. Planten kunnen dit metaal opnemen en zo kan Cd de voedselketen binnenkomen. Op die manier is het een bedreiging voor de gezondheid van de mens en is het belangrijk om Cd toxiciteit te onderzoeken in planten. Cadmium kan een verhoogde productie van ROS veroorzaken wat leidt tot oxidatieve stress. Verhoogde ROS levels kunnen schade veroorzaken aan macromoleculen zoals DNA. Planten bezitten een uitgebreid anti oxidatief verdedigingssysteem om zichzelf te beschermen tegen oxidatieve schade. Een voorbeeld van een anti oxidatief metaboliet is glutathion (GSH). Naast zijn rol in anti oxidatieve verdediging, is glutathion ook betrokken in de regulatie van de celcyclus. In planten bestaat er een alternatieve versie van de klassieke celcyclus, endoreduplicatie.

Uit voorgaand onderzoek blijkt dat externe stressfactoren, zoals Cd-blootstelling, zowel de celdeling als endoreduplicatie in *A. thaliana* blaadjes inhiberen. Glutathion is gekend om zijn rol in celcyclus regulatie en plant responses op Cd-blootstelling. Daarom is de hypothese van dit onderzoeksproject dat GSH betrokken is in regulatie van de celcyclus als respons op Cd-blootstelling in *A. thaliana* blaadjes. Om deze hypothese te onderzoeken worden plant responsen tussen WT planten en GSH-deficiënte *cadmium-sensitieve 2-1* (*cad2-1*) mutanten vergeleken. Verschillende parameters betrokken bij de regulatie van de celcyclus, oxidatieve stress en DNA schade worden vergeleken tussen WT en *cad2-1 A. thaliana* planten gegroeid in hydrocultuur en blootgesteld aan 0 of 5 µM Cd. Hiernaast wordt ook een screeningsmethode om Cd-sensitieve *A. thaliana* mutanten in 96-well platen te identificeren geoptimaliseerd.

Parameters gerelateerd aan de celcyclus en genexpressie levels gerelateerd aan oxidatieve stress, DNA schade en celcyclus werden significant beïnvloed door Cd blootstelling in de mutant vergeleken met het WT. Dit benadrukt de betrokkenheid van GSH in de DNA schade respons en celcyclus regulatie. Gelijkaardige responsen werden teruggevonden in andere GSH-deficiënte mutanten. Dit betekent dat de effecten te wijten zijn aan de verminderde GSH levels. Hiernaast blijkt dat de 96-well setup geschikt is als screeningsmethode. Verschillende Cd-sensitieve parameters reageren sterk op Cd blootstelling. Omwille van de verschillen in de Cd-geïnduceerde reacties tussen WT en mutant, is het mogelijk om Cd-sensitieve mutanten te identificeren via dit systeem.

1. INTRODUCTION

Many regions worldwide, including the Campine region in North-East Belgium, are affected by metal pollution of the soil. An example of a metal contaminating the environment is cadmium (Cd) (1). Although Cd is a rare element naturally present in the environment, Cd contamination is mainly caused by anthropogenic activities such as mining and smelting in metal working industries and the use of phosphate fertilizers (2)(3). If Cd accumulates in the body, it can pose a serious risk to human health, causing kidney damage, lung cancer and bone demineralization (4). Furthermore, it is classified as a class I carcinogen to humans (5). Cadmium can enter the food chain via uptake in plants from contaminated soils, thereby posing a threat to human health (6). Therefore, it is important to investigate the effects of Cd toxicity in plants.

Although Cd is a non-essential element, it can be taken up by plants via transporters for essential elements such as calcium (Ca), iron (Fe) and zinc (Zn) and thereby disturb plant growth and development (7)(8). Despite its non-redox active nature, Cd indirectly causes an increased production of reactive oxygen species (ROS), resulting in oxidative stress, defined as an imbalance between pro- and anti-oxidants in favor of the former (3)(6).

Cadmium can indirectly induce ROS production via several mechanisms. It can bind to thiol groups of certain enzymes, disturbing their function. Furthermore, it can replace redox-active elements and inhibit the activity of antioxidative enzymes and metabolites. In addition, Cd increases enzymatic ROS production via the induction of NADPH oxidase and enhances subcellular ROS production in mitochondria, peroxisomes and chloroplasts (6). Additionally, Cd can cause a decrease of cellular glutathione (GSH) levels due to an increased phytochelatin synthesis (9).

Although ROS play an important role in cellular signaling at low concentrations, elevated ROS levels can significantly damage cellular macromolecules including proteins, membrane lipids and DNA (6). In order to protect themselves against oxidative damage, plants possess an extensive antioxidative defense system, composed of both antioxidative enzymes and metabolites (6)(10)(11)(12).

Examples of antioxidative enzymes are superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (13). Superoxide dismutase is a metallo-enzyme that converts superoxide (O_2^-•) into hydrogen peroxide (H_2O_2). Subsequently, CAT can neutralize H_2O_2 to water (H_2O) and oxygen (O_2). Similarly, APX catalyzes the ascorbic acid (AsA)-dependent oxidation of H_2O_2 (9). The antioxidative metabolites are either hydrophobic (lipid-soluble) such as vitamin E, which protects cell membranes from lipid peroxidation or hydrophilic (water-soluble) such as AsA and GSH (14)(15).

Glutathione (GSH) is a tripeptide composed of the three amino acids glutamate (Glu), cysteine (Cys) and glycine (Gly). The GSH biosynthesis pathway consists of two ATP-dependent steps. In the first step, γ-glutamylcysteine synthetase (GSH1) forms a peptide bond between Glu and Cys, synthesizing γ-glutamylcysteine. Subsequently, Gly is added by glutathione synthetase (GSH2) in the second step, resulting in the formation of GSH (9)(16).

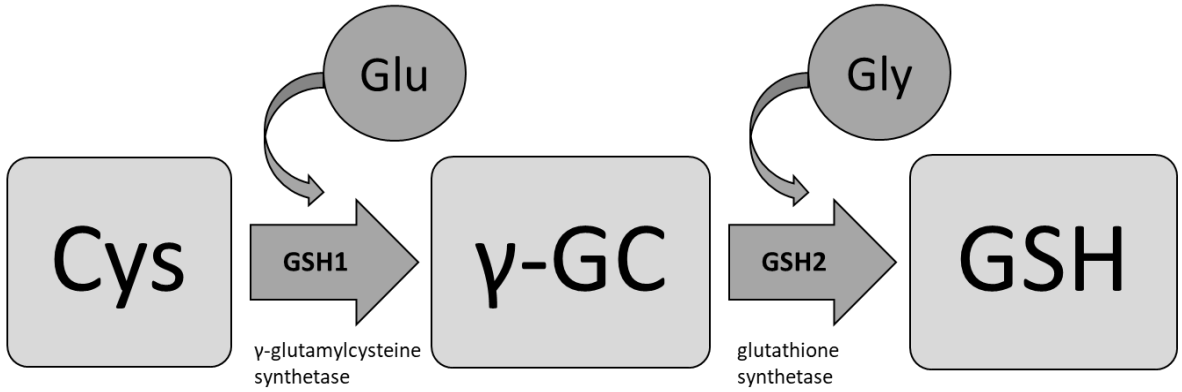

Figure 1: Schematic overview of glutathione biosynthesis.
Abbreviations: Cys, cysteine; Glu: glutamate; GSH1: γ-glutamylcysteine synthetase; γ-GC: γ-glutamylcysteine; Gly: glycine; GSH2: glutathione synthetase; GSH: glutathione.

Glutathione is involved in the antioxidative defense system removing excess hydrogen peroxide (H_2O_2) via several pathways: (1) direct detoxification of H_2O_2 via non-enzymatic GSH oxidation, (2) indirect neutralization of H_2O_2 via the AsA-GSH cycle where AsA and GSH get oxidized and reduced to allow AsA peroxidase (APx) to neutralize H_2O_2 or (3) H_2O_2 detoxification via the redoxin cycle where the two thiol-redox enzymes, glutaredoxin (GRx) and thioredoxin (TRx) recycle peroxiredoxin (PRx) that in his turn will neutralize H_2O_2. As a consequence, GSH is oxidized to glutathione disulfide (GSSG), which can be again reduced by glutathione reductase (GR), using NADPH as the electron donor (9)(17).

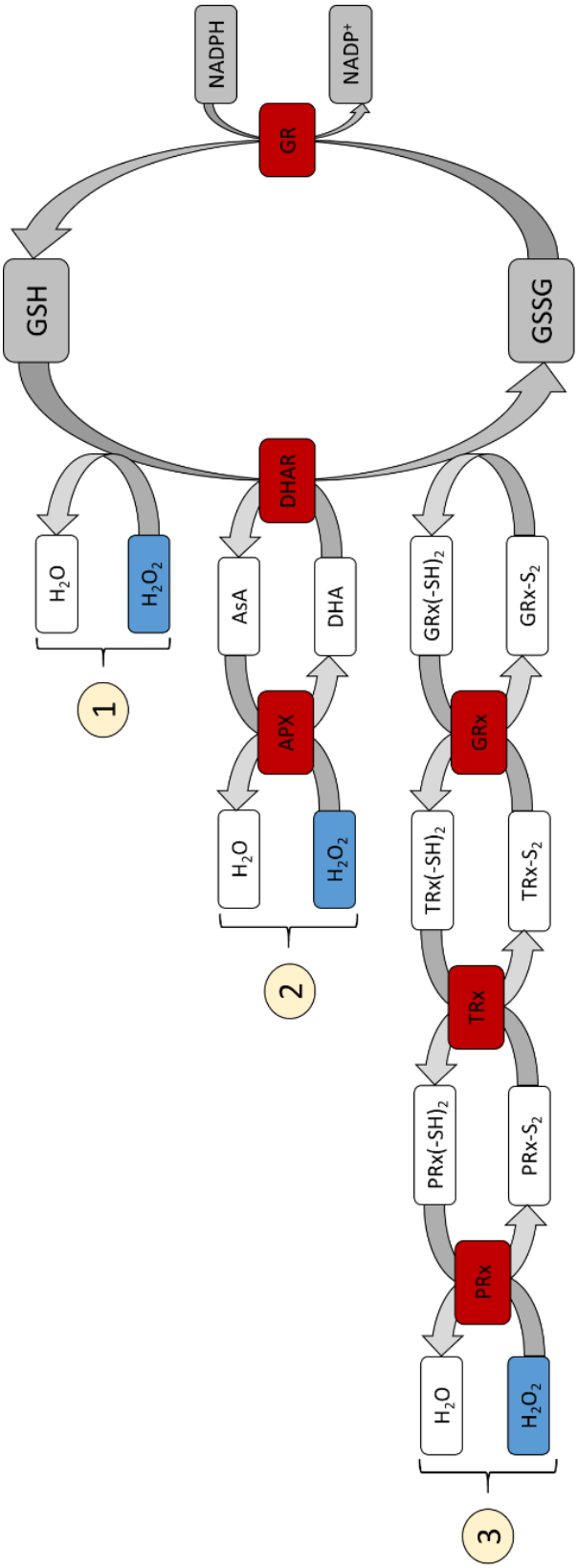

Figure 2: Roles of glutathione in antioxidative defense. Adapted from Jozefczak et al. (2012) (9).
1: Direct, non-enzymatic conversion. 2: AsA-GSH cycle. 3: Redoxin cycle. Abbreviations: GSH: glutathione, GSSG: glutathione disulfide, H_2O_2: hydrogen peroxide, GR: glutathione reductase, APX: ascorbate peroxidase, AsA: ascorbic acid, DHA: dehydroascorbate, DHAR: dehydroascorbate reductase. PRx: peroxiredoxin, TRx: thioredoxin, GRx: glutaredoxin.

Glutathione is not only involved in the neutralization of Cd-induced ROS, but also plays an important role in Cd chelation, as it is the precursor for phytochelatin (PC) synthesis (18). Phytochelatins (PCs) are composed of multiple GSH molecules polymerized by phytochelatin synthase (PCS). They can chelate several metal ions, including Cd, due to the presence of multiple thiol groups. Cadmium has a high affinity for the thiol group of the GSH cysteine (9)(18)(19).

Besides its role in antioxidative defense and metal chelation, GSH is also involved in cell cycle regulation. As shown by Diaz-Vivancos et al. (2010), antioxidants such as GSH have an important role in establishing an appropriate redox environment, which is necessary for cell cycle progression (20). Indeed, the recruitment of GSH into the nucleus during the G1 phase of the cell cycle has an important effect on the redox state of the cytoplasm and the expression of redox-related genes (21)(22). Furthermore, an increase of the total cellular GSH level is essential for the progression of cells from the G1 to the S-phase of the cell cycle (22). In addition, the *rml1* mutant, characterized by GSH levels of only 5% of WT levels, does not develop an active apical root meristem (23). This is due to low GSH levels that are responsible for a decreased level of cyclins and CDKs necessary for progression from the G2 to M phase (20).

The classical cell cycle consists of four phases: a Gap 1 (G1), Synthesis (S), Gap 2 (G2) and Mitosis (M) phase. During the S phase, nuclear DNA is replicated, whereas during the M phase, the cell divides into two daughter cells (24). The cell cycle is regulated by the activity of cyclin-dependent kinases (CDKs). These serine-threonine protein kinases associate with cyclins, forming heterodimers that phosphorylate target proteins essential for progression throughout the cell cycle (25)(26).

In plants, an alternative version of the classical cell cycle, endoreduplication, exists. During an endoreduplication cycle (endocycle), nuclear DNA is replicated (S phase) without intervening mitosis (M phase). As a consequence, cells become polyploid (27).

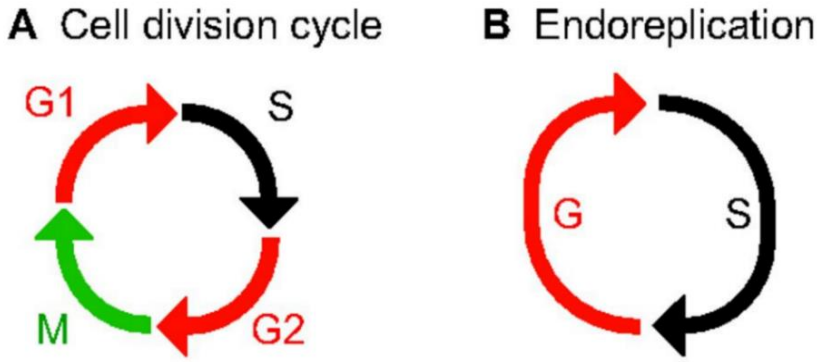

Figure 3 : Comparison of the classical cell cycle (A) and the endocycle (B). The classical cell cycle contains four phases (G1, S, G2 and M), whereas the endocycle only consists of a G and S phase without intervening mitosis.

The ploidy level that can be reached, depends on the organism and cell type. Cells of *Arabidopsis thaliana* - the model organism used in this project - can reach ploidy levels up to 32C (28). Whereas endoreduplication plays an important role in normal plant growth and development, it is also often affected by environmental (stress) conditions such as temperature, water status, sunlight and soil quality (29).

Similar to the classical cell cycle, endoreduplication is also regulated by cyclin-CDK complexes. In order for endoreduplication to take place, mitotic CDK activity must be suppressed. This can be achieved in three ways: (1) transcriptional downregulation of mitotic CDKs and cyclins; (2) proteolytic degradation of mitotic cyclins and (3) inhibition of mitotic CDK activity by CDK inhibitors such as SIAMESE-RELATED (SMR) proteins (29).

In order to prevent DNA replication and division of cells with damaged DNA, the cell cycle contains two checkpoints: the G1/S checkpoint and the G2/M checkpoint. When DNA damage is detected, checkpoint kinases are activated in order to transiently inhibit cell cycle progression to allow for DNA repair. When the damage is repaired, cells can resume DNA replication and cell division. In case the damage cannot be repaired, however, cells will enter a permanent cell cycle arrest and eventually undergo apoptosis (30).

Different types of DNA damage exist, including point mutations, single- and double-strand breaks, base pair mutations, deletions and inserts, rearrangements and oxidized bases. In order to maintain genome integrity, plants have developed a wide array of DNA repair mechanisms. Examples of DNA repair pathways are homologous recombination, non-homologous endjoining, mismatch repair and base excision repair. Each pathway is specific for the repair of a certain type of DNA damage (31).

Preliminary data of our research group show that Cd exposure inhibits cell division and endoreduplication in *A. thaliana* leaves. As GSH is known to be involved in both cell cycle regulation and plant responses to Cd, the hypothesis of this project is that GSH is involved in cell cycle regulation in response to Cd exposure in *A. thaliana* leaves. To this end, plant responses are compared between WT plants and the GSH-deficient *cadmium-sensitive 2-1* (*cad2-1*) mutant. This mutant has 70 to 85 % decreased GSH levels as compared to WT plants as a result of a mutation in the gene encoding GSH1 (33). As a consequence, it displays an increased sensitivity to Cd.

The goal of this project is to compare several parameters related to cell cycle regulation, oxidative stress and DNA damage between WT and *cad2-1* mutant *A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM Cd. In order to confirm the involvement of GSH in these processes related to cell cycle, oxidative stress and DNA damage, similar experiments were performed on three other GSH-deficient mutants. The *phytoalexin-deficient 2-1* mutant (*pad2-1*), this mutant has lower GSH levels due to a substitution and the *regulator of APX2 1-1* mutant (*rax1-1*) which has an impaired cysteine binding due to a missense mutation (33)(34).

Additionally, this project aims to optimize a screening method for Cd-sensitive *A. thaliana* mutants using 96-well plates.

2. MATERIALS AND METHODS

2.1. HYDROPONIC PLANT CULTIVATION

2.1.1. Plant cultivation and harvest

Wild-type and *cad2-1* mutant *A. thaliana* seeds were surfaced-sterilized in a 0.1% sodium hypochlorite and Tween 80 solution. Next, they were stored for 2 nights at 4°C to synchronize germination. Plants were grown in hydroponic culture as described by Keunen et al. (2011) (35). Growth conditions were set at a 12 h photoperiod, 65% relative humidity and day and night temperatures of 22°C and 18°C, respectively. A combination of blue, red and far-red LED light is used to simulate the spectrum of photosynthetic active radiation (PAR) in sunlight. A modified Hoagland solution provides nutrients (Table 1). This Hoagland solution was refreshed every 3 to 4 days. On day 19 after sowing, the plants were exposed to 5 µM Cd by addition of $CdSO_4$ to the Hoagland solution. For flow cytometric analysis, separate leaves were harvested after one week of Cd exposure. For gene expression analysis, samples were harvested after 24 h of 72 h of Cd exposure. All samples were snap-frozen in liquid nitrogen and stored at the -70°C freezer until further analysis.

Table 1: Composition of the modified Hoagland solution.

	CONCENTRATION
Macro elements	
KNO_3	505 µM
$Ca(NO_3)_2 . 4H_2O$	150 µM
NH4H2PO4	100 µM
$MgSO_4 . 7H_2O$	100 µM
Fe solution	
$FeSO_4 . 7H_2O$	1.64 µM
$Na_2 . EDTA$	0.81 µM
Micro elements	
H_3BO_3	4.63 µM
$MnCl_2 . 4H_2O$	0.91 µM
$CuSO_4 . 5H_2O$	0.03 µM
$H2MoO_4 . H_2O$	0.06 µM
$ZnSO_4 . 7 H_2O$	0.08 µM

2.1.2. Flow cytometry

In order to determine nuclear ploidy levels, separate leaves (4, 6, 8 and 10) were harvested. The analysis was performed using the CyStain® PI Absolute P kit (Sysmex-Partec, Kobe, Hyogo, Japan). Each leaf was chopped and incubated in 500 µL extraction buffer and filtered over a 50 µm Celltrics filter (Sysmex-Partec, Kobe, Hyogo, Japan). Subsequently, 2 mL staining solution – consisting of 2 mL staining buffer, 12 µL propidium iodide (PI) and 6 µL RNase – was added. Samples were incubated at 4°C in the dark for at least 1 hour and analyzed using a CyFlow® Cube 8 Flow cytometer (Sysmex-Partec, Kobe, Hyogo, Japan). Propidium iodide-stained nuclei were excited using a 488 nm laser and PI fluorescence was measured in the FL-2 channel. The percentage of nuclei corresponding to each ploidy level was determined using FCS Express 4 software (De Novo Software, California, United States). The endoreduplication factor (EF), which represents the average number of endocycles per cell, was calculated using the following formula: EF = [(0 x % 2C) + (1 x % 4C) + (2 x % 8C) + (3 x % 16C)] / 100.

2.1.3. Gene expression

RNA extraction

The extraction of RNA was performed using the RNaqueous® kit (Thermofisher Scientific, Waltham, United States) following the protocol of the manufacturer. The leaf samples were crushed using the Mixer Mill MM400 (Retsch, Haan, Germany). The concentration and purity of the RNA samples were checked using the Nanodrop ND-1000 (ThermoFisher Scientific, Waltham, United States), whereas the RNA integrity was determined using gel electrophoresis. The RNA samples were stored at -70°C.

cDNA synthesis

An input of 1 µg RNA was used for each sample for cDNA synthesis. To remove any contaminating genomic DNA, the TURBO DNA-free™ Kit (ThermoFisher Scientific, Waltham, United States) was used according to the protocol of the manufacturer. The PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara; Clontech, Leusden, The Netherlands) was used for the synthesis of cDNA. The PCR conditions used are shown in Table 2. After the PCR run, the samples were 10x diluted in 1/10 Tris-EDTA (TE) buffer and stored at -20°C.

Table 2: PCR program for cDNA synthesis.

	Step 1	*Step 2*	*Step 3*
Temperature	37°C	85°C	4°C
Time	15 min	5s	∞

Real time quantitative PCR

For the analysis of gene expression levels, real-time quantitative PCR (qPCR) was performed. A master mix containing Quantinova SYBR Green Mastermix, RN ROX, forward primer (300 nM final), reverse primer (300 nM final) and RNase-free water was prepared for the total amount of cDNA samples and No Template Controls (NTCs). Primer concentrations can be doubled/tripled to increase reaction efficiency. The sequences of the primers used are shown in Table 3.

Table 3: Overview of the primer sequences (5' - 3') of the forward (F) and reverse (R) primers used for qPCR analysis of the genes of interest.

UPOX: Upregulated By Oxidative Stress; *SOG1*: Suppressor of Gamma Radiation 1; *PARP2*: Poly(ADP-Ribose) Polymerase 2; *BRCA1*: Breast Cancer Susceptibility 1; *XRCC1*: Homologue of X-ray Repair Cross Complementing 1; *SMR*: SIAMESE-RELATED; *RBOHC*: Respiratory Burst Oxidase Homologue C; *AOX1*: Alternative Oxidase 1; *ERF1*: Ethylene Response Factor 1.

Gene	Forward primer	Reverse primer
AT1G05340	TCGGTAGCTCAGGGTAAAGTGG	CCAGGGCACAACAGCAACA
AT1G19020	GAAAATGGGACAAGGGTTAGACAAA	CCCAACGAAAACCAATAGCAGA
AT1G57630	ACTCAAACAGGCGATCAAAGGA	CACCAATTCGTCAAGACAACACC
UPOX	GACTTGTTTCAAAAACACCATGGAC	CACTTCCTTAGCCTCAATTTGCTTC
AT2G43510	ATGGCAAAGGCTATCGTTTCC	CGTTACCTTGCGCTTCTATCTCC
SOG1	AGTGGTGTGGAAGAGCAACC	GCAATCCTGGCCAATCATCAA
PARP2	ATCGGAGGTGATTGATCGGTATG	AAATCATGAGGTATCACTGTGTAGAACTCT
BRCA1	GTGAACCTGTCTCTGCGGAT	TCCGGCTTCTTGTCAACTCC
XRCC1	TGGGCCAGGGATGACCTAAG	CCGCAGCTATTCGCTTGATTT
SMR4	TGATGGTGGTGAGAAAACGAGA	TCTCTTCGAGGCTGTGCGTAG
SMR5	CAGCATATCCGCCTTGTCCA	CTGCTACCACCGAGAAGAACAAGT
SMR7	ACATCGATTCGGGCTTCACTAA	CCGTGGGAGTGATACAAATTCC
RBOHC	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
AOX1a	CTCTTCGTTGGCCTACCGAT	AACCATTCCAGGTACTGCTGCTAC
AOX1d	GCAGCCACCGTCTCTAGCAA	ACCGTTCAAACTCTGAAAATACCG
ERF1	TCCTCGGCGATTCTCAATTTT	CAACCGGAGAACAACCATCCT

Reactions containing 8 µL master mix and 2 µL cDNA sample (RNase free water for the NTC) were added to a 96 well plate and run in the 7500 Fast Real-Time PCR System (Bio-Rad, California, United States) according to the cycling conditions described in Table 4. The results were analyzed using the $2^{-\Delta Cq}$ method. Data were normalized using reference genes (Table 5) selected by the GrayNorm algorithm (36).

Table 4: Cycling conditions for the 7500 Fast Real-Time PCR System.

Initial denaturation	2 min at 95°C
40 cycles Denaturation Annealing and elongation	 5 s at 95°C 25 s at 60°C
Melting curve stages	15 s at 95°C 60 s at 60°C 15 s at 95°C 15 s at 60°C

Table 5: Overview of the primer sequences (5' - 3') of the forward (F) and reverse (R) primers used to determine the expression of the reference genes.
UBC21: ubiquitin conjugating enzyme 21; *MON1*: monensin sensitivity 1; *TIP41*: tonoplast intrinsic protein 41-like; *YSL8*: yellow-leaf-specific gene 8.

Reference gene	Forward primer	Reverse primer
UBC21	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
MON1	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
TIP41	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
YSL8	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT

2.1.4. Element determinations

During harvest, leaf samples were rinsed twice with distilled water. Root samples were incubated for 15 minutes in ice-cold 10 mM lead nitrate ($PbNO_3$) and rinsed two times in distilled water. The samples containing fresh plant material were dried at 60°C. When completely dry, they were weighed and digested in a heat block using 70% HNO_3 and 37% HCl suprapur solutions. Element concentrations were measured using inductively coupled plasma-optical emission spectrometry (ICP-OES 710, Agilent Technologies, Australia).

2.2. OPTIMIZATION OF 96-WELL CULTURE SYSTEM

2.2.1. Plant cultivation and harvest

In a second part of the project, a screening system using *A. thaliana* seedlings grown in 96-well plates was optimised. To this end, *A. thaliana* seeds were surface-sterilised in a 0.1% hypochlorite solution and sown in 96-well plates containing Murashige and Skoog (MS) growth medium. To synchronise germination, they were stored at 4°C for 2 nights. Growth conditions were the same as described for the hydroponic plant cultivation. After one week, plants were transferred to new plates filled with fresh medium either or not containing $CdSO_4$. To avoid fungal contamination, plants in 96-well plates were only handled under a laminar air flow cabinet. After harvest, samples were snap-frozen in liquid nitrogen and stored at -70°C until further analysis. Both flow cytometric and gene expression analysis were performed as described for hydroponics, except for the fact that a different RNA extraction method was used. Several plants were pooled for both analyses.

The optimization was divided in three different experiments. The aim of the first experiment was to determine the concentration of the MS growth medium yielding an optimal germination. To this end, WT plants were grown in 96-well plates containing three different MS concentrations (1/2, 1/4 and 1/8 MS medium) and the percentage of germination was determined. Furthermore, the minimal weight required for gene expression analysis and flow cytometry was assessed.

The objective of the second experiment was to investigate whether nuclear ploidy levels and the expression of oxidative stress, DNA damage and cell cycle-related (marker) genes could be used as sensitive markers for Cd exposure. To this end, WT plants were exposed to 0, 20 or 50 µM Cd one week after sowing. Samples for gene expression analysis were harvested after 72 h and one week of exposure, whereas samples for flow cytometric analysis were only harvested after one week of exposure. Eventually only the time point of one week was selected to use in further experiments because one time point is more convenient in the context of a fast and easy screening method.

The aim of the third experiment was to test whether the 96-well system could be used to identify mutants with an increased Cd sensitivity. Therefore, WT and *cad2-1* plants were exposed to 0, 20 or 50 µM Cd one week after sowing and harvested for both gene expression analysis and flow cytometric analysis after one week of exposure.

2.2.2. Gene expression

RNA extraction

RNA was extracted according to the protocol described by Valledor et al. (2014) (37). First, the samples were shredded at 30 Hz for 1.5 minutes. The pellet was dissolved in 400 µL of pellet solubilization buffer (PSB: 7 M Guanidine HCl, 2% v/v Tween 20, 4% v/v NP-40, 50 mM Tris/HCl pH 7.5, 1% v/v β-mercaptoethanol) and incubated in a thermal shaker (750 rpm) at 30°C for at least 15 minutes. Subsequently, the samples were centrifuged at 14000 x g for 3 minutes and the supernatant containing the RNA was transferred to a new tube. For precipitation of RNA on the column, 300 µL acetonitrile was added to the supernatant and mixed thoroughly. This mixture was transferred to a silica column in a Eppendorf tube and centrifuged at 12000 x g for 2 minutes. Afterwards, the column was washed once with wash buffer 1 (2 mM Tris/HCl pH7.5, 20 mM NaCl, 0.1 mM EDTA, 90% ethanol) and twice with wash buffer 2 (2 mM Tris/HCl pH7.5, 20 mM NaCl, 0.1 mM EDTA, 70% ethanol). The RNA was eluted using preheated RNase free water. The concentration and purity of the RNA samples were checked using the Nanodrop (Thermo Scientific, Waltham, United States), whereas the RNA integrity was determined using gel electrophoresis. The RNA samples were stored at -70°C.

2.3. STATISTICAL ANALYSIS

The statistical analysis of the obtained data was performed using R software (The R Foundation for Statistical Computing). In case the assumptions of normality and homoscedasticity (respectively tested with Shapiro-Wilk an Bartlett test) of the data were met, a parametric two-way ANOVA and *post-hoc* Tukey-Kramer test were performed. When necessary, data were transformed (log x, $\sqrt{x}$, x^{-1}, e^x). If the assumptions were not met, a non-parametric Kruskall-Wallis test was used, followed by the *post hoc* Wilcoxon Rank Sum test for multiple comparison. Gene expression data were standardly log transformed.

3. RESULTS

The goal of this study was to investigate the role of glutathione in cell cycle regulation and the DNA damage response in Cd-exposed *A. thaliana* plants. To this end, several parameters related to oxidative stress, DNA damage and the cell cycle were compared between leaves of wild-type *A. thaliana* plants and *cad2-1* mutants grown in hydroponics and exposed to 5 µM Cd for 24 h, 72 h or 8 days starting from day 19 after sowing. Additionally, a screening method using *A. thaliana* plants grown in 96-well plates was optimized.

3.1. EFFECTS OF CADMIUM ON CELL CYCLE REGULATION, OXIDATIVE STRESS AND DNA DAMAGE IN *ARABIDOPSIS THALIANA* LEAVES

3.1.1. The role of glutathione in cell cycle regulation in cadmium-exposed Arabidopsis thaliana leaves

3.1.1.1. Growth responses

Wild-type and *cad2-1 A. thaliana* seedlings were grown in hydroponics and exposed to a sublethal Cd concentration of 5 µM for 8 days starting from day 19 after sowing.

After exposure to cadmium for 8 days, the rosette fresh weight was determined. The results show that upon Cd exposure the fresh leaf weight was significantly lower as compared to the control condition in both genotypes. The effect in the Cd-sensitive mutant, *cad2-1*, however, was more pronounced as compared to that in WT plants (Fig. 4A).

Root fresh weight was significantly lower in Cd-exposed as compared to control plants of both genotypes. This effect was more pronounced in the GSH-deficient *cad2-1* mutant. In contrast, root fresh weight did not differ between both genotypes under control conditions (Fig 4B).

Furthermore, the rosette diameter was also determined. Under control conditions, the rosette diameters of WT and mutant plants did not differ. Cadmium exposure negatively affected the rosette diameter of both genotypes. This effect was more pronounced in the GSH-deficient mutant (Fig 4C).

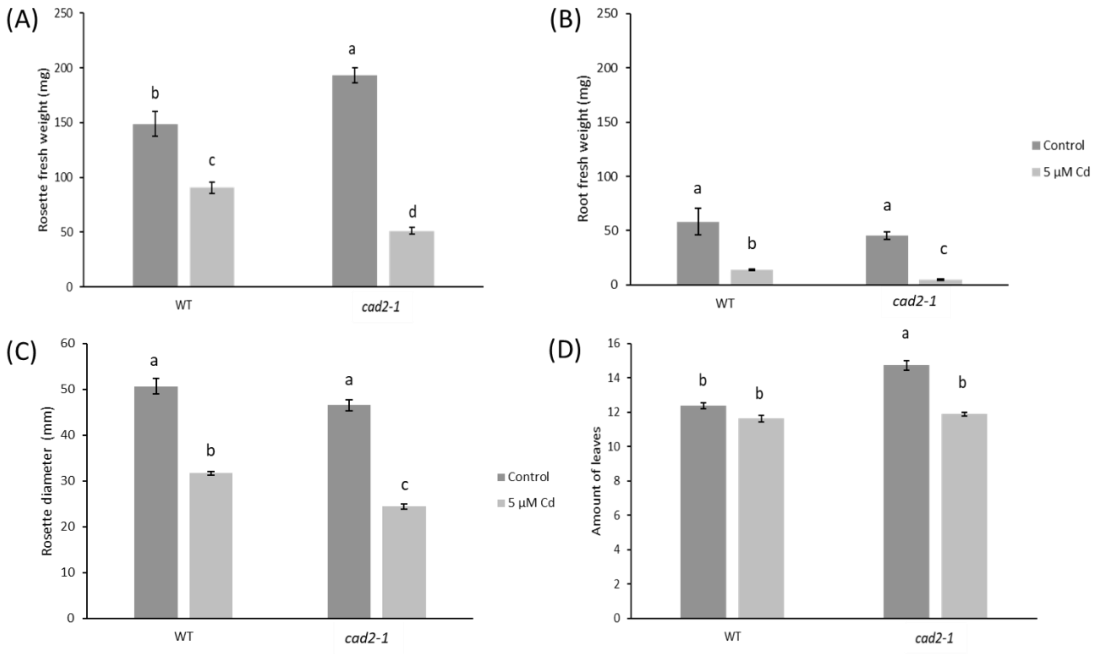

Figure 4. Rosette fresh weight (mg) (A), root fresh weight (mg) (B) rosette diameter (mm) (C) and number of leaves (D) of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

Additionally , the number of leaves was determined and compared between both genotypes. Under control conditions, the GSH-deficient mutant *cad2-1* has a significantly lower number of leaves as compared to the WT. The leaf number of Cd-exposed *cad2-1* mutant plants is significantly lower than that of their control counterparts. In contrast, Cd exposure did not influence the number of leaves in WT plants (Fig. 4D).

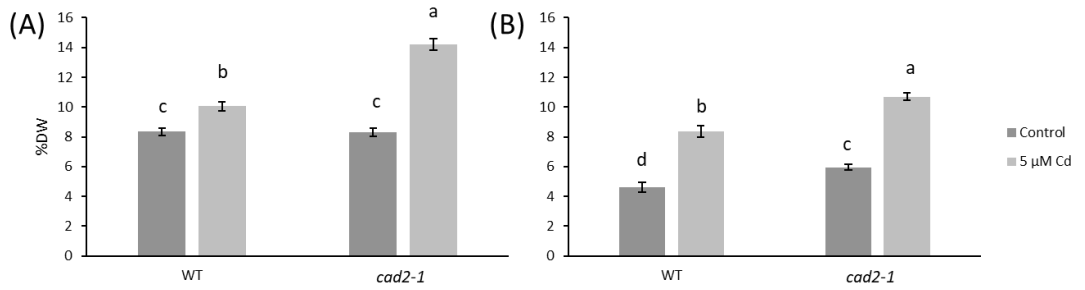

Figure 5. Percentage of dry weight of leaf (A) and root (B) samples of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the average ± S.E. of 3 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

When the samples were completely dry after approximately 3 weeks at 60°C, the dry weight was determined. The results show that upon Cd exposure the percentage of dry weight in leaves was significantly higher as compared to the control condition in both genotypes. The effect in the Cd-sensitive mutant, *cad2-1*, however, was more pronounced as compared to WT plants (Fig. 5A).

The percentage of dry weight in leaves was also significantly higher in Cd-exposed as compared to control plants of both genotypes. This effect was more pronounced in the GSH-deficient *cad2-1* mutant. The percentages of leaf dry weight differed significantly between both genotypes under control conditions (Fig 5B).

In addition, the surface area of leaf 4, leaf 6, leaf 8 and leaf 10 was determined to investigate the effect of Cd exposure on leaf growth. The surface area of the older leaves (4 and 6) was significant lower in Cd-exposed as compared to control leaves (Fig. 6A, 6B). This effect was more pronounced in the *cad2-1* mutant. Whereas the surface area of leaves 8 and 10 was not affected by Cd exposure in WT plants, it was negatively affected in the GSH-deficient mutant (Fig 6C, 6D).

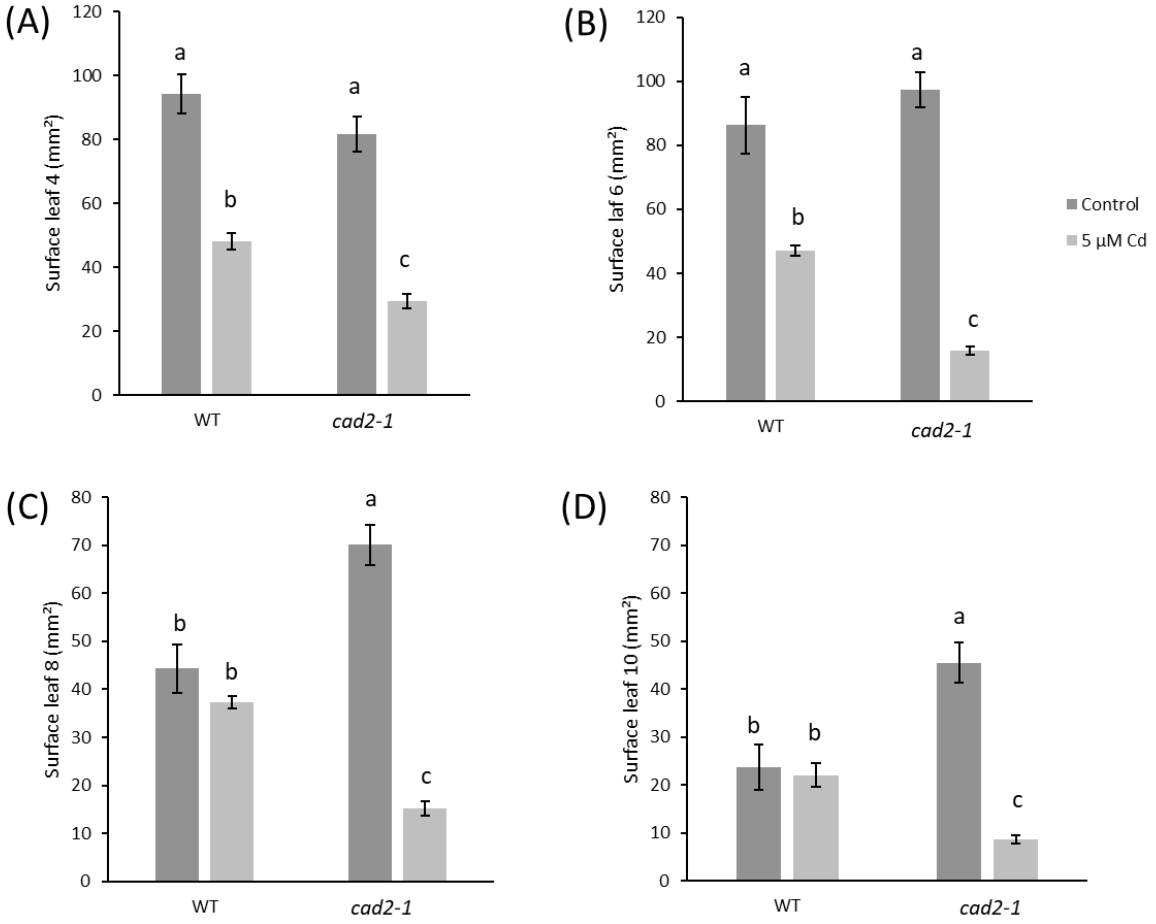

Figure 6. Leaf surface of leaf 4 (A), leaf 6 (B), leaf 8 (C) and leaf 10 (D) of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

3.1.1.2. Cadmium concentrations

As this study aims to investigate the effects of Cd exposure, root and leaf Cd concentrations were determined using ICP-OES. The results show that root Cd concentrations did not differ between both genotypes. The concentration in leaves, on the other hand, was lower in the GSH-deficient *cad2-1* mutant, as compared to the WT (Fig. 7).

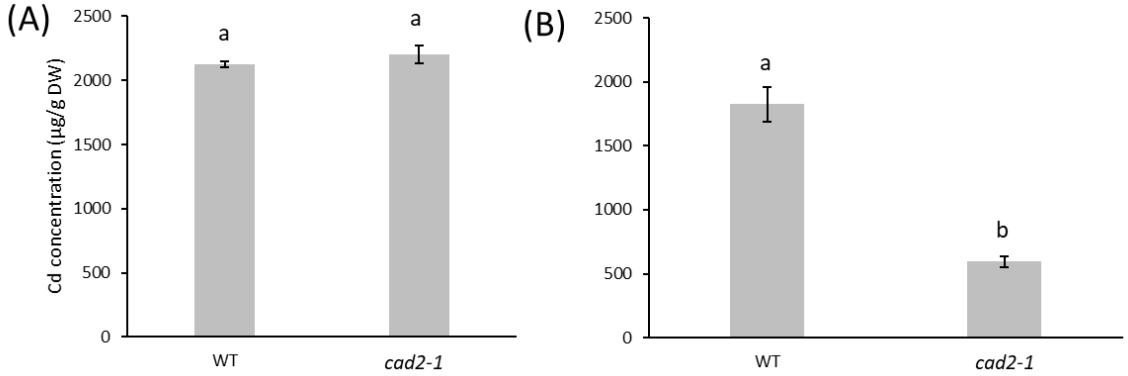

Figure 7. Cadmium concentrations (µg/g DW) in leaves (A) and roots (B) of wild-type (WT) and *cad2-1 A. thaliana* seedlings exposed to 5 µM Cd for 8 days. Data represent the average ± S.E. of 3 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (1-way ANOVA).

3.1.1.3. Element concentrations

Element concentrations in both roots and leaves were measured using ICP-OES in WT and mutant plants grown under control and Cd-exposed conditions. To this end, root and leaf dry weight (DW) was first determined (Fig. 2) as the concentrations were expressed as µg/g DW. Root and leaf element concentrations are presented in Table 6.

The leaves showed a decreased Ca concentration upon Cd exposure in both genotypes, although this response was more pronounced in the mutant. Root Ca concentrations were not affected by Cd exposure in the WT, whereas they increased in the mutant. Furthermore, a Cd-induced increase in root Cu concentrations was observed in roots of both genotypes, whereas Cu concentration in leaves remained unchanged. Both K and Mg concentrations significantly decreased upon Cd exposure in leaves of the *cad2-1* mutant, whereas this response was absent in WT plants. Concentrations of K and Mg in roots, on the other hand, decreased in both genotypes and this effect was more pronounced in the mutant. The P concentration in leaves was significantly decreased by Cd exposure in both genotypes. In addition, Cd exposure caused a decrease of the root P concentration in both genotypes.

Furthermore, Cd exposure caused an increase and a decrease in leaf S concentrations in respectively WT and mutant, whereas S concentration in roots remained unchanged. Leaf and root concentrations of the micro-element Mn decreased after Cd exposure in both genotypes. The effect was stronger in the mutant for leaf samples and stronger in the WT for root samples. In leaf samples, there was no significant change in Na concentrations. Root Na concentrations did not change upon Cd exposure in the WT, whereas they significantly decreased in the mutant. Leaf Zn concentration only decreased significantly in the mutant upon Cd exposure whereas the concentration of the WT did not changed significantly. In root samples, the Zn concentration remained unchanged.

Table 6: Metal concentrations (µg/g DW) in leaves and roots of wild-type (WT) and *cad2-1 A. thaliana* seedlings exposed to 5 µM Cd for 8 days. Data represent the mean ± S.E. of 3 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).
Ca: calcium: Cu: copper; K: potassium; Mg: magnesium; P: phosphorus; S: sulphur; Mn: manganese; Na: sodium; Zn: zinc.

Leaves	WT		*cad2-1*	
	0 µM Cd	5 µM Cd	0 µM Cd	5 µM Cd
Macro-elements				
Ca	38097.12 ± 750.96 ^{a}	30655.84 ± 1726.55 ^{b}	34530.10 ± 1417.10 ^{a}	22925.03 ± 883.53 ^{c}
Cu	2.84 ± 0.79 ^{a}	1.45 ± 0.63 ^{a}	3.20 ± 0.40 ^{a}	1.37 ± 0.26 ^{a}
K	29475.86 ± 1227.86 ^{a}	30565.48 ± 838.88 ^{a}	29312.11 ± 1796.75 ^{a}	20404.67 ± 218.88 ^{b}
Mg	8177.95 ± 224.60 ^{a}	7413.78 ± 205.89 ^{a}	7425.35 ± 263.55 ^{a}	4972.75 ± 144.44 ^{b}
P	11492.47 ± 132.25 ^{a}	8758.23 ± 323.28 ^{b}	11715.22 ± 178.53 ^{a}	8244.48 ± 331.16 ^{b}
S	9370.48 ± 133.35 ^{b}	14598.61 ± 432.28 ^{a}	8872.96 ± 421.74 ^{b}	6769.70 ± 144.89 ^{c}
Micro-elements				
Mn	234.45 ± 6.29 ^{a}	184.92 ± 8.16 ^{b}	216.71 ± 2.88 ^{a}	137.84 ± 4.52 ^{c}
Na	365.36 ± 28.94 ^{a}	427.70 ± 25.40 ^{a}	371.35 ± 6.94 ^{a}	380.65 ± 8.52 ^{a}
Zn	43.97 ± 2.26 ^{a}	46.07 ± 3.45 ^{a}	39.66 ± 0.65 ^{a}	27.01 ± 0.85 ^{b}
Roots	WT		*cad2-1*	
	0 µM Cd	5 µM Cd	0 µM Cd	5 µM Cd
Macro-elements				
Ca	1195.19 ± 50.24 ^{c}	1361.00 ± 35.53 ^{bc}	1376.28 ± 31.24 ^{b}	1668.58 ± 27.26 ^{a}
Cu	7.62 ± 0.43 ^{c}	60.11 ± 1.10 ^{b}	10.20 ± 2.34 ^{c}	99.29 ± 1.22 ^{a}
K	38362.07 ± 3953.18 ^{a}	23280.90 ± 2435.55 ^{b}	38445.03 ± 295.48 ^{a}	14410.04 ± 916.36 ^{c}
Mg	960.84 ± 83.05 ^{b}	703.30 ± 48.16 ^{d}	1104.14 ± 13.05 ^{a}	705.47 ± 6.80 ^{c}
P	12419.60 ± 61.45 ^{ab}	10213.30 ± 266.42 ^{c}	13128.37 ± 203.30 ^{a}	12127.04 ± 235.01 ^{b}
S	11077.64 ± 1096.46 ^{a}	12241.37 ± 414.61 ^{a}	10480.92 ± 570.65 ^{a}	9564.15 ± 169.33 ^{a}
Micro-elements				
Mn	210.10 ± 53.14 ^{a}	27.07 ± 1.90 ^{c}	275.01 ± 75.74 ^{a}	40.59 ± 0.75 ^{b}
Na	387.90 ± 36.06 ^{a}	253.46 ± 30.58 ^{ab}	391.42 ± 46.34 ^{a}	161.67 ± 21.78 ^{b}
Zn	80.41 ± 7.75 ^{a}	80.92 ± 4.77 ^{a}	86.29 ± 5.94 ^{a}	77.25 ± 1.28 ^{a}

3.1.1.4. Cell-cycle related parameters

A flow cytometric analysis of leaf 4, 6, 8 and 10 was performed to assess the extent of cell division and endoreduplication in leaves of WT and *cad2-1* mutant *A. thaliana* plants exposed to 5 µM Cd for 8 days.

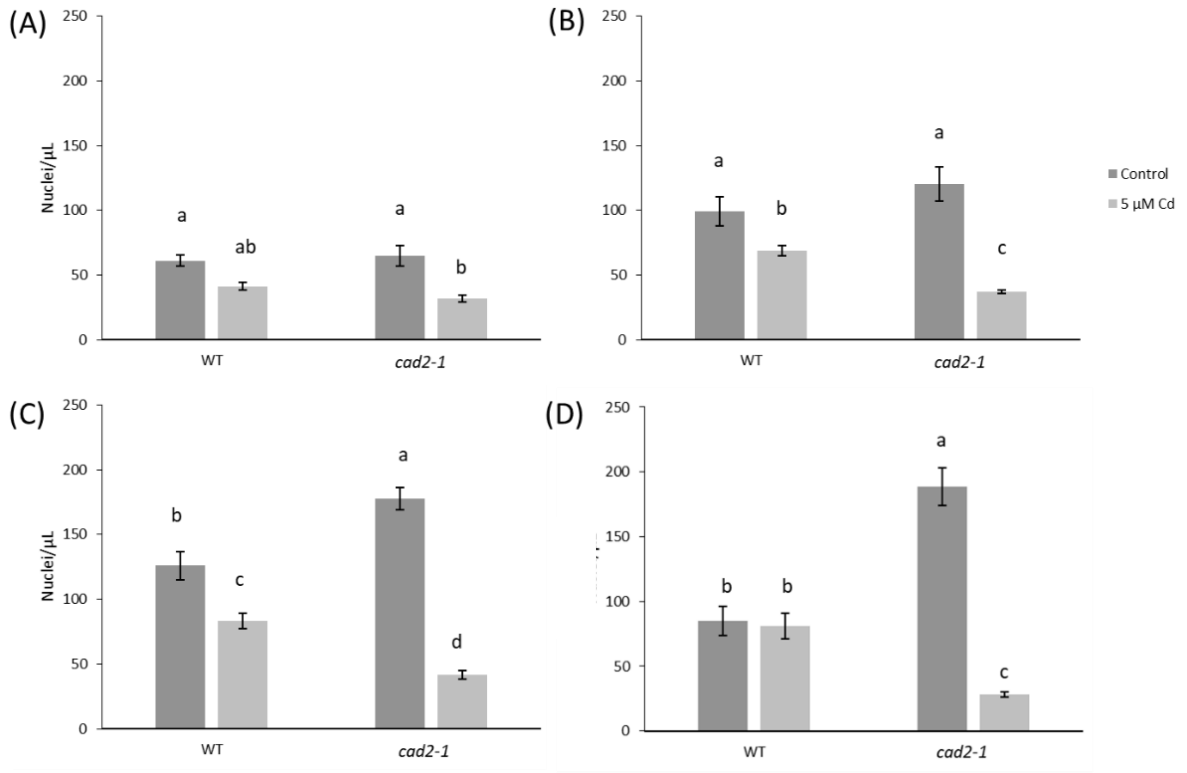

Figure 8. Nuclei/µL in leaf 4 (A), leaf 6 (B), leaf 8 (C) and leaf 10 (D) of wild-type (WT) and *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the mean ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

The concentration of nuclei determined via flow cytometric analysis was used as a proxy to analyse the effects of Cd exposure on the extent of cell division. Under control conditions, this parameter did not differ in leaf 4 of both genotypes. Furthermore, it was not affected by Cd exposure in leaf 4 of the WT. However, the concentration of nuclei was significantly lower in leaf 4 of Cd-exposed *cad2-1* mutant plants as compared to the same leaf of their control counterparts (Fig. 8A). In leaf 6, a leaf slightly younger than leaf 4, Cd exposure caused a significant decrease in the concentration of nuclei in flow cytometry extracts of both genotypes, but this effect was more pronounced in the *cad2-1* mutant (Fig. 8B).

Cadmium exposure caused a significant decrease in the concentration of nuclei/µL flow cytometry extract in both genotypes of leaf 8, but this effect was more pronounced in the *cad2-1* mutant. Furthermore, the nuclear concentration also significantly differed between both genotypes under control conditions. The nuclear concentration of the mutant is higher under control conditions as compared to the WT (Fig. 8C). Leaf 10, the youngest leaf studied, showed no difference in the concentration of nuclei in flow cytometry extracts between both conditions of the WT. The GSH-deficient mutant, *cad2-1*, showed a clear significant decrease in the amount of particles/µL after Cd exposure (Fig. 8D).

In the *cad2-1* mutant, Cd exposure increased the percentage of nuclei with lower ploidy levels (2C, 4C and 8C) and decreased the relative number of nuclei with the higher ploidy levels (16C, 32C) in leaf 4, the oldest leaf studied. In the same leaf of WT plants, a significantly increased proportion of 2C and 32C nuclei was observed upon Cd exposure (Fig. 9A)(Supplementary Fig. 1). As a result, the EF was significantly reduced by Cd exposure in leaf 4 of the mutant, whereas it was not affected in WT plants (Fig. 9B).

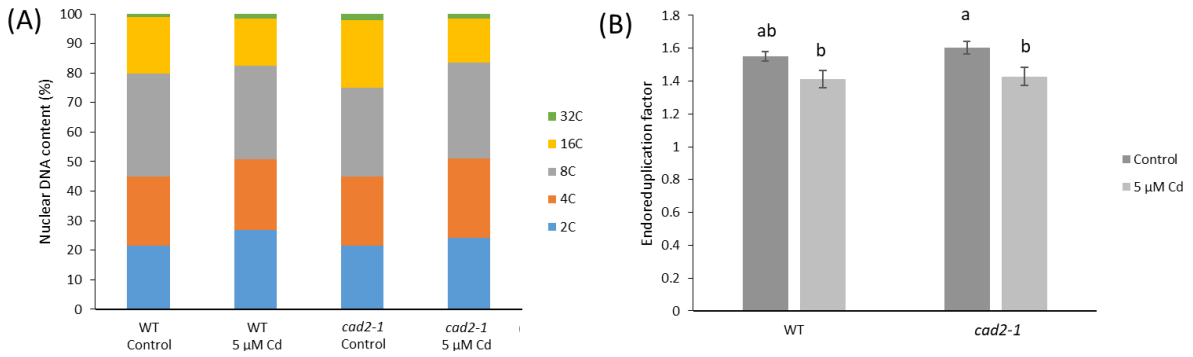

Figure 9. Nuclear DNA content (A) and endoreduplication factor (B) in leaf 4 of wild-type (WT) and *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

In leaf 6 of both the GSH-deficient *cad2-1* mutant and the WT, an increase in the level of 2C and 4C nuclei was observed after Cd exposure. The levels of 8C, 16C and 32C nuclei, on the other hand, showed a decrease (Fig. 10A)(Supplementary Fig. 2). The EF of leaf 6 significantly decreased in the *cad2-1* mutant after Cd exposure, whereas this response was not observed in the WT (Fig. 10B).

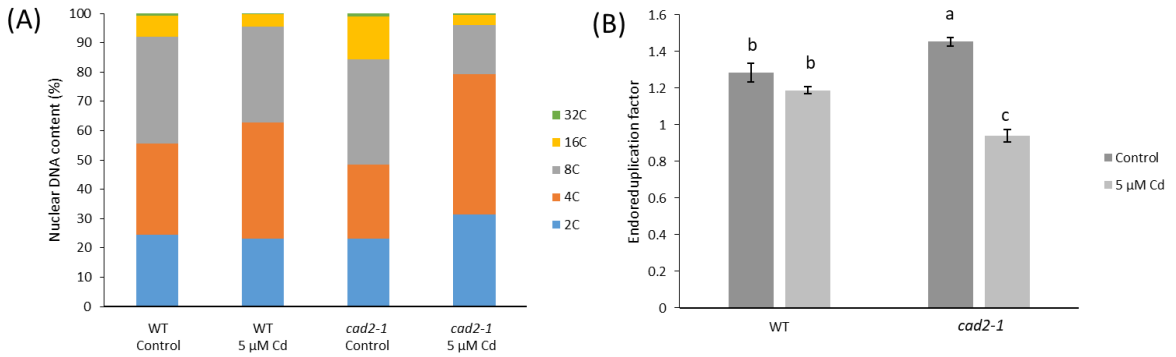

Figure 10. Nuclear DNA content (A) and endoreduplication factor (B) in leaf 6 of wild-type (WT) and *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

In leaf 8 of the *cad2-1* mutant, Cd exposure caused an increase in the percentage of nuclei with lower ploidy levels (2C and 4C). As a consequence, the relative amount of nuclei with higher ploidy levels (8C, 16C and 32C) showed a decrease. In leaf 8 of the WT, the opposite trend was observed, with the number of nuclei with lower and higher ploidy levels decreasing and increasing, respectively (Fig. 11A)(Supplementary Fig. 3). The EF in leaf 8 of the WT did not differ between control and Cd-exposed conditions. In contrast, the EF significantly decreased upon Cd exposure in leaf 8 of the *cad2-1* mutant (Fig. 11B).

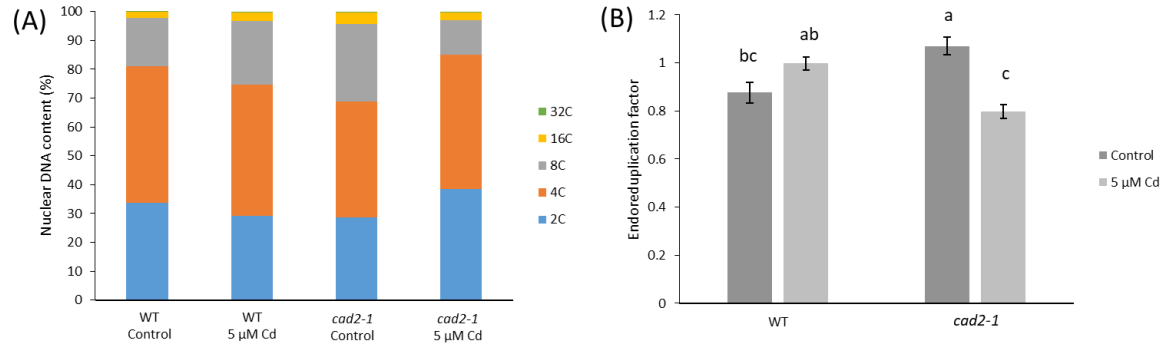

Figure 11. Nuclear DNA content (A) and endoreduplication factor (B) in leaf 8 of wild-type (WT) and *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

The percentage of 2C nuclei decreased upon Cd exposure in leaf 10 of WT plants, whereas the percentage of nuclei with higher ploidy levels showed an increase. In the mutant, the opposite effect was observed, with the relative number of 2C nuclei increasing and the percentage of nuclei with higher ploidy levels decreasing (Fig. 12A)(Supplementary Fig.4). The EF differed significantly between leaf 10 of control and Cd-exposed plants of both genotypes. Whereas the EF of leaf 10 increased in WT plants, it decreased in the *cad2-1* mutant (Fig. 12B).

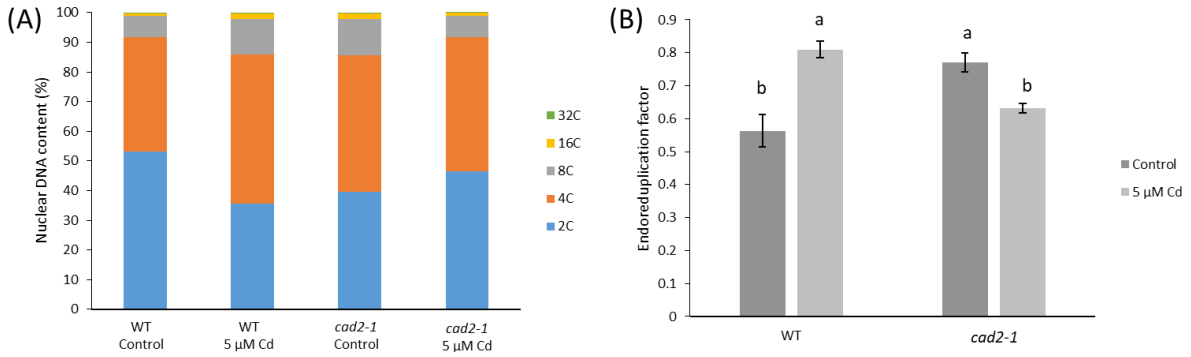

Figure 12. Nuclear DNA content (A) and endoreduplication factor (B) in leaf 10 of wild-type (WT) and *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

3.1.2. The role of glutathione in the DNA damage response in cadmium-exposed *Arabidopsis thaliana* leaves

3.1.2.1. Growth responses

In order to investigate the involvement of GSH in the Cd-induced DNA damage response, WT and *cad2-1 A. thaliana* seedlings were grown in hydroponics and exposed to a sublethal Cd concentration of 5 µM for 24 h of 72 h starting from day 19 after sowing. The leaf fresh weight as determined during harvest is shown in Fig. 13.

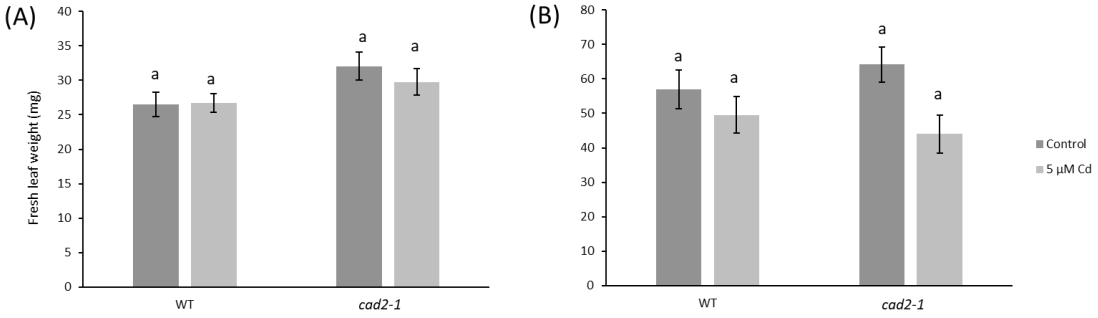

Figure 13. Fresh leaf weight (mg) of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings exposed to 0 or 5 µM Cd for respectively 24 h (A) and 72 h (B) starting from day 19 after sowing. Data represent the average ± S.E. of 5 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

Results show that leaf fresh weight was not significantly affected by Cd in either WT or mutant plants after 24 or 72 h of exposure. However, a decreasing trend in rosette fresh weight was observed in the mutant after 72 h (Fig. 13).

3.1.2.2. Gene expression levels

To determine the role of GSH in the Cd-induced DNA damage response in *A. thaliana* leaves, differences were assessed in the responses of WT and GSH-deficient *cad2-1* mutant plants to Cd exposure. An analysis of expression levels of genes related to oxidative stress and signaling, DNA damage and repair and cell cycle regulation was performed in WT and *cad2-1* mutant *A. thaliana* seedlings exposed to 5 µM Cd for 24 h or 72 h.

Table 7. Gene expression levels in leaves of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings exposed to 0 or 5 µM Cd for 24 h and 72 h starting from day 19 after sowing. Data represent the average ± S.E. of 5 biological independent replicates, expressed relative to the control of the same genotype (Supplementary table 1, 2). Asterisks (*) indicate a significantly different Cd-induced fold change between both genotypes. Green and red colors indicate significant Cd-induced upregulations and downregulations, respectively ($p < 0.05$)(2-way ANOVA). Data were normalized against the expression of *AT5G25760 (UBC), AT2G28390 (MON1) and AT4G34270 (TIP41)*[1].

	24 h		72 h	
	WT	*cad2-1*	WT	*cad2-1*
OXIDATIVE STRESS AND SIGNALING				
AT1G05340	19.97 ± 3.59	8.08 ± 1.20*	4.33 ± 1.52	35.86 ± 5.82*
AT119020	25.72 ± 2.06	42.70 ± 2.89	2.33 ± 0.39	27.39 ± 0.48*
AT1G57630	26.85 ± 1.65	25.97 ± 1.84	6.77 ± 0.83	17.16 ± 2.13*
UPOX	4.76 ± 0.35	7.30 ± 0.78	3.87 ± 0.59	1.54 ± 0.06*
AT2G43510	10.73 ± 1.00	15.08 ± 1.86*	8.12 ± 2.86	30.25 ± 3.44*
AOX1a	6.16 ± 0.51	6.81 ± 0.43	2.20 ± 0.21	2.66 ± 0.24
AOX1d	178.5 ± 27.77	167.79 ± 13.26	9.33 ± 2.82	96.09 ± 18.33*
ERF1	143.68 ± 8.84	116.19 ± 10.03	14.17 ± 3.74	78.26 ± 8.17*
RBOHC	43.73 ± 9.72	146.83 ± 10.63*	2.89 ± 0.52	2.37 ± 0.08
DNA DAMAGE AND REPAIR				
SOG1	1.97 ± 0.06	1.03 ± 0.03*	1.73 ± 0.09	1.65 ± 0.05
PARP2	1.83 ± 0.03	0.80 ± 0.05*	8.92 ± 1.02	1.78 ± 0.12*
BRCA1	0.84 ± 0.05	0.60 ± 0.06*	4.18 ± 0.48	1.17 ± 0.15*
XRCC1	2.22 ± 0.08	1.81 ± 0.04	1.22 ± 0.04	1.61 ± 0.04*
CELL CYCLE – CDK INHIBITORS				
SMR4	1.68 ± 0.12	0.68 ± 0.06*	2.42 ± 0.23	1.31 ± 0.05*
SMR5	1.62 ± 0.12	0.92 ± 0.04*	6.76 ± 0.82	1.88 ± 0.15*
SMR7	0.83 ± 0.16	0.39 ± 0.03*	44.17 ± 6.5	1.56 ± 0.16*

Results show that all oxidative stress marker genes and genes involved in oxidative signalling were significantly upregulated after 24 h of Cd exposure in the WT. In the mutant, these inductions were even stronger. Similarly, a Cd-induced increased expression of genes involved in DNA damage and repair was also observed in plants exposed for 24 h.

[1] *UBC21*: ubiquitin conjugating enzyme 21; *MON1*: monensin sensitivity 1; *TIP41*: tonoplast intrinsic protein 41-like; *UPOX*: Upregulated By Oxidative Stress; *AOX1*: Alternative Oxidase 1; *ERF1*: Ethylene Response Factor 1; *RBOHC*: Respiratory Burst Oxidase Homologue C; *SOG1*: Suppressor of Gamma Radiation 1; *PARP2*: Poly(ADP-Ribose) Polymerase 2; *BRCA1*: Breast Cancer Susceptibility 1; *XRCC1*: Homologue of X-ray Repair Cross Complementing 1; *SMR*: SIAMESE-RELATED.

In the mutant, it was observed that the inductions of most of these genes were smaller or even absent as compared to the WT. Expression of the SIAMESE-RELATED (SMR) genes, involved in cell cycle regulation in response to DNA damage were upregulated in the WT and down regulated in the mutant after 24 h exposure (table 7). All genes analysed were significantly upregulated in the WT after 72 h of Cd exposure. In the mutant, the inductions of several oxidative stress related genes upon Cd exposure were much stronger as compared to the WT. On the other hand, the induction of almost all DNA damage and repair related genes and the SMRs were much smaller after Cd exposure in the mutant as compared to the WT (table 7).

3.1.2.3. Growth responses of other GSH-deficient mutants

In order to confirm the involvement of GSH in the Cd-induced DNA damage response, a similar experiment was performed in three different GSH-deficient mutants: the *cadmium-sensitive 2-1* mutant (*cad2*-1), the *phytoalexin-deficient 2-1* mutant (*pad2-1*) and the *regulator of APX2 1-1* mutant (*rax1-1*). They were grown in hydroponics and exposed to 5 µM Cd for 72 h.

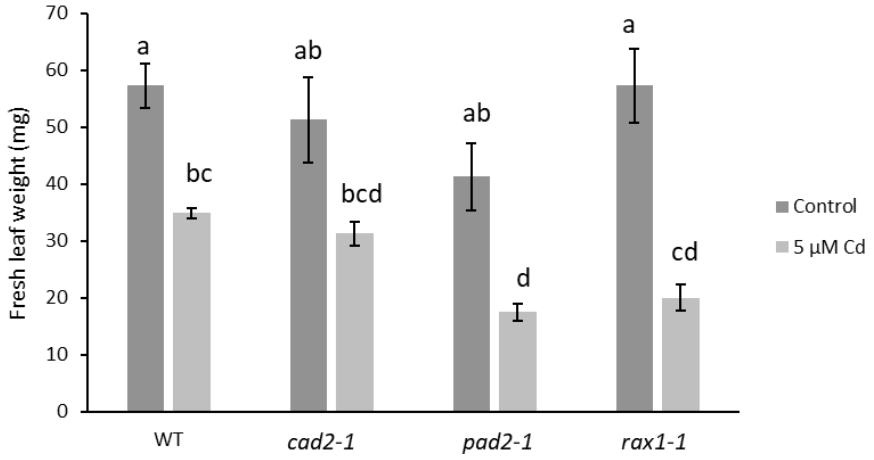

Figure 14. Fresh leaf weight (mg) of wild-type (WT) seedlings and 3 other *A. thaliana* genotypes (*cad2-1, pad2-1, rax1-1*) exposed to 0 or 5 µM Cd for 72 h starting from day 19 after sowing. Data represent the average ± S.E. of 5 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

During harvest, the rosette fresh weight of both control and Cd-exposed *A. thaliana* plants was determined. Results show that rosettes of Cd-exposed plants of all genotypes had a lower fresh weight as compared to their control counterparts (Fig. 14).

3.1.2.4. Gene expression levels of other GSH-deficient mutants

In addition, Cd-induced effects on the expression of a limited number of genes involved in the DNA damage response and cell cycle regulation were compared between WT plants and the three GSH-deficient genotypes, *cad2-1*, *pad2-1* and *rax1-1*. To this end, *A. thaliana* seedlings were exposed to 5 µM Cd for 72 h.

Table 8. Gene expression levels of wild-type (WT) seedlings and 3 other *A. thaliana* genotypes (*cad2-1, pad2-1, rax1-1*) exposed to 5 µM Cd for 72 h. Data represent the average ± S.E. of 4 biological independent replicates expressed relative to the control of the same genotype (Supplementary table 3). Asterisks (*) indicate a significantly different Cd-induced fold change between both genotypes. Green and red colors indicate significant Cd-induced upregulations and downregulations, respectively ($p < 0.05$)(2-way ANOVA). Data were normalized against the expression of AT5G25760 (UBC), AT4G34270 (TIP41) and AT5G08290 (YSL8)[2].

	WT	*cad2-1*	*pad2-1*	*rax1-1*
DNA DAMAGE AND REPAIR				
PARP2	4.53 ± 1.18	1.46 ± 0.17*	1.57 ± 0.07*	1.70 ± 0.27*
BRCA1	2.11 ± 0.32	1.06 ± 0.04	1.24 ± 0.07	1.30 ± 0.24
CELL CYCLE – CDK INHIBITORS				
SMR4	1.51 ± 0.06	0.65 ± 0.03*	0.97 ± 0.01*	1.41 ± 0.16
SMR5	2.28 ± 0.21	2.22 ± 0.11	2.24 ± 0.20	1.64 ± 0.28
SMR7	6.80 ± 0.53	1.24 ± 0.12*	1.40 ± 0.13*	1.09 ± 0.02*

Gene expression levels are shown in Table 8. In the WT, all genes analysed were significantly upregulated. In the three GSH-deficient mutants, *cad2-1*, *pad2-1* and *rax1-1*, the inductions of genes related to DNA damage and repair and were much smaller or they even disappeared. The same conclusion can be made for the CDK inhibitors.

[2] *UBC21*: ubiquitin conjugating enzyme 21; *TIP41*: tonoplast intrinsic protein 41-like; *YSL8*: yellow-leaf-specific gene 8; *PARP2*: Poly(ADP-Ribose) Polymerase 2; *BRCA1*: Breast Cancer Susceptibility 1; *SMR*: SIAMESE-RELATED.

3.2. OPTIMIZATION OF A 96-WELL SCREENING METHOD

3.2.1. Germination

In the first experiment aiming to optimize a 96-well screening method, the concentration of the MS growth medium yielding an optimal germination of the *A. thaliana* seedlings was assessed. To this end, three different MS concentrations were tested and the percentage of germination was calculated at three time points after sowing. The results are shown in Fig. 15 and show that plants germinate optimally on the 1/4 MS growth medium.

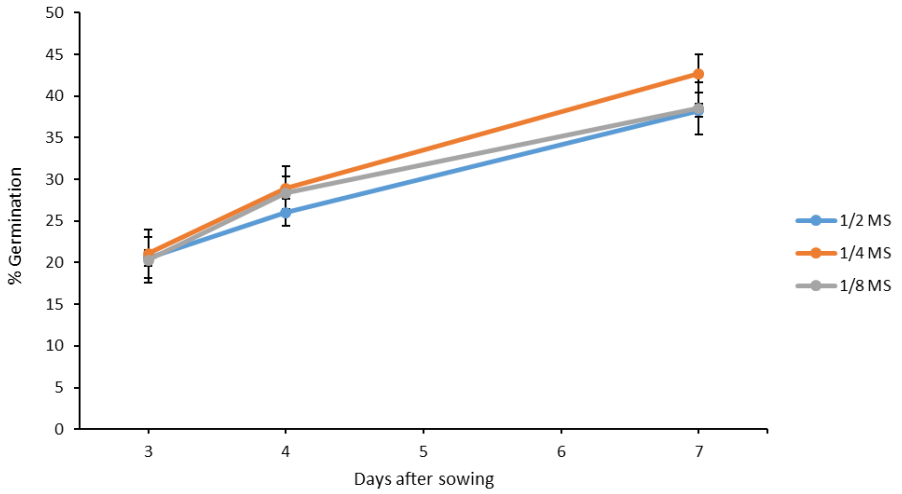

Figure 15. Percentage of germination of wild-type (WT) *A. thaliana* seedlings grown in 96-well plates containing three different concentrations of the Murashige and Skoog (MS) growth medium (1/2, 1/4 and 1/8). Data represent the average ± S.E. of 4 biological independent replicates.

3.2.2. Selection of cadmium-sensitive parameters

The goal of the second experiment was to identify Cd-sensitive parameters to be used in the 96-well system. To this end, WT plants were grown in 96-well plates and exposed to three different Cd concentrations (0, 20 and 50 µM Cd) for 72 h and 7 days. Ploidy levels and the concentration of nuclei determined via flow cytometric analysis together with gene expression levels of genes related to oxidative stress and signalling, DNA damage and repair and the cell cycle were assessed as these parameters were affected upon Cd exposure in hydroponics.

The number of nuclei per µL measured via flow cytometry was used as a proxy to display the effects of Cd exposure on cell division. They were compared between WT plants exposed to three Cd concentrations for 7 days. Results show that exposure to 50 µM Cd caused a significant decrease in this parameter (Fig. 16).

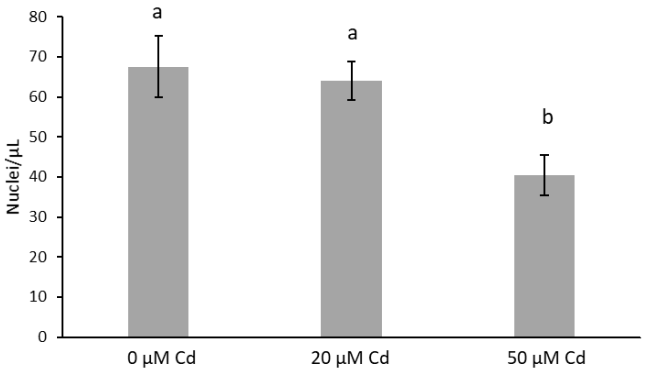

Figure 16. Number of nuclei per µL in flow cytometry extracts of wild-type (WT) *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 7 days. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

The percentage of nuclei with a 2C ploidy level decreased with an increasing Cd concentration. This response was concomitant with an increased level of 32C nuclei (Fig. 17A)(Supplementary Fig. 5). In addition, the EF showed an increasing trend with increasing Cd concentrations and was significantly increased after exposure to 50 µM Cd for 7 days (Fig. 17B).

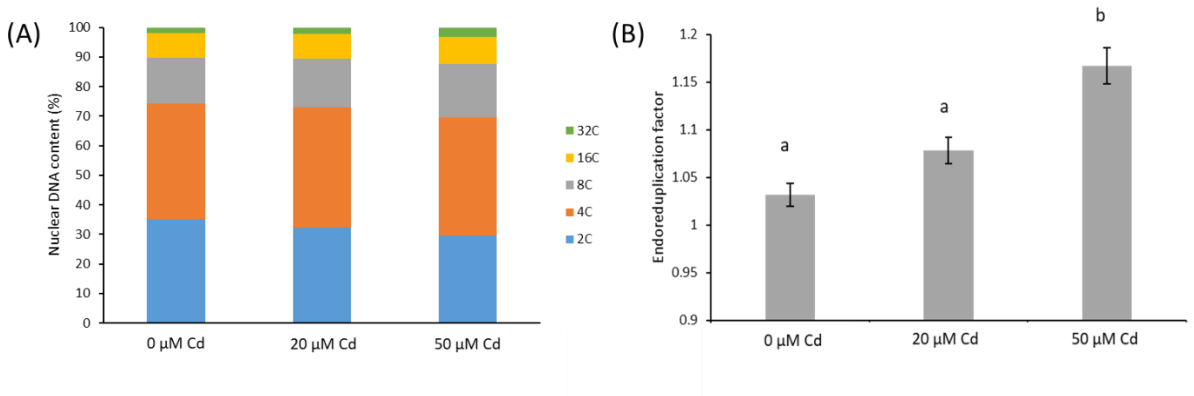

Figure 17. Nuclear DNA content (A) and endoreduplication factor (B) of wild-type (WT) *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 7 days. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (1-way ANOVA).

Furthermore, an analysis of the expression of genes related to oxidative stress and signaling, DNA damage and repair and cell cycle regulation was performed in WT *A. thaliana* plants exposed to 0, 20 or 50 µM Cd for 72 h or 7 days (Table 9).

Table 9. Gene expression levels of wild-type (WT) *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 72 h and 7 days. Data represent the average ± S.E. of 4 biological independent replicates expressed relative to the control of the same genotype. Asterisks (*) indicate a significantly different Cd-induced fold change between both genotypes. Green and red colors indicate significant Cd-induced upregulations and downregulations, respectively ($p < 0.05$)(2-way ANOVA). Data were normalized against the expression of *AT4G34270 (TIP41), AT5G08290 (YSL8) and AT4G26410 (RHIP1)*[3].

	72 h		7 d	
	20 µM Cd	50 µM Cd	20 µM Cd	50 µM Cd
OXIDATIVE STRESS AND SIGNALING				
AT1G05340	0.48 ± 0.06	0.80 ± 0.08	0.58 ± 0.02	1.45 ± 0.27
AT119020	0.27 ± 0.01	0.53 ± 0.03	0.82 ± 0.08	1.26 ± 0.18
AT1G57630	0.23 ± 0.03	0.66 ± 0.08	0.64 ± 0.06	1.98 ± 0.25
UPOX	2.31 ± 0.31	2.40 ± 0.16	1.65 ± 0.08	2.68 ± 0.25
AT2G43510	0.37 ± 0.04	0.85 ± 0.07	0.44 ± 0.08	1.89 ± 0.28
ERF1	0.62 ± 0.10	2.35 ± 0.21	1.11 ± 0.23	2.89 ± 0.28
DNA DAMAGE AND REPAIR				
SOG1	1.05 ± 0.04	0.98 ± 0.05	0.98 ± 0.03	0.56 ± 0.01
PARP2	1.94 ± 0.06	1.50 ± 0.04	1.97 ± 0.21	1.14 ± 0.08
BRCA1	1.23 ± 0.09	0.81 ± 0.08	1.03 ± 0.05	0.43 ± 0.04
CELL CYCLE – CDK INHIBITORS				
SMR4	1.57 ± 0.08	1.51 ± 0.07	1.20 ± 0.08	0.87 ± 0.08
SMR5	0.96 ± 0.15	0.65 ± 0.03	1.22 ± 0.06	0.57 ± 0.13
SMR7	0.93 ± 0.01	0.85 ± 0.05	1.81 ± 0.08	0.64 ± 0.05

Especially in the category of oxidative stress and signaling, there was a changed expression of the genes after Cd exposure for 72 h. Almost all genes were downregulated after exposure to 20 µM Cd. One gene, *UPOX* was upregulated in both the 20 and 50 µM Cd-exposed condition. In the two other categories, *PARP2* as marker gene for DNA damage and repair and *SMR4*, a CDK inhibitor were upregulated with exposure to respectively 20 and 50 µM Cd (Table 9).

After 7 days of Cd exposure, a similar pattern is observed in genes of all three categories. Exposure to 20 µM Cd caused a downregulation of one oxidative stress marker gene, whereas with exposure to 50 µM Cd there are already more genes with a changed expression as compared to the control condition (Table 9).

[3] *TIP41*: tonoplast intrinsic protein 41-like; *YSL8*: yellow-leaf-specific gene 8; RHIP1: RGS1-HXK1 interacting protein 1; *UPOX*: Upregulated By Oxidative Stress; *ERF1*: Ethylene Response Factor 1; *SOG1*: Suppressor of Gamma Radiation 1; *PARP2*: Poly(ADP-Ribose) Polymerase 2; *BRCA1*: Breast Cancer Susceptibility 1; *SMR*: SIAMESE-RELATED.

3.2.3. Use of the 96-well system to identify cadmium-sensitive mutants

The ultimate goal of this part of the project was to develop a screening system to identify mutants with an increased Cd sensitivity. Therefore, Cd-induced effects were compared between WT plants and the GSH-deficient *cad2-1* mutant, known for its increased Cd-sensitivity, after 7 days of exposure.

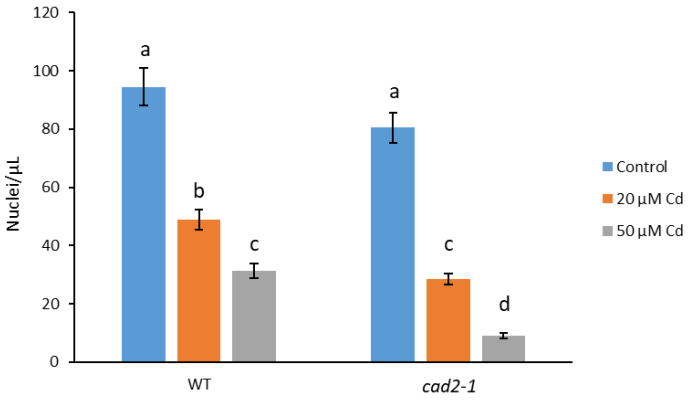

Figure 18. Number of nuclei per µL in flow cytometry extracts of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 7 days. Data represent the mean ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

The number of nuclei per µL measured using flow cytometry showed a decreasing trend with increasing Cd concentrations in both genotypes, but this effect was more pronounced in the *cad2-1* mutant (Fig. 18).

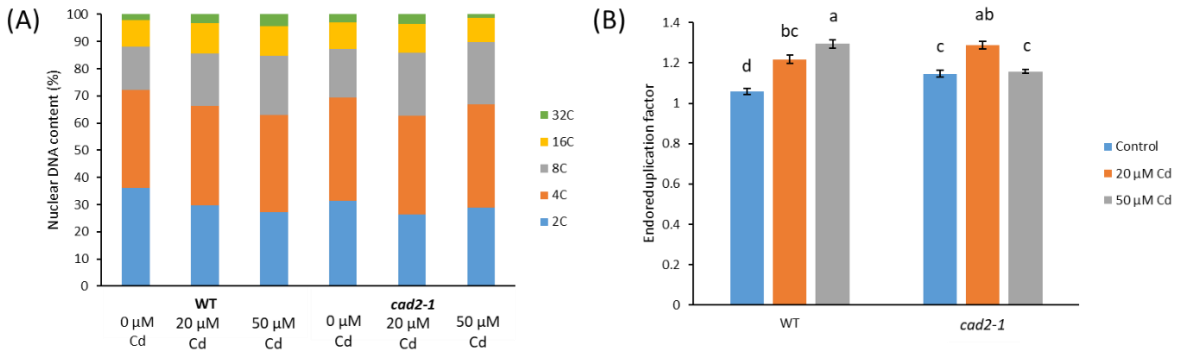

Figure 19. Nuclear DNA content (A) and endoreduplication factor (B) of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 8 days. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

In WT seedlings, the percentage of nuclei with a 2C DNA content decreased, whereas that of nuclei with higher ploidy levels (8C, 16C and 32C) decreased upon Cd exposure (both 20 and 50 µM). The ploidy levels in the *cad2-1* mutant decreased from 0 to 20 µM Cd and from 20 to 50 µM Cd, they increased again. (Fig. 19A)(Supplementary Fig. 6).

As a result, the EF in WT plants significantly increased with an increasing Cd concentration. In contrast, the EF in the *cad2-1* mutant was increased by exposure to 20 µM Cd, but remained unaltered after exposure to 50 µM Cd (Fig. 19B).

In addition, expression levels of genes related to oxidative stress and signaling, DNA damage and repair and cell cycle regulation were analyzed in WT and *cad2-1 A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 7 days (Table 10).

Table 10. Gene expression levels of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 7 days. Data represent the average ± S.E. of 5 biological independent replicates relative to the control of the same genotype (Supplementary table 4). Asterisks (*) indicate a significantly different Cd-induced fold change between both genotypes. Green and red colors indicate significant Cd-induced upregulations and downregulations, respectively ($p < 0.05$)(2-way ANOVA). Data were normalized against the expression of *AT5G25760 (UBC21), AT4G34270 (TIP41) and AT5G08290 (YSL8)*[4].

	WT		*cad2-1*	
	20 µM Cd	50 µM Cd	20 µM Cd	50 µM Cd
OXIDATIVE STRESS AND SIGNALING				
AT1G05340	2.97 ± 0.12	8.55 ± 0.24	1.06 ± 0.08*	2.63 ± 0.18*
AT119020	3.15 ± 0.33	8.88 ± 0.50	0.93 ± 0.06*	1.34 ± 0.10*
AT1G57630	7.74 ± 0.67	43.88 ± 4.01	0.84 ± 0.14*	1.22 ± 0.09*
UPOX	1.39 ± 0.08	6.10 ± 0.25	1.90 ± 0.14	2.01 ± 0.05
AT2G43510	2.84 ± 0.23	15.59 ± 1.28	4.57 ± 0.33	6.24 ± 0.40
AOX1a	1.81 ± 0.13	3.63 ± 0.13	1.07 ± 0.10*	2.50 ± 0.18
AOX1d	14.97 ± 2.11	579.73 ± 30.66	6.08 ± 0.57	17.22 ± 1.57
ERF1	5.59 ± 0.87	63.82 ± 6.11	5.66 ± 0.58	7.30 ± 0.65
RBOHC	0.68 ± 0.14	0.59 ± 0.04	0.76 ± 0.02	0.66 ± 0.05
DNA DAMAGE AND REPAIR				
SOG1	1.08 ± 0.12	0.84 ± 0.02	0.74 ± 0.03*	0.76 ± 0.01
PARP2	1.21 ± 0.13	1.13 ± 0.12	0.93 ± 0.06	0.47 ± 0.03*
BRCA1	0.78 ± 0.09	0.71 ± 0.06	0.82 ± 0.08	0.51 ± 0.05
CELL CYCLE – CDK INHIBITORS				
SMR4	0.96 ± 0.03	0.84 ± 0.06	0.90 ± 0.03	0.70 ± 0.06
SMR5	1.14 ± 0.07	1.02 ± 0.02	0.71 ± 0.03*	0.97 ± 0.06
SMR7	0.85 ± 0.05	0.54 ± 0.03	0.62 ± 0.10	0.39 ± 0.03

Genes related to oxidative stress and signalling were significantly upregulated by Cd exposure in both WT and mutant plants. However, this response was generally less pronounced in the mutant as compared to the WT. The expression of genes related to DNA damage and repair was not affected by Cd exposure in WT plants, but was significantly decreased in the *cad2-1* mutant upon exposure to 50 µM Cd. In general, the expression of the *SMR* genes was decreased upon Cd exposure in both genotypes. This response was more pronounced in the GSH-deficient *cad2-1* mutant (Table 10).

[4] *UBC21*: ubiquitin conjugating enzyme 21; *TIP41*: tonoplast intrinsic protein 41-like; *YSL8*: yellow-leaf-specific gene 8; *UPOX*: Upregulated By Oxidative Stress; *AOX1*: Alternative Oxidase 1; *ERF1*: Ethylene Response Factor 1; *RBOHC*: Respiratory Burst Oxidase Homologue C; *SOG1*: Suppressor of Gamma Radiation 1; *PARP2*: Poly(ADP-Ribose) Polymerase 2; *BRCA1*: Breast Cancer Susceptibility 1; *SMR*: SIAMESE-RELATED.

4. DISCUSSION

This study aimed to investigate the role of GSH in Cd-induced effects on cell cycle regulation and the DNA damage response in leaves of *A. thaliana*. To this end, the extent of cell division and endoreduplication and the transcription of genes related to oxidative stress, DNA damage and the cell cycle were compared between leaves of wild-type *A. thaliana* plants and the GSH-deficient *cad2-1* mutant exposed to 5 µM Cd. Furthermore, a screening method for the identification of Cd-sensitive *A. thaliana* mutants in 96-well plates was optimized.

4.1. EFFECTS OF CADMIUM ON CELL CYCLE REGULATION, OXIDATIVE STRESS AND DNA DAMAGE IN *A. THALIANA* LEAVES ARE ALTERED IN A GSH-DEFICIENT MUTANT

In the first part is this study, the effects of Cd exposure on cell cycle regulation, oxidative stress and DNA damage and repair were determined in WT and GSH-deficient *cad2-1* mutant *A. thaliana* plants grown in hydroponics to estimate the role of GSH in these processes.

4.1.1. Glutathione influences the effects of cadmium exposure on cell cycle regulation

Glutathione is known to be involved in both cell cycle regulation and plant responses to Cd. Therefore, this project aimed to investigate whether GSH is involved in cell cycle regulation in response to Cd exposure in *A. thaliana* leaves. Cadmium exposure is known to disturb plant growth and development (3), but the Cd-induced effects on the cell cycle are not yet fully described. Cui et al. (2017) reported an arrest of the cell cycle mediated by Cd-induced DNA damage in *Arabidopsi*s root tips (38).

4.1.1.1. Glutathione deficiency affects growth responses after cadmium exposure

The rosette and root fresh weight, rosette diameter and the number of leaves of WT and *cad2-1* mutant plants exposed to 5 µM Cd for 8 days were determined to estimate the extent of Cd toxicity. Both leaf and root fresh weight were significantly lower in Cd-exposed as compared to control plants of both genotypes. This effect is due to the fact that exposure inhibits plants growth. However, the effect in the GSH-deficient *cad2-1* mutant was more pronounced as compared to that in WT plants. This observation is due to the fact that this mutant has a higher Cd sensitivity as compared to the WT (32). Furthermore, the rosette diameter was also determined to assess influences of Cd exposure on plant growth. Under control conditions, the rosette diameters of WT and mutant plants did not differ.

Upon Cd exposure, the rosette diameter of both genotypes was negatively affected. This effect was more pronounced in the GSH-deficient mutant, again confirming the higher Cd sensitivity of this mutant (32). Under control conditions, the GSH-deficient mutant *cad2-1* developed significantly more leaves as compared to the WT. This is an indication that the lower GSH-levels have an influence on the growth and development of plants in the absence of external stress conditions like Cd. This is in agreement with the results of Ogawa et al. (2001) who reported a higher number of rosette leaves in the *cad2-1* mutant as compared to the WT (39). Together, these data suggest that the leaves of the *cad2-1* mutant emerged in an earlier stage as compared to those of the WT. The leaf number of Cd-exposed *cad2-1* mutant plants was significantly lower as compared to that of their control counterparts, whereas the leaf number of the WT did not differ significantly. This response could also be explained by the increased Cd sensitivity of the mutant (32). In addition, the percentage of root and leaf dry weight of control and Cd-exposed WT and *cad2-1* mutant plants was also determined. This parameter was higher in Cd-exposed as compared to control plants of both genotypes. This increased percentage of dry weight after Cd exposure could be explained in two ways. Firstly, it could be due to a larger amount of plant dry matter. Secondly, it could also be caused by water loss, as Cd is known to affect the water household in plants (40). The raise in percentage dry weight was even more pronounced in the *cad2-1* mutant, highlighting its increased Cd sensitivity (32). Additionally, the surface area of leaves 4, 6, 8 and 10 was determined. Leaf 4 and 6 are older leaves and they were already present at the start of the Cd exposure. Leaf 8, on the other hand, had just emerged when the Cd exposure started. Furthermore, leaf 10 still had to emerge when the Cd exposure started. The surface area of the older leaves analysed (leaves 4 and 6) was significant lower after Cd exposure. These leaves were already present at the start of the exposure and are therefore exposed longer as compared to the other leaves, leading to a more extensive effect on the leaf area. This effect was even more pronounced in the *cad2-1* mutant due to its increased Cd sensitivity. Also the fact that the leaves of the mutant emerged earlier an could therefore be longer exposed as compared to the WT could be a possible explanation. The surface area of the younger leaves analysed (leaves 8 and 10) was not affected by Cd exposure in WT plants. The surface area of leaf 8 and 10 was negatively affected in the GSH-deficient mutant explaining its difficulty to cope with Cd-exposure.

4.1.1.2. Cadmium concentrations are altered in a GSH-deficient mutant

Because this study aimed to assess the effects of Cd exposure, root and leaf Cd concentrations were investigated using ICP-OES and compared between WT plants and the GSH-deficient *cad2-1* mutant exposed to 5 µM Cd for 7 days.

The obtained results show that Cd concentrations in roots did not differ between both genotypes. On the other hand, the Cd concentrations in leaves were lower in the GSH-deficient *cad2-1* mutant, as compared to the WT. This could be explained by a lower Cd translocation from roots to shoots in the mutant. This is in agreement with a study of Sobrino-Plata et al. (2014), who reported similar results about the lower Cd translocation in GSH-deficient mutants (41).

4.1.1.3. Glutathione deficiency affects element concentrations after cadmium exposure

Cadmium toxicity can be displayed by a disturbance of the homeostasis of several essential elements (2). Therefore, element concentrations in both roots and leaves of WT and *cad2-1* mutant plants grown under control and Cd-exposed conditions were measured using ICP-OES. Preliminary data have indicated a disturbance of the Cu homeostasis in *A. thaliana* plants upon Cd exposure (42)(43). An increase in Cu concentrations was observed after Cd exposure in roots of both genotypes, whereas Cu concentration in leaves remained unchanged. This could be explained by the fact that roots stimulate Cu uptake upon Cd exposure. In contrast, leaves show a Cu deficiency upon Cd exposure. This effect is even more pronounced in the mutant, possibly as a result of its increased Cd sensitivity. These results are in agreement with those of Gielen et al. (2016), who reported Cd induced Cu deficiency in leaves and a significant increase of the Cu concentration in roots of *A. thaliana* plants (WT + *cad1-3* mutant) exposed to 5 µM Cd for 24 h and 72 h (43). Furthermore, the leaves of Cd-exposed *cad2-1* mutant plants had decreased K levels as compared to their control counterparts. Root K concentrations significantly decreased in both gentoypes. This could be attributed to stress-induced membrane leakage. The effect was stronger in the mutant, again pointing towards its increased Cd sensitivity. This decrease of K levels in roots of both genotypes was also observed by Jozefczak et al. (2015) (44). Additionally, cadmium exposure caused an increase and a decrease in leaf S concentrations in WT and mutant plants, respectively. In contrast, root S concentrations remained unchanged. In general, plants increase S assimilation in leaves as a defense mechanism under Cd exposure conditions. Sulfur is an important compound of GSH and an substrate for phytochelatins synthesis which are both involved in responses to stress conditions like Cd exposure (45). The obtained results suggest that this mechanism is activated in the WT, whereas it is absent in *cad2-1* mutant plants due to their increased Cd sensitivity. The concentration in roots probably remained unchanged, as S assimilation predominantly takes place in chloroplasts, present in leaves (44). In addition, leaf Zn levels decreased upon Cd in the *cad2-1* mutant, but not in WT plants. This response is likely due to the fact that Cd is taken up through Zn transporters, thereby reducing Zn uptake. In contrast to WT plants, the GSH-deficient *cad2-1* mutant might not be able to counteract this competition between Cd and Zn as a result of its increased Cd sensitivity, ultimately resulting in a decreased Zn concentration.

4.1.1.4. Glutathione levels influence cadmium-induced effects on the cell cycle

Glutathione is known to be involved in both cell cycle regulation and in the responses of *A. thaliana* to Cd stress. To unravel the combined role of GSH in Cd-induced effects on cell division and endoreduplication, WT and GSH-deficient *cad2-1* mutant *A. thaliana* plants were exposed to 5 µM Cd for 7 days, starting from day 19 after sowing.

The nuclear DNA content in flow cytometry extracts of leaves 4, 6, 8 and 10 were determined. The older leaves (4 and 6) were already present at the start of Cd exposure. Leaf 8, however, had just emerged when the Cd exposure started, whereas leaf 10 had not yet emerged at the moment when Cd exposure was initiated. The concentration of nuclei in leaf extracts measured via flow cytometric analysis was used as a proxy to determine the effects of Cd exposure on the extent of cell division. In WT plants, this parameter showed a decreasing trend which was not statistical significant after Cd exposure in leaf 4, the oldest leaf analysed. In leaf 6, a slightly younger leaf, a small decrease after Cd exposure was observed, whereas in leaf 8, this decrease was more pronounced. The number of nuclei per µL measured in extracts of leaf 10 was not affected by Cd exposure in WT plants. Preliminary data show that the effects of Cd on the cell cycle increase throughout time. It is possible that leaf 10 is still too young and that is was not yet possible for Cd to have a significant effect on the cell cycle. In the *cad2-1* mutant, on the other hand, the concentration of nuclei in flow cytometry extracts was decreased by Cd exposure in all leaf positions analysed. This decrease was most pronounced in younger leaves. In these younger leaves (8 and 10), the concentration of nuclei was higher under control conditions in the *cad2-1* mutant as compared to the WT. This can be linked back to the fact that the mutant has a higher amount of leaves compared to the WT and therefore the conclusion can be made that the leaves of the mutant emerge earlier as those of the WT. Therefore the leaves of the mutant are slightly older and the cells have already experienced more cell divisions. Taken together, these results about the nuclear DNA content of WT and mutant plants indicate that GSH levels influence the effect of Cd exposure on cell division. Upon Cd exposure, the EF of leaves 4, 6 and 8 remained unchanged in the WT and decreased in the mutant. In contrast, the EF of leaf 10 increased in the WT and also decreased in the mutant after Cd exposure. The decrease of the extent of endoreduplication in the mutant can be explained by the fact that the GSH-deficient mutant is unable to cope with the external stress factor of Cd exposure. The EF of the WT of leaf 4 and 6 was probably not affected by Cd exposure because these leaves were approaching maturity at the start of Cd exposure and cells might no longer be undergoing endoreduplication. In younger leaves of the WT (8 and 10), on the other hand, the EF increased as this is a defense mechanism for plants against stress conditions like Cd exposure. They use the process of endoreduplication as an adaptive response to handle the effects of Cd-induced stress.

Plasticity in their nuclear ploidy levels can provide a mechanism to accommodate to their changing environment (46). In the *cad2-1* mutant, this defense mechanism does not seem to be activated, possibly due to its increased Cd sensitivity. These cell cycle related data can be linked back to the data on the surface area of the different leaves. If the leaves are having less cells and therefore a lower extent of endoreduplication which can be correlated to the cell size, the leaves are expected to be smaller. In the older leaves (4 and 6), the leaf surface of both WT and mutant decreased upon Cd exposure whereas the EF also decreased in both cases. For the younger leaves, this correlation can only be made for the mutant, where both the EF and the leaf surface were negatively affected by Cd.

In conclusion and based on the differences in responses between WT and mutant, these data confirm the hypothesis that GSH levels influence the effects of Cd exposure on cell cycle regulation.

4.1.2. Glutathione influences the cadmium-induced DNA damage response

The cell cycle, as assessed in the first part of the project, is known to be affected by DNA damage and oxidative stress. Interestingly, these processes can also be influenced by GSH, which plays an important role in antioxidative defence. Therefore, oxidative stress and the DNA damage response are investigated in a second part of the project. Other studies demonstrated that Cd can induce DNA damage as observed by Silveira et al. (2017), who reported DNA damage in meristematic cells of *Allium cepa* and *L. sativa* root tips upon exposure to 25 µM Cd (47). These finding are in agreement with those of Gichner et al. (2004), who stated Cd can induce DNA damage in tobacco roots (48). Cadmium can induce oxidative stress in *A. thaliana* (7). According to Dixit et al. (2000), the enhanced level of lipid peroxidation and the increased levels of H_2O_2 in the tissues demonstrate that Cd is capable of inducing oxidative stress in pea plants (49).

4.1.2.1. Short-term cadmium exposure does not affect leaf growth

The second part of this project aimed to investigate the involvement of GSH in the Cd-induced DNA damage response. To this end, WT and *cad2-1* mutant *A. thaliana* seedlings were grown in hydroponics and exposed to 5 µM Cd for 24 h of 72 h, starting from day 19 after sowing. During harvest, leaf fresh weight was determined. The results show that this parameter is not affected by short-term exposure in either of the genotypes studied. However, a decreasing trend in leaf fresh weight is observed after 72 h of Cd exposure. It is likely that the time frame of exposure is too short to cause significant effects on leaf fresh weight. These data can be supplemented with those of Jozefczak et al. (2015), who observed a significant reduction of leaf fresh weight of the mutant after 72 h of exposure to 5 µM Cd. After 24 h of exposure they also did not observed a significant change of the leaf fresh weight of both genotypes (44).

4.1.2.2. Glutathione deficiency affects the cadmium-induced DNA damage response

An analysis of expression levels of genes related to oxidative stress, DNA damage and repair and cell cycle regulation was performed in WT and *cad2-1* mutant *A. thaliana* plants exposed to 5 µM Cd for 24 h or 72 h in order to determine the role of GSH in the Cd-induced DNA damage response. The expression of genes related to oxidative stress was significantly upregulated after 24 and 72 h of Cd exposure in the WT and this induction was even more pronounced in the mutant. The oxidative stress hallmark genes (*AT1G05340*, *AT119020*, *AT1G57630*, *UPOX* and *AT2G43510*) are genes that are characterized by a strong upregulation upon oxidative stress (50). These genes were upregulated in both genotypes after 24 h Cd exposure. After 72 h, there was still an upregulation in both genotypes and the induction was even stronger in the mutant whereas the induction of the WT was smaller as compared to 24 h Cd exposure. This is an indication that the mutant still experiences oxidative stress after 72 h of Cd exposure whereas it diminishes for the WT. Jozefczak et al. reported similar results concerning the upregulation of oxidative stress hallmark genes in both the WT and the mutant (44). An upregulation of the genes *AOX1a* and *AOX1d*, alternative oxidases involved in oxidative defense, was displayed in both genotypes after 24 and 72 h of Cd exposure. Also the expression of ethylene responsive factor 1 (*ERF1*) was significantly upregulated in both genotypes after 24 h. After 72 h, this induction falls away in the WT, whereas it was still present in the mutant. Because *ERF1* is involved in oxidative signaling, this is a second indication that the GSH-deficient mutant was still suffering from oxidative stress after 72 h whereas this was not the case for the WT. The induction of DNA repair and cell cycle marker genes was also significantly upregulated after Cd exposure in the WT. In contrast, this response was less pronounced or even absent in the mutant, both after 24 h and 72 h of exposure. The WT showed an upregulation of *SMR4* and *SMR5* after 24 h of Cd exposure. After 72 h of Cd exposure, *SMR4*, *SMR5* and *SMR7* were upregulated. In the mutant, this induction was less pronounced or absent. Also the DNA damage marker genes, *PARP2* and *BRCA1* were upregulated in the WT exposed to Cd whereas this Cd-induced upregulation was not observed in the mutant (51). Yi et al. (2014) reported that an upregulation of *SMR* genes can be caused by ROS-induced DNA damage (52). As both the induction of SMRs and DNA damage marker genes falls away in the GSH-deficient mutant, this implies that GSH has an influence on the DNA damage response regulated via the SMRs. The absence of a Cd-induced DNA damage response in the mutant can have two possible explanations. It is possible that there is less DNA damage in the mutant or that the mutant does not respond to the DNA damage. The DNA damage in this study was measured indirectly via the transcription of DNA damage related genes. In the future, the extent of DNA damage can be directly measured via the comet assay. The findings taken together and the differences in responses between WT and mutant implicate that GSH is indeed involved in the Cd-induced DNA damage response.

4.1.2.3. Short-term cadmium exposure influences leaf growth of other GSH-deficient mutants

To confirm the involvement of GSH in the Cd-induced DNA damage response and to make sure that the obtained results are due to the decreased GSH levels of the mutant, a similar experiment was performed in three different GSH-deficient mutants: *cad2*-1, *pad2-1* and *rax1-1*. These mutants (*pad2-1*, *rax1-1*) had a similar mutation in the GSH1 biosynthesis gene as the *cad2-1* mutant (33)(34).

Plants of all genotypes were grown in hydroponics and exposed to 5 µM Cd for 72 h. This time point was chosen as DNA damage and repair and cell cycle related genes reacted strongly in the WT after 72 h. The rosette fresh weight of the Cd-exposed plants of all three genotypes was lower as compared to that of the control plants. This response is highly similar to that previously observed in the *cad2-1* mutant, where the fresh rosette weight showed a decreasing trend after 72 h, confirming that the effects seen in the *cad2-1* mutant are due to its decreased GSH levels. Similar results were observed by Jozefczak et al. (2015), who reported a significant reduction of root and leaf fresh weight after 72 h of Cd exposure in all mutants, except the *rax1-1* mutant, which was not affected (44).

4.1.2.4. Glutathione levels affect the cadmium-induced DNA damage response in other GSH-deficient mutants

Furthermore, a few of the genes measured in the *cad2-1* experiment related to DNA damage and repair and cell cycle regulation were also measured in the other GSH-deficient mutants. Results show that all genes analysed were significantly upregulated in the WT. In the three GSH-deficient mutants, the inductions were less pronounced or even absent. Similar responses were observed when analysing the expression of *SMR4, SMR5* and *SMR7.*

Taken together, these results support the hypothesis that GSH is involved in the Cd-induced DNA damage response. This could be linked back to the data of the part concerning the cell cycle regulation. The fact that GSH is involved in the Cd-induced DNA damage response can possibly lead to different effects of Cd on the cell cycle in the GSH-deficient mutant. Further research will be necessary to explore in what way GSH is involved in this process.

4.2. OPTIMIZATION OF A 96-WELL SCREENING METHOD TO IDENTIFY CADMIUM-SENSITIVE *A. THALIANA* MUTANTS

The aim of the second main part of this study was to optimize a 96-well screening method to identify Cd-sensitive *A. thaliana* mutants.

4.2.1. The use of a 1/4 MS growth medium yields an optimal germination of the *A. thaliana* seedlings

In the first experiment of this part of the project, the MS concentration yielding an optimal germination of the plants was determined. To this end, WT plants were grown in 96-well plates containing 1/2, 1/4 or 1/8 MS medium and the percentage of germination was calculated at three time points after sowing. Although there were no significant differences in plant germination between the three MS concentrations, the 1/4 MS concentration was selected to use in further experiments, as this concentration was already used for root growth analysis on vertical agar plates. The concentration should be sufficiently high to ensure plants take up sufficient nutrients, but an excessively high MS concentrations should be avoided, as high nutrient levels will decrease the extent of Cd uptake, thereby preventing the detection of Cd-induced effects on the parameters studied.

4.2.2. Cell cycle-related parameters and gene expression levels related to oxidative stress, DNA damage and cell cycle can be used as cadmium-sensitive parameters

The second experiment aimed to identify Cd-sensitive parameters to be used in the 96-well system. To this end, WT plants were grown in 96-well plates and exposed to 0, 20 and 50 µM Cd for 72 h and 7 days. Nuclear ploidy levels and the concentration of nuclei determined via flow cytometric analysis and expression levels of genes related to oxidative stress, DNA damage and the cell cycle were assessed, as these parameters were shown to be affected by Cd exposure in hydroponics.

The number of nuclei per µL measured via flow cytometric analysis was used as a proxy to determine the effects of Cd on cell division after 7 days of exposure. Results show that only the highest Cd concentration used caused a significant decrease of this parameter, although a decreasing trend was also observed after exposure to 20 µM Cd. In contrast, the EF showed an increasing trend after exposure to 20 µM Cd and was significantly increased by exposure to 50 µM Cd. It seems that the plants grown in the 96-well system compensate for their decreased extent of cell division with an increased extent of endoreduplication.

The results are in agreement with those of Adachi et al. (2011), who reported that endoreduplication is induced in *Arabidopsis* upon double-strand DNA breaks, another stress factor (53). As described by Scholes et Al. (2015), this higher level of endoreduplication can be seen as a defense mechanism to cope with different stress factors like Cd exposure and DNA breaks (46).

Furthermore, expression levels of genes related to oxidative stress, DNA damage and cell cycle regulation were measured in WT plants exposed to 0, 20 and 50 µM Cd for 72 h or 7 days to determine which genes could be used as biomarkers for Cd-induced stress. Cadmium-induced effects on the expression of these genes showed a similar pattern after both exposure durations. Most of the genes related to oxidative stress were downregulated after exposure to both 20 and 50 µM Cd, whereas *UPOX* expression was induced by exposure to both Cd concentrations. In other categories, the pattern was the same for 20 and 50 µM Cd. The oxidative stress hallmark genes were strongly induced upon both Cd concentrations after both exposure durations meaning that both Cd concentrations were sufficient high to cause oxidative stress. Also some DNA damage marker genes like *PARP2* and *BRCA1* and the *SMR* genes were induced upon Cd exposure. Therefore these genes can be used as biomarkers for Cd-induced stress.

Although effects on the extent of cell division and endoreduplication were only significant after exposure to 50 µM Cd, both 20 and 50 µM Cd were used in further experiments. There was already a clear trend visible with exposure to 20 µM Cd and this trend will possibly be even more distinct in mutants with an increased Cd sensitivity. Because this 96-well system needs to be used as a fast and easy screening method, harvesting was limited to only one time point, namely after 7 days of exposure, in further experiments. It is known that the effect of Cd on the cell cycle accumulate in time, so it is expected that these effects will be more pronounced after a longer exposure. Results also display that the expression of genes measured on both time points show a similar pattern, so continuing with one time point for harvesting will be suitable. A last argument is that the plants weight more after a longer exposure because they are further in their development, because of that less plant material will be necessary per sample to perform the analyses.

4.2.3. The 96-well screening method can be used to identify cadmium-sensitive *Arabidopsis thaliana* mutants

The ultimate goal of this part of the project was to develop a screening method to identify cadmium-sensitive *Arabidopsis thaliana* mutants. To this end, WT plants and the GSH-deficient *cad2-1* mutant, known for its increased Cd sensitivity, were grown in 96-well plates and exposed to 0, 20 and 50 µM Cd. After 7 days of exposure, Cd-induced effects were compared between both genotypes.

As cell cycle related parameters and gene expression levels related to oxidative stress, DNA damage and cell cycle were identified as Cd-sensitive parameters in the second experiment, these parameters were measured again in a similar setup including a Cd-sensitive mutant.

As seen in the second experiment, the nuclear DNA content was decreased by Cd exposure in the WT. The negative influence of Cd-exposure on the number of nuclei in flow cytometry extracts is even more pronounced in the *cad2-1* mutant, highlighting its increased Cd sensitivity. Furthermore, the EF increased significantly in the WT with increasing Cd concentrations, which was again in agreement with the results obtained in the second experiment of this part of the project. In the mutant however, the EF was also increased upon exposure to 20 µM Cd, but was decreased after exposure to 50 µM Cd. It seems that the Cd-sensitive mutant has too much difficulties coping with the higher Cd concentration. The connection can be made to a similar effect seen in the younger leaves in hydroculture where the EF also increased. Plants increase their extent of endoreduplication as a defense mechanism to adapt themselves to stress conditions like Cd exposure (46). The *cad2-1* mutant does not seem to induce this defense mechanism because of its increased Cd sensitivity.

Additionally, expression levels of genes related to oxidative stress, DNA damage and cell cycle regulation were analyzed in WT and *cad2-1* mutant *A. thaliana* plants grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 7 days. Genes related to oxidative stress showed a significant upregulation after Cd exposure in both genotypes, but the effect was less pronounced in the mutant as compared to the WT. This is in contrast with the results of the hydroculture, where the induction of the oxidative stress related genes is more pronounced in the mutant as compared to the WT. The expression of DNA repair genes was not affected by Cd exposure in the WT, but it significantly decreased in the mutant after exposure to 50 µM Cd. The expression of genes related to cell cycle regulation was decreased by Cd exposure in both genotypes and the response was more pronounced in the mutant. These data are in agreement with the results from the hydroculture experiment. In general, the induction of all of these genes was less pronounced in the mutant as compared to the WT in this 96-wel system. It is possible that the responses in the mutant occur at an earlier time point as the WT.

The major part of the Cd-sensitive parameters as identified in the second experiment reacted differently upon Cd exposure in the Cd-sensitive *cad2-1* mutant cultured via the 96-well system. Therefore the conclusion can be made that this 96-well screening system can possibly be used to identify Cd-sensitive mutants. However, further research will be necessary with additional Cd-sensitive mutants.

5. CONCLUSION AND SYNTHESIS

In a first part of this study, the effects of Cd exposure on cell cycle regulation, oxidative stress and DNA damage were determined in *A. thaliana* leaves. To unravel the role of GSH in these processes, WT plants and a GSH-deficient *cad2-1 A. thaliana* mutant were grown in hydroponics and exposed to 5 µM Cd. Growth responses showed that the *cad2-1* mutant was more sensitive to Cd as compared to the WT, emphasising the importance of GSH in the plant growth and development. Furthermore, also cell cycle-related parameters and gene expression levels related to oxidative stress, DNA damage and cell cycle regulation were differentially affected by Cd exposure in the mutant, indicating the involvement of GSH in the Cd-induced DNA damage response and cell cycle regulation. Other GSH-deficient mutants showed similar responses as compared to the *cad2-1* mutant, ensuring that the observed effects were due to decreased GSH levels. In future experiments, it would be interesting to further investigate the role of GSH in the DNA-damage response by for example determining the extent of DNA damage via the comet assay.

A second part of this study aimed to optimize a 96-well screening system to identify Cd-sensitive *Arabidopsis thaliana* mutants. Based on the data obtained throughout the different experiments, the conclusion can be made that this system can indeed be used as a fast and simple screening method to identify Cd-sensitive mutants. Cell cycle related parameters and the expression levels of genes related to oxidative stress, DNA damage and cell cycle regulation responded strongly upon Cd exposure and can therefore be used as Cd-sensitive parameters. Furthermore, the differences in Cd-induced responses between the WT and GSH-deficient *cad2-1* mutant determined via this 96-well system emphasize its ability to identify Cd-sensitive mutants. The system can be further tested via the use of other Cd-sensitive mutants like *cad2-1.* Further optimization can be needed concerning other stress factors, like for example other heavy metals, herbicides or nutrient deficiencies.

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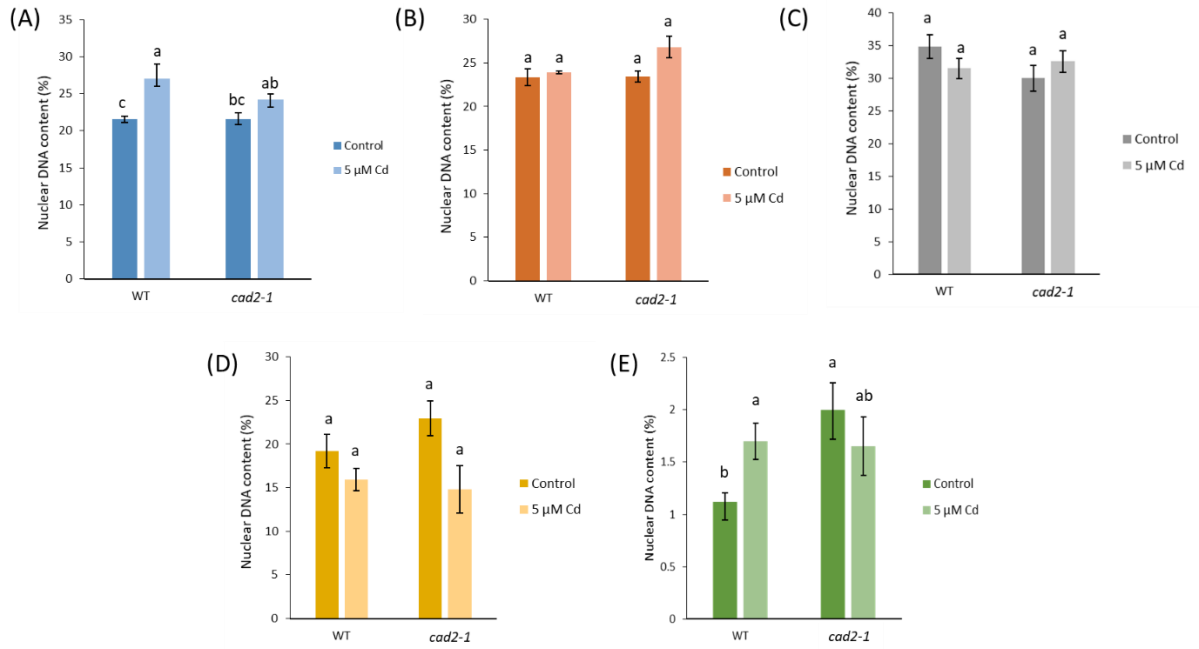
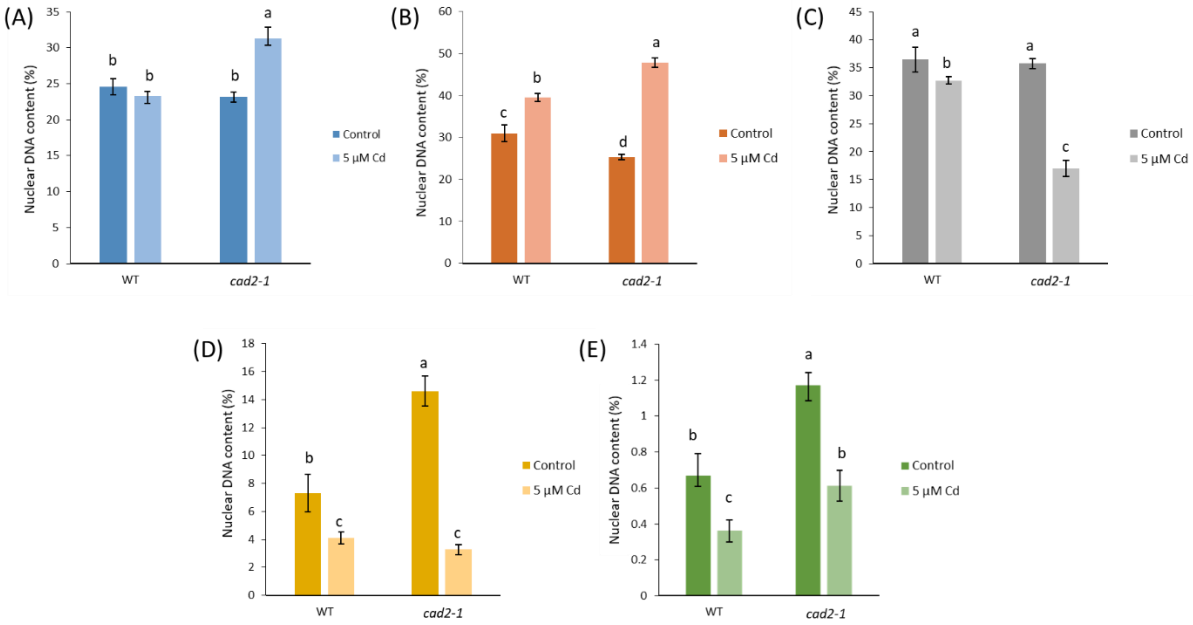
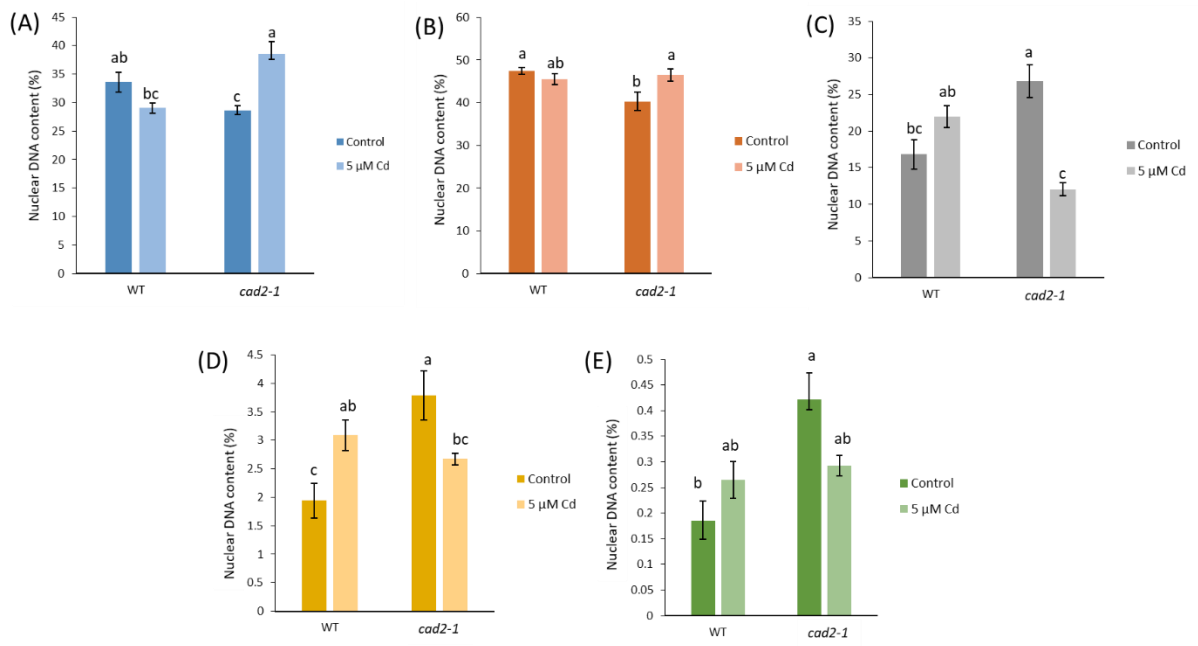
SUPPLEMENTARY DATA



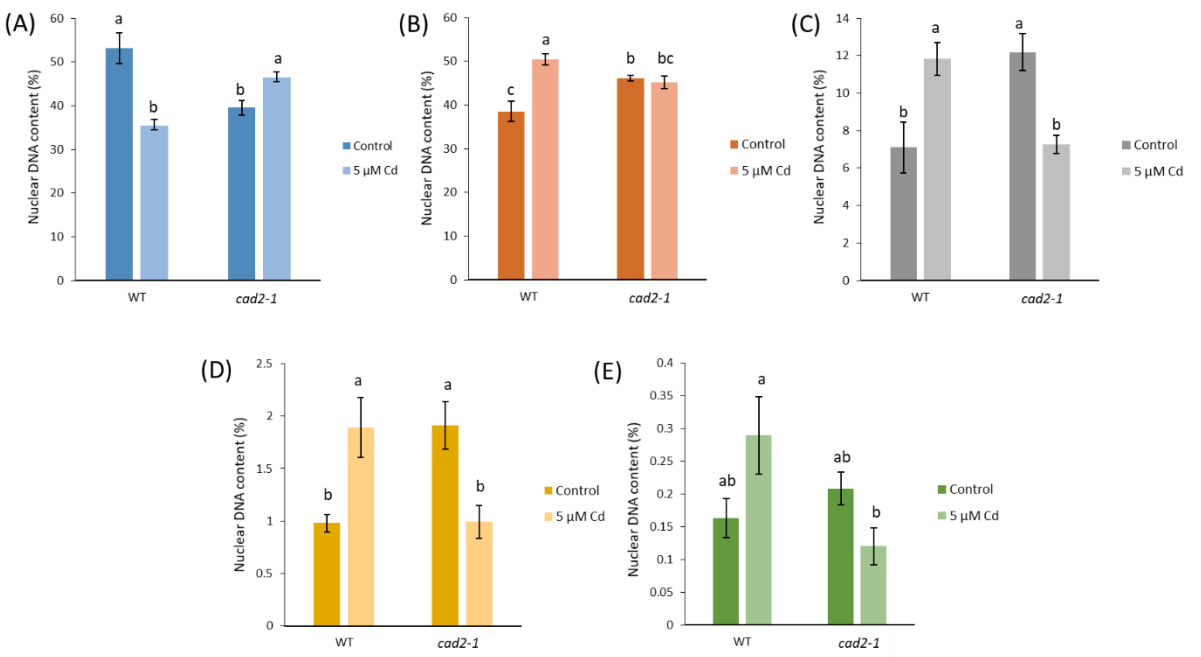
Supplementary Figure 1. Nuclear ploidy levels in leaf 4 of wild-type (WT) *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. (A) Percentage of 2C nuclei; (B) Percentage of 4C nuclei; (C) Percentage of 8C nuclei; (D) Percentage of 16C nuclei; (E) Percentage of 32C nuclei. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).



Supplementary figure 2. Nuclear ploidy levels in leaf 6 of wild-type (WT) *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. (A) Percentage of 2C nuclei; (B) Percentage of 4C nuclei; (C) Percentage of 8C nuclei; (D) Percentage of 16C nuclei; (E) Percentage of 32C nuclei. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).



Supplementary figure 3. Nuclear ploidy levels in leaf 8 of wild-type (WT) *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. (A) Percentage of 2C nuclei; (B) Percentage of 4C nuclei; (C) Percentage of 8C nuclei; (D) Percentage of 16C nuclei; (E) Percentage of 32C nuclei. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).



Supplementary figure 4. Nuclear ploidy levels in leaf 10 of wild-type (WT) *cad2-1 A. thaliana* seedlings exposed to 0 or 5 µM Cd for 8 days, starting from day 19 after sowing. (A) Percentage of 2C nuclei; (B) Percentage of 4C nuclei; (C) Percentage of 8C nuclei; (D) Percentage of 16C nuclei; (E) Percentage of 32C nuclei. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

Supplementary Table 1. Gene expression levels of wild-type (WT) and *cad2-1 A. thaliana* seedlings grown under control conditions. Data represent the average ± S.E. of 5 biological independent replicate, expressed relative to the WT. Green and red colors indicate a significantly higher and lower expression in the mutant as compared to the WT, respectively ($p < 0.05$)(1-way ANOVA). Data were normalized against the expression of *AT5G25760 (UBC21), AT2G28390 (MON1)* and *AT4G34270 (TIP41).*

UBC21: ubiquitin conjugating enzyme 21; *MON1*: monensin sensitivity 1; *TIP41*: tonoplast intrinsic protein 41-like.

	24 h	
	WT	*cad2-1*
OXIDATIVE STRESS AND SIGNALING		
AT1G05340	1.00 ± 0.13	1.07 ± 0.11
AT119020	1.00 ± 0.24	0.94 ± 0.10
AT1G57630	1.00 ± 0.21	0.76 ± 0.12
UPOX	1.00 ± 0.05	1.78 ± 0.04
AT2G43510	1.00 ± 0.12	1.11 ± 0.10
AOX1a	1.00 ± 0.05	1.03 ± 0.06
AOX1d	1.00 ± 0.34	0.71 ± 0.09
ERF1	1.00 ± 0.18	0.83 ± 0.14
RBOHC	1.00 ± 0.16	0.33 ± 0.09
DNA DAMAGE AND REPAIR		
SOG1	1.00 ± 0.02	1.01 ± 0.06
PARP2	1.00 ± 0.05	1.34 ± 0.04
BRCA1	1.00 ± 0.02	1.04 ± 0.04
XRCC1	1.00 ± 0.01	0.93 ± 0.02
CELL CYCLE – CDK INHIBITORS		
SMR4	1.00 ± 0.06	1.05 ± 0.09
SMR5	1.00 ± 0.04	1.13 ± 0.02
SMR7	1.00 ± 0.05	1.53 ± 0.11

Supplementary table 2. Gene expression levels of wild-type (WT) and *cad2-1 A. thaliana* seedlings grown under control conditions. Data represent the average ± S.E. of 5 biological independent replicate, expressed relative to the WT. Green and red colors indicate a significantly higher and lower expression in the mutant as compared to the WT, respectively ($p < 0.05$)(1-way ANOVA). Data were normalized against the expression of *AT5G25760 (UBC21), AT2G28390 (MON1)* and *AT4G34270 (TIP41).*

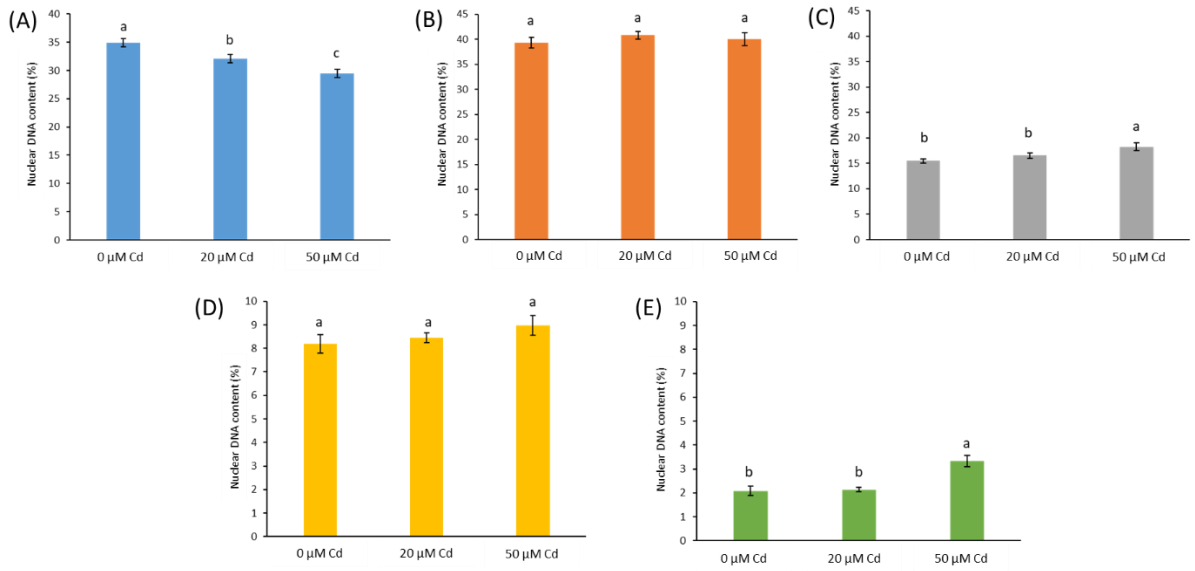
UBC21: ubiquitin conjugating enzyme 21; *MON1*: monensin sensitivity 1; *TIP41*: tonoplast intrinsic protein 41-like.

	72 h	
	WT	*cad2-1*
OXIDATIVE STRESS AND SIGNALING		
AT1G05340	1.00 ± 0.23	0.19 ± 0.02
AT119020	1.00 ± 0.85	0.13 ± 0.02
AT1G57630	1.00 ± 0.31	0.82 ± 0.18
UPOX	1.00 ± 0.07	1.78 ± 0.19
AT2G43510	1.00 ± 0.02	1.19 ± 0.19
AOX1a	1.00 ± 0.07	0.87 ± 0.01
AOX1d	1.00 ± 0.39	0.41 ± 0.16
ERF1	1.00 ± 0.44	0.86 ± 0.24
RBOHC	1.00 ± 0.30	2.93 ± 1.25
DNA DAMAGE AND REPAIR		
SOG1	1.00 ± 0.05	1.11 ± 0.05
PARP2	1.00 ± 0.01	1.31 ± 0.11
BRCA1	1.00 ± 0.04	1.01 ± 0.06
XRCC1	1.00 ± 0.04	0.97 ± 0.04
CELL CYCLE – CDK INHIBITORS		
SMR4	1.00 ± 0.12	1.11 ± 0.11
SMR5	1.00 ± 0.12	1.03 ± 0.04
SMR7	1.00 ± 0.06	1.88 ± 0.21

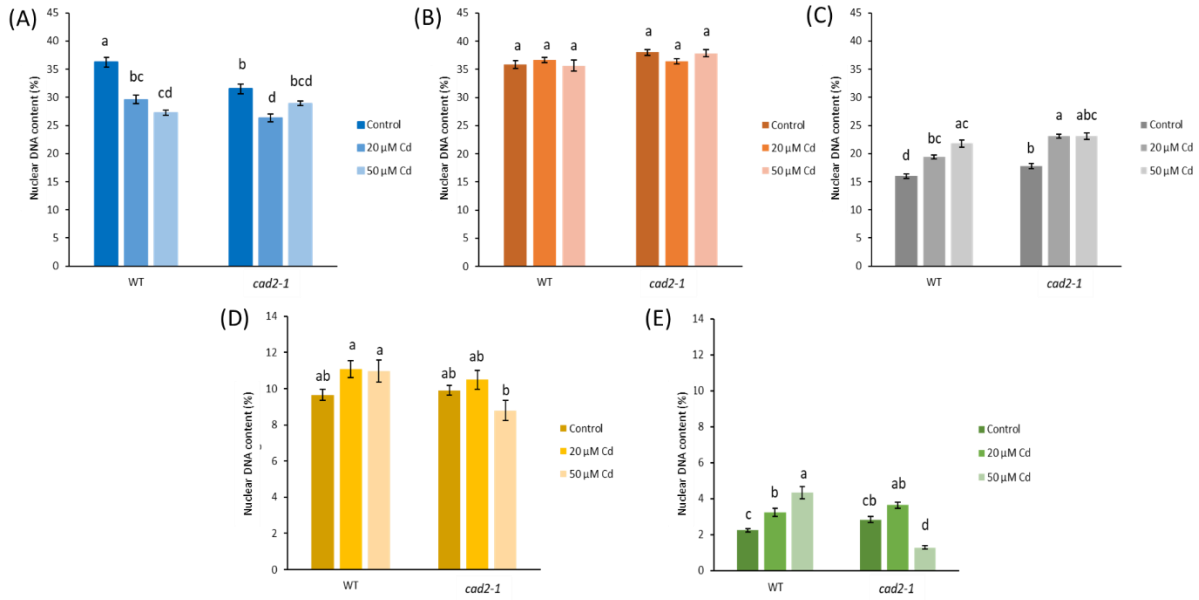
Supplementary table 3. Gene expression levels of wild-type (WT) *A. thaliana* seedlings and three other GSH-deficient *A. thaliana* genotypes grown under control conditions. Data represent the average ± S.E. of 5 biological independent replicate, expressed relative to the WT. Green and red colors indicate a significantly higher and lower expression in the mutant as compared to the WT, respectively. ($p < 0.05$)(1-way ANOVA). Data were normalized against the expression of *AT5G25760 (UBC21), AT4G34270 (TIP41)* and *AT5G08290 (YSL8).*

UBC21: ubiquitin conjugating enzyme 21; *TIP41*: tonoplast intrinsic protein 41-like; *YSL8*: yellow-leaf-specific gene 8.

	WT	*cad2-1*	*pad2-1*	*rax1-1*
DNA DAMAGE AND REPAIR				
PARP2	1.00 ± 0.05	1.24 ± 0.10	1.13 ± 0.10	1.12 ± 0.12
BRCA1	1.00 ± 0.08	1.21 ± 0.08	0.84 ± 0.07	1.13 ± 0.13
CELL CYCLE – CDK INHIBITORS				
SMR4	1.00 ± 0.10	1.03 ± 0.09	1.03 ± 0.10	1.12 ± 0.12
SMR5	1.00 ± 0.03	1.12 ± 0.13	1.25 ± 0.08	1.23 ± 0.11
SMR7	1.00 ± 0.05	1.23 ± 0.19	1.46 ± 0.12	1.35 ± 0.05



Supplementary figure 5. Nuclear ploidy levels of wild-type (WT) *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 8 days. (A) Percentage of 2C nuclei; (B) Percentage of 4C nuclei; (C) Percentage of 8C nuclei; (D) Percentage of 16C nuclei; (E) Percentage of 32C nuclei. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (1-way ANOVA).



Supplementary figure 6. Nuclear ploidy levels of wild-type (WT) and *cad2-1* mutant *A. thaliana* seedlings grown in 96-well plates and exposed to 0, 20 or 50 µM Cd for 8 days. (A) Percentage of 2C nuclei; (B) Percentage of 4C nuclei; (C) Percentage of 8C nuclei; (D) Percentage of 16C nuclei; (E) Percentage of 32C nuclei. Data represent the average ± S.E. of 8 biological independent replicates. Statistical significance is expressed using lower case letters ($p < 0.05$) (2-way ANOVA).

Supplementary table 4. Gene expression levels of wild-type (WT) and *cad2-1 A. thaliana* seedlings grown under control conditions. Data represent the average ± S.E. of 5 biological independent replicate, expressed relative to the WT. Green and red colors indicate a significantly higher and lower expression in the mutant as compared to the WT, respectively. ($p < 0.05$)(1-way ANOVA). Data were normalized against the expression of *AT5G25760 (UBC21), AT4G34270 (TIP41)* and *AT5G08290 (YSL8).*

UBC21: ubiquitin conjugating enzyme 21; *TIP41*: tonoplast intrinsic protein 41-like; *YSL8*: yellow-leaf-specific gene 8.

	WT	*cad2-1*
	0 µM Cd	0 µM Cd
OXIDATIVE STRESS AND SIGNALING		
AT1G05340	1.00 ± 0.11	1.00 ± 0.12
AT119020	1.00 ± 0.13	1.70 ± 0.25
AT1G57630	1.00 ± 0.16	1.31 ± 0.36
UPOX	1.00 ± 0.03	1.22 ± 0.06
AT2G43510	1.00 ± 0.18	0.61 ± 0.16
AOX1a	1.00 ± 0.06	1.18 ± 0.05
AOX1d	1.00 ± 0.20	0.91 ± 0.25
ERF1	1.00 ± 0.05	1.77 ± 0.19
RBOHC	1.00 ± 0.07	0.85 ± 0.16
DNA DAMAGE AND REPAIR		
SOG1	1.00 ± 0.07	1.08 ± 0.03
PARP2	1.00 ± 0.07	1.27 ± 0.08
BRCA1	1.00 ± 0.09	1.05 ± 0.03
CELL CYCLE – CDK INHIBITORS		
SMR4	1.00 ± 0.05	1.23 ± 0.03
SMR5	1.00 ± 0.09	0.88 ± 0.06
SMR7	1.00 ± 0.07	1.37 ± 0.14

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