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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Cadmium-induced effects on developmental processes in a glutathione-deficient *Arabidopsis thaliana* mutant

Promotor :
Prof. dr. Ann CUYPERS

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

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ABBREVIATIONS

Cd	Cadmium
CDK	Cyclin-dependent kinase
CYC	Cyclin
DSBs	Double-strand breaks
EF	Endoreduplication factor
GSH	Glutathione
PC	Phytochelatin
ROS	Reactive oxygen species
SSBs	Single-strand breaks
WT	Wild-type/wildtype

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ABSTRACT

Worldwide, many regions cope with cadmium (Cd) contamination of soils. Cadmium is uptaken by plants, entering the food chain and posing a risk to human health. In plants, Cd increases reactive oxygen species (ROS) production, resulting in oxidative stress. To protect themselves against ROS, plants have developed an extensive antioxidative defense system. An important antioxidative metabolite is glutathione (GSH), which is also involved in cell cycle regulation. In addition to the classical cell cycle, an alternative version of the cell cycle – endoreduplication – exists, consisting of DNA replication without intervening mitosis, resulting in endopolyploidy. This process is affected by external stress factors, including Cd exposure. As GSH plays an important role in plant defense against Cd, it was hypothesized that Cd-induced effects on developmental processes are altered in a GSH-deficient *Arabidopsis thaliana* (*A. thaliana*) mutant.

Nuclear ploidy level, transcription of cell cycle-related genes, cell number and size, and vegetative and reproductive growth and survival, were compared between wild-type (WT) and *cadmium-sensitive* (*cad2-1*) mutant *A. thaliana* plants exposed to 5 µM Cd. Additionally, a toxicity screening method using *A. thaliana* plants grown in 96-well plates was optimized by testing gene expression and flow cytometric parameters.

Gene expression of mitosis stimulators, such as *CYCLIN-DEPENDENT KINASE B2;1* (*CDKB2;1*) and *CYCLIN A2;3* (*CYCA2;3*), differed significantly between WT and *cad2-1* mutant leaves after 72 hours of exposure. Endoreduplication tended to decrease in leaf 4 of both genotypes exposed to 5 µM Cd for 72 hours and 12 days. In leaf 1 of the mutant, however, the nuclear ploidy level showed an increasing trend after 72 hours of exposure. Phenotypically, both vegetative and reproductive growth occurred sooner in the mutant under control conditions than in wild-type plants, while the opposite was true in Cd-exposed plants. All plants reached reproductive maturity. The oxidative stress hallmark genes and CDK inhibitors showed the strongest response to Cd in both WT and *cad2-1* mutant plants, so they can be used as biomarkers for Cd stress.

It can be concluded that the *cad2-1* mutant responds differently to Cd in an array of developmental and defense-related processes. Understanding these mechanisms can aid in optimizing phytoremediation strategies to clean contaminated areas. Furthermore, the 96-well plate setup is a suited method to screen for Cd toxicity in *A. thaliana* mutants and could be used to screen other contaminants in other *A. thaliana* mutants in the future.

SAMENVATTING

Wereldwijd kampen vele regio's met bodemvervuiling door Cd. Cadmium wordt opgenomen door planten en kan zo de voedselketen binnentreden, waardoor het een risico vormt voor de menselijke gezondheid. In planten verhoogt Cd de productie van reactieve zuurstofsoorten, wat resulteert in oxidatieve stress. Planten hebben een uitgebreid antioxidatief verdedigingssysteem ontwikkeld om zichzelf te beschermen tegen deze reactieve zuurstofsoorten. Een belangrijk antioxidatief metaboliet is glutathion (GSH), ook betrokken bij de regulatie van de celcyclus. Bovenop de klassieke celcyclus, bestaat er ~~ook~~ een alternatieve versie, namelijk endoreduplicatie. Endoreduplicatie wordt gekenmerkt door DNA-replicatie zonder tussenkomende mitose, wat resulteert in polyploïdie. Dit proces wordt beïnvloed door externe stressfactoren, zoals blootstelling aan Cd. Omdat GSH een belangrijke rol speelt in de verdediging tegen Cd, luidt de hypothese dat Cd-geïnduceerde effecten op ontwikkelingsprocessen anders zijn in een GSH-deficiënte *A. thaliana* mutant.

Het nucleaire ploïdieniveau, de transcriptie van celcyclus gerelateerde genen, het aantal cellen en hun grootte en de vegetatieve en reproductieve groei en overleving werden vergeleken tussen WT en *cad2-1 A. thaliana* planten die werden blootgesteld aan 5 µM Cd. Daarenboven werd een toxiciteitstest geoptimaliseerd door het meten van genexpressie en flowcytometrie parameters in *A. thaliana* opgegroeid in 96-well platen.

Genexpressie van mitose stimulatoren, zoals *CDKB2;1* en *CYCA2;3*, verschilden significant tussen blaadjes van het WT en de *cad2-1* mutant na 72 u blootstelling. Endoreduplicatie vertoonde een verlaging in blad 4 van beide genotypes, blootgesteld aan 5 µM Cd gedurende 72 u en 12 dagen. In blad 1 van de mutant, vertoonde het nucleaire ploïdieniveau een verhoging na 72 u blootstelling. Fenotypisch gebeurde zowel vegetatieve als reproductieve groei vroeger in de mutant onder controleomstandigheden dan in het WT, terwijl het tegenovergestelde werd waargenomen in de planten blootgesteld aan Cd. Alle planten bereikten het stadium van reproductieve volwassenheid. De oxidatieve stress merkergenen en CDK-inhibitoren vertoonden de sterkste respons in zowel WT als *cad2-1* planten, dus deze kunnen worden gebruikt als biomerker voor Cd-stress.

Er kan worden besloten dat de *cad2-1* mutant anders reageert op Cd in verschillende ontwikkelings- en verdedigingsprocessen. Het begrijpen van deze mechanismen kan helpen bij het optimaliseren van fytoremediatiestrategieën om gecontamineerde gebieden schoon te maken. Verder is de 96-well plaat

opzet geschikt als screeningsmethode voor Cd-toxiciteit in *A. thaliana* en kan het gebruikt worden om voor andere contaminanten in andere *A. thaliana* mutanten te screenen in de toekomst.

1. INTRODUCTION

Due to historical and current agricultural and industrial activities, many regions worldwide are affected by metal contamination of the soil. Waste incineration, traffic and the use of soil amendments and metal-containing phosphate fertilizers, for example, have caused an increase of metal concentrations in agricultural and other soils (1, 2). In humans, cadmium (Cd) is classified as a class I carcinogen and is known to disturb bone, kidney, lung and cardiovascular function (3-5). Cadmium can be taken up by plants from contaminated soils, thereby entering the food chain and posing a risk to human health (6). Therefore, it is important to investigate the effects of Cd exposure in plants.

Although Cd is a non-essential element, it is taken up in plants via transporters for essential nutrients such as calcium (Ca), iron (Fe), copper (Cu) and zinc (Zn) and negatively influences plant growth and development (7, 8). One of the most important effects of Cd exposure in plants is the induction of oxidative stress, which is defined as an imbalance between pro- and antioxidants in favor of the former. Although Cd is not redox-active, it indirectly stimulates the production of reactive oxygen species (ROS) via several mechanisms. First of all, Cd increases the activity of NADPH oxidases, producing superoxide ($O_2^{\bullet-}$). Conversion of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) – both spontaneously and enzymatically through the action of superoxide dismutase (SOD) – leads to the characteristic increase in H_2O_2 levels after Cd exposure (7). Furthermore, Cd can also disturb the function of certain enzymes by binding to their thiol groups, thereby causing an imbalance of the cellular redox state. In addition, Cd can deplete cellular GSH levels as a consequence of an increased phytochelatin synthesis (9).

Although ROS play beneficial roles as signal molecules at low concentrations, they can cause damage to macromolecules such as proteins, DNA and membrane lipids when present in high concentrations. In order to prevent oxidative damage, plants have developed an extensive antioxidative defense system, consisting of both enzymes and metabolites (1, 6, 10). Antioxidative enzymes such as SOD, catalase (CAT) and ascorbate peroxidase (APX) convert ROS to less harmful intermediates and eventually oxygen and water (2). Antioxidative metabolites are either lipid-soluble or water-soluble. Examples of lipid-soluble antioxidants are carotenoids and tocopherols (2, 6). Water-soluble antioxidants include ascorbate (AsA) and glutathione (GSH).

Glutathione is a tripeptide consisting of cysteine (Cys), glutamate (Glu) and glycine (Gly). In the first, rate-limiting step of GSH biosynthesis, y-glutamylcysteine (y-EC) is synthesized from cysteine (Cys) and glutamate (Glu) in a reaction catalyzed by y-glutamylcysteine synthetase (GSH1). Subsequently, glycine (Gly) is added by GSH synthetase (GSH2), thereby producing GSH (9). Glutathione plays an important role in antioxidative defense, as it is involved in H_2O_2 detoxification via several mechanisms: (1) direct, non-enzymatic conversion of H_2O_2, (2) conversion of H_2O_2 via the AsA-GSH cycle and (3) conversion of H_2O_2 via the redoxin cycle, all ultimately yielding H_2O and the oxidized form of GSH, glutathione disulfide (GSSG) (Figure #). This oxidized form of GSH can be reduced by GSH reductase (GR), using NADPH as an electron donor (9, 11). Glutathione not only neutralizes Cd-induced ROS, but is also involved in direct Cd detoxification via sequestration by phytochelatins (PCs). Phytochelatins are polymers of GSH molecules that chelate Cd via their large number of thiol groups and are synthesized by phytochelatin synthase (PCS). The importance of GSH and PCs in the response of *A. thaliana* to Cd are emphasized by the increased Cd sensitivity of the GSH-deficient *cadmium-sensitive (cad2-1)* and the PC-deficient *cad1-3* mutant. The *cad2-1* mutant is characterized by a mutation in the *GSH1* gene, resulting in a decreased GSH level of approximately 30 % of wild-type (WT) levels (12). The *cad1-3* mutant is deficient in PCS1 and is therefore unable to produce PCs (13).

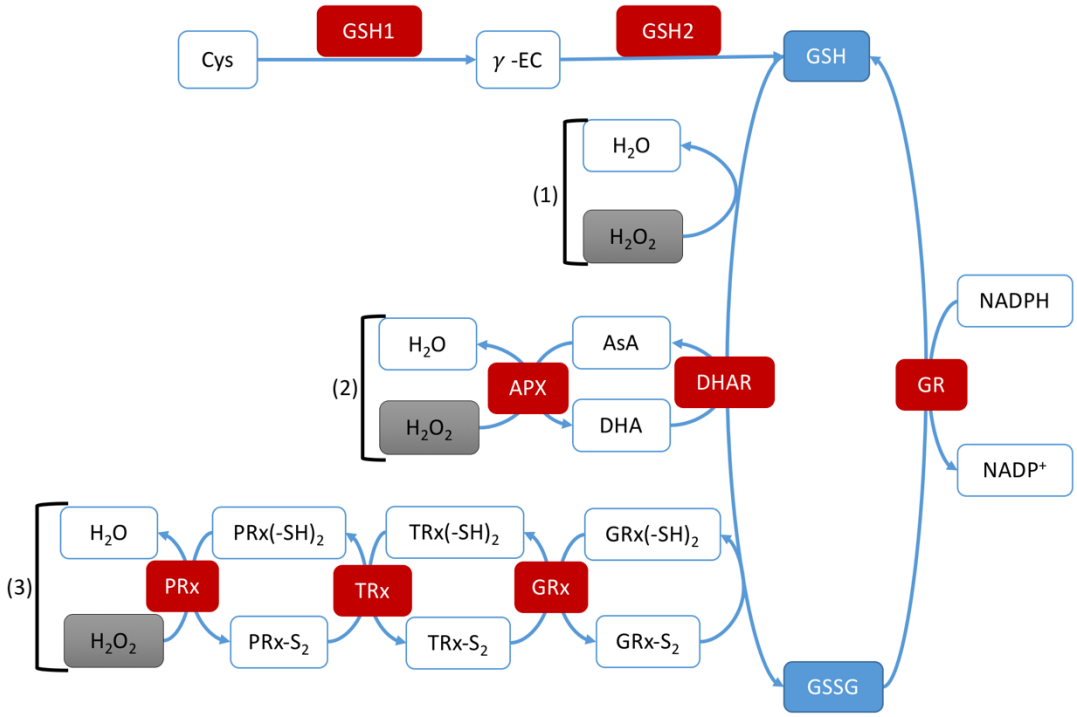

Figure 1: *Glutathione biosynthesis and roles in antioxidative defense.* (1) Direct, non-enzymatic conversion. (2) AsA-GSH cycle. (3) Redoxin cycle. Abbreviations: Cys, cysteine; GSH1, y-glutamylcysteine synthetase; y-EC, y-glutamylcysteine; GSH2, GSH synthetase; GSH, glutathione; H_2O_2, hydrogen peroxide; GR, glutathione reductase; APX, ascorbate peroxidase; AsA, ascorbic acid; DHA, dehydroascorbate; DHAR, DHA reductase; PRx, peroxiredoxin; TRx, thioredoxin; GRx; glutaredoxin; GSSG, GSH disulfide. Adapted from Jozefczak *et al.* 2012.

In addition to its role in antioxidative defense, GSH also plays a role in cell cycle regulation (potters 2002). The classical plant cell cycle consists of four phases: a first gap phase (G1), a DNA replication phase (S), a second gap phase (G2) and a mitotic phase (M). During G1 and G2, successful completion of the previous phase is verified and the cell prepares for the next phase. During the S phase, the cell replicates its nuclear DNA, whereas the cell divides into two daughter cells during mitosis (M phase). Regulation of the cell cycle is established by cyclin-dependent kinases (CDKs), which form heterodimers with their regulatory cyclins in order to become activated. Cyclin-dependent kinases phosphorylate proteins are essential for cell cycle progression at two checkpoints: one at the transition between the G1 and S phase and one between the G2 phase and M phase (14, 15). When DNA damage is detected, cell cycle progression is inhibited to allow for DNA repair.

Oxidative stress can cause DNA damage in a direct or indirect fashion. Direct damage to DNA molecules results from hydroxyl radical attack of guanine, forming 8-oxoguanine adducts. Furthermore, ROS can also attack the DNA sugar backbone, thereby causing single strand breaks (SSBs). Indirect DNA damage can be caused by molecules generated as a result of oxidative damage, such as byproducts of lipid peroxidation (16). Plants possess different DNA repair mechanisms specific to a certain type of DNA damage. In *A. thaliana*, a number of DNA damage marker genes have been identified. These include *BRCA1*, involved in double strand break (DSB) repair, and *PARP1* and *PARP2*, both involved in single strand damage repair. As reported by Mannuss *et al.* (2012), the expression of these genes is strongly induced upon genotoxic stress (16).

In plants, an alternative version of the cell cycle – endoreduplication – exists. During an endoreduplication cycle or endocycle, nuclear DNA is replicated (S phase) without intervening mitosis (M phase). As a result, nuclear ploidy levels increase. It is interesting to note that correlations between ploidy levels and cell size are often reported (17). Cells of WT *A. thaliana* plants have been shown to reach ploidy levels up to 32C, with C representing the haploid DNA content of the cell (15, 18). For plant cells to enter endoreduplication, mitotic CDK activity needs to be suppressed (Figure 2). This can be established by three mechanisms: (1) inhibition of CDK activity by CDK inhibitors, (2) proteolytic degradation of mitotic cyclins and (3) transcriptional downregulation of genes encoding mitotic cyclins and CDKs. Known inhibitors of CDK activity are SIAMESE-RELATED proteins (SMRs. Interestingly, Yi *et al.* 2014 demonstrated that H_2O_2-induced DNA damage causes transcriptional activation of *SMR5* and *SMR7*, thereby inhibiting cell cycle progression.

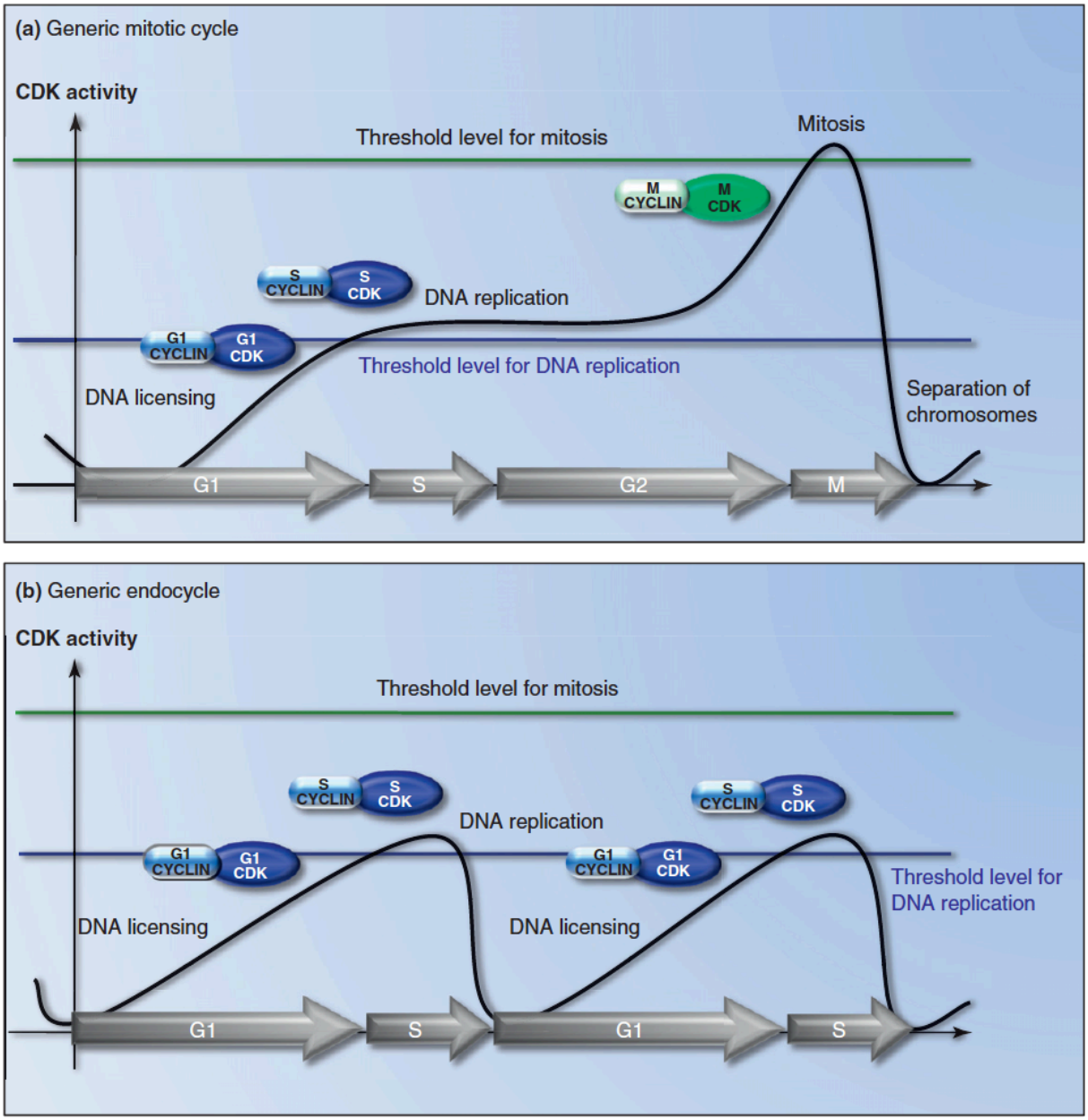

Figure 2: *CDK activity during (a) a generic mitotic cycle and (b) an endocycle*. For endoreduplication to occur, the activity of mitotic CDKs needs to be suppressed. De Veylder *et al.* 2011.

Endoreduplication plays an important role in plant growth and development, usually as a part of the final differentiation of specialized tissues. Trichomes of *A. thaliana*, for example, consist of a single cell that completes up to four endocycles to achieve its characteristic branched structure. As increased nuclear ploidy levels are related to cell growth (17), endoreduplication is considered as one of the key players in the determination of final cell size. Correspondingly, it is also thought to function as a mechanism to manage the increasing metabolic demand of growing cells, as it increases the copy number of genes involved in cellular metabolism (14, 19).

In addition to its role in plant growth and development, endoreduplication is affected by a number of environmental stress factors, such as low light, UV-B irradiation, soil quality and water availability (20). Cells of etiolated leaves of *A. thaliana*, for example, increase their ploidy levels in order to enlarge the overall leaf surface area to capture more light (18, 21, 22). Furthermore, DSBs caused by excessive UV-B irradiation were shown to increase the extent of endoreduplication (23). Several authors hypothesize this is a coping strategy of the cell to avoid transmission of damaged DNA to daughter cells, which would take place during mitosis (14, 19, 24-26).

Preliminary data of our research group have shown that several parameters related to oxidative stress and cell cycle regulation differ significantly between *A. thaliana* WT plants and the *cad2-1* mutant. Since it is known that GSH plays a very important role in the response of *A. thaliana* to Cd stress, it is hypothesized that Cd-induced effects on developmental processes are altered in this GSH-deficient mutant. To test this hypothesis, WT and *cad2-1* mutant *A. thaliana* plants are grown in hydroponics and exposed to either 0 or 5 µM Cd. Subsequently, Cd-induced effects on nuclear ploidy levels, cell number and cell surface area and the transcription of genes related to oxidative stress, cell cycle regulation and DNA damage and repair are compared between both genotypes. Furthermore, vegetative and reproductive growth and survival are investigated. In addition, a toxicity screening system using *A. thaliana* seedlings grown in 96-well plates is optimized in the context of this study.

2. Materials and methods

2.1 Plant cultivation and harvest

2.1.1 Hydroponic culture

Wild-type, *cad2-1* and *cad1-3* mutant *A. thaliana* seeds were surface-sterilized using a 0.1 % sodium hypochlorite solution and Tween 80. In order to synchronize germination, the seeds were stored in the dark at 4°C for 2 days. Next, they were grown in hydroponic culture as previously described by Keunen *et al.* (27). The growth conditions were set at a 12 h photoperiod, a relative humidity of 65 % and day and night temperatures of 22°C and 18°C, respectively. A combination of blue, red and far-red LED lights was used to simulate the spectrum of photosynthetic active radiation (PAR) in sunlight, with 170 µmol m^{-2} s^{-1} at the leaf level. The composition of the modified Hoagland solution is shown in Table 1. After 3 weeks, the plants were exposed to either 0 or 5 µM Cd, supplied to the roots as $CdSO_4$. For gene expression analysis, roots and shoots were harvested after 24 and 72 h of Cd exposure. For flow cytometric and microscopic analysis, separate leaves were harvested after 72 h and 12 d or after 72 h of exposure, respectively. Samples for all analyses, except for microscopy, were snap-frozen in liquid nitrogen and stored at -70°C until further analysis.

Table 1: *Composition of the modified Hoagland solution.*

HOAGLAND		FINAL CONCENTRATION (µM)
MACRO-ELEMENTS	KNO_3	505
	$Ca(NO_3)_2.4H_2O$	150
	$NH_4H_2PO_4$	100
	$MgSO_4.7H2O$	100
IRON	$FeSO_4.7H_2O$	1.64
	Na_2-EDTA	0.81
MICRO-ELEMENTS	H_3BO_3	4.63
	$MnCl_2.4H_2O$	0.91
	$CuSO_4.5H_2O$	0.03
	$H_2MoO_4.H_2O$	0.06
	$ZnSO_4.7H_2O$	0.08

2.2 Gene expression

2.2.1 RNA extraction

RNA was extracted using the RNAqueous® kit (AM1912, Thermo Fisher Scientific; Waltham, US-MA), following the manufacturer's protocol. RNA purity and concentration were checked on the NanoDrop ND-1000 (Thermo Fisher Scientific; Waltham, US-MA) and RNA integrity was assessed using the Experion (Bio-Rad; Hercules, US-CA).

2.2.2 cDNA synthesis

First, genomic DNA was removed from the samples using the TUBRO DNA-*free*™ kit (AM1907, Thermo Fisher Scientific; Waltham US-MA), following manufacturer's instructions. Next, cDNA was synthesized with 1 µg of RNA input using the PrimeScript™ RT Reagent Kit (Perfect Real Time) (RR0337A, TaKaRa Bio Inc.; Leusden, the Netherlands), following the manufacturer's protocol. cDNA Samples were diluted 10x in 1/10 Tris-EDTA (TE) buffer.

2.2.3 Real-time quantitative PCR

Gene expression levels were measured by real-time quantitative PCR using the SYBR Green chemistry. Reactions contained 8 µL master mix (5 µL Fast SYBR Green Master Mix, 0.3 µL forward primer (300 nM final), 0.3 µL reverse primer (300 nM final) and 2.4 µL RNase-free water) and 2 µL diluted cDNA sample and were run in the 7500 Fast Real-Time PCR System (Applied Biosystems). Cycling conditions are presented in Table 2. Primer sequences are shown in Table 3. Reference genes for normalization of the results using the $2^{-\Delta Cq}$ method were selected by the GrayNorm algorithm (28).

Table 2: *Real-time PCR cycling conditions.*

	TEMPERATURE (°C)	TIME (S)
INITIAL DENATURATION	95	20
DENATURATION AND	95	3
ELONGATION (40 CYCLES)	60	30

Table 3: *Primer sequences for real-time PCR.*

GENE	FORWARD PRIMER (5'→3')	REVERSE PRIMER (5'→3)
FBOX	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
UBC	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
PPR	AAGACAGTGAAGGTGCAACCTTACT	AGTTTTTGAGTTGTATTTGTCAGAGAAAG
YLS8	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
UBIQUITIN	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
SAND	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
TIP41-LIKE	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
EF1A	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA
AT4G26410	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
ACT2	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
UPOX	GACTTGTTTCAAAAACACCATGGAC	CACTTCCTTAGCCTCAATTTGCTTC
AT2G43510	ATGGCAAAGGCTATCGTTTCC	CGTTACCTTGCGCTTCTATCTCC
AT1G19020	GAAAATGGGACAAGGGTTAGACAAA	CCCAACGAAAACCAATAGCAGA
AT1G05340	TCGGTAGCTCAGGGTAAAGTGG	CCAGGGCACAACAGCAACA
AT1G57630	ACTCAAACAGGCGATCAAAGGA	CACCAATTCGTCAAGACAACACC
RBOHD	AACTCTCCGCTGATTCCAACG	TGGTCAGCGAAGTCTTTAGATTCCT
RBOHC	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
FSD1	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
CAT1	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
CAT2	AACTCCTCCATGACCGTTGGA	TCCGTTCCCTGTCGAAATTG
CAT3	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA
LOX1	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC
ERF1	TCCTCGGCGATTCTCAATTTT	CAACCGGAGAACAACCATCCT
AOX1A	CTCTTCGTTGGCCTACCGATT	AACCATTCCAGGTACTGCTGCTAC
SMR4	TGATGGTGGTGAGAAAACGAGA	TCTCTTCGAGGCTGTGCGTAG
SMR5	CAGCATATCCGCCTTGTCCA	CTGCTACCACCGAGAAGAACAAGT
SMR7	ACATCGATTCGGGCTTCACTAA	CCGTGGGAGTGATACAAATTCC
CDKB2;1	CTGGCAAGAACATTCCAACCC	TGTGCAATATCCCGTGACCA
CYCA2;3	CCACTGGACCCAACAAGAGG	AATCCATGACCGCGTCCTTT
CYCB1;1	CACGATCTCAAAATCCCACGC	TTCCCAGCCACTTTCTTCGG
CYCA3;1	CGTCTACTGTGGCTGCTTCA	TCACCCGCTTTGTATCTTGTGT
CYCA3;2	AGACGGTTTACTAGAGTTGCACA	AGAAGCAGCCAACAGAGACG
CDKB1;1	CGTGACTGGCATGTTTACCC	TCGGCTGGATTGTACTTGAGC
PARP1	TGCATTGGGAGAAATACATGAGC	CCGAGCCCTTTGGTCGAG
PARP2	ATCGGAGGTGATTGATCGGTATG	AAATCATGAGGTATCACTGTGTAGAACTCT
BRCA1	GTGAACCTGTCTCTGCGGAT	TCCGGCTTCTTGTCAACTCC
LIG4	TGATGTATCGGATATCAAGGGCA	GAATGGGACCGAGGCACG
HIS4	AGGAGGAAAAGGGTTAGGCAAA	GACGAATCGCTGGCTTTGTG
E2FA	GATCCAGATGAAGCGGCTGA	CTGCACCACTCCCATTTGTG
XRCC1	TGGGCCAGGGATGACCTAAG	CCGCAGCTATTCGCTTGATTT

2.3 Flow cytometric analysis of nuclear ploidy levels

Flow cytometric analysis of nuclear ploidy levels of leaves 1 (the oldest leaf), 4 and 11 was performed using the CyStain® PI Absolute P kit (Partec-Sysmex; Kobe, Hyogo, Japan). First, frozen leaves were chopped and incubated in 500 µL extraction buffer. The solution was filtered over a 50 µm Celltrics® filter (Partec-Sysmex; Kobe, Hyogo, Japan) and 2 mL staining solution (2 mL staining buffer, 12 µL propidium iodide (PI) and 6 µL RNase) was added. The samples were incubated in the dark at 4°C for at least 1 h before analysis on the Sysmex CyFlow® Cube 8 (Partec-Sysmex; Kobe, Hyogo, Japan). The nuclear DNA content of 5000 to 15000 nuclei was measured. Nuclei were excited with a 488 nm laser and the PI fluorescence intensity was measured in the FL-2 channel (580/30 nm) and plotted against the forward scatter. Using the FCS Express software (FCS Express 4; De Novo Software), the percentage of nuclei corresponding to each ploidy level was determined. The endoreduplication factor (EF) was calculated using the following formula: [(0 x % 2C) + (1 x % 4C) + (2 x % 8 C) + (3 x % 16C) + (4 x % 32C)] / 100.

2.4 Microscopic analysis of epidermal leaf cell area and number

Leaves 1 to 6 of both control and Cd-exposed plants were scanned using a conventional flatbed scanner and immediately cleared in 100 % ethanol for 24 h. Next, they were further cleared in lactic acid and stored in the dark until microscopic analysis. If necessary, excessive starch was cleared by incubation in Hoyer solution (10 mL dH_2O, 20 mL glycerol and 80 g chloral hydrate) for 2 h. The leaf was placed on a microscopic slide with a drop of lactic acid and covered with a coverslip. Two pictures of the abaxial epidermis were taken using a Leica DM2500 microscope in differential interference contrast (DIC) mode equipped with a DFR 450C camera. One picture was taken at 25 % and one at 75 % between the leaf apex and base, halfway between the midrib and the leaf margin. Using the ImageJ software (NIH; New York, USA), the average cell surface area and the leaf surface area were determined. Based on these values, an estimation of the number of abaxial epidermal cells per leaf was made.

2.5 Phenotypic analysis of survival and reproductive potential

A hydroponic culture of WT and *cad2-1* mutant *A. thaliana* plants was set up as described above. The plants were followed up phenotypically from day 20 (start of exposure) until day 55 (35 days of exposure) after sowing. The number of leaves, rosette diameter, inflorescence emergence, inflorescence height and flower and silique formation were assessed and coupled to the growth stages as described by Boyes *et al.* (Table 4) (29).

Table 4: Arabidopsis thaliana *growth stages.* Adapted from Boyes *et al.* 2001 and Keunen *et al.* 2011.

Stage	***Description***
Principal growth stage 1	Leaf development
1.06	6 rosette leaves > 1 mm in length
Principal growth stage 3	Rosette growth
3.50	Rosette measures 50% of its final size
3.70	Rosette measures 70% of its final size
3.90	Rosette growth is finished
Principal growth stage 5	Emergence of inflorescence
5.10	Emergence of the inflorescence meristem

2.6 Optimization of 96-well plate plant culture

In order to optimize a screening method for *A. thaliana*, seeds were surface-sterilized using 0.1% HCl and Tween 80 and subsequently sown in a 96-well plate filled with ½ Murashige and Skoog (MS) medium (Greiner Bio-One B.V.B.A.; Kremsmünster, Austria). The plates were incubated in the dark at 4°C for two days for stratification of the seeds. The plants were grown under the same conditions as described for hydroponic culture. The ½ MS medium was refreshed after six days.

In the first experiment, only WT plants were used and samples for gene expression analysis were harvested 7, 8, 11 and 12 days after sowing. In the second experiment, WT plants were exposed to 0 or 5 µM Cd 7 days after sowing by addition of $CdSO_4$ to the medium. Samples for gene expression analysis were harvested after 24, 48, 72 and 148 h of exposure. In the third experiment, WT and *cad2-1* seedlings were exposed to 0, 5 or 10 µM Cd 7 days after sowing and harvested for gene expression analysis 24 and 72 h after exposure.

For the last optimization experiment, WT and *cad2-1* plants were exposed to either 0, 10 or 20 µM Cd 7 days after sowing. Samples for gene expression were harvested after 24 h; samples for flow cytometric analysis were harvested after 72 h. All samples were snap-frozen in liquid nitrogen and stored at -70°C until further analysis. Flow cytometric and gene expression analysis were performed as described for hydroponic culture. For both analysis, complete plants were used.

2.7 Statistical analysis

Microscopy data were analyzed using a Student's t-test, while a two-way ANOVA was performed on flow cytometry and gene expression data. A *post-hoc* Tukey-Kramer test was used for pairwise multiple comparisons. Normality and homoscedasticity of the data were verified using the Shapiro-Wilk test and Bartlett test, respectively. If necessary, data were transformed (logarithm, inverse, exponent or square root). Gene expression data were standardly log transformed. Data that did not meet normality and homoscedasticity assumptions were analyzed using a non-parametric Kruskal-Wallis test, followed by a *post-hoc* Wilcoxon Rank Sum test for multiple comparison. All statistical analyses were performed in R, version 3.2.3 (The R Foundation for Statistical Computing, Auckland, New Zealand).

3. RESULTS

In this study, the involvement of GSH in Cd-induced the effects on developmental processes in *A. thaliana* was investigated by measuring several molecular, cellular and phenotypic parameters. Additionally, a toxicity screening method based on growth of *A. thaliana* in 96-well plates was optimized in the context of this study.

3.1 Effects of cadmium exposure on oxidative stress, cell cycle regulation and DNA damage in *Arabidopsis thaliana* leaves

3.1.1 Cadmium-induced effects on gene expression in leaves of wild-type, cad2-1 and cad1-3 mutant plants

In order to determine the differences in response to 5 µM Cd between WT and *cad2-1* mutant *A. thaliana* plants, a gene expression analysis was performed. The transcription of an array of genes related to oxidative stress, cell cycle regulation and DNA damage and repair was measured after 24 h (Table 5) and 72 h (Table 6) of exposure. All oxidative stress marker genes were significantly upregulated after 24 h in both genotypes. After 72 h, they were still upregulated in the *cad2-1* mutant, whereas in the WT only *AT2G43510* remained upregulated.

The expression of the S phase marker genes *HISTONE H4* (*HIS4*), *CYCLIN A3;1* (*CYCA3;1*), *CYCA3;2* and *E2Fa* showed a decreasing trend in all genotypes after 24 h of Cd exposure. After 72 h, the transcription of these genes slightly increased in the WT, whereas it showed a decreasing trend in the mutant. The expression of mitotic CDKS and cyclins *CDKB2;1*, *CDKB1;1*, *CYCA2;3* and *CYCB1;1* was significantly downregulated in both genotypes after 24 h. After 72 h, they showed an increasing trend in the WT, but a decreasing trend in the *cad2-1* mutant. The expression of S phase marker genes and mitotic CDKs and cyclins was slightly increased in the mutants under control conditions at 72 h as compared to the WT, whereas this increasing trend was not observed after 24 h. Expression of CDK inhibitors *SIAMESE-RELATED 4* (*SMR4*), *SMR5* and *SMR7* was increased in the WT at both time points. In the *cad2-1* mutant, their expression was slightly decreased after 24 h, but also showed an increasing trend after 72 h.

The DNA repair genes *POLY ADP-RIBOSE POLYMERASE 2* (*PARP2*), *DNA LIGASE IV* (*LIG4*) and *X-RAY REPAIR CROSS COMPLEMENTING 1* (*XRCC1*) were significantly upregulated in the WT after 24 h, whereas *PARP1* expression was downregulated. In the *cad2-1* mutant, *PARP1* and *PARP2* were significantly downregulated after 24 h. After 72 h, the expression of DNA damage markers *PARP2* and *BREAST CANCER SUSCEPTIBILITY 1* (*BRCA1*) was upregulated more strongly in the WT than in the mutants.

To determine whether the differences between WT and *cad2-1* mutant plants were due to the deficiency in GSH or PCs, gene expression analysis of the PC-deficient *cad1-3* mutant was performed after 72 h of exposure to 5 µM Cd. All oxidative stress hallmark genes, except for *AT1G57630*, showed an increasing trend, similar to the *cad2-1* mutant. The S phase markers and mitotic cyclins and CDKs showed a decreasing trend similar to the *cad2-1* mutant. Like the *cad2-1* mutant, the *cad1-3* mutant responded to Cd exposure by slightly downregulating the expression of *SMR4* and *SMR7*, and increasing *SMR5* transcription. The DNA repair genes *PARP2* and *LIG4* showed an increasing trend similar to WT and *cad2-1* mutant plants, while *XRCC1*, *BRCA1* and *PARP1* were slightly downregulated in the *cad1-3* mutant. In general, it can be concluded that the gene expression of the *cad1-3* mutant responded in a similar fashion as compared to that of the *cad2-1* mutant.

Gene	Cd (µM)	Wild-type	*cad2-1*
Oxidative stress			
UPOX	0	1.00 ± 0.07	1.26 ± 0.10
	5	5.92 ± 0.75	9.82 ± 1.88
AT2G43510	0	1.00 ± 0.28	1.07 ± 0.32
	5	25.81 ± 1.22	27.11 ± 10.76
AT1G19020	0	1.00 ± 0.04	1.49 ± 0.35
	5	101.52 ± 24.12	107.68 ± 52.48
AT1G05340	0	1.00 ± 0.09	1.06 ± 0.02
	5	77.08 ± 13.73	20.53 ± 12.19*
AT1G57630	0	1.00 ± 0.39	1.21 ± 0.32
	5	103.77 ± 21.47	50.76 ± 18.78
Cell cycle			
S phase markers			
CYCA3;1	0	1.00 ± 0.02	0.88 ± 0.04
	5	0.57 ± 0.01	0.55 ± 0.05
CYCA3;2	0	1.00 ± 0.01	0.88 ± 0.03
	5	0.69 ± 0.03	0.61 ± 0.11
HIS4	0	1.00 ± 0.00	0.95 ± 0.04
	5	0.72 ± 0.09	0.79 ± 0.08
E2Fa	0	1.00 ± 0.10	0.94 ± 0.09
	5	0.78 ± 0.05	0.49 ± 0.04*
G2-M markers			
CDKB2;1	0	1.00 ± 0.22	1.35 ± 0.21
	5	0.54 ± 0.07	0.60 ± 0.17
CDKB1;1	0	1.00 ± 0.08	1.07 ± 0.08
	5	0.54 ± 0.10	0.74 ± 0.11
CYCB1;1	0	1.00 ± 0.10	0.96 ± 0.01
	5	0.42 ± 0.08	0.42 ± 0.01
CYCA2;3	0	1.00 ± 0.09	0.89 ± 0.08
	5	0.54 ± 0.06	0.41 ± 0.05
CDK inhibitors			
SMR4	0	1.00 ± 0.02	1.07 ± 0.07
	5	3.02 ± 0.48	0.64 ± 0.02*
SMR5	0	1.00 ± 0.36	0.84 ± 0.08
	5	2.43 ± 0.27	1.56 ± 0.01
SMR7	0	1.00 ± 0.01	1.22 ± 0.11
	5	7.35 ± 4.03	0.67 ± 0.16
DNA damage and repair			
PARP1	0	1.00 ± 0.03	0.90 ± 0.04
	5	0.61 ± 0.07	0.54 ± 0.05
PARP2	0	1.00 ± 0.06	1.44 ± 0.12
	5	2.72 ± 0.30	0.73 ± 0.01*
BRCA1	0	1.00 ± 0.24	0.75 ± 0.05
	5	0.84 ± 0.21	0.55 ± 0.09
LIG4	0	1.00 ± 0.10	0.96 ± 0.05
	5	2.18 ± 0.24	1.23 ± 0.01*
XRCC1	0	1.00 ± 0.10	1.11 ± 0.01
	5	3.52 ± 0.39	1.39 ± 0.22*

Table 5: *Gene expression in leaves of WT and cad2-1 mutant A. thaliana plants exposed to 5 µM $CdSO_4$ for 24 h, starting from day 19 after sowing.* Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration. Colors indicate significant differences as compared to the control of the same genotype. Green and orange colors represent upregulations and downregulations, respectively ($p < 0.05$; 2-way ANOVA). Data represent the average ± S.E. of four biological independent replicates and were normalized based on the expression of *AT5G15710* (*FBOX*), *AT5G25760* (*UBC*) and *AT5G55840* (*PPR*).

Gene	Cd (µM)	Wild-type	*cad2-1*	*cad1-3*
Oxidative stress				
UPOX	0	1.00 ± 0.04	1.56 ± 0.12	0.98 ± 0.06
	5	1.46 ± 0.11	3.02 ± 0.52*	2.80 ± 0.53*
AT2G43510	0	1.00 ± 0.25	0.66 ± 0.12	2.13 ± 0.34*
	5	4.35 ± 0.48	8.07 ± 1.88	25.87 ± 3.28*
AT1G19020	0	1.00 ± 0.43	0.51 ± 0.19	1.32 ± 0.47
	5	2.18 ± 0.34	4.80 ± 1.60	2.87 ± 0.39
AT1G05340	0	1.00 ± 0.41	0.39 ± 0.18	2.79 ± 0.92
	5	2.68 ± 0.93	2.69 ± 0.51	11.12 ± 4.08*
AT1G57630	0	1.00 ± 0.51	0.27 ± 0.17	1.56 ± 0.61
	5	1.07 ± 0.14	1.68 ± 0.49	0.95 ± 0.16
Cell cycle				
S phase markers				
CYCA3;1	0	1.00 ± 0.10	1.98 ± 0.12*	1.35 ± 0.16
	5	1.68 ± 0.09	1.34 ± 0.06	0.93 ± 0.17*
CYCA3;2	0	1.00 ± 0.11	1.44 ± 0.09	1.26 ± 0.07
	5	1.06 ± 0.05	1.02 ± 0.03	0.70 ± 0.14*
HIS4	0	1.00 ± 0.08	1.78 ± 0.14*	1.29 ± 0.11
	5	1.53 ± 0.11	1.61 ± 0.16	1.22 ± 0.21
E2Fa	0	1.00 ± 0.15	2.44 ± 0.20*	1.46 ± 0.14
	5	1.29 ± 0.10	1.20 ± 0.07	0.65 ± 0.12*
G2-M markers				
CDKB2;1	0	1.00 ± 0.08	1.38 ± 0.04	1.22 ± 0.13
	5	1.32 ± 0.06	1.07 ± 0.11	0.87 ± 0.14*
CDKB1;1	0	1.00 ± 0.10	1.88 ± 0.09*	1.41 ± 0.21
	5	1.95 ± 0.11	1.72 ± 0.22	1.36 ± 0.24
CYCB1;1	0	1.00 ± 0.09	1.52 ± 0.05	1.52 ± 0.26
	5	1.51 ± 0.11	1.23 ± 0.13	0.76 ± 0.17*
CYCA2;3	0	1.00 ± 0.13	2.18 ± 0.20	1.34 ± 0.19
	5	1.84 ± 0.11	1.36 ± 0.27	0.68 ± 0.20*
CDK inhibitors				
SMR4	0	1.00 ± 0.10	1.17 ± 0.13	1.06 ± 0.15
	5	1.26 ± 0.08	1.07 ± 0.14	0.43 ± 0.09*
SMR5	0	1.00 ± 0.12	1.02 ± 0.07	1.06 ± 0.08
	5	1.78 ± 0.15	2.54 ± 0.43	4.26 ± 1.02*
SMR7	0	1.00 ± 0.10	2.54 ± 0.30	1.04 ± 0.06
	5	4.06 ± 0.05	2.14 ± 0.67*	0.91 ± 0.14*
DNA damage and repair				
PARP1	0	1.00 ± 0.07	1.58 ± 0.10*	1.25 ± 0.04
	5	1.25 ± 0.05	1.25 ± 0.08	0.68 ± 0.09*
PARP2	0	1.00 ± 0.05	1.79 ± 0.13*	0.98 ± 0.12
	5	3.02 ± 0.11	2.29 ± 0.17	1.07 ± 0.14*
BRCA1	0	1.00 ± 0.11	2.21 ± 0.23*	1.24 ± 0.17
	5	2.41 ± 0.17	2.28 ± 0.22	0.90 ± 0.19*
LIG4	0	1.00 ± 0.11	1.28 ± 0.11	1.34 ± 0.11
	5	1.22 ± 0.05	1.78 ± 0.08	1.44 ± 0.27
XRCC1	0	1.00 ± 0.10	1.03 ± 0.08	1.43 ± 0.07
	5	1.24 ± 0.07	1.44 ± 0.11	0.99 ± 0.14

Table 6: : *Gene expression in leaves of wild-type and cad2-1 mutant A. thaliana plants exposed to 5 µM $CdSO_4$ for 72 h, starting from day 19 after sowing.* Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration. Colors indicate significant differences as compared to the control of the same genotype. Green and orange colors represent upregulations and downregulations, respectively ($p < 0.05$; 2-way ANOVA). Data represent the average ± S.E. of four biological independent replicates and were normalized based on the expression of *UBC*, *AT4G34270* (*TIP41*), *AT5G08290* (*YLS8*) and *AT4G26410*.

3.1.2 Cadmium-induced effects on ploidy levels in leaves of wild-type and cad2-1 mutant plants

To assess the differences in ploidy levels between WT and *cad2-1* mutant *A. thaliana* plants exposed to 0 or 5 µM Cd for 72 h and 12 days, a flow cytometric analysis of leaves 1, 4 and 11 was performed. In leaf 1, the oldest leaf, the proportion of 8C and 16C nuclei was significantly higher in the mutant under control conditions, as compared to the WT (Figure 3a). The *cad2-1* mutant also showed a significantly higher proportion of 32C nuclei after 72 h of exposure, compared to the WT. As shown in Figure 1a, Cd exposure slightly increased the proportion of 32C nuclei in WT plants, whereas it mainly affected the 16C content in the *cad2-1* mutant. The endoreduplication factor (Figure 3b) did not differ significantly between leaves of control and exposed plants. In both conditions, the ploidy level was slightly higher in the mutant, compared to the WT.

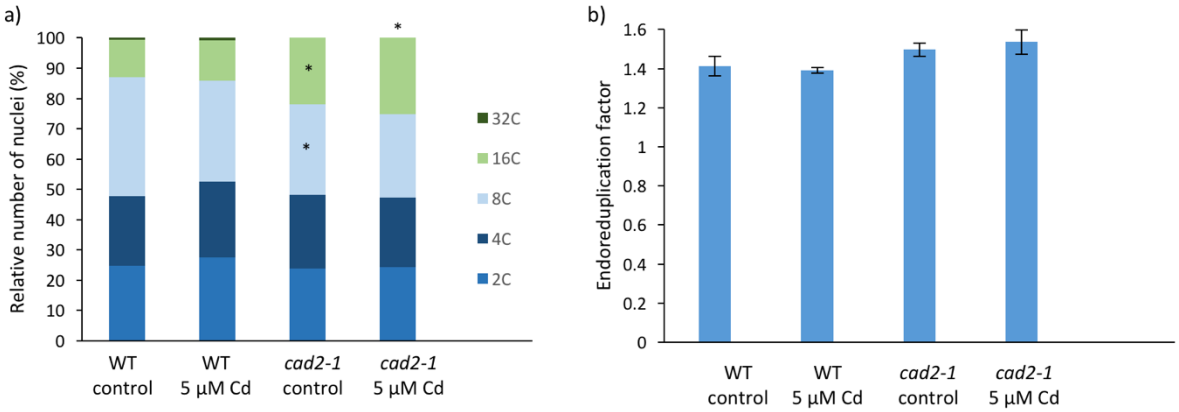

Figure 3: *Ploidy levels and endoreduplication factor in leaf 1 of WT and cad2-1 mutant A. thaliana plants exposed to 5 µM $CdSO_4$ for **72 h**, starting from day 20 after sowing.* (a) Ploidy levels. (b) Endoreduplication factor. Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration ($p < 0.05$; two-way ANOVA). Data represent the average ± S.E. of four biological independent replicates.

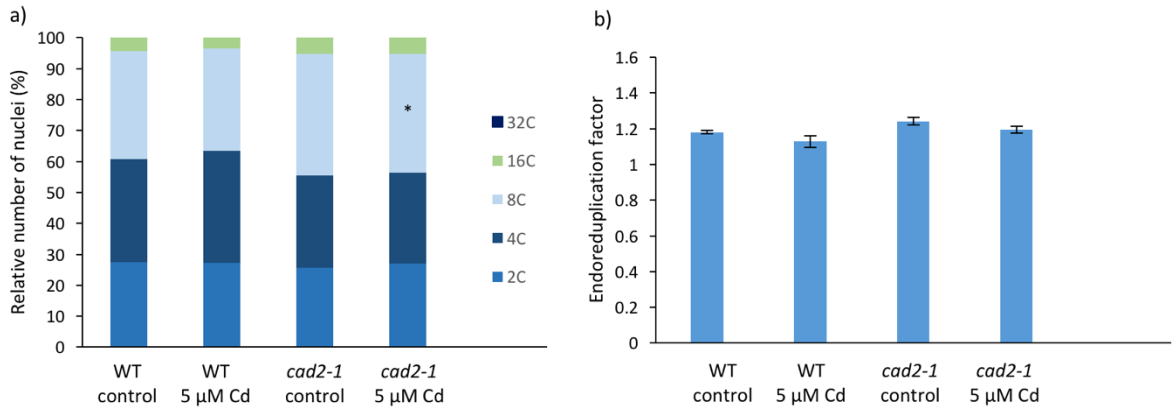

Figure 4: *Ploidy levels and endoreduplication factor in leaf 4 of WT and cad2-1 mutant A. thaliana plants exposed to 5 µM $CdSO_4$ for **72 h**, starting from day 20 after sowing.* (a) Ploidy levels. (b) Endoreduplication factor. Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration ($p < 0.05$; two-way ANOVA). Data represent the average ± S.E. of four biological independent replicates.

In leaf 4, a younger leaf, the endoreduplication factor showed a slightly decreasing trend after Cd exposure in both genotypes (Figure 4b). The percentage of 8C and 16C nuclei showed a decreasing trend in both genotypes when exposed to Cd, in contrast to the number of 4C nuclei, which showed an increasing trend. The proportion of 8C nuclei was significantly higher in the *cad2-1* mutant, as compared to the WT, after 72 h of exposure (Figure 4b).

After 12 days of exposure, the decreasing trend in the endoreduplication factor of leaf 4 became more pronounced (Figure 5b). Cadmium exposure induced a decreasing trend in the percentage of 8C, 16C and 32C nuclei, whereas it slightly increased the proportion of 4C nuclei in leaves of both genotypes. In the WT, the 2C nuclei percentage was significantly increased after Cd exposure (Figure 5a).

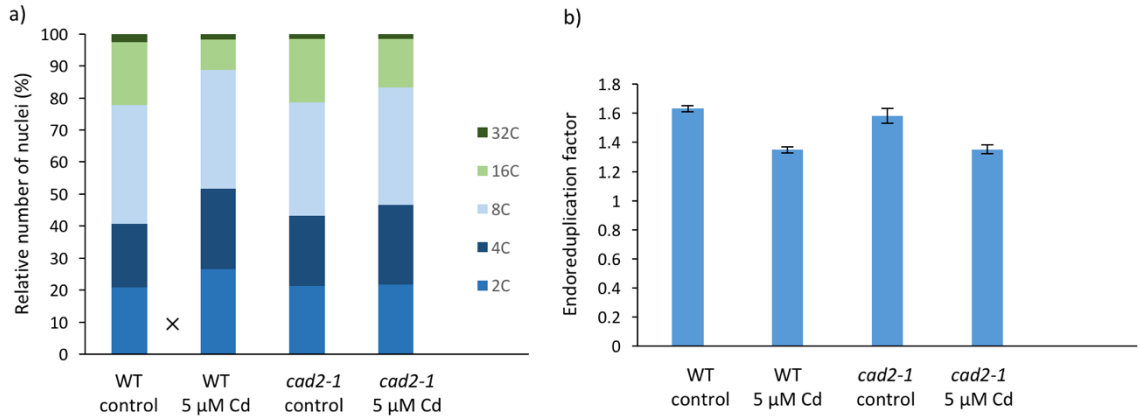

Figure 5: *Ploidy levels and endoreduplication factor in leaf 4 of WT and cad2-1 mutant A. thaliana plants exposed to 5 µM $CdSO_4$ for* ***12 d****, starting from day 20 after sowing.* (a) Ploidy levels. (b) Endoreduplication factor. Significant differences as compared to the control of the same genotype are indicated by "×" ($p < 0.05$; Kruskal-Wallis). Data represent the average ± S.E. of four biological independent replicates.

The same analysis was also performed for leaf 11 after 12 days of exposure, to gain knowledge on the effects of Cd exposure in younger leaves of the plants which had not yet emerged at the start of exposure. The results are shown in Figure 6. Here, the mutant showed a different response to Cd exposure as compared to the wildtype. While WT plants showed a slightly increasing trend in endoreduplication factor, the mutant showed a decreasing trend in endoreduplication factor when exposed to Cd (Figure 6b). This can also be seen in the ploidy levels (Figure 6a). While the proportion of 8C and 16C nuclei remained constant in WT plants, a decreasing trend was observed in the *cad2-1* mutant when exposed to Cd. The number of 4C nuclei showed an increasing trend when exposed to Cd, while the number of 8C, 16C and 32C slightly decreased in both genotypes after Cd exposure. In WT, the proportion of 2C nuclei decreased significantly after Cd exposure. In leaf 11 of *cad2-1* mutant under control conditions, the 2C nuclei percentage was significantly lower, whereas the number of 8C nuclei was significantly higher, as compared to the WT. As shown in Figure 6b, the EF was significantly higher in the *cad2-1* mutant as compared to the WT under control conditions.

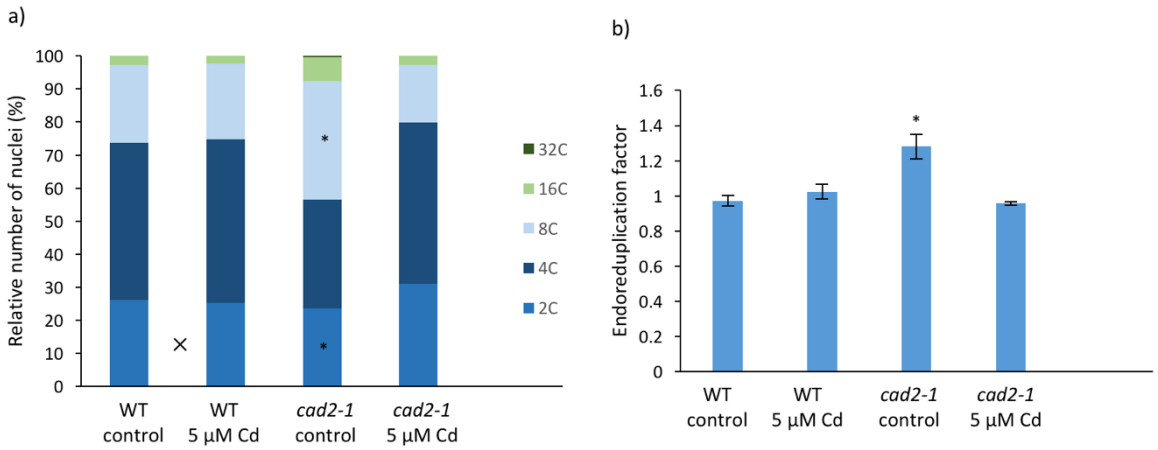

Figure 6: *Ploidy levels and endoreduplication factor in leaf 11 of WT and cad2-1 mutant A. thaliana plants exposed to 5 µM $CdSO_4$ for* ***12 d****, starting from day 20 after sowing.* (a) Ploidy levels. (b) Endoreduplication factor. Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration. Significant differences as compared to the control of the same genotype are indicated by "×" ($p < 0.05$; Kruskal-Wallis). Data represent the average ± S.E. of four biological independent replicates.

3.1.3 Cadmium-induced effects on the number of cells and cell surface area of wild-type plants

In order to determine Cd-induced effects on cell division in leaves of WT plants, the number of abaxial epidermal cells per leaf was calculated based on microscopic data of abaxial cell surface area and leaf surface area. Data of leaves 1 and 2 were combined into one average, as they emerge at the same time point. The obtained results are shown in Figure 7. As shown in Figures 7a and 7c, the cell surface area increased with leaf age, while the cell number decreased with leaf age. Leaves 3 and 5 of Cd-exposed plants had a significantly lower number of cells and a smaller cell surface area as compared to those of control plants. A similar trend was also observed for the other leaves analyzed. Figure 7b shows that leaves of Cd-exposed plants had a significantly smaller surface area in comparison to those of control plants. It is important to note, however, that the leaf surface area showed a similar trend with increasing leaf age in control and Cd-exposed plants.

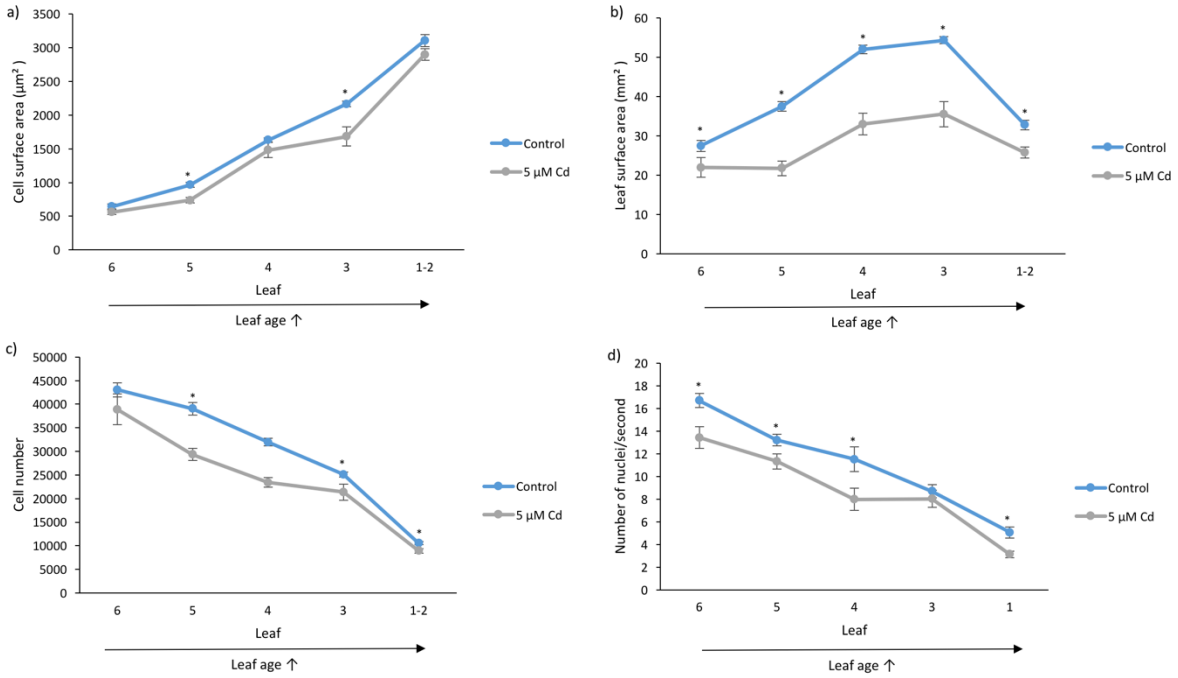

***Figure 7**: Cell surface area, leaf surface area and cell number of WT A. thaliana plants exposed to 5 µM $CdSO_4$ for **72 h**, starting from day 19 after sowing.* (a) Abaxial epidermal cell surface area. (b) Leaf surface area. (c) Abaxial epidermal cell number. (d) Number of nuclei measured per second using flow cytometry. Data represent the average of at least eight biological replicates. Significant differences between control and Cd-exposed leaves are indicated with an asterisk ($p < 0.05$; Student's t-test).

Although no microscopic analysis was performed on leaves of the *cad2-1* mutant, results of this study indicate that the Cd-induced effect on cell number can be estimated based on the number of nuclei that is analyzed using flow cytometry in a specific time range, using the same extraction method and flow cytometer settings for each sample. Indeed, Figure 7d shows that the number of nuclei measured per second shows a decreasing trend with increasing leaf age in WT plants. Furthermore, the number of nuclei measured per second is generally lower in Cd-exposed as compared to control leaves. These

data strongly correspond to those obtained using DIC microscopy (Figure 7c), implying that they can be used to estimate Cd-induced effects on cell number.

Therefore, the number of nuclei measured per second was compared between leaves of WT and *cad2-1* mutant plants grown under control conditions or exposed to 5 µM Cd for 72 h or 12 d. As depicted in Figure 8, the number of nuclei showed a decreasing trend in all leaves of the WT after exposure to Cd. This decrease was most pronounced in leaf 11 after 12 d of Cd exposure. The number of nuclei also showed a decreasing trend in leaves 4 and 11 of the *cad2-1* mutant after 12 d of Cd exposure. Under both control and Cd exposure conditions, the number of nuclei in leaves 1 and 4 of the mutant was slightly higher as compared to the WT. The opposite trend was observed in leaf 11.

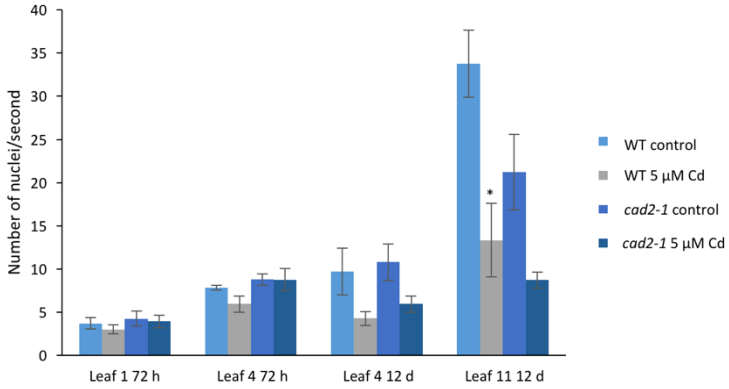

Figure 8: *Number of nuclei measured per second using flow cytometry in leaves 1, 4 and 11 of WT and cad2-1 mutant A. thaliana plants exposed to 5 µM CdSO4 for* ***72 h*** *or* ***12 d****.* Data represent the average of four biological replicates. Asterisks (*) indicate significant differences as compared to the control of the same genotype ($p < 0.05$; two-way ANOVA).

3.2 Long-term effects of cadmium exposure on *Arabidopsis thaliana* plants

3.2.1 Influence of cadmium exposure on vegetative growth of wild-type and cad2-1 mutant plants

To determine the influence of Cd on the vegetative growth of *A. thaliana* plants, 20 day-old seedlings were exposed to 5 µM Cd and followed up phenotypically. The rosette diameter (Figure 9a) was clearly influenced by exposure to Cd. While the rosette diameter of the control plants of both genotypes showed a clear increasing trend of rosette diameter, reaching a plateau phase on day 39, the Cd-exposed plants seemed to have reached this plateau phase at the start of Cd exposure. The final rosette diameter also clearly differed between both conditions and genotypes. The Cd-induced decrease of the final rosette diameter was much more pronounced in the *cad2-1* mutant as compared to WT plants. Under control conditions, the vegetative growth was comparable between both genotypes, although the *cad2-1* mutant reached a larger final rosette diameter.

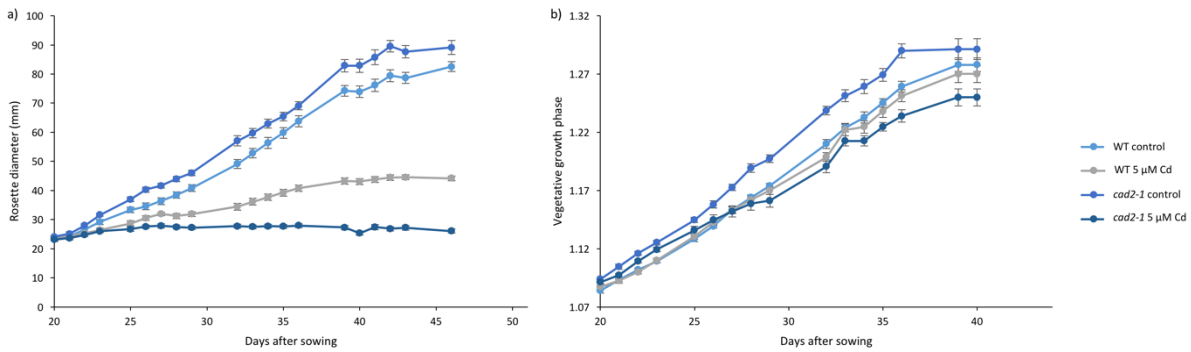

Figure 9: *Vegetative growth of WT and cad2-1 mutant Arabidopsis thaliana plants exposed to 5 µM $CdSO_4$, starting from day 20 after sowing.*(a) *Rosette diameter.* (b) *Leaf development.* Data represent the average ± S.E. of 15 biological independent replicates.

Leaf development showed a similar trend in plants of all conditions, but the final number of leaves differed between conditions (Figure 9b). Cadmium exposure did not clearly affect the vegetative growth phase of WT plants. In contrast, the final number of leaves reached by the *cad2-1* mutant was strongly decreased by Cd exposure. These results show that vegetative growth is affected by Cd exposure. Although both genotypes showed a similar trend when exposed to Cd, the Cd-induced effect was much more pronounced in the GSH-deficient *cad2-1* mutant.

3.2.2 Influence of cadmium on reproductive growth

To determine the influence of Cd on reproductive growth, the plants were further followed up until they reached the stage of silique formation. The moment at which the plants reached growth stage 5.10 (the emergence of the inflorescence meristem) was affected by Cd in a similar way as compared to the other growth stages. Wild-type plants all showed an inflorescence meristem by day 41 (control) or day 43 (Cd-exposed). In the *cad2-1* mutant, an inflorescence meristem was observed in all control plants by day 35, while 100 % of the Cd-exposed plants had one by day 54. These data indicate that, under control conditions, the *cad2-1* mutant showed an earlier inflorescence emergence as compared to the WT. Under Cd exposure conditions, however, WT plants developed an inflorescence meristem earlier than mutant plants. Under control conditions, the height of the inflorescence increased faster in the mutant plants as compared to the WT (Figure 10a). The the opposite effect was observed under Cd exposure conditions, as WT plants showed a sharper increase in inflorescence height over time than the *cad2-1* mutant. Whereas the inflorescence height of the Cd-exposed *cad2-1* mutants had already reached a plateau phase at the end of the experiment, the inflorescence of the other plants was still increasing in height. The final inflorescence height was, again, more strongly decreased by Cd exposure in the mutant as compared to the WT.

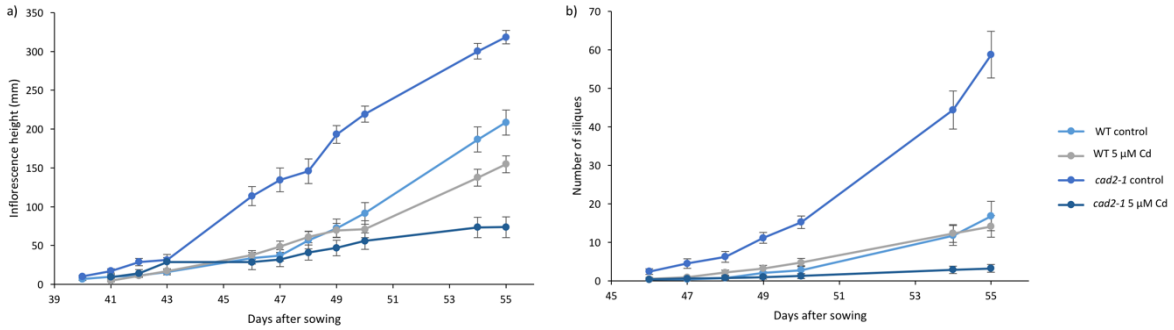

***Figure 10**: Reproductive growth of WT and cad2-1 mutant Arabidopsis thaliana plants exposed to 5 µM $CdSO_4$, starting from day 20 after sowing.* (a) *Inflorescence height.* (b) *Silique formation.* Data represent the average ± S.E. of 15 biological independent replicates.

Silique formation is shown in Figure 10b and showed a trend similar to that of the inflorescence height. Whereas Cd exposure only had a very small decreasing effect on the number of siliques in the WT, the effect was much more pronounced in the *cad2-1* mutant. Under control conditions, the *cad2-1* mutant showed a higher silique formation as compared to the wildtype, whereas the opposite effect was observed under Cd exposure conditions. These findings show that the influence of Cd on the vegetative and reproductive growth of the *cad2-1* mutant is larger as compared to that on WT plants.

3.3 Optimization of a 96-well plate screening system for *Arabidopsis thaliana* plants

3.3.1 Growth of Arabidopsis thaliana in 96-well plates

To determine whether *A. thaliana* seedlings can be grown in 96-well plates for a fast and simple screening of contaminant effects, WT and *cad2-1* mutant *A. thaliana* seedlings were grown in ½ MS medium. In all experiments, the germination percentage was approximately 70% (data not shown). In the first experiment, only WT plants were grown under control conditions to determine the amount of plant material needed for gene expression analysis. In the second experiment, a fungal contamination occurred, after which it was decided to perform future experiments in 96-well plates under a laminar air flow cabinet. In the third experiment, WT and *cad2-1* mutant plants were exposed to 0, 5 and 10 µM Cd. Gene expression analysis of genes related to oxidative stress and cell cycle regulation was performed at different time points to determine when the responses were most pronounced. As the most clear-cut responses were observed after 24 h, this time point was selected for the last experiment. In the fourth and last experiment, the seedlings were exposed to 0, 10 or 20 µM Cd, starting from day 7 after sowing and harvested after 24 h for transcriptional and flow cytometric analysis. All plants survived the treatment, despite being in an early developmental stage as shown in Figure 11. These results show that *A. thaliana* can be grown in a 96-well plate setup.

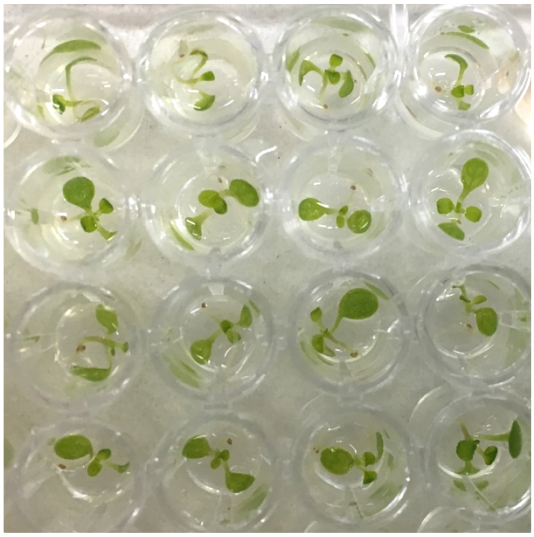

Figure 11: *A. thaliana seedlings in ½ MS medium in 96-well plates.*

3.3.2 Use of 96-well plate growth system for ecotoxicity screening

A first parameter that was measured, was the transcription of genes involved in oxidative stress signaling, cell cycle regulation and DNA repair. Gene expression of WT plants and *cad2-1* mutant *A. thaliana* plants exposed to 0, 10 or 20 µM Cd for 24 h was compared. As shown in Table 7, the oxidative stress hallmark genes *UPOX*, *AT2G43510*, *AT1G19020*, *AT1G05340* and *AT1G57630* were all significantly upregulated in the *cad2-1* mutant exposed to 20 µM Cd, whereas their expression did not change in WT plants. Transcription of the pro-oxidative *RESPIRATORY BURST OXIDASE HOMOLOGUE C* (*RBOHC*) and *RBOHD* was not affected by Cd exposure. The pro-oxidative *LIPOXYGENASE 1* (*LOX1*) was upregulated in *cad2-1* mutant plants exposed to 20 µM Cd. A downregulation of *ALTERNATIVE OXIDASE 1a* (*AOX1a*), involved in oxidative defense, was observed in WT plants exposed to 20 µM Cd. The H_2O_2-neutralizing enzyme *CATALASE 3* (*CAT3*) was significantly downregulated in the *cad2-1* mutant under 20 µM Cd, as was *IRON SUPEROXIDE DISMUTASE 1* (*FSD1*). The expression of *ETHYLENE*

RESPONSIVE FACTOR 1 (*ERF1*), involved in oxidative signaling, was significantly increased in WT plants after exposure to 10 and 20 µM Cd. A similar trend was observed in the *cad2-1* mutant.

SIAMESE-RELATED 4 (*SMR4*) and *SMR5* were significantly downregulated in the *cad2-1* mutant after exposure to 10 and 20 µM Cd. For *SMR7*, a similar decreasing trend was observed in the mutant, although only significant after 20 µM Cd. Expression levels of *SMR4*, *SMR5* and *SMR7* were not affected

***Table 7**: Gene expression of WT and cad2-1 mutant A. thaliana plants exposed to 10 or 20 µM CdSO4 for **24 h**, starting from day 7 after sowing.* Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration. Colors indicate significant differences as compared to the control of the same genotype. Green and orange colors represent upregulations and downregulations, respectively ($p < 0.05$; 2-way ANOVA). Data represent the average ± S.E. of four biological independent replicates and were normalized based on the expression of FBOX, UBC and PPR.

Gene	Cd (uM)	Wild-type	*cad2-1*	Gene	Cd (uM)	Wild-type	*cad2-1*
Oxidatives stress and signaling				**Oxidatives stress and signaling (continued)**			
UPOX	0	1.00 ± 0.03	1.29 ± 0.10	*CAT3*	0	1.00 ± 0.05	1.34 ± 0.07
	10	0.86 ± 0.06	1.30 ± 0.11		10	0.79 ± 0.06	1.17 ± 0.11*
	20	0.92 ± 0.04	2.03 ± 0.36*		20	0.92 ± 0.05	0.76 ± 0.07
AT2G43510	0	1.00 ± 0.24	1.04 ± 0.15	*FSD1*	0	1.00 ± 0.05	1.02 ± 0.13
	10	0.96 ± 0.08	1.18 ± 0.04		10	1.04 ± 0.03	1.05 ± 0.06
	20	0.99 ± 0.14	2.27 ± 0.26*		20	1.13 ± 0.09	0.59 ± 0.10*
AT1G19020	0	1.00 ± 0.11	1.32 ± 0.10	*ERF1*	0	1.00 ± 0.31	1.53 ± 0.16
	10	1.10 ± 0.08	1.84 ± 0.12*		10	2.13 ± 0.06	2.93 ± 0.31
	20	1.03 ± 0.13	4.11 ± 0.15*		20	2.18 ± 0.38	2.93 ± 0.34
AT1G05340	0	1.00 ± 0.12	1.10 ± 0.09	**Cell cycle**			
	10	0.86 ± 0.08	1.31 ± 0.07	*CDK inhibitors*			
	20	0.79 ± 0.05	2.26 ± 0.29*	*SMR4*	0	1.00 ± 0.05	1.22 ± 0.05
AT1G57630	0	1.00 ± 0.12	0.93 ± 0.11		10	1.02 ± 0.02	1.06 ± 0.06
	10	1.14 ± 0.07	1.25 ± 0.18		20	1.11 ± 0.04	0.57 ± 0.13*
	20	1.10 ± 0.14	2.51 ± 0.62*	*SMR5*	0	1.00 ± 0.03	1.06 ± 0.06
RBOHC	0	1.00 ± 0.04	1.06 ± 0.03		10	0.59 ± 0.02	0.49 ± 0.07
	10	1.07 ± 0.06	1.09 ± 0.06		20	0.48 ± 0.02	0.24 ± 0.09*
	20	1.16 ± 0.04	1.00 ± 0.09	*SMR7*	0	1.00 ± 0.12	2.48 ± 0.21*
RBOHD	0	1.00 ± 0.04	1.07 ± 0.05		10	0.84 ± 0.08	1.42 ± 0.22
	10	0.95 ± 0.10	1.06 ± 0.05		20	0.80 ± 0.06	0.88 ± 0.22
	20	0.96 ± 0.03	1.20 ± 0.05	**DNA damage and repair**			
LOX1	0	1.00 ± 0.10	1.33 ± 0.14	*PARP1*	0	1.00 ± 0.01	1.14 ± 0.10
	10	1.00 ± 0.10	1.46 ± 0.09		10	0.85 ± 0.03	0.99 ± 0.04
	20	1.13 ± 0.05	2.92 ± 0.22*		20	1.00 ± 0.01	0.83 ± 0.08
AOX1a	0	1.00 ± 0.06	1.32 ± 0.17	*PARP2*	0	1.00 ± 0.12	1.24 ± 0.00
	10	0.70 ± 0.05	1.20 ± 0.07*		10	0.82 ± 0.03	1.25 ± 0.03*
	20	0.67 ± 0.03	1.70 ± 0.16*		20	0.93 ± 0.05	0.88 ± 0.07
CAT1	0	1.00 ± 0.03	1.29 ± 0.12	*BRCA1*	0	1.00 ± 0.06	1.15 ± 0.11
	10	0.82 ± 0.03	1.13 ± 0.09*		10	0.90 ± 0.03	0.92 ± 0.11
	20	0.87 ± 0.02	1.33 ± 0.11*		20	0.82 ± 0.00	0.82 ± 0.06
CAT2	0	1.00 ± 0.00	0.94 ± 0.06				
	10	1.04 ± 0.10	0.96 ± 0.13				
	20	1.11 ± 0.11	0.77 ± 0.15				

by Cd exposure in WT plants. Transcription of *PARP1* and *PARP2* was significantly downregulated in

the *cad2-1* mutant exposed to 20 µM Cd. The expression of *BRCA1* was not affected by Cd exposure in either of the genotypes.

The second parameter measured in plants grown in 96-well plates, was the ploidy level. The results of the flow cytometric analysis can be found in Figure 12. No significant differences were observed between the different exposure conditions and genotypes.

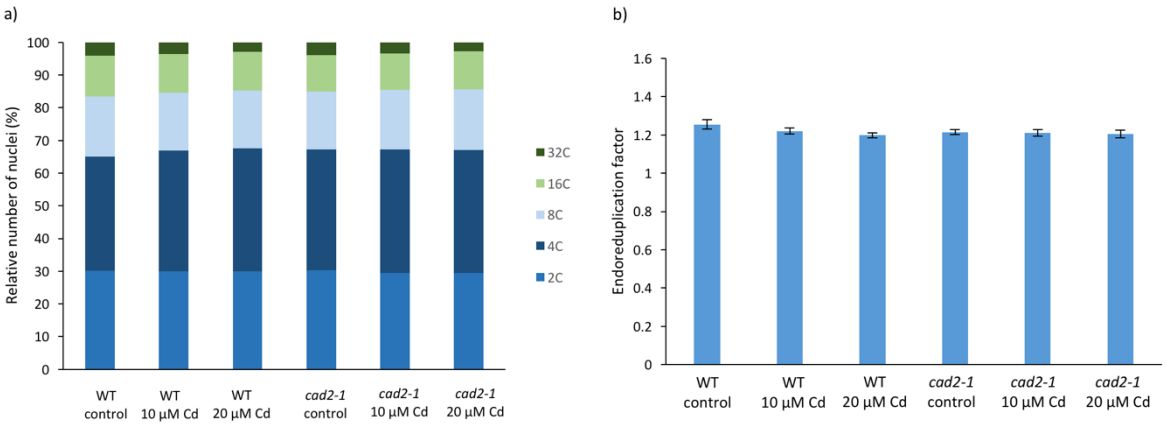

Figure 12: *Ploidy levels and endoreduplication factor in WT and cad2-1 mutant A. thaliana plants exposed to 10 or 20 µM $CdSO_4$ for **72 h**, starting from day 7 after sowing*. (a) *Ploidy levels.* (b) *Endoreduplication factor.* Data represent the average ± S.E. of eight biological independent replicates.

To relate the ploidy levels to the number of cells present in the plants, the number of nuclei measured per second using flow cytometry was calculated (Figure 13). Whereas the number of nuclei was not affected by Cd exposure in the *cad2-1* mutant, it showed an increasing trend in WT plants.

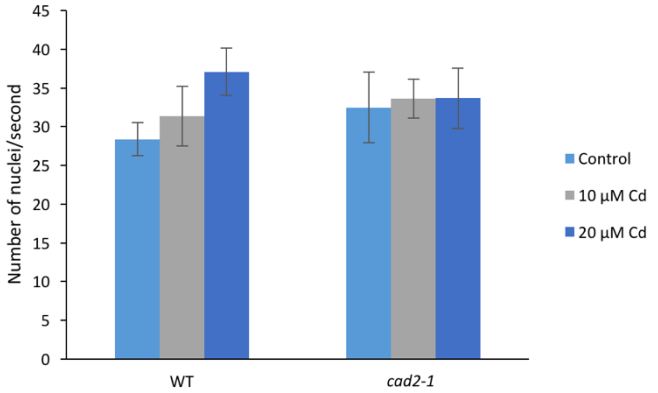

Figure 13: *Number of nuclei measured per second using flow cytometry in WT and cad2-1 mutant A. thaliana plants exposed to 10 or 20 µM $CdSO_4$ for **72 h**, starting from day 7 after sowing.* Data represent the average ± S.E. of eight biological independent replicates.

4. DISCUSSION

This study aimed the involvement of GSH in Cd-induced effects on developmental processes in *A. thaliana*. To this end, the effect of Cd exposure on the transcription of genes related to oxidative stress, cell cycle regulation and DNA damage and repair, nuclear ploidy levels and phenotypic parameters was determined and compared between WT plants and the GSH-deficient *cad2-1* mutant. Furthermore, a toxicity screening assay using *A. thaliana* plants grown in 96-well plates was optimized in the context of this study.

4.1 Effects of cadmium exposure on *Arabidopsis thaliana* leaves

In a first part of the project, effects of Cd exposure on (1) the expression of genes related to oxidative stress, cell cycle regulation and DNA damage and repair and (2) the extent of endoreduplication and cell division were investigated in WT and *cad2-1* mutant *A. thaliana* plants.

4.1.1 Cadmium-induced oxidative stress and its effects on cell cycle regulation and DNA repair are altered in a GSH-deficient and a PC-deficient mutant

To investigate the differences in response to 5 µM Cd between WT, *cad2-1* and *cad1-3* mutant *A. thaliana* leaves, a gene expression analysis of genes related to oxidative stress, cell cycle regulation and DNA damage and repair was performed.

The oxidative stress hallmark genes – characterized by a more than 5-fold upregulation under different oxidative stress-inducing conditions (Gadjev *et al*., 2006) – were significantly upregulated by Cd exposure in leaves of both WT and *cad2-1* mutant plants after 24 h (30). After 72 h, they were still upregulated in the *cad2-1* mutant, but not in the WT (Table 6). This upregulation of the oxidative stress hallmark genes in the *cad2-1* mutant was also observed by Jozefczak *et al.* (2015) and indicates that the mutant still experiences oxidative stress after 72 h of exposure, as opposed to the WT (31). These data indicate that GSH is involved in mitigating oxidative stress under Cd exposure conditions. In the *cad1-3* mutant, the oxidative stress hallmark genes (except *AT1G57630*) were also upregulated after 72 h of exposure, implying that the role of GSH in attenuating oxidative stress is mainly due to its function as a precursor for PC synthesis.

The expression of S phase and G_2-M phase marker genes showed a decreasing trend in leaves of both WT and *cad2-1* mutant plants after 24 h exposure to 5 µM Cd (Table 5). After 72 h of Cd exposure, their expression slightly increased in WT, but remained decreased in the mutant. The effect of Cd exposure on the transcription of these genes in the PC-deficient *cad1-3* mutant was comparable to

that in the *cad2-1* mutant. These results could indicate an inhibition of progression through the cell cycle in all genotypes after 24 h. After 72 h, cell cycle activity is restored – and even slightly increased – in the WT, whereas it remains suppressed in the mutants. A potential explanation for these observations is that GSH is (indirectly) involved in cell cycle regulation under Cd exposure conditions, possibly as a consequence of its function as a precursor of PC synthesis. The increased extent of oxidative stress in the mutants might lead to a larger extent of DNA damage, thereby affecting cell cycle progression. However, it is important to note that changes in the expression of genes encoding cyclins and CDKs are not necessarily reflected by changes in CDK activity. Therefore, future studies should aim to investigate Cd-induced effects on CDK activity and to compare these between WT, *cad2-1* and *cad1-3* mutant plants.

The expression of *SMR4*, *SMR5* and *SMR7* increased in leaves of WT plants after 24 and 72 h of Cd exposure (Table 5 and 6). Yi *et al.* have shown that ROS-induced DNA damage causes an upregulation of these *SMR* genes (32). The higher expression of the oxidative stress hallmark genes and DNA damage marker genes – *PARP2* and *BRCA1* – in leaves of WT plants exposed to 5 µM Cd suggests that these plants suffer from DNA damage and that the pathway described by Yi *et al.* (2014) is also activated under Cd exposure conditions. In the *cad2-1* mutant, *SMR4* and *SMR7* were downregulated at both time points, as was the case for the *cad1-3* mutant at 72 h. Interestingly, the Cd-induced upregulation of DNA damage marker genes was not observed in the GSH- and PC-deficient mutants, implying that GSH and/or PC levels affect this SMR-regulated DNA damage response.

Taken together, these findings suggest that GSH is either directly or indirectly involved in the regulation of the oxidative stress-induced DNA damage response and consequent cell cycle arrest under Cd stress. This effect is likely related to its role as a PC precursor.

4.1.2 Glutathione influences the effect of cadmium exposure on the number of endocycles

To determine the effects of Cd exposure on the extent of endoreduplication in WT and *cad2-1* mutant *A. thaliana* plants, nuclear ploidy levels of leaves 1, 4 and 11, exposed to 5 µM Cd for 72 h and 12 d, were determined using flow cytometry. In leaf 1 – the oldest leaf – Cd exposure did not affect the ploidy levels (Figure 3a). As leaf 1 was already mature at the start of exposure, cell cycle activity in this leaf was very low, possibly explaining why it was not affected by Cd exposure. In contrast, the EF of leaf 4 showed a decreasing trend in both genotypes after 72 h of Cd exposure (Figure 4b). This effect became more pronounced after 12 d of exposure. In general, ploidy levels of leaf 4 were higher after 12 d as compared to 72 h of exposure in both control and exposed plants (Figure 4a and 5a). This indicates that, although Cd-exposed plants had lower ploidy levels than control plants, they were still capable of endoreduplication. These data are also in agreement with the fact that the extent of endoreduplication increases with leaf age (preliminary data; (33, 34)). In leaf 11 of WT plants, nuclear ploidy levels were slightly increased after exposure to 5 µM Cd for 12 d (Figure 6a). The opposite effect was observed in leaf 11 of the mutant.

The general decrease in ploidy levels after Cd exposure can possibly be explained by the fact that DNA replication is stalled in order to repair Cd-induced DNA damage. This hypothesis is supported by the observed upregulation of DNA repair genes and by the increased expression of *SMR* genes, which can inhibit CDK activity and thereby inhibit cell cycle progression (Table 5 and 6). However, the data obtained in this project are in contrast to those of Adachi *et al.* (2011), who observed that DSB-inducing agents such as zeocin increased the extent of endoreduplication in roots of *A. thaliana* (23). The current experiment, however, considered a different organ and used a different culture system. Furthermore, it is possible that Cd exposure induces a different type of DNA damage than DSBs, causing a different effect on endoreduplication. In this context, it is important to note that DNA damage was not directly measured in this study, but rather indirectly by measuring the transcription of DNA damage marker genes and DNA repair genes. Future studies should therefore aim to directly measure the extent and type of DNA damage induced by Cd exposure in *A. thaliana* leaves.

Furthermore, data indicate that the ploidy levels in leaves of the *cad2-1* mutant are higher as compared to those in leaves of WT plants under control conditions. This could be related to the fact that the leaves of the mutant are slightly older as they emerge earlier than those of the WT, as was observed in the long-term follow-up. As ploidy levels are known to increase with increasing leaf age, an earlier emergence of the leaves could explain their higher ploidy levels (33, 34).

Taken together, these results show that Cd exposure affects the extent of endoreduplication in leaves of WT and *cad2-1* mutant *A. thaliana* plants. However, Cd-induced effects on nuclear ploidy levels differ between both genotypes, confirming that GSH is involved in cell cycle regulation.

4.1.3 Cadmium exposure decreases cell number, cell surface area and leaf surface area in wild-type and cad2-1 mutant A. thaliana plants

To assess the effect of Cd for 72 h on the extent of cell division in leaves of WT plants, the number of abaxial epidermal cells per leaf was calculated based on microscopic data of abaxial cell surface area and leaf surface area. The number of cells of WT and *cad2-1* mutant leaves was also estimated based on the number of nuclei analyzed using flow cytometry in a specific time range. In leaves of both exposed and control plants, the cell number decreased with increasing leaf age. Furthermore, data show that the number of cells was lower in leaves of Cd-exposed plants as compared to control plants of both genotypes, suggesting a Cd-induced inhibition of cell division (Figure 7). These results are in agreement with the hypothesis that Cd stress induces DNA damage, thereby blocking or slowing down cell cycle progression.

Furthermore, results show that the cell surface area increased with increasing leaf age. As cell surface area is known to correlate strongly with nuclear ploidy levels, these data suggest that the extent of endoreduplication is higher in older as compared to younger leaves (17, 18, 35). This is in agreement with data available in literature (36) and is supported by the results obtained using flow cytometry. In addition, leaves of Cd-exposed plants had a smaller cell surface area as compared to those of control plants, suggesting that Cd exposure reduces the extent of endoreduplication. Again, this is supported by the data obtained by flow cytometric analysis of nuclear ploidy levels.

Results also indicate that leaf surface area increased with leaf age until leaf 3. At day 22 after sowing, leaf 1 and 2 were generally smaller than the younger leaves. This can be explained by the fact that older leaves, such as leaf 1 and 2, switch to endoreduplication earlier during their development and therefore undergo fewer cell divisions than younger leaves. In contrast, cells in younger leaves continue dividing for a longer period of time as compared to leaves 1 and 2, thereby obtaining a higher number of cells that can subsequently increase in size via endoreduplication. This could clarify why younger leaves reach a larger leaf surface area as compared to leaves 1 and 2.

In the *cad2-1* mutant, the number of nuclei was mainly affected after 12 d of exposure. Leaves 4 and 11 of the Cd-exposed *cad2-1* mutant showed a decreased number of nuclei after 12 d of exposure (Figure 5 and 6). The number of nuclei of leaves 1 and 4 after 72 h of Cd exposure are not affected,

which corresponds to the results obtained by flow cytometry. These data suggest that cell division and endoreduplication are not influenced by Cd exposure. Leaf 11 of the *cad2-1* mutant had a lower number of nuclei as compared to the WT under control conditions. This can be explained by the higher endoreduplication factor observed, as this leaf of the mutant may have switched to endoreduplication earlier than the WT. As mentioned before, the leaves of the mutant emerge earlier, possibly explaining why the extent of endoreduplication is higher in leaf 11 of the *cad2-1* mutant than in that of the WT under control conditions. These findings, again, confirm a role for GSH as a regulator of the cell cycle and plant development, both under control and Cd exposure conditions.

Taken together, these data suggest that the Cd-induced disturbance of leaf growth and development is – at least partially – due to an inhibition of cell division and endoreduplication. So far, the effect of Cd on the plant cell cycle has only been investigated in cell cultures. Sobkowak *et al*., for example, showed that Cd exposure causes DNA damage and decreases DNA synthesis in soy bean cell cultures, which is accordance with the findings of this study (37).

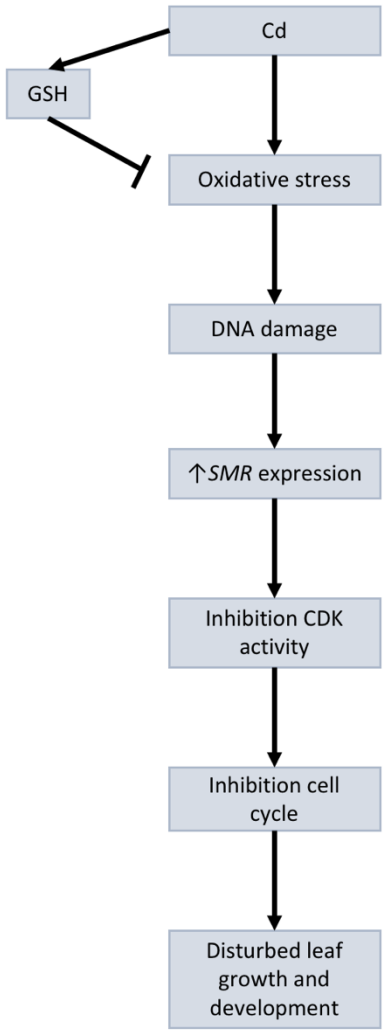

Figure 14: *Proposed working model for Cd-induced effects on developmental processes in A. thaliana.*

Based on the results obtained in this part of the project, a working model is proposed (Figure 14). This model hypothesizes that Cd exposure causes oxidative stress, which leads to DNA damage. This in turn results in an upregulation of *SMR* expression, which suppresses CDK activity. As a result, cell cycle progression is inhibited, ultimately leading to a disturbed leaf growth and development. In WT plants, GSH alleviates the Cd-induced oxidative stress, both directly by sequestering Cd and indirectly by detoxification of ROS. In the GSH-deficient mutant used, however, this DNA damage response and upregulation of *SMR* expression were not observed, suggesting a regulatory role for GSH in this pathway.

4.2 Long-term effects of cadmium exposure on *Arabidopsis thaliana* plants

4.2.1 Cadmium severely compromises vegetative growth in the cad2-1 mutant

In order to determine the effect of Cd exposure on the vegetative growth of WT and *cad2-1* mutant *A. thaliana* plants, 20 day-old seedlings were exposed to 5 µM Cd and followed up phenotypically. Both rosette diameter and leaf development were compromised in Cd-exposed plants. This effect was observed in both genotypes, but the effect was more pronounced in the *cad2-1* mutant. The results obtained in WT plants were in accordance to those reported by Keunen *et al.* (2011), who demonstrated a Cd-induced inhibition of vegetative growth in *A. thaliana*. The stronger effect of Cd exposure in the mutant can be explained by its increased Cd sensitivity (12, 31, 38). These results also confirm that GSH is indeed an important regulator of developmental processes in Cd-exposed plants. Under control conditions, the *cad2-1* mutant had a larger rosette diameter and developed more leaves than the WT, which indicates that its lowered GSH content does not compromise leaf growth and development in absence of Cd. The leaves of the mutant also emerged earlier than those of the WT. These results are in accordance to those of Ogawa *et al*. (2001), who reported a higher number of rosette leaves in *cad2-1* mutant as compared to WT plants grown under control conditions (39). Furthermore, Jozefczak *et al*. (2015) demonstrated that the rosette fresh weight of the *cad2-1* mutant was higher than that of the WT (31). Taken together, these data support the hypothesis that GSH regulates vegetative development of *A. thaliana*, both under control and Cd exposure conditions.

4.2.2 Cadmium-exposed cad2-1 mutants reach reproductive maturity

To assess the influence of Cd exposure on the reproductive growth, WT and *cad2-1 A. thaliana* plants were exposed to 5 µM Cd from day 22 after sowing and phenotypically followed up until the stage of silique formation. In addition to the vegetative growth, the emergence of the inflorescence meristem was also influenced by Cd. The control plants showed an inflorescence meristem by day 41 (WT) or day 35 (*cad2-1* mutant), whereas in the Cd-exposed plants the inflorescence meristem emerged by day 43 (WT) or day 54 (*cad2-1* mutant). Genotype- and Cd-dependent effects on inflorescence height and silique formation were similar as compared to those on vegetative growth. In general, the mutant developed faster than the WT under control conditions, whereas it developed much slower when exposed to Cd. Although its growth was severely compromised by Cd exposure, the mutant still reached the reproductive state. This indicates that even though their antioxidative defense system is weakened, GSH deficiency does not prevent *cad2-1* mutants from reaching the reproductive stage.

4.3 Optimization of a 96-well plate screening system for *Arabidopsis thaliana* plants

4.3.1 Oxidative stress hallmark and SIAMESE-RELATED genes as biomarkers for Cd stress

To determine which genes could be used as biomarkers for Cd stress or for the identification of Cd-sensitive mutants in a quick ecotoxicity screening, WT and *cad2-1* mutant plants were grown in ½ MS medium in 96-well plates and exposed to 10 or 20 µM Cd for 24 h. Phenotypically, none of the plants showed a Cd-induced growth inhibition (data not shown). Therefore, it is proposed to use higher Cd concentrations and a larger range of concentrations in future experiments.

The effect of Cd exposure on the expression of the oxidative stress hallmark genes and *SMR* genes clearly differed between both genotypes. Indeed, all oxidative stress hallmark genes were upregulated in the mutant exposed to 20 µM Cd, while remaining unaffected in the WT. This response was expected, as the mutant is known for its increased sensitivity to Cd. The expression of the *SMR* was generally downregulated in the mutant exposed to 10 and 20 µM Cd. In the WT, this effect was only observed for *SMR5*. These effects were similar to those observed in hydroponic culture, validating this technique to differentiate Cd-induced responses of *cad2-1* mutants from those of WT plants. These findings suggest that the expression of the oxidative stress hallmark genes and *SMR* genes can be used as a biomarker for oxidative stress in the 96-well screening system. As the *SMR* genes responded strongly to Cd exposure in the mutant, we suggest to also study *SMR*-deficient *A. thaliana* mutants in this system. Indeed, it would be interesting to use a wide array of mutants in order to further optimize the proposed toxicity screening system.

4.3.2 Short-term cadmium exposure does not alter ploidy levels in Arabidopsis thaliana grown in 96-well plates

To identify whether the extent of endoreduplication could be used as a biomarker for Cd stress in plants grown in 96-well plates, nuclear ploidy levels of WT and *cad2-1* mutant plants, exposed to 10 or 20 µM Cd for 72 h were measured using flow cytometry. As opposed to the effects observed in plants grown in hydroponics, no clear effects of Cd exposure were seen in the 96-well set-up. There are several potential explanations for the discrepancy between results obtained in hydroponics and 96-well plates. First of all, a cell cycle takes approximately 24 h to complete, which could explain why no effects were observed after 72 h. This could be solved by exposing the plants for a longer time period or from an earlier time point during their development. In addition, the used Cd concentrations may not have been high enough to induce effects on endoreduplication in the 96-well cultivation system. Therefore, it could be interesting to use higher Cd concentrations and a broader concentration range. Furthermore, the flow cytometric analysis was performed on entire plants instead of separate leaves – as is done in hydroponics. Therefore, it could be useful to separate leaves from roots and analyze

ploidy levels in both organs separately. Finally, the plants grown in 96-well plates are in a different developmental stage as compared to those grown in hydroponics at the time of harvesting. As the extent of endoreduplication is strongly related to the leaf developmental stage, this could also explain the difference in response between plants grown in hydroponics and plants grown in 96-well plates.

In addition, the number of nuclei measured within a specific time range using flow cytometry was calculated to give an estimation of the number of cells present in the samples analyzed. The number of nuclei was not affected by Cd exposure in the mutant, whereas it showed an increasing trend in the WT. This indicates a Cd-induced increase of cell division in the WT. Although the effect is small, the increase in cell number suggests an attempt to compensate for the slightly decreased extent of endoreduplication.

In general, ploidy levels could possibly be used as a biomarker for Cd stress, although further optimization is required.

5. CONCLUSION

To conclude, the mechanism by which Cd causes cell cycle arrest and subsequent inhibition of leaf growth and development is hypothesized to be caused by oxidative stress-induced DNA damage. Cadmium is suggested to lead to DNA damage via oxidative stress induction. This DNA damage causes an increase in *SMR* expression and a subsequent inhibition of CDK activity, efficiently blocking cell cycle progression. This inhibition of the cell cycle could then disturb leaf growth and development. Glutathione alleviates the Cd-induced oxidative stress, both directly and indirectly. The GSH-deficient *cad2-1* mutant, however, did not show this DNA damage response to Cd nor the upregulation of *SMR*, suggesting that GSH is an important regulator is this pathway. For future experiments, it would be interesting to measure DNA damage directly in plants exposed to cadmium to further establish the link between Cd and the cell cycle as well the role of GSH therein.

From the second part of the study, it is concluded that *A. thaliana* grown in 96-well plates can indeed be used for Cd toxicity screening. In this experiment, the oxidative stress hallmark genes and *SMR* genes, responded most strongly in the *cad2-1* mutant and are therefore suggested as biomarkers for Cd toxicity. It would be interesting to include for example *SMR*-deficient mutants in future experiments for a more in-depth validation of the system. Moreover, further optimization is needed for the ploidy levels to be used as an effective biomarker in this setup.

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7. SUPPLEMENTAL DATA

7.1 Figure S1: Ploidy levels of leaf 1 of WT and *cad2-1* mutant *A. thaliana* exposed to 5 µM Cd for 72 h

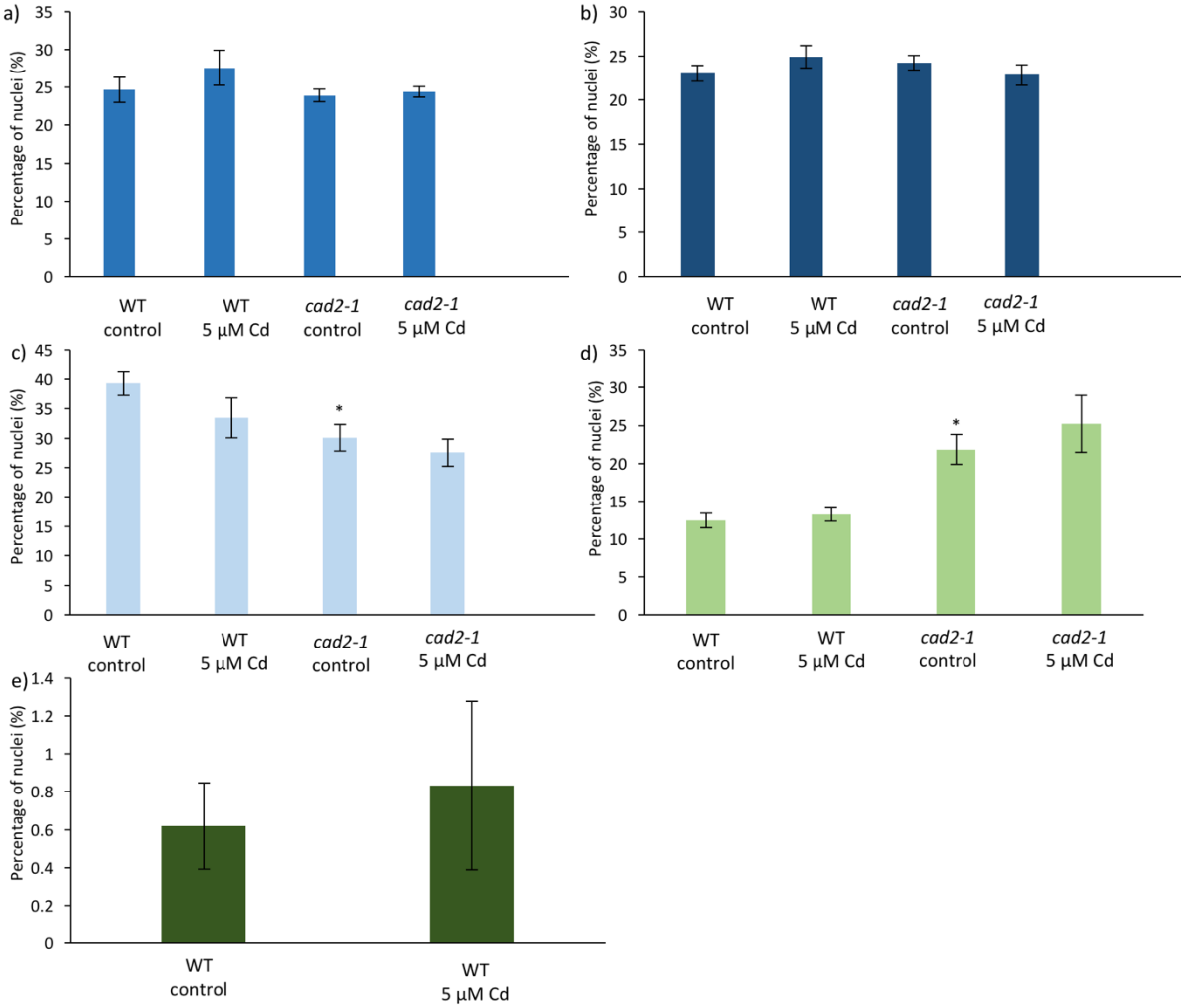

Figure 15: *Ploidy levels of leaf 1 of WT and cad2-1 mutant A. thaliana exposed to 5 µM Cd for* ***72 h****, starting from day 20 after sowing*. (a) Percentage of 2C nuclei. (b) Percentage of 4C nuclei. (c) Percentage of 8C nuclei. (d) Percentage of 16C nuclei. (e) Percentage of 32C nuclei. Data represent the average ± S.E. of 4 biological independent replicates. Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration ($p < 0.05$; two-way ANOVA).

7.2 Figure S2: Ploidy levels of leaf 4 of WT and *cad2-1* mutant *A. thaliana* exposed to 5 µM Cd for 72 h

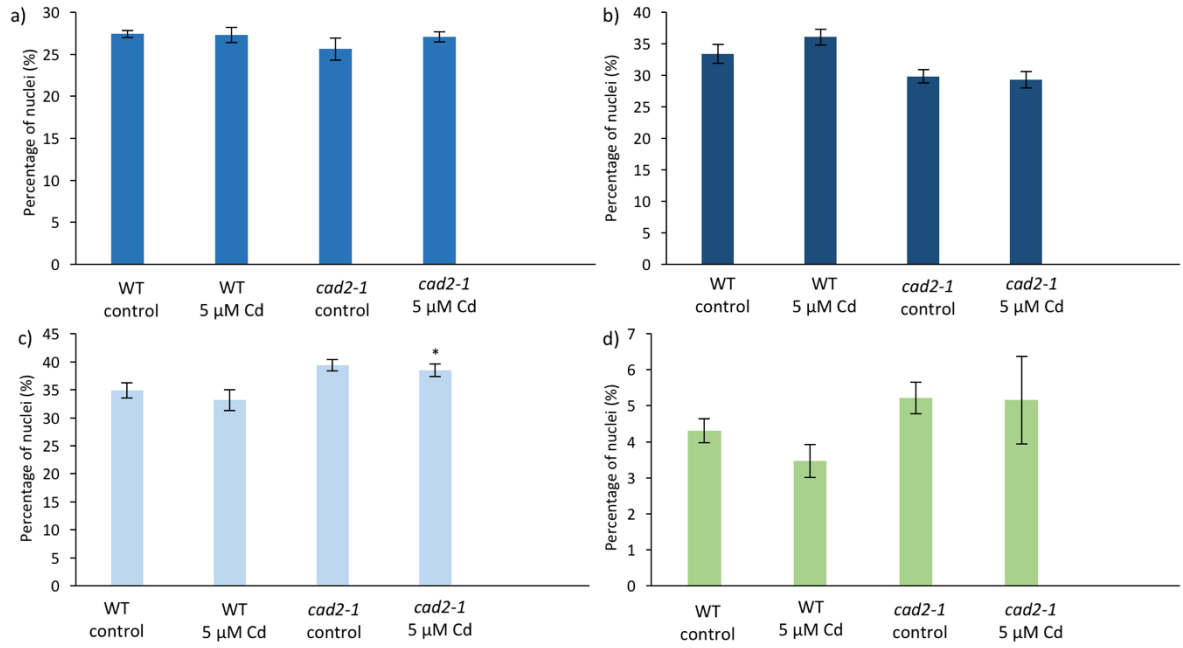

Figure 16: *Ploidy levels of leaf 4 of WT and cad2-1 mutant A. thaliana exposed to 5 µM Cd for* ***72 h****, starting from day 20 after sowing.* (a) Percentage of 2C nuclei. (b) Percentage of 4C nuclei. (c) Percentage of 8C nuclei. (d) Percentage of 16C nuclei. Data represent the average ± S.E. of 4 biological independent replicates. Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration ($p < 0.05$; two-way ANOVA).

7.3 Figure S3: Ploidy levels of leaf 4 of WT and *cad2-1* mutant *A. thaliana* exposed to 5 µM Cd for 12 d

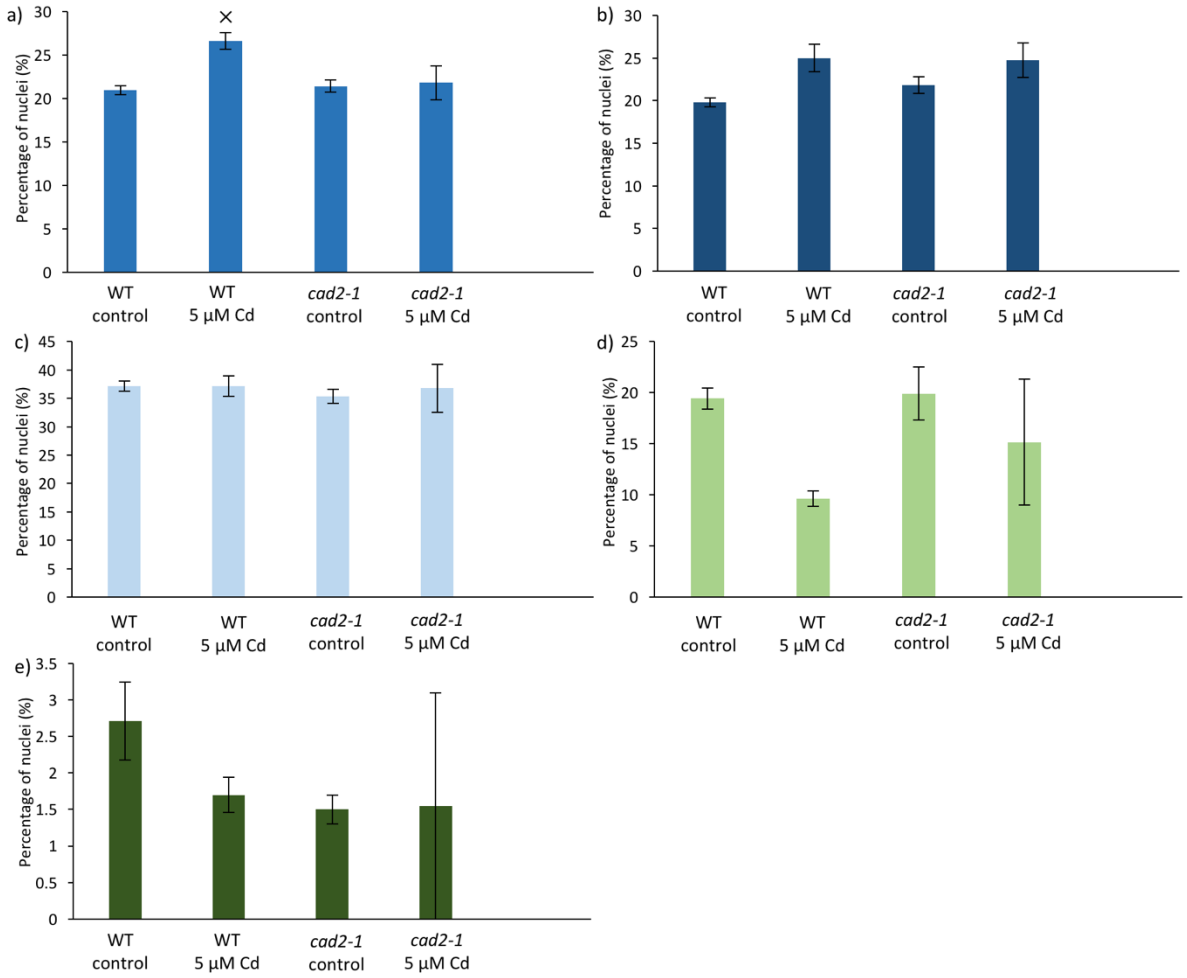

Figure 17: *Ploidy levels of leaf 4 of WT and cad2-1 mutant A. thaliana exposed to 5 µM Cd for* ***12 d****, starting from day 20 after sowing.* (a) Percentage of 2C nuclei. (b) Percentage of 4C nuclei. (c) Percentage of 8C nuclei. (d) Percentage of 16C nuclei. (e) Percentage of 32C nuclei. Data represent the average ± S.E. of 4 biological independent replicates. Significant differences as compared to the control of the same genotype are indicated by "×" ($p < 0.05$; Kruskal-Wallis).

7.4 Figure S4: Ploidy levels of leaf 11 of WT and *cad2-1* mutant *A. thaliana* exposed to 5 µM Cd for 12 d

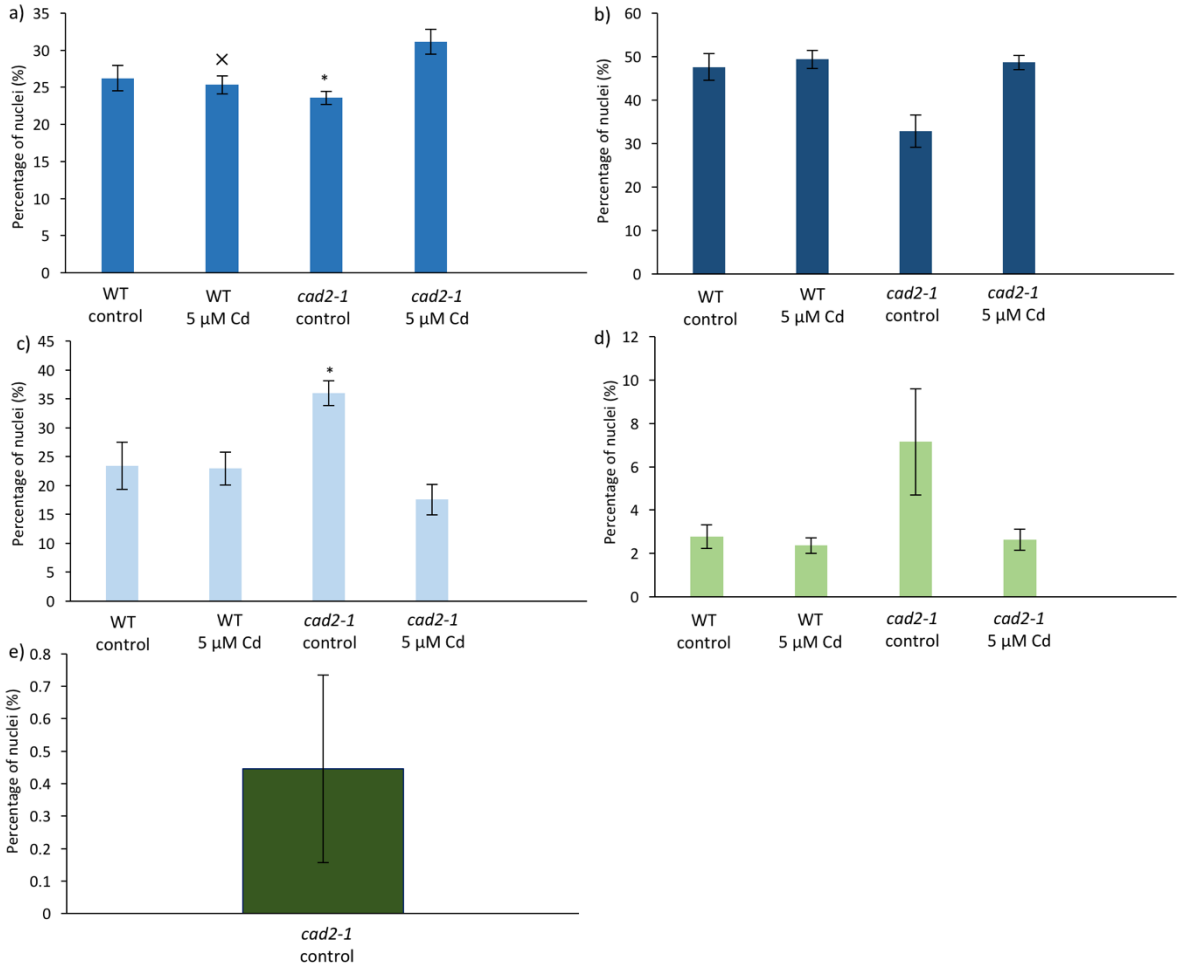

Figure 18: *Ploidy levels of leaf 4 of WT and cad2-1 mutant A. thaliana exposed to 5 µM Cd for* ***12 d****, starting from day 20 after sowing*. (a) Percentage of 2C nuclei. (b) Percentage of 4C nuclei. (c) Percentage of 8C nuclei. (d) Percentage of 16C nuclei. (e) Percentage of 32C nuclei. Data represent the average ± S.E. of 4 biological independent replicates. Asterisks (*) indicate significant differences as compared to the WT exposed to the same Cd concentration. Significant differences as compared to the control of the same genotype are indicated by "×" ($p < 0.05$; Kruskal-Wallis).

7.5 Figure S5: Ploidy levels of WT and *cad2-1* mutant *A. thaliana* plants exposed to 10 or 20 µM Cd for 72 h

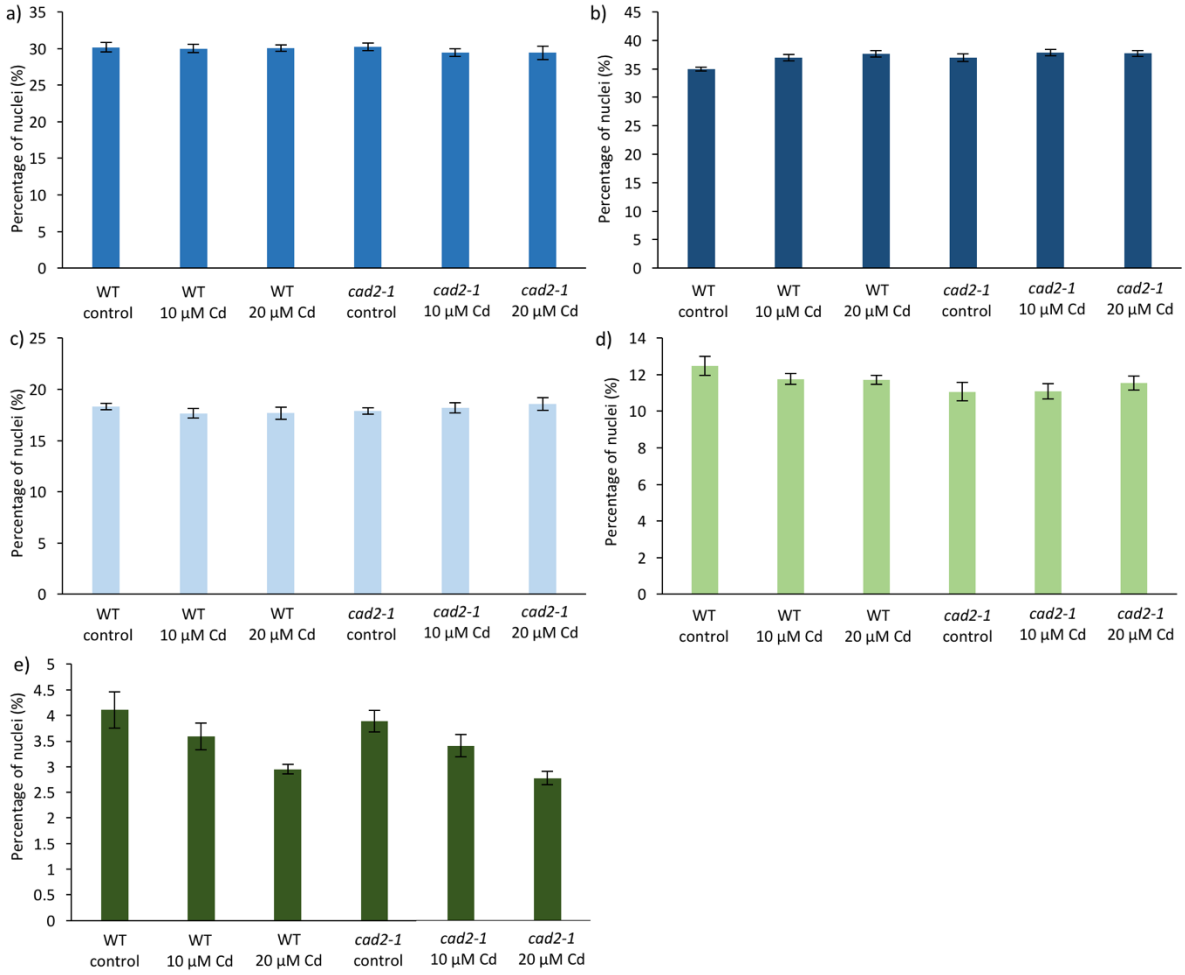

***Figure 19**: Ploidy levels of WT and cad2-1 mutant A. Thaliana plants exposed to 10 or 20 µM Cd for 72 h, starting from day 7 after sowing*. (a) Percentage of 2C nuclei. (b) Percentage of 4C nuclei. (c) Percentage of 8C nuclei. (d) Percentage of 16C nuclei. (e) Percentage of 32C nuclei. Data represent the average ± S.E. of 8 biological independent replicates.

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