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Faculty of Medicine and Life Sciences
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Master's thesis

The role of phytochelatins in reproductive growth of Arabidopsis thaliana during cadmium exposure

Laura Naulaerts

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

SUPERVISOR :

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The role of phytochelatins in reproductive growth of *Arabidopsis thaliana* during cadmium exposure

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*Running title: *phytochelatin effects during cadmium exposure*

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ABSTRACT

BACKGROUND: Metal-chelating phytochelatins and their precursor glutathione play an important role in the mediation of cadmium (Cd)-induced (oxidative) stress. Effects of this Cd-induced stress have mainly been investigated during the vegetative growth phase. Despite evidence of Cd-induced effects on the reproductive growth of plants, the exact mechanism by which Cd affects reproductive growth is currently limited. This study aims to elucidate the effects of Cd exposure on the molecular processes relating to reproductive growth in *Arabidopsis thaliana* and to investigate the role of phytochelatins in these effects.

METHODS: To determine Cd-induced effects, wildtype (WT) *A. thaliana* and a phytochelatin synthesis disrupted mutant (*cad1-3*) were cultivated. Expression profiles of reproductive growth genes were analyzed in hydroponics-grown *A. thaliana* exposed to 0, 0.5, or 2 µM $CdSO_4$. Levels of membrane peroxidation and antioxidative capacity were determined in addition to a phenotypical follow-up of vegetative and reproductive growth. Finally, vertical agar plate cultures were used to determine Cd sensitivity.

RESULTS: In Cd-exposed *A. thaliana*, the onset of reproductive growth was earlier in *cad1-3* compared to the WT plants in addition to a decrease in inflorescence length. Vegetative growth was similarly affected both phenotypically and in gene expression. Compared to WT, stress markers and antioxidative capacity were increased in the *cad1-3* mutant. None of the analyzed reproductive genes were significantly affected at 24 h, 72 h, or 1 week.

CONCLUSIONS: In *A. thaliana*, phytochelatin levels are important in reproductive growth during Cd exposure, the genetic basis of this needs to be further elucidated.

INTRODUCTION

Cadmium (Cd) is a soil pollutant that is present in soils worldwide. The main sources of this pollution are the old processes for smelting zinc ores, but smaller sources such as the use of phosphate-based fertilizers also contribute. Two factors contribute to the high environmental persistence of Cd in soil: it is not biodegradable and in addition, Cd can strongly bind to the organic matter in soil, efficiently retaining it in the soil (1).

As Cd is a nonessential element, plant root systems do not contain Cd-specific transporters. Despite this, plants growing on these polluted soils can take up Cd. This occurs through root-based transporters for essential elements such as zinc (Zn^{2+}), iron (Fe^{2+}), and manganese (Mn^{2+}) (2, 3). After uptake, different processes transport Cd from roots to shoots (4). This risks entry of Cd, a known human carcinogen, into the food chain (5).

Cadmium exposure is not only a threat to humans but also affects plant growth. Even at low concentrations of Cd, effects such as growth retardation and decreases in biomass can be identified (2, 4). In addition, effects on the reproductive growth can be identified as evidenced by the earlier shifts towards

reproductive growth, decreases in inflorescence length, and diminished silique generation (4). The effects on vegetative growth can partially be explained by the indirect generation of oxidative stress during Cd exposure. As Cd displaces bound elements out of other proteins, this increases the concentration of redox-active elements such as Fe^{2+} leading to a higher concentration of reactive oxygen species (ROS) (6-8). This increase in the concentration of ROS equals an imbalance between pro-oxidants such as the hydroxyl radicals and antioxidant molecules (8, 9). These ROS can cause damage at different sites of the plant cell. There are three main targets for Cd-induced damage: DNA, proteins, and lipids. At the protein level, Cd has a dual mechanism of action: it can displace chemically bound metals in the active site of the protein, or it can bind to the structural sites of proteins. This causes a conformational change, inactivating the enzymatic capacity of these proteins. At the DNA level, hydroxyl radicals can damage the cyclical component of the DNA base through attacking present double bonds, as well as targeting the sugar group of the DNA. This damage can be repaired by DNA repair mechanisms. However if left unrepaired, this can cause the formation of DNA mutations (6, 8, 10). At the lipid level, ROS target double bonds within lipid molecules of the cell membranes. This causes the formation of lipid radicals, highly active radicals that attack nearby lipid molecules. This resulting chain reaction damages the structure and fluidity of the cell membrane in a process called membrane peroxidation. During this process malondialdehyde (MDA) is formed (2, 6, 11).

Plants have an antioxidant response to mediate the damage caused by this redox imbalance. This response consists of both enzymes such as catalases and superoxide dismutases, and antioxidative molecules (2, 6) such as ascorbate (AsA) and glutathione (GSH). Glutathione is a tripeptide of glutamate (Glu), cysteine (Cys), and glycine (Gly) and has a dual role in the plant defense: (1) it is part of the AsA-GSH cycle which is responsible for the detoxification of H_2O_2 (6, 11); (2) a secondary role for GSH in Cd defense is the generation of phytochelatins (PCs). These PCs are polymers of two to eleven γ-glutamyl-cysteine (γ-Glu-Cys) molecules. The generation of PCs is a multi-step reaction catalyzed by multiple enzymes. The main enzyme is *A. thaliana* phytochelatin synthase 1 (AtPCS1), a γ-glutamylcysteine dipeptidyl transpeptidase. This enzyme catalyzes the following reaction in the presence of Cd and other metals (12, 13).

$$\gamma\text{-Glu-Cys-Gly} \rightarrow \gamma\text{-Glu-Cys+Gly}$$
$$\rightarrow \gamma\text{-Glu-Cys+}(\gamma\text{-Glu-Cys})_n\text{-Gly}$$

These polymers can be between two (PC2) and eleven (PC11) polymers long (12, 13). In this reaction, GSH functions as a donor of γ-Glu-Cys. After generation, the -SH sidechains present on Cys chelate Cd molecules to form a PC-Cd complex. These PC-Cd complexes are translocated to plant vacuoles for storage. Phytochelatins are thus important in preventing Cd-induced damage by preventing the interaction of Cd with cellular components. This alleviates oxidative stress (2, 12, 14). The production of phytochelatins is an early response to Cd exposure, with detectable PC production as early as 2 h after exposure to Cd and production continues at long-term exposure to Cd for at least 21 days (15, 16). Less is currently known about the effects of PCs during long-term exposure to Cd, nor how it affects reproductive growth.
To investigate this, *cad1-3* one of the most Cd-sensitive mutants will be used. This *cad1-3* mutant has a loss-of-function mutation in the *ARABIDOPSIS THALIANA PHYTOCHELATIN SYNTHASE 1* gene. This mutant is incapable of producing PCs (17).

While the effects of Cd exposure on vegetative growth, as described prior, has been studied more extensively, few studies are available regarding the effects of Cd exposure on the reproductive growth of *A. thaliana*. Available studies indicate that during Cd exposure an earlier onset of reproductive growth occurs, associated with decreases in reproductive growth capabilities (4).

In non-exposed *A. thaliana*, reproductive growth is regulated by an intricate network of transcription factors, microRNAs, and epigenetic mechanisms (18).

There are four main pathways of regulation of flowering time: vernalization, long day, autonomous, and gibberellic acid (GA) dependent pathways. Both vernalization and long-day pathways regulate flowering dependent on the plant environment, taking into account light and temperature. Autonomous and GA pathways are dependent on endogenous conditions (19). These pathways do not act completely independently of each other, but integrate through three main flowering pathway integrators: *FLOWERING LOCUS T* (*FT*), *LEAFY (LFY)*, and

SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*SOC1*) (19).

Initiation of the reproductive growth occurs through the development of the inflorescence meristem. This requires the vegetative meristem which gives rise to the initial rosette, to change to an inflorescence meristem (20, 21). This change is mediated by a complex of *FT* and *FLOWERING LOCUS D* (*FD*) that together activates the transcription factor *SOC1* which controls numerous genes related to meristem identity and the development of the floral meristem (18, 22). To retain inflorescence meristem identity, expression of *TERMINAL FLOWER 1* (*TFL1*) is important. *TERMINAL FLOWER 1* competes with *FT* to form a complex with *FD*, hereby preventing further change to a floral meristem (21, 23). The negative regulation of this vegetative phase change has been attributed to *AGAMOUS-LIKE 18* (*AGL18*), which represses the expression of *FT* (24).

The floral meristem forms on the side of the inflorescence meristem. This occurs through the expression of *LFY* and *APETALA*1 (*AP1)* through the *FT-FD* complex. The protein *PENNYWISE* (*PNY*) has a role in the patterning of the floral meristem, determining the branching of the meristem by restricting the expression of genes responsible for branching. At these floral meristems, the development of flowers gets regulated by *SEPTALA3* (*SEP3*) which is responsible for the expression of so-called 'floral organ identity genes', which are necessary for the identity of flower parts such as the sepals, petals, stamen, and carpel. The expression of *SEP3* is dependent on the repression of *SHORT VEGETATIVE PHASE* (*SVP*) and *SOC1*, which prevent *SEP3* production. This repression of *SVP* and *SOC1* is dependent on *AP1* production (22).

This study aims to elucidate the role of phytochelatins in the mediation of oxidative stress during the vegetative growth of *A. thaliana* during Cd exposure. In addition, the role of PCs in the mediation of Cd-induced changes to reproductive growth will be studied to determine changes to reproductive growth.

EXPERIMENTAL PROCEDURES

Plant cultivation and cadmium exposure – Seeds of wildtype (WT) and *cad1-3* mutant *A. thaliana* plants (Columbia ecotype) were used after the genotype was verified using PCR. Seeds were surface sterilized in a 70% ethanol solution and stored in H_2O in the dark at 4 °C for two days to ensure equal germination. Plants were grown in a 12 h photoperiod at 65 % relative humidity. During day and night conditions, plants were kept at 22 and 18 °C, respectively. Light was provided by Philips Green-Power LED modules, generating a mixture of blue, red, and far-red light with a photosynthetic flux density of 170 µmol m^{-1} s^{-1} at the rosette level, to stimulate the sunlight's natural photosynthetically active radiation of the sun (PAR).

Two different methods for the cultivation of *A. thaliana* were employed, the first one being growth on vertical agar plates (VAPs) to determine sensitivity to Cd exposure. Plants were sown on germination plates containing 1/4 Murashige and Skoog medium (Table 1), supplemented with 5 g l^{-1} sucrose. After seven days of growth, plants were transferred to exposure plates containing 1/4 MS medium supplemented with a concentration of 0, 20, 40, or 60 µM $CdSO_4$. To ensure a constant sulfate concentration in all exposure plates, $CdSO_4$ stocks were supplemented with K_2SO_4. Root length at the time of exposure was marked. After five days of exposure, plates were scanned at 300 dpi, using the Epson V300 scanner.

Table 1 - Components of 1/4 Murashige and Skoog medium.

Component	Concentration (mg l^{-1})
NH_4NO_3	412.5
H_3BO_3	1.55
$CaCl_2$	83.05
$CoCl_2.6H_2O$	0.00625
$CuSO_4.5H_2O$	0.00625
$FeSO_4.5H_2O$	6.95
$MgSO_4$	45.175
$MnSO_4.H_2O$	4.225
$Na_2MoO4.H_2O$	0.0625
KI	0.2075
KNO_3	475

For all other analyses, a modified hydroponics culture system was used, as described by Keunen *et al*. (4). Plants were exposed to 0, 0.5, or 2 µM of $CdSO_4$ through supplementation of the Hoagland solution after 20 days of growth under control conditions. After 24 h, 72 h, or one week of exposure, root and leaf samples were collected. All samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Determination of antioxidative capacity – Plant samples were shredded in a Retsch Mixer Mill MM 400 at 30 Hz for 90 seconds. Shredded samples were then dissolved in ice-cold 80% ethanol, mixed in a vortex mixer, and centrifuged (14 000 rpm, 4 °C, 30 min). The resulting supernatant was transferred to a 96-well plate in triplicate. A standard series of 6 hydroxy-2,5,7,8 tetramethylchrommane-2-carboxylic acid (TROLOX) dissolved in 80% ethanol, ranging from 0 to 10 nmol per well, was transferred to the same 96-well plate in duplicate. Measurements were performed using a reaction mix consisting of 0,3 M acetate buffer at a pH of 3.6, 10 mM 2,4,6 Tris(2 pyridyl)s-triazine (TPTZ), and 20 mM $FeCl_3$ with which samples and standards were incubated at 4 °C in the dark for 20 min. Afterward, absorption at 600 nm was determined using a FLUOstar Omega plate reader. Sample concentrations were calculated based on the TROLOX standard series.

Membrane peroxidation assay – Plant samples were shredded in a Retsch Mixer Mill MM 400 at 30 Hz for 90 seconds. Thereafter, shredded samples were dissolved in ice-cold 80% ethanol, vortexed, and centrifuged (14 000 rpm, 4 °C, 30 min). Blank samples, consisting of 80% ethanol were included. To all samples, 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid (TCA) was added in a 2/1 ratio of TBA/sample. Samples were incubated at 90 °C for one hour, after which the reaction was stopped by placing the samples on ice. Once cold, samples were centrifuged (5 000 rpm, 4 °C, 1 min). The supernatant was transferred to a 96-well plate in duplicate, and the absorbance was analyzed at 400, 532, and 600 nm using a FLUOstar Omega plate reader. Sample absorbances were blank corrected, and concentrations were determined based on the Lambert-Beer law. The extinction coefficient for the TBA-MDA complexes was 0.155 μM^{-1} cm^{-1}.

Gene expression analysis – Frozen root and leaf samples were ground with the Retsch Mixer Mill MM 400 (30 Hz, 90 s) after addition of two stainless beads. After shredding, RNA extraction was performed using the RNaqueous™ Total RNA Isolation Kit, following a modified protocol. The elution of RNA was performed using RNase-free H_2O instead of a heated elution buffer as per the manufacturer's instructions. The concentration and purity of the extracted RNA was determined using the NanoDrop™ ND-1000 spectrophotometer. The integrity of the extracted RNA was confirmed through gel electrophoresis. Before cDNA synthesis, the TURBO DNA-free™ Kit was used to remove residual genomic DNA, following the manufacturer's instructions. The PrimeScript™ RT Reagent Kit was used to convert 1 µg RNA to cDNA following the manufacturer's instructions. The resulting cDNA was diluted to a 1/10 concentration in 1/10 TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Undiluted and diluted cDNA samples were stored at -20 °C. Following cDNA synthesis, RT-qPCR analysis was performed using the QuantStudio™ 3 Real-Time PCR system (ThermoFisher Scientific). For analysis, a mastermix containing 5 µL of SYBR™ Green, 0.05 µL Quantinova ROX reference dye, 0.3 µL of 10 µM forward and reverse primer, and 2.35 µL RNase-free H_2O was used. Gene expression levels were determined using the $2^{-\Delta Cq}$ method and were normalized against the expression levels of *ACTIN2* (*ACT2*), *UBIQUITIN (UBQ10)*, and *RGS1-HXK1 INTERACTING PROTEIN* (*RHIP)*. Optimal reference genes were determined using the GrayNorm algorithm (25). Genes related to oxidative stress, cell cycle, and reproductive growth were analyzed (Table S 1). Primers were designed using Primer3Plus. Primer specificity was analyzed using TAIR BLAST 2.9.0+ against the Araport11 genomic locus sequences (DNA) and Araport11 transcripts. Primer efficiency was confirmed to be between 90 – 110 %.

Cadmium sensitivity analysis – Scans of VAPs plates after five days of Cd exposure were analyzed using RootNav software. Root length prior to exposure and root growth during Cd exposure was determined (26).

Phenotypical follow-up – Starting at 13 days after sowing, growth parameters of WT and *cad1-3 plants* were followed up trice weekly until rosette growth had reached a plateau. At each of the follow-up moments, the number of rosette leaves was determined, and rosette diameter was determined using digital calipers. In addition, the presence of the inflorescence bud was determined. After emergence, inflorescence length was determined using digital calipers.

Statistical analysis – Statistical analysis was performed in RStudio (version 1.4.1106, R version: 4.1.2, "*bird hippie"*). The Shapiro-Wilk test was used to test the normal distribution of the data and the Bartlett's test was performed to determine homoscedasticity of variances. If these assumptions were not met, data were transformed

(square root, inverse, exponent, or logarithm). If assumptions were met, a two-way ANOVA and Tukey-Kramer *posthoc* test were used to analyze data. If the assumptions of normality or homoscedasticity were not met, a non-parametric Kruskal-Wallis and pairwise Wilcoxon rank-sum tests were used. For both tests, a significance level of 5% was used. The extreme studentized deviate method ($\alpha = 0.05$) was used in GraphPad to detect outliers in the sample population.

RESULTS

Cadmium exposure decreases root growth – Cadmium-exposed *A. thaliana* root growth was significantly decreased in a Cd-dependent manner (Figure 1). The *cad1-3* mutant displayed a more pronounced growth reduction represented by the root length at each Cd concentration compared to the WT plants of the same condition. In the highest exposure condition, 60 µM, *cad1-3* mutant plants did not grow after transfer to exposure plates. At this exposure condition, however, WT plants continued to grow after transfer.

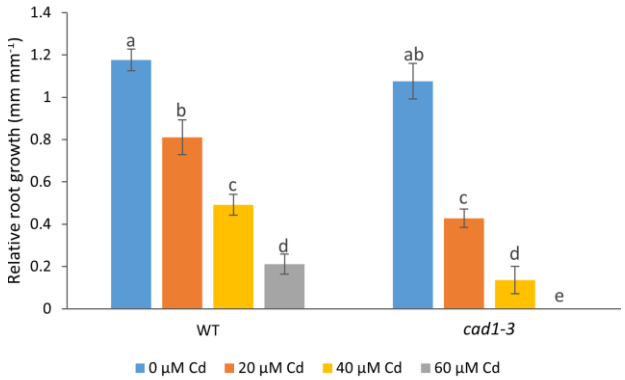

Figure 1 - Relative root growth of wildtype (WT) and *cad1-3 Arabidopsis thaliana* after 5 days of exposure to 0 (■), 20 (■), 40 (■), or 60 (■) µM $CdSO_4$ relative to 7 days growth on ¼ MS medium without Cd supplementation. Data shows averages ± S.E. of 10 biological replicates. Different letters indicate significant differences ($p < 0.05$; two-way ANOVA).

Influence of cadmium exposure on vegetative growth – Three parameters of vegetative growth (fresh weight, rosette diameter, and the number of rosette leaves) were followed-up in WT and *cad1-3* mutant *A. thaliana*. Changes were confirmed in gene expression of cell cycle genes.

At harvest, rosette fresh weight was determined after 24 h, 72 h, and 1 week of Cd exposure. Cadmium exposure affected rosette fresh weight in a time-dependent manner (Figure 2A). During the two shortest investigated timeframes, the fresh weight of WT *A. thaliana* was not impacted by Cd exposure. Only at 1 week of exposure, Cd exposure negatively affected fresh weight independently of exposure conditions. This is reflected in the appearance of the harvested rosettes. At 72 h of Cd exposure (Figure 2B) rosettes showed few phenotypical signs of Cd-induced stress. After 1 week, (Figure 2C) especially the plants exposed to the highest Cd conditions started to show some signs of stress: rosettes appeared visually smaller and displayed chlorosis, especially along the leaf edges. These Cd-induced effects are genotype-dependent. Contrary to the WT fresh weights, rosettes of the *cad1-3* mutant showed a more pronounced effect of Cd exposure. Even at the earliest exposure timeframe, fresh weight was decreased between control and 2 µM Cd exposed condition. At 72 h of exposure, a Cd concentration-dependent decrease in fresh weight was present in all exposure conditions. This decrease was exacerbated at 1 week of Cd exposure but was not dependent on Cd concentrations. Visually, harvested rosettes of *cad1-3* mutant plants were smaller compared to non-exposed rosettes both at 72 h (Figure 2B) and 1 week (Figure 2C) of exposure. Starting at 72 h, but especially at 1 week of Cd exposure harvested rosettes show signs of stress, more than their WT counterparts. In both 0.5 and 2 µM Cd exposure conditions, rosette leaves show signs of chlorosis. At 1 week of exposure, a strong purple discoloration was present in both 0.5 and 2 µM conditions, but most pronounced at 2 µM.

Secondly, rosette growth was followed up. In both genotypes, Cd exposure significantly decreased the final rosette diameter compared to control conditions (Figure 3A). Cadmium-induced effects were genotype-dependent: rosette size decreases were dependent on Cd concentration in *cad1-3*, but not in WT plants. The *cad1-3* rosette size decreased more compared

to WT plants. The final rosette diameter of the *cad1-3* mutant at 0.5 µM Cd exposure was nearly half the size of the WT plants; similar effects were seen in the 2 µM Cd exposed plants. The growth of *cad1-3* plants stagnated almost immediately after exposure to Cd, especially in the 2 µM condition, while WT plants reached their plateau only a few days before control conditions. These effects are similar to those in rosette fresh weight, which decreased in all exposed conditions for the *cad1-3* mutant, except at 0.5 µM Cd exposure for 24 h.

Changes to the number of rosette leaves (Figure 3B) differed from those seen in rosette fresh weight and rosette diameter. There is a genotype-dependent decrease in rosette leaf count, comparing *cad1-3* to WT plants. In both WT and *cad1-3* mutant plants, only the highest concentration of Cd exposure caused a decrease in leaf count.

The changes that were seen in these vegetative parameters, could also be identified in the expression patterns of cell cycle-related genes at 24 h of Cd exposure (Table 2). Two out of three cell cycle genes were downregulated in *cad1-3* plants compared to their control conditions under Cd exposure. These effects, however, were independent of Cd concentration.

Table 2 - Gene expression patterns of cell cycle genes in wild-type (WT) and *cad1-3 Arabidopsis thaliana* plants exposed to 0, 0.5, and 2 µM for 24 h. Table indicates average data of 5 biological replicates ± SE. Data are represented as the fold change relative to the control of the same genotype set at 1. Red color indicates a significant downregulation compared to the genotype control condition, asterisks indicate significant changes compared to the same exposure condition on the other genotype ($p < 0.05$; two-way ANOVA). Wildtype (WT), *A-type Cyclin-cependent Kinase (CDKA1), A2-type Cyclin* (*CYC2;3), Histone H4 (HIS4)*

Timepoint	Gene	Cd exposure	WT	*cad1-3*
24 h	*CDKA1*	0.5 µM	0.78 ± 0.07	0.89 ± 0.06
		2 µM	0.74 ± 0.09	0.96 ± 0.12
	CYCA2;3	0.5 µM	0.82 ± 0.10*	0.68 ± 0.08*
		2 µM	0.92 ± 0.12*	0.42 ± 0.10*
	HIS4	0.5 µM	0.80 ± 0.15*	0.56 ± 0.09*
		2 µM	0.99 ± 0.25*	0.54 ± 0.05*

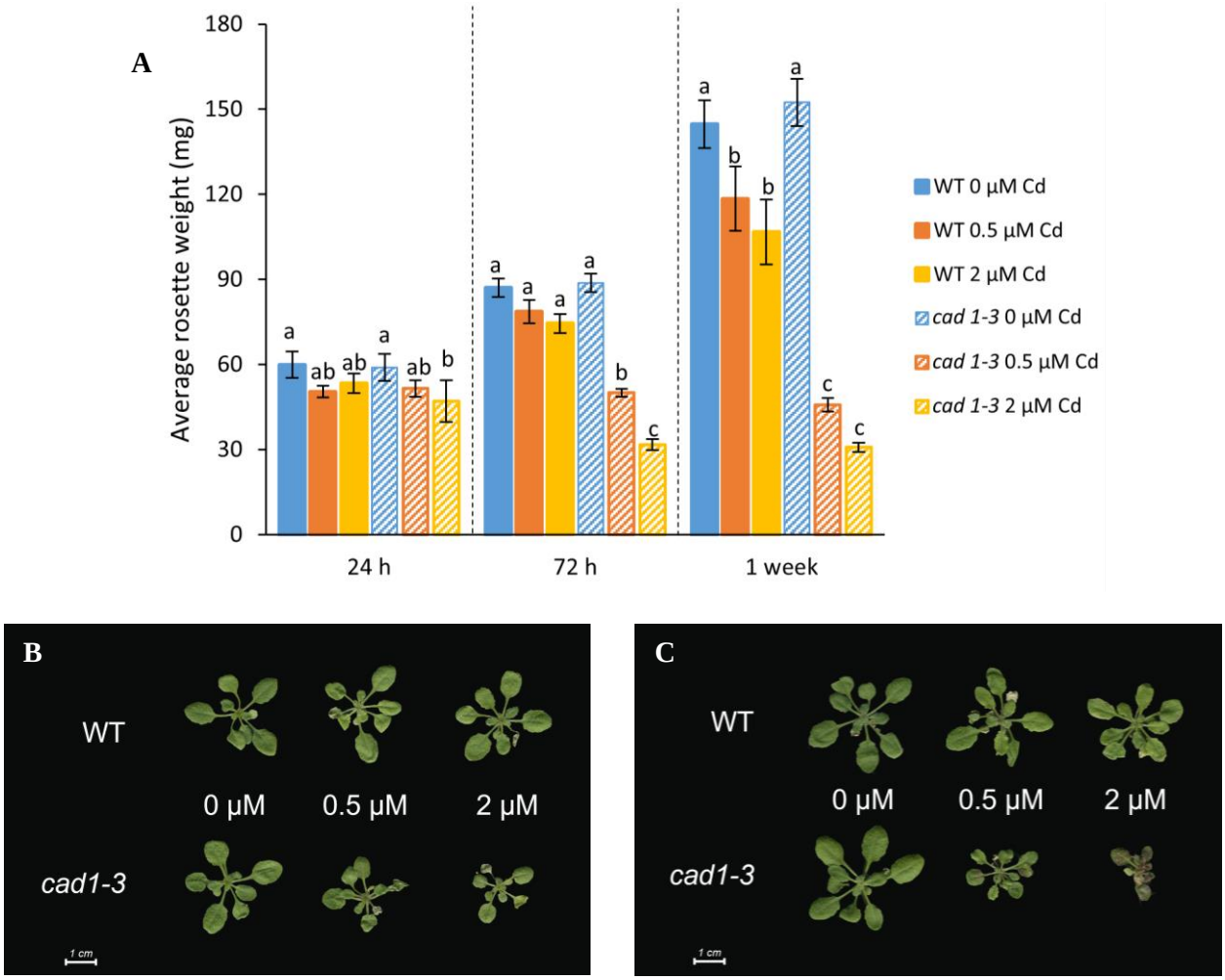

Figure 2 - Fresh weight (A) and visual appearance (B, C) of harvested wildtype (WT) (■) and *cad1-3* (▨) *A. thaliana* rosettes during Cd exposure. Plants were exposed to 0 (■), 0.5 (■), or 2 (■) µM $CdSO_4$ starting at 20 days after sowing for a duration of 24 hours, 72 hours, or 1 week. Fresh weights (A) represent averages ± S.E. of 5 biological replicates at all timepoints. Different letters indicate statistical differences per timepoint (two-way-ANOVA, $p < 0.05$). Scale bar indicates 1 cm.

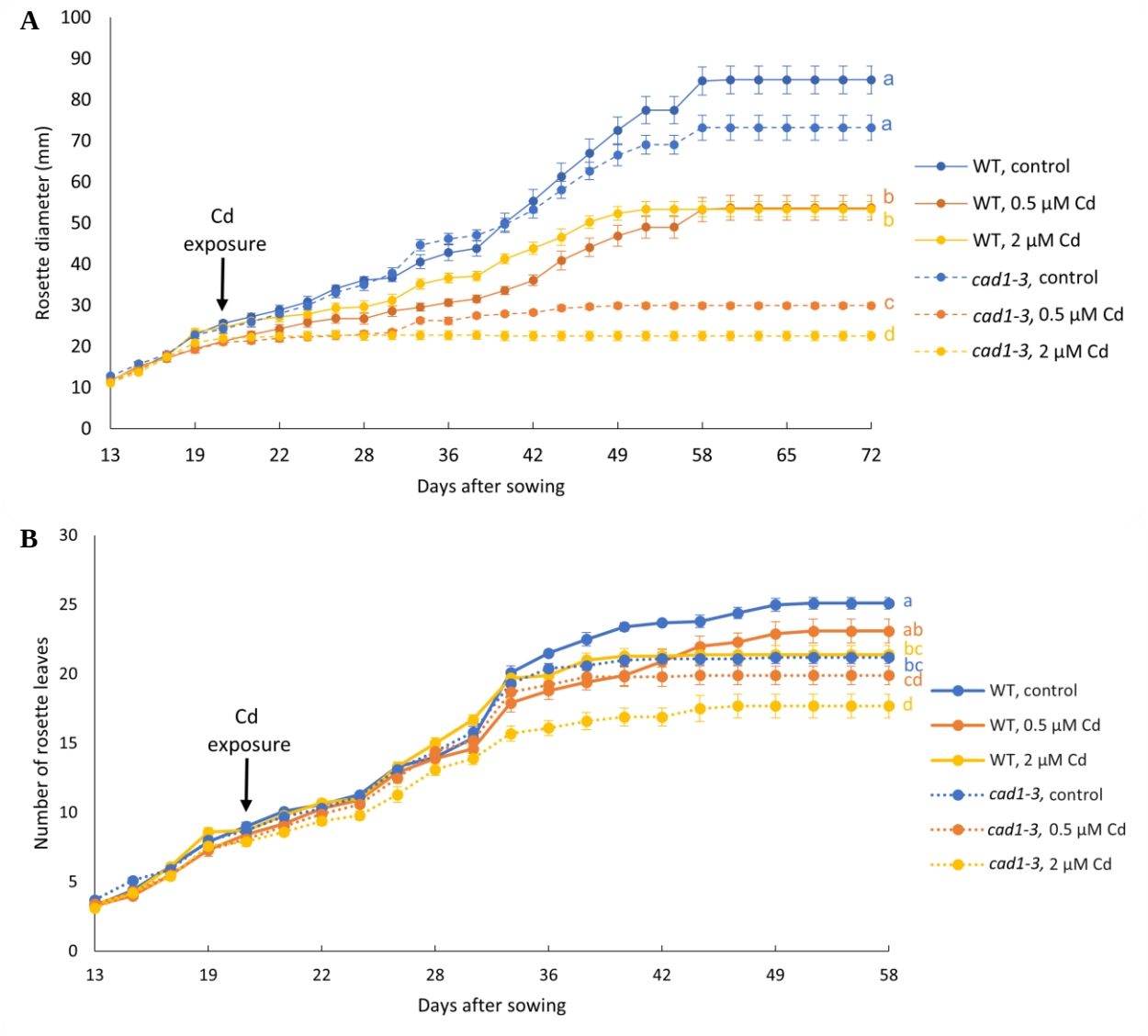

Figure 3 - Phenotypical parameters of vegetative growth of *A. thaliana* during cadmium exposure. Rosette diameter (A) and number of rosette leaves (B) follow-up starting at 13 days after sowing (DAS) until a plateau was reached. The WT (—) and *cad1-3* (····) were exposed to 0 ( ) , 0.5 ( ), or 2 ( ) µM $CdSO_4$ starting at 20 DAS. Data represents average ± S.E. of 10 biological replicates. Different letters indicate statistical differences in endpoint data (two-way ANOVA, $p < 0.05$).

Cadmium-induced effects on the oxidative balance – To investigate membrane peroxidation, levels of TBA-reactive elements (including MDA) were determined in both WT and *cad1-3 A. thaliana* plants after 1 week of exposure to 0 or 2 µM Cd. Levels of TBA-reactive elements were significantly increased in Cd exposed *cad1-3* mutants compared to all other conditions (Figure 4A) This increase was not present in WT conditions.

The ferric reducing ability (FRAP) results were similar to TBA-reactive elements (Figure 4B). In *cad1-3* plants exposed to 2 µM Cd, FRAP increased significantly compared to the other conditions.

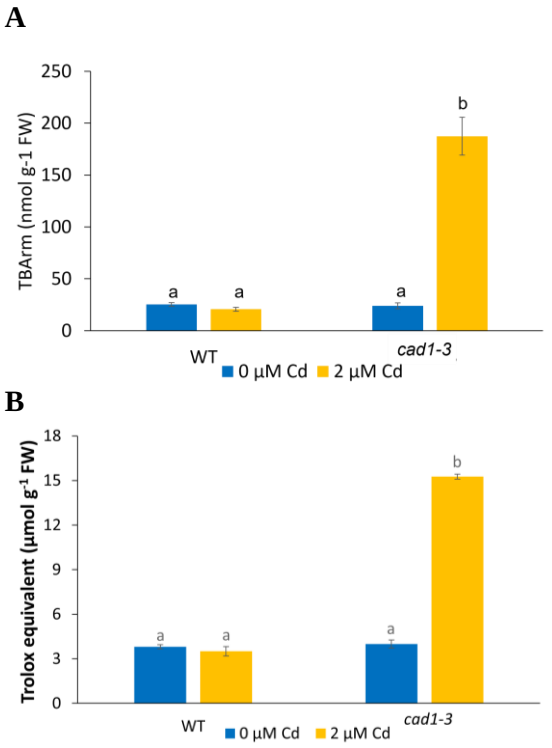

Figure 4 - Membrane peroxidation levels (A) and antioxidative stress mediation (B) in wildtype (WT) and *cad1-3 Arabidopsis thaliana* exposed to 0 (■) or 2 µM (■) $CdSO_4$ for 1 week, starting at 20 days after sowing. Data represents average ± S.E. of 8 biological replicates. Different letters indicate significantly different conditions (two-way ANOVA, $p \leq 0.05$). TBA reactive molecules (TBArm)

The results of the membrane peroxidation assay are corroborated by gene expression analysis data (Table 3). In the *cad1-3* plants, three oxidative stress markers were upregulated in 0.5 µM Cd exposed plants, and one in 2 µM Cd exposed plants. In WT plants, however, two out of five genes were upregulated at 2 µM Cd exposure.

Table 3 - Gene expression patterns of oxidative stress markers in wild-type (WT) and *cad1-3 Arabidopsis thaliana* plants exposed to 0, 0.5, and 2 µM for 24 h. Table indicates average data of 5 biological replicates ± SE. Data are represented as the fold change relative to the control of the same genotype set at 1. Green color indicates a significant upregulation compared to the genotype control condition; asterisks indicate significant changes compared to the same exposure condition on the other genotype ($p < 0.05$; two-way ANOVA). *TIR - Toll Interleukin Receptor (TIR), Upregulated By Oxidated Stress* (*UPOX*)

Gene	Cd exposure	WT	*cad1-3*
AT1G05340	0.5 µM	2.60 ± 0.51*	1.91 ± 0.53*
	2 µM	2.48 ± 1.12*	1.99 ± 0.27*
AT1G19020	0.5 µM	**2.12 ± 0.59***	1.18 ± 0.11*
	2 µM	**2.54 ± 0.48***	1.50 ± 0.35*
TIR	0.5 µM	1.76 ± 0.39*	2.31 ± 0.39*
	2 µM	2.94 ± 0.66*	1.49 ± 0.36*
UPOX	0.5 µM	1.89 ± 0.21*	2.38 ± 0.25*
	2 µM	1.97 ± 0.28	1.75 ± 0.36
DEFENSIN-LIKE	0.5 µM	**3.46 ± 0.73***	**11.30 ± 1.40***
	2 µM	**3.14 ± 0.80***	**23.2 ± 3.20***

Cadmium-induced changes in reproductive growth parameters – The emergence of the inflorescence bud (Figure 5A) started at 34 days after sowing, two weeks past the start of exposure. Plants exposed to $CdSO_4$ showed a delay in the development of inflorescence buds. This delay was more pronounced in the *cad1-3* mutant plants compared to WT. Upon exposure to 2 µM of Cd in WT and *cad1-3* plants 40 and 50% of the plants had, respectively, developed inflorescence buds.

Shortly after the emergence of the inflorescence bud, growth of the inflorescence started (Figure 5B). During the follow-up timeframe, *cad1-3* plants exposed to Cd had reached final inflorescence size. All WT plants and control conditions of *cad1-3* mutants had not reached final inflorescence size. Both WT and *cad1-3* plants under control conditions had taller inflorescences compared to Cd-exposed plants. Inflorescence lengths were smaller upon exposure to Cd, but this decrease was not dependent on Cd concentration. Size decreases were dependent on genotype, the final inflorescence size in Cd exposed *cad1-3* plants was smaller than the length of WT inflorescences

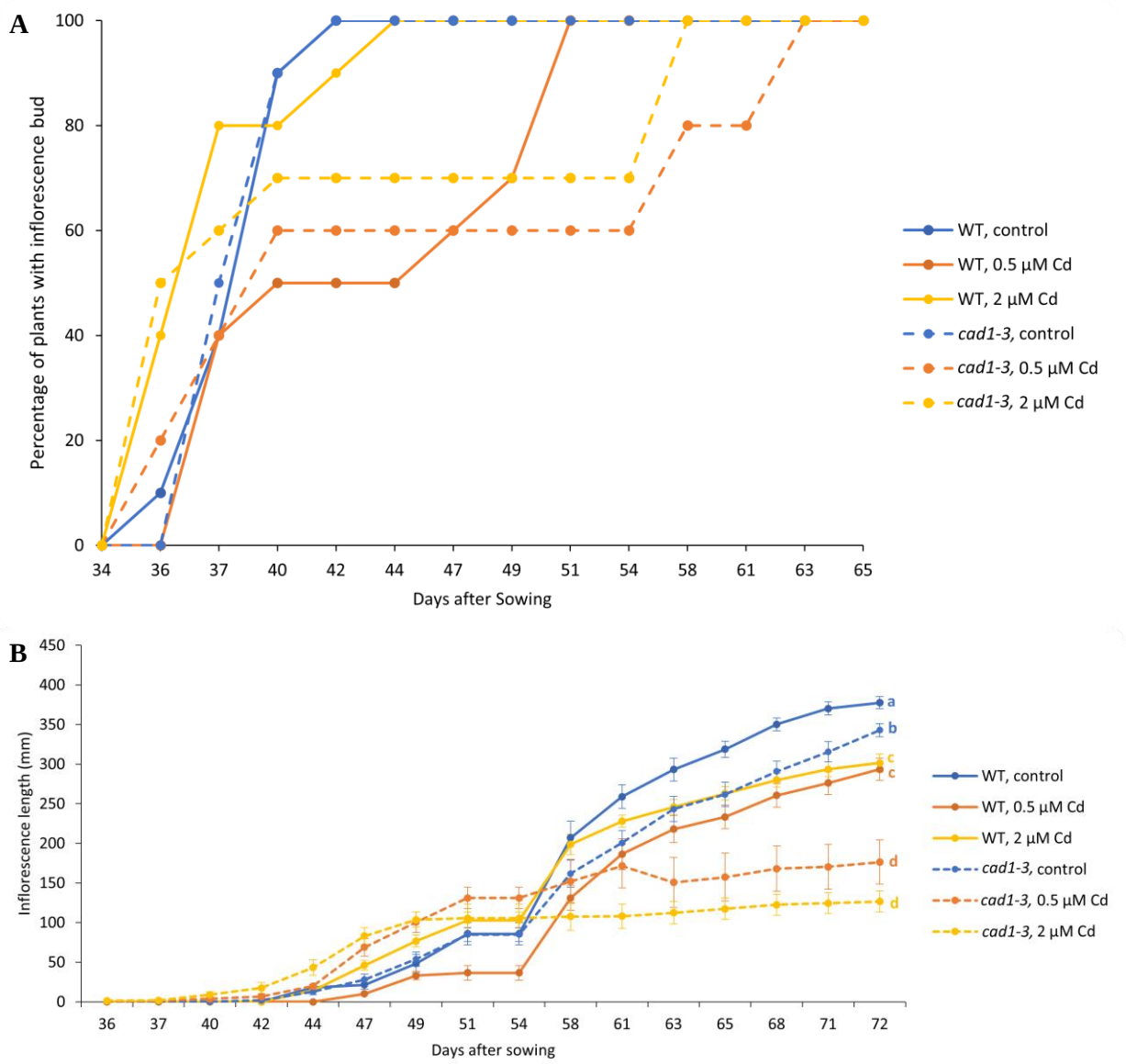

Figure 5 - Emergence of inflorescence bud (A) and length of inflorescence (B). Wildtype (—) and *cad1-3* (·····) *Arabidopsis thaliana* was exposed to 0 (■), 0.5 (■) or 2 (■) µM $CdSO_4$ starting from 20 days after sowing. Data represent average ± S.E. of biological replicates.

at the end of the follow-up timeframe.

Gene expression of genes related to the reproductive growth was also determined in plants exposed to 0, 0.5, or 2 µM of Cd for 1 week. None of the investigated genes were significantly up- or downregulated compared to either the control condition or other Cd exposed conditions (Table 4). There was a trend toward downregulation of *SVP*, and *SOC1*.

Table 4 - Gene expression patterns of reproductive growth genes in wild-type (WT) and *cad1-3 Arabidopsis thaliana* plants exposed to 0, 0.5, and 2 µM for 24 h. Table indicates average data of 5 biological replicates ± S.E. Data are represented as the fold change relative to the control of the same genotype set at 1. Green color indicates a significant upregulation compared to the genotype control condition; asterisks indicate significant changes compared to the same exposure condition on the other genotype ($p < 0.05$; two-way ANOVA).
WT: wildtype, Aguamous-like 18 (AGL18), PENNYWISE (PNY), SCHLAFFMUTZE (SMZ), SUPPRESOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), and *TOE2*

Gene	Cd exposure	WT	*cad1-3*
AGL18	0.5 µM	1.02 ± 0.13	1.07 ± 0.10
	2 µM	0.88 ± 0.18	1.15 ± 0.22
PNY	0.5 µM	0.93 ± 0.09	0.96 ± 0.15
	2 µM	0.85 ± 0.14	1.20 ± 0.27
SMZ	0.5 µM	1.32 ± 0.24	1.64 ± 0.42
	2 µM	0.26 ± 0.03	1.75 ± 0.45
SOC1	0.5 µM	0.87 ± 0.11	0.57 ± 0.11
	2 µM	0.50 ± 0.07	0.61 ± 016
SVP	0.5 µM	0.73 ± 0.05	0.47 ± 0.06
	2 µM	0.70 ± 0.10	0.63 ± 0.09
TOE2	0.5 µM	0.85 ± 0.07	1.31 ± 0.19
	2 µM	0.87 ± 0.06	1.43 ± 0.23

DISCUSSION

Cadmium sensitivity is increased in cad1-3 A. thaliana – In the VAPs culture experiment, the phytochelatin deficient cad1-3 plants exposed to Cd showed marked Cd concentration-dependent decreases in growth after transfer compared to the WT plants at the same exposure. This indicates that the *cad1-3* mutant is more sensitive to Cd exposure than WT plants. These findings are confirmed by previous studies. Howden *et al.* (17) found *cad1-3* to be more sensitive to Cd exposure than WT, even at concentrations of 0.3 µM Cd when studying whole seedlings grown in agar culture similar to the set-up of this study. Kühnlenz *et al.* found similar effects in hydroponics-grown *cad1-3* exposed to 0.5 µM Cd, where root lengths were shorter in *cad1-3* exposed to Cd than in WT plants exposed to the same levels of Cd concentration (13). In the *cad1-3* mutant, phytochelatin synthesis is disrupted, leading to a deficiency in PCs and therefore lack of the ability to chelate Cd molecules. This deficiency could lead to an increased concentration of free Cd molecules – and an associated increase in Cd-associated damage.

Phytochelatin deficiency increases oxidative stress during Cd exposure – To determine Cd-induced changes in the redox balance, parameters of both oxidative stress and oxidative defense were analyzed in WT and *cad1-3* plants exposed to 0 and 2 µM Cd for 1 week. Cadmium exposure did not affect the total ferric reducing capacity of WT plants. In the *cad1-3* mutant, total antioxidative capacity was increased upon exposure to 2 µM Cd compared to the control. A possible explanation for this was posed by Howden *et al.* who found that in liquid medium grown WT and *cad1-3*, levels of GSH increased in *cad1-3* plants exposed to 30 µM Cd (17). Production of GSH increases upon exposure to Cd to aid in the mitigation of Cd-induced stress. In WT plants, GSH is used to generate PCs, decreasing the amount of available GSH. In *cad1-3* plants, this process is impaired and GSH levels do not deplete leading to higher levels of GSH. During Cd exposure, levels of membrane damage increased in *cad1-3* but not in WT conditions.

To estimate membrane damage, the level of TBA reactive molecules was determined after 1 week of exposure to 2 µM Cd. In WT plants, no increase in membrane damage was observed. Existing literature mainly focuses on Cd exposure during shorter timeframes and at higher exposure

levels such as 5 and 10 µM of Cd. Similar effects were seen by Jozefczak *et al*. during 72h of exposure to 5 µM Cd in a similar hydroponics set-up. At these lower levels of Cd concentration – WT plants seem to be capable of mitigating oxidative stress to determine. In WT plants, expression of oxidative stress markers was increased only during exposure to 2 µM of Cd for 1 week. However, *cad1-3* plants had increased expression of stress markers at both 0.5 and 2 µM Cd after 1 week of exposure. These results indicate that a deficiency in PC levels increases sensitivity to Cd-induced oxidative stress. Similar effects were seen in VAPs culture – as described prior.

Combined, this indicates that the *cad1-3* plants had a decreased capacity of dealing with oxidative stress compared to WT plants. This decreased ability to cope with oxidative stress compared to WT could be related to the deficiency in PCs. In WT plants, PCs help mediate oxidative stress by chelating Cd molecules and transporting them to vacuoles. In *cad1-3*, no Cd molecules are chelated, this could increase the levels of free Cd in the plant (12, 13) leading to the observed higher degrees of ROS, and ROS damage as determined via membrane peroxidation. These higher levels of ROS cause increased production of antioxidative molecules, as determined via the FRAP assay to control the damage potential that these reactive oxygen species cause. However, as evidenced by the increased ROS damage, this antioxidative response is insufficient to prevent damage in PC deficient plants. This indicates that in the Cd-induced oxidative stress response, the production of PCs is vital in the control of the oxidative stress response upon Cd exposure.

Phytochelatin deficiencies exacerbate cadmium-induced vegetative growth inhibition – During Cd exposure, vegetative growth and expression of cell cycle genes are inhibited more in *cad1-3* compared to WT plants. These effects were dependent on Cd concentration for rosette fresh weight and rosette diameter. This suggests that phytochelatin deficiencies do not affect all parameters equally during Cd exposure.

During Cd exposure, the findings of our study demonstrate downregulated expression of *HIS4* and *CYC2;3* in *cad1-3* but not WT plants. However, *CDKA1*, an important factor in the overall cell cycle was not affected. The expression of *HIS4* increases during the G1/S phase of the cell cycle due to their role in the replication of DNA (27), the downregulation of these genes indicates an inhibition of the cell cycle during Cd exposure. A possible explanation for the observed inhibition of the cell cycle, and thus decreased growth potential lies in the increase of oxidative stress in *cad1-3* plants compared to WT plants. During the cell cycle, expression of *CYCA2;3* is dependent on an environment with low levels of ROS (28). During Cd exposure, as ROS levels rise, transcription factors required for regulation of *CYC2;3* expression are activated less – possibly leading to the observed decreased expression of genes vital to the cell cycle. Due to the phytochelatin deficiency in *cad1-3* plants, free Cd levels in these plants could be increased as previously described. In addition to the oxidative stress-mediated decreases in *CYCA2;3*. Secondarily, prior studies have suggested that the inhibitions in vegetative growth are a plant protective mechanism. Cadmium uptake in these plants is decreased, leading to shorter

A third possible explanation, that cannot be verified within the experimental set-up of this study, but evidenced by Kumar *et al.* in *cad1-3* plants exposed to up to 1 µM Cd in a hydroponics setup, is the decreased uptake of essential elements such as Fe, K, S, and Mg during Cd exposure (29, 30). Uptake and translocation of Cd occur through transporters for essential elements, thereby decreasing uptake of these essential elements and causing potential deficiencies (2, 3). Larsson *et al.* found that in *cad1-3* plants, uptake of essential elements was decreased compared to WT conditions with increasing Cd concentrations (29). These deficiencies in essential elements might be explained by the shorter root lengths of *cad1-3* plants exposed to Cd compared to WT plants. Deficiencies in essential elements are known to affect vegetative growth in *A. thaliana*, as these essential elements are co-factors of enzymes, but also in the synthesis of amino acids which are both required for plant growth to occur (30). Further research is necessary to elucidate the exact mechanism behind the vegetative growth inhibition. Combined, this suggests the vegetive growth inhibition of *A. thaliana*.

Reproductive growth during Cd exposure is limited in cad1-3 – Reproductive growth parameters were significantly affected by Cd exposure. Inflorescence lengths were smaller in *cad1-3* compared to WT plants exposed to the same Cd concentrations. In addition, the emergence of inflorescence buds was earlier in

cad1-3 exposed to Cd compared to WT plants. Keunen *et al*. studied reproductive and vegetative growth hydroponics-grown WT plants exposed to 5 or 10 µM Cd. Their findings identified an earlier emergence of visible inflorescence meristem and smaller inflorescences in Cd-exposed conditions compared to control conditions, confirming the effects this study found in WT plants{Keunen, 2011 #290}. The observed shorter inflorescences at 0.5 and 2 µM of Cd for WT plants were not as extreme as the results by Keunen *et al.*, this indicates that the phenotypical effects are dependent on Cd concentration. Keunen *et al.* did not propose possible mechanisms behind these observed changes (4).

To explain the observed phenotypical changes, the expression of reproductive growth genes was analyzed. Gene expression analysis was performed at 1 week of Cd exposure, 27 days after sowing (DAS). The emergence of the inflorescence buds occurred at 34 DAS. The function of *AGL18, SVP, PNY,* and *TOE2* are associated with the development and maintenance of floral meristem and initiation of flowering (20). As the emergence of the flower buds occurs at a later time point than gene expression analysis was measured (27 DAS), it is likely that these two processes were not yet activated. The absence of changes in gene expression despite significant effects at the phenotypical level could thus possibly be explained by the chosen time points. Further investigation at a later stage of reproductive growth is required to fully identify whether the observed phenotypical changes are also reflected in the expression of reproductive growth genes. Our study did identify a trend toward downregulation of *SOC1*. Despite this, prior research indicates *SOC1* is an earlier mediator which positively regulates the change from vegetative to reproductive growth, specifically the growth of the inflorescence meristem (18). Contrary to our results, an upregulation of gene expression would explain the earlier emergence of the inflorescence bud in Cd-exposed plants (16, 20).

Knowledge regarding how these Cd-induced effects occur is limited. Our results could not explain how these Cd-induced changes occurred during Cd exposure. However, a clear stronger inhibition and earlier effects were seen in mutant *cad1-3* plants compared to WT plants. As described previously within this discussion – the deficiency in PCs increases oxidative stress by a possible increase in free Cd concentration compared to WT plants. In addition, studies suggest in *cad1-3* plants the uptake of essential nutrients was limited compared to WT plants during very short-term exposure to Cd (29). These nutrient changes could affect the overall reproductive growth during Cd exposure, however, this would need to be investigated during the reproductive growth phases. Another plausible hypothesis is that prolonged exposure to oxidative stress is in part responsible for these effects as during these chronic exposures, antioxidant capacity could have depleted leading to more damage. Data at chronic time points is not currently available and requires further investigation to fully identify how these changes are determined.

CONCLUSION

The results of this study indicate that phytochelatin deficiencies in *Arabidopsis thaliana* exacerbated oxidative stress and associated damage compared to WT plants, thereby confirming the hypothesis that phytochelatin production is important in the control of oxidative stress during Cd exposure. In addition, Cd-induced inhibition to vegetative and reproductive growth parameters was increased in *cad1-3* compared to WT plants, confirming that phytochelatins mediate Cd-induced toxicity not only during short, but also longer-term exposures to Cd. The mechanism by which these changes occur, could not be elucidated by this study – further research is required to address possible mechanisms by which long-term exposure to Cd impacts reproductive growth.

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SUPPLEMENTAL DATA

Table S 1 - Forward and reverse primers used for RT-qPCR analysis of gene expression.

Gene	**Forward primer (5' -> 3')**	**Reverse primer (5' -> 3')**
CDKA1	CCAGAGCATTCGGTATCCCTG	ATGCACCCCACAGACCAAAT
CYC2;3	CCACTGGACCCAACAAGAGG	AATCCATGACCGCGTCCTTT
HIS4	AGGAGGAAAAGGGTTAGGCAAA	GACGAATCGCTGGCTTTGTG
AT1G05340	TCGGTAGCTCAGGGTAAAGTGG	CCAGGGCACAACAGCAACA
AT1G19020	GAAAATGGGACAAGGGTTAGACAAA	CCCAACGAAAACCAATAGCAGA
TIR	ACTCAAACAGGCGATCAAAGGA	CACCAATTCGTCAAGACAACACC
UPOX	GACTTGTTTCAAAAACACCATGGAC	CACTTCCTTAGCCTCAATTTGCTTC
DEFENSIN-LIKE	ATGGCAAAGGCTATCGTTTCC	CGTTACCTTGCGCTTCTATCTCC
AGL18	GCCTCAAGATTCTAGCCCAGA	TCTCTTTGTGCAATACCCCGT
PNY	TGCTGTTACTGTTCTAAGGGCT	TCCAAACCCTAACTCTTGCGT
SMZ	GCTGCTATCAAATTCCGTGGTC	TCTCTCGCCTAAGTGTTTGCA
SOC1	GCAACAGCTTGAGAAAAGTGTCA	CGCTTTCATGAGATCCCCACT
SVP	CGCAACTAACGGAAGAGAACG	TTCCTCGTACACAGCAGCG
TOE2	ACTTTCTATCGGAGGACAGGC	GGAATTTGACAGCAGCTCGATC
ACT	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
UBQ10	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
RHIP	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC