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List of Abbreviations

AsA	Ascorbate (vitamin C)
EIN	Ethylene insensitive
ERF	Ethylene responsive transcription factor
GC	γ-glutamylcysteine synthetase
GSH	Glutathione (reduced)
GST	GSH-S-transferase
H_2O_2	Hydrogen peroxide
MT	Metallothioneins
NADPH	Nicotinamide adenine dinucleotide phosphate
$O_2^{\bullet -}$	Superoxide radical
$^{\bullet}OH$	Hydroxyl radical
PC	Phytochelatin
PCS	Phytochelatin synthase
PRX	Peroxidases
ROS	Reactive oxygen species

Acknowledgement

During the writing of this thesis I have learned a lot about science but also about myself. I had to learn to accept that not everything always goes as planned. This because nothing went as planned, and looking back on these past 8 months I realise how much appreciation I have for the people that helped me along. First of all, I want to thank Dr Els Keunen. You saved the day, or better said, my entire year. You supported me and told me to keep my head up high. This thesis would not have been possible without all of your efforts and support. I want to thank Prof. Dr Ann Cuypers, who is always so openminded and ready to help and point you in the right direction. Next to this, I want to thank Sophie Hendrix, who has had to patience to work with me, even though I might be a bit wearing sometimes. And the same can be said for Amber Mertens. You laughed at my bad jokes, sat with me through endless hours of talking and became an amazing friend who I wish to see more often. Finally, I want to thank my best friends; Marieke, Stefanie and Jana. You have supported me through this (sometimes not so easy) year with laughter, adventures, support and being the wonderful creatures that you are!

Abstract

Cadmium (Cd) is a non-essential element able to induce the production of reactive oxygen species (ROS), resulting in a phytotoxic effect on plant growth and quality. This translates into diminished crop yield and a general decrease in arable land availability. The gaseous hormone ethylene is involved in physiological processes related to plant growth and development, but recent discoveries linking ethylene to Cd stress have stimulated research towards the involvement of ethylene signalling in defence mechanisms such as glutathione (GSH) replenishment and subsequently phytochelatin (PC) production.

Short-term (72h) exposure of wildtype (WT) and GSH deficient *cadmium sensitive2-1* (*cad2-1*) mutant *Arabidopsis thaliana* plants to 5 µM Cd showed a higher upregulation of ethylene biosynthesis and signalling gene expression in GSH deficient conditions. This confirms the link between GSH and ethylene signalling to stimulate defence mechanisms during Cd stress. The involvement of ethylene-initiated defence mechanisms to cope with Cd phytotoxicity was further explored by HPLC measurements of Cd-chelating PCs in ethylene-insensitive *ein2-1* mutants as compared to WT plants. While ethylene signalling is impaired, and the production of GSH delayed, the immediate increase in PCs was persistent and parallel in leaves of both mutants and WT plants. This indicates a bypass to alleviate the defective ethylene signalling, possibly explaining the insensitivity of *ein2-1* mutants to short-term Cd exposure at the biomass level.

Investigation of long-term (>72h) exposure to 5 µM Cd of WT versus *ein2-1* plants was performed to further elucidate previous findings within this study. Phenotypic results measuring daily vegetative and reproductive phases of plant growth showed a reproductive growth delay in the mutant, although a similar response to Cd exposure was noted for both genotypes. This confirms the presence of bypass mechanisms to compensate ethylene signalling impairment. Finally, results of nuclear ploidy levels in this study deviated from other studies, with an increase in ploidy levels in leaves of *ein2-1* mutants under control and 72 h Cd exposure, yet a decrease after 12 days in Cd exposure condition. This indicates an interplay between ethylene signalling and activation of endoreduplication under moderate Cd exposure.

Samenvatting

Cadmium (Cd) is een niet-essentieel element dat de productie van reactieve zuurstof vormen (RZV) induceert, wat leidt tot een fytotoxisch effect op de plantgroei en kwaliteit. Cd-vervuiling resulteert samengevat in een verlaagde gewas opbrengst en een algemene daling van cultiveerbare gronden. Het vluchtig plant hormoon ethyleen speelt een rol in fysiologische processen gerelateerd aan de plantgroei en ontwikkeling, maar recente ontdekkingen omtrent de connectie tussen ethyleen en Cd-geïnduceerde toxiciteit stimuleren het onderzoek naar de rol van ethyleen in antioxidatieve beschermingsmechanismen zoals de aanmaak van glutathion (GSH) en vervolgens productie van fytochelatines.

Bij een korte (72h) blootstelling van wildtype (WT) *Arabidopsis thaliana* en de GHS deficiënte *cad2-1* mutant aan 5 µM Cd, was de genexpressie van ethyleen biosynthese- en signaleringsgenen verhoogd in de mutant. Dit bevestigt de link tussen GSH en ethyleen signalering om beschermingsmechanismen te stimuleren tijdens Cd-stress. Verder werd de betrokkenheid van ethyleen-geïnitieerde verdedigingsmechanismen onderzocht door Cd-chelerende fytochelatines te meten in de ethyleen-ongevoelige *ein2-1* mutant m.b.v. HPLC in vergelijking met WT planten. Hoewel ethyleen signalering afwezig is en de GSH-productie vertraagd, bleef de initiële verhoging van fytochelatines zowel in WT als mutant aanwezig. Dit duidt op een alternatief mechanisme dat het defect in ethyleen signaal omzeilt. Dit is een mogelijke verklaring voor de ongevoeligheid van de *ein2-1* mutant op niveau van biomassa na korte Cd-blootstelling.

Hiernaast werden ook de effecten van een lange (>72h) blootstelling aan 5µM Cd in WT versus *ein2-1* planten onderzocht. Dagelijkse metingen van de vegetatieve en reproductieve fasen van plant ontwikkeling toonden een reproductieve vertraging/achterstand in de mutant. Cd-stress induceerde echter een gelijkaardige respons in beide genotype. Dit bevestigt nogmaals de aanwezigheid van een omzeiling van het defecte ethyleen signaal. Ten slotte, resultaten van deze studie omtrent het nucleaire ploidie niveau weken af van andere studies. Zo stegen de ploidie niveaus in bladeren van *ein2-1* mutanten onder controle en 72 h Cd-blootstelling, maar daalden na 12 dagen Cd-bloostelling. Dit duidt mogelijk op een wisselwerking tussen ethyleen signalering en activatie van endoreduplicatie onder blootstelling aan Cd.

1 Introduction

The global population and with it the demand for safe and high-quality food and feed supply is growing rapidly. With the current state of increasing soil contamination, however, this demand cannot be met. The increased contamination can be linked to anthropogenic activities (1). Energy production and innovative industry mark modern society, but contribute to an increased soil and air pollution as well as competition with the food industry for the use of available land. Drought, mining activities, excessive use of pesticides and a decline of soil nutrition (phosphorus and nitrates) additionally contribute to an increase in marginal soils unsuitable for plant biomass production (2,3). Together these activities cause a decrease in arable land. This has stimulated the significance of research towards the rehabilitation of contaminated soils concerning crop production and quality. Noteworthy is the contribution of metal pollution due to its persistent and harmful nature to both plants and humans (4). Researchers are investigating different approaches to transform metal-contaminated soils into suitable land for agriculture. In order to grow safe crops with profitable yield, plant growth optimisation and a decrease in metal bioavailability for the uptake by plant roots is essential. However, performing phytoremediation strategies on contaminated soils can only be successful when investigating the underlying toxicity mechanisms (5).

One of the metals building up and persisting in soils is the non-essential element cadmium (Cd). As a cation (Cd^{2+}), its resemblance with essential elements such as calcium causes plants to take it up easily. Via absorption by the root system, Cd can easily translocate to the shoot and accumulate in plant tissues. In particular, the Northern Campine Region in Limburg, Belgium, is challenged with historic Cd pollution due to non-ferrous metal industry, the main industrial activity in the 19th century in this region (6). Multiple studies have shown that Cd poses a threat to human health through accumulation in the food chain, accumulating in and causing damage to multiple organs such as kidneys, lungs, liver and bones (6,7). Also, Cd was shown to negatively affect crop growth and quality (4,8). In leaves of Cd-exposed plants, for example, multiple studies indicate an interfered photosynthesis, which might underlie the overall decrease in plant growth (9,10). Cadmium exposure induces phytotoxicity in different manners as described by Sharma and Dietz (2009): (1) by replacing co-factors due to high similarities in chemical properties, e.g. replacement of and thereby increasing the free fraction of iron (Fe) and copper (Cu), (2) by binding on functional groups of biomolecules such as sulfhydryl groups, hereby causing inactivation or even denaturation of the molecule and (3) by disturbing the cellular redox balance. Although Cd is a non-redox-active metal, it is able to indirectly increase the production of reactive oxygen species (ROS) (11).

1.1 Cadmium-induced oxidative stress

Under physiological circumstances, ROS take part in cellular signalling (12,13). Examples of ROS are the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), the latter being highly reactive and harmful (14,15). Hydrogen peroxide, unlike the other two, is a non-radical and therefore less reactive. These oxygen-derived reactive molecules are mainly by-products of cell organelles with a respiratory electron transport chain and/or a high oxidising-related metabolism such as mitochondria, chloroplasts and peroxisomes (16). Complexes I and III of the mitochondrial respiratory chain are prone to electron leakage, thereby stimulating the formation of the reactive $O_2^{\bullet -}$ molecule (17).

Next to the formation of ROS as by-products, plants have specialized enzymes that actively generate ROS under normal plant functioning (e.g. apoptosis, proliferation) as well as in response to biotic or abiotic stresses (18). For example, NADPH oxidases form a group of multi-component enzymes known as the NOX family and are localized in the apoplast. Superoxide is produced via the transfer of electrons by these oxidases from intracellular NADPH to extracellular O_2. In this way, ROS can be used to indicate stress-inducing threats which are then localized and eliminated if possible. Since H_2O_2 is the reduced form of $O_2^{\bullet -}$ with a low reactivity, it plays a major role in oxidative signalling cascades. Apart from its low reactivity, it has a longer lifespan and can easily cross cellular membranes. In the presence of free redox-active metals such as Cu and Fe, formation of highly reactive and harmful $^{\bullet}OH$ radicals takes place. These reactions are referred to as Fenton and Haber-Weiss reactions, in which respectively H_2O_2 and $O_2^{\bullet -}$ are used as substrates (Figure 1) (14).

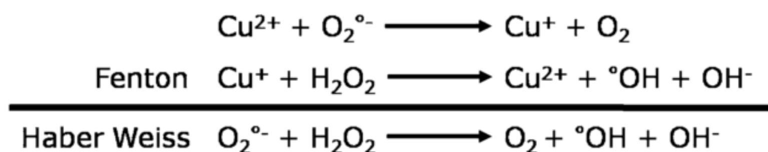


Figure 1: Fenton and Haber-Weiss reactions finally leading to the formation of hydroxyl radicals (13).

Whenever pro-oxidants are favoured due to a disturbance in the redox balance, ROS production exceeds the physiological range. This tipping of the scale towards pro-oxidants resulting in tissue damage is called oxidative stress. To keep the production of ROS within physiological ranges, tightly coordinated detoxification mechanisms of plant cells are required to minimize harmful and deleterious effects on tissues and macromolecules. These mechanisms are referred to as the antioxidant defence system (Figure 2), consisting of both antioxidative enzymes (e.g. peroxidases) and metabolites such as ascorbate (AsA) and glutathione (GSH) (19).

Starting from the formation of $O_2^{\bullet-}$, specialized enzymes called superoxide dismutases (SODs) are activated to catalyse the conversion of $O_2^{\bullet-}$ into H_2O_2 and H_2O. This dismutation is a high-rate and effective way to protect the cell against reactive $O_2^{\bullet-}$ radicals. Superoxide dismutases are classified by their active centre which contains different metals (e.g. Fe-SOD using Fe as a cofactor). After the formation of H_2O_2, further detoxification is required and executed by peroxidases (PRX) such as AsA peroxidase (APX) and/or catalase (CAT). Using AsA as a reducing substrate, APX is known for its fine-tuning of ROS signalling. Catalase, on the other hand, reduces H_2O_2 into H_2O without the need for a redox equivalent and is mainly found in the peroxisomes.

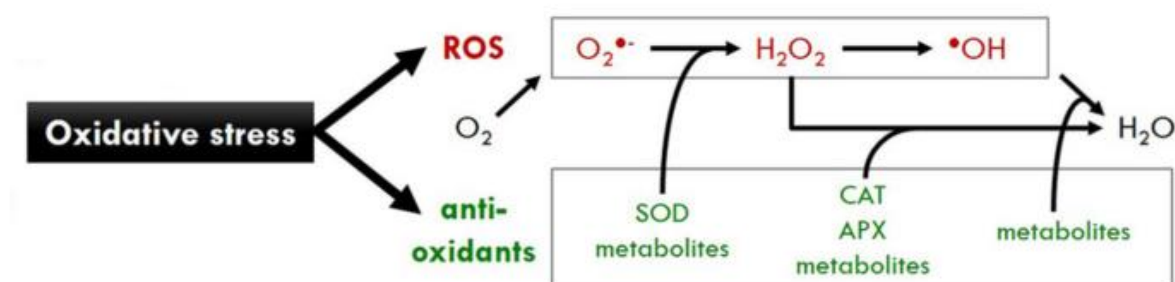


Figure 2: ROS production and antioxidative defence as a consequence of metal-induced oxidative stress (20).

Ascorbate is a well-known metabolite that exerts an antioxidative function. Through evolution, humans have lost the capacity to produce AsA themselves due to the lack of a key oxidative enzyme of the AsA biosynthesis, and therefore are dependent on dietary intake. Plants, however, produce AsA themselves and successfully use it to scavenge ROS. A second important antioxidative metabolite is the primary intracellular antioxidant and chelating agent GSH (21). When it comes to Cd stress, this tripeptide is one of the most important antioxidants because of the thiol (-SH) group in its cysteine residue. Via this functional group, GSH can directly chelate Cd and can be used as a substrate for detoxifying GSH-S-transferases (GSTs) and PRXs. For this reason, it is essential to maintain the pool of reduced GSH within cells by the action of glutathione reductase (GR) (22). In addition, GSH serves as a precursor for phytochelatins (PCs) (23,24). These enzymatically synthesised peptides consist of 2 up to 11 oligomers of GSH molecules. Whenever plant cells sense metal phytotoxicity, there is an activation of the enzyme phytochelatin synthase (PCS) and an increase in the production of PCs. Whenever metals enter the cell, PCs in the cytosol bind and ship them to the vacuole. Especially under Cd toxicity, PC production is elevated, indicating a high sensitivity of these enzymes to Cd uptake (25,26). Finally, an important interplay exists between antioxidative enzymes and metabolites, called the AsA-GSH cycle. During this interplay, APX cooperates with AsA and GSH to detoxify H_2O_2. The reducing capacity of the cycle is ultimately derived from NADPH, by which GSH and AsA can be back-reduced in a cyclic loop. Eventually, H_2O_2 is detoxified to H_2O (27,28).

It has been shown that ROS production is elevated in plants under abiotic stress such as Cd exposure, causing oxidative stress and damage in plants from root to leaf (29). As mentioned before, Cd is a non-essential, non-redox-active element. This indicates that it induces ROS production in an indirect manner. By replacing redox-active elements such as Cu and Fe, there is an increased induction of the Fenton and Haber-Weiss reactions, ultimately resulting in increased $^{\bullet}OH$ production (Figure 1). Also, studies investigating the effect of Cd on antioxidative defence showed a significant correlation between the uptake of Cd and depletion of GSH. As discussed before, GSH plays a major role in maintaining the oxidative balance by scavenging H_2O_2. The high affinity of Cd for the cysteine-rich thiol group of GSH results in a quick binding of Cd to the cellular GSH reservoirs during acute Cd toxicity (30), thereby leaving the cell short on one of its most essential antioxidants. This has direct consequences for the antioxidative defence mechanisms of plants. For example, the AsA-GSH cycle is highly dependent on the GSH pool and is disrupted upon its depletion. Besides GSH, other small thiol-containing peptides called metallothioneins (MTs) help to scavenge Cd (24,31). Due to its high binding capacity with MTs and GSH, Cd remains bound, explaining the long half-life of the element in organisms. After prolonged exposure to Cd, however, this GSH depletion is counteracted by a compensatory increase of GSH production mainly in the leaves, with GSH being transported to the roots (25,32,33).

1.2 Cadmium phytotoxicity is accompanied by increased ethylene production

The induction of ROS signalling and oxidative stress has an effect on the production of phytohormones. These play an important role by controlling normal developmental processes such as cell proliferation and differentiation, regulating osmotic balance, inducing senescence etc. However, when a plant suffers from biotic or abiotic stress, there also seems to be a correlation with the production of certain phytohormones as a defence reaction (34,35).

A highly important player in Cd-induced phytotoxicity is the stress hormone ethylene (C_2H_4). This gaseous plant hormone exerts its effects on many processes involved in plant growth and development such as fruit ripening, abscission, responses to biotic and abiotic stresses, seed germination and root elongation (36,37). The simple biosynthesis pathway of ethylene starts with the conversion of methionine into S-adenosylmethionine (SAM) by SAM synthetase, first described by Yang in 1984 (38). Next, SAM is used as a substrate by 1-aminocyclopropane-1-carboxylic acid synthase (ACS), forming 1-aminocyclopropane-1-carboxylic acid (ACC). This is the rate-limiting step within the ethylene biosynthesis pathway. Additionally, ACS also produces 5'-methylthioadenosine (MTA), which is recycled back to methionine in the methionine cycle. Further oxidation of ACC by ACC oxidase (ACO)

is required to obtain ethylene with CO_2 and cyanide as by-products. Cyanide, being a harmful substance, is detoxified to β-cyanoalanine by β-cyanoalanine synthase to prevent toxicity (39–42).
The general mechanism of activation is mostly preserved among the various responses induced by ethylene signalling. This mechanism is driven by the sensing of ethylene by transmembrane receptors. They consist of an N-terminal ethylene-binding domain, a C-terminal domain containing the histidine kinase subunit and a domain responsible for protein-protein interactions. The receptors are situated at the endoplasmic reticulum (ER) and five receptors can be distinguished in *A. thaliana*: ETHYLENE RESPONSE SENSOR 1 and 2 (ERS1 and ERS2), ETHYLENE RESISTANT 1 and 2 (ETR1 and ETR2) and ETHYLENE INSENSITIVE 4 (EIN4) (43).

In the absence of ethylene (Figure 3A), the signalling process is actively inhibited at the beginning of the pathway via a negative regulator (44). This regulator, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) causes the phosphorylation of ETHYLENE INSENSITIVE 2 (EIN2) which leads to its degradation and consequently the inhibition of the pathway (Figure 3A). The name ETHYLENE INSENSITIVE 2 is related to the complete insensitivity of the plant for ethylene after a loss-of-function mutation in its encoding gene. However, when the production of ethylene is stimulated, e.g. under Cd toxicity (29), the hormone binds to a transmembrane receptor which induces a cleavage of the C-terminus domain. After transfer to the nucleus, it activates a group of transcription factors (TFs) belonging to the ETHYLENE INSENSITIVE 3 (EIN3) family. These in turn will activate other TFs such as ETHYLENE RESPONSE FACTOR 1 (ERF1) that induce the expression of ethylene-responsive genes (Figure 3B) (45,46).

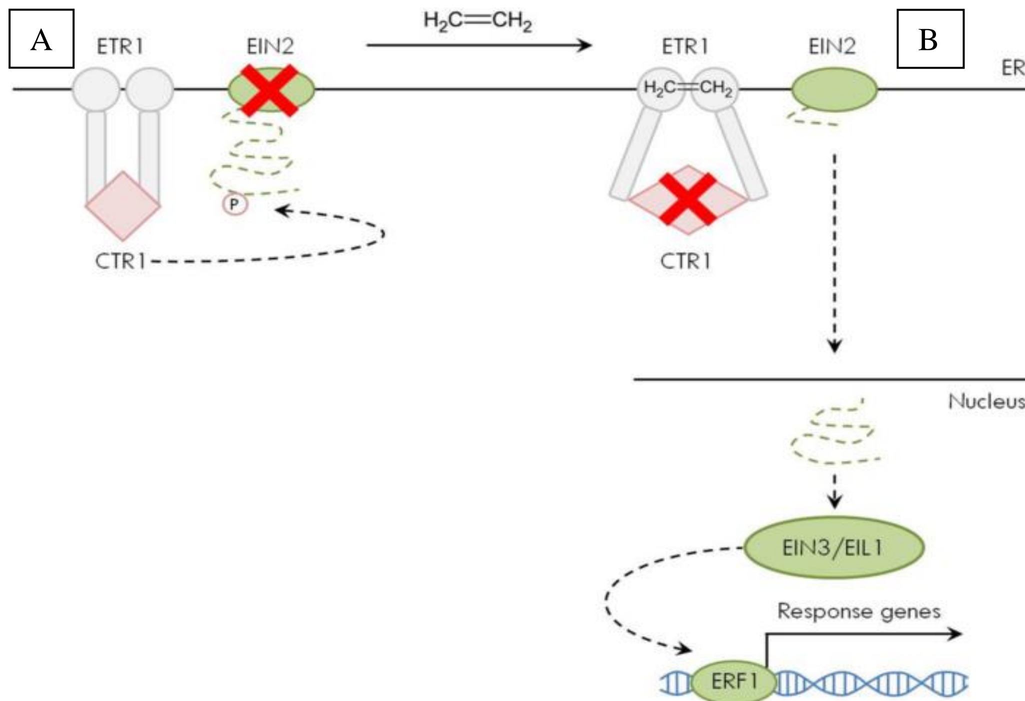


Figure 3: Ethylene signal transduction pathway. (A) Ethylene is absent and the negative regulator CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) inhibits the signalling cascade by the phosphorylation of ETHYLENE INSENSITIVE 2 (EIN2). (B) In the presence of ethylene, receptor binding inactivates the negative regulator CTR1, and downstream signalling occurs through translocation of the C- terminal domain of EIN2 to the nucleus. Transcription factors of the family ETHYLENE INSENSITIVE 3 (EIN3)/EIN3-LIKE 1 (EIL1) and ETHYLENE RESPONSIVE FACTOR 1 (ERF1) induce the transcription of ethylene-responsive genes, resulting in multiple outcomes. Adapted from Keunen et al. (47).

Cadmium, as mentioned previously, has been correlated to ethylene production due to a transcriptional upregulation of ethylene biosynthesis pathway compounds, more specifically an upregulation of *ACS* and *ACO* gene expression (48). This has been shown in recent studies, where short-term Cd exposure (24 h) in *A. thaliana* plants increased the ethylene production via upregulation of *ACS2/6* genes. From these results, the link between ethylene production, signalling and eventually increased oxidative stress was hypothesised (49). After 72 h of Cd exposure however, plants showed a decreased production of ethylene as compared to 24 h, indicating its potential role in acute responses to Cd stress.

1.3 Results indicate a link between ethylene signalling and GSH responses

Studies using GSH deficient *cadmium sensitive 2-1 (cad2-1) Arabidopsis thaliana* mutants indicated the essential role of GSH since phytotoxic effects, including increased ROS production, were elevated after Cd exposure as compared to wild-type (WT) plants (25). In addition, an important link connecting GSH and ethylene production has been suggested (47). Studies showed that when ethylene production is increased, the GSH pool seems to be positively influenced (29,50). Schellingen *et al*. investigated the effects of moderate (5 µM) Cd exposure in ethylene biosynthesis (*acs2-1/acs6-1*, 2015a) and signalling (*ein2-1*, 2015b) *A. thaliana* mutants, respectively (29,50). Related to GSH metabolism, there were

significant differences between WT and *acs2-1/acs6-1* mutant plants after 24 h Cd exposure, which faded out after 72 h. More specifically, lower expression levels of GSH biosynthesis genes resulted in a decreased amount of GSH in leaves of the *acs2-1/acs6-1* mutant after 24 h as compared to WT plants. Although this could not be observed in the roots, these results do point out the importance of ethylene-mediated responses during acute Cd stress (29). As compared to the ethylene biosynthesis mutant, leaves of the *ein2-1* signalling mutants had lower total and reduced GSH concentrations upon Cd exposure as compared to WT plants after both 24 and 72 h exposure. In addition, transcript levels of genes encoding GSH biosynthesis enzymes as well as oxidative stress marker genes were less or not increased after 24 h Cd exposure as compared to Cd-exposed WT plants (50). However, more prolonged Cd exposure (72 h) did not show this effect between exposure and control conditions in the *ein2-1* mutant. These results indicate that GSH responses in the *ein2-1* mutant are delayed, therefore linking ethylene signalling and GSH responses necessary for replenishment of the GSH pool. However, the prolonged increased *ERF1* expression in plants exposed to Cd for several days indicates a potential role for ethylene in long-term responses to Cd stress as well (unpublished results).

Another important link between GSH and ethylene production, is the common incorporation of cysteine during the biosynthesis of these compounds. Cysteine is the main source of sulphur, which is necessary for the Cd-chelating actions of GSH and subsequently PCs. It is therefore hypothesised that ethylene signalling might stimulate the upregulation of sulphur metabolism in order to obtain the observed effects on the maintenance of the GSH pool under Cd stress (51,52).

1.4 Linking ethylene to endoreduplication under Cd exposure

Ethylene has been shown to regulate different processes involved in plant growth and development. One such process could be endoreduplication (also called endoreplication or endocycle), an alternative form of the regular cell cycle (53). Unlike the normal cell cycle where after DNA replication, cell division results in two daughter cells with a 2C ploidy level, endoreduplication involves multiple rounds of DNA replication without the occurrence of subsequent chromosome separation and cytokinesis (54). Next, increases in cell size rather than proliferation takes place. Hereby, cells with higher ploidy levels and massive cell expansion are common in metabolically active, highly specialised tissues where endoreduplication is trivial (e.g. fruits of tomato plants, cereal endosperm, etc.) (55).

The process of endoreduplication has been indicated to play a crucial role in the growth and development of plants (53). The specificities of this role, however, and the importance in physiological processes remains to be unravelled. Mainly, attention on the subject points towards DNA protection by endoreduplication in cells undergoing (a)biotic stresses. As such, damaged DNA after the insult is protected by the limitation of cell proliferation, thereby confining the passing along of mutations to

daughter cells. More research is performed around the connection between Cd toxicity and DNA damage, linking all these processes together in a complex network (56). Also, the increase of ploidy levels in metabolically active cells might indicate a stimulation of gene expression necessary to answer to physiological demands (57).

Generally, it has been pointed out that phytohormones play a role in the coordination of the cell cycle and thus cell division and endoreduplication processes. As such, ethylene has shown to have a general posttranscriptional suppressive effect on cell proliferation and cytokinesis, for example by inhibiting cyclin-dependent kinase A (CDKA) activity and influencing mediator CDKB of the cell cycle. The effect of ethylene on endoreduplication is mainly reported as positive, having an overall stimulatory effect (58–61).

1.5 Research objectives

Within the current study, the ultimate aim is to further unravel the role of ethylene within plant responses to Cd stress. Firstly, the potential link between ethylene and GSH under sublethal stress conditions is further studied by measuring the expression of genes related to ethylene biosynthesis (*ACS6/2*) and signaling (*ERF1*) in GSH deficient *cad2-1* mutants. Sulphur metabolism is hypothesised to play a role in the interaction between ethylene and GSH. Therefore, the concentrations of PCs, dependent on sulphur and GSH supply, are determined in both WT and *ein2-1* mutant plants under acute Cd exposure. Furthermore, as previous research in relation to ethylene signalling mostly focussed on short-term Cd exposure, long-term effects are investigated within a phenotypical experiment. Finally, the prominent role of ethylene in plant growth and development might be related to endoreduplication as well. Therefore, ploidy levels are determined after short- (72 h) and long-term (12 days) Cd exposure in leaves of WT versus *ein2-1* mutant plants.

2 Materials and Methods

To investigate the modulating role of ethylene during Cd exposure in *A. thaliana*, this project was divided into two subparts. In the first part, the focus lies on short-term (72 h) exposure effects of Cd related to ethylene biosynthesis and signalling in GSH deficient *cad2-1* mutant versus WT plants. This mutant only contains around 30 % of WT GSH levels due to its deficiency in γ-glutamylcysteine synthetase activity (62). In addition, Cd-induced changes in PC production were compared between WT and ethylene insensitive *ein2-1* mutant plants. The latter are known for their insensitivity to ethylene due to a mutated *EIN2* gene, resulting in impaired signalling (63). Next, long-term exposure effects (> 72 h) were compared between WT plants and *ein2-1* mutants. Besides a phenotypical follow-up experiment, the occurrence of endoreduplication was investigated to link ethylene to plant growth and development under control and Cd exposure conditions in a prolonged time frame. All plants were grown using a well-established hydroponic cultivation system (64).

2.1 Plant culture

Prior to sowing, seeds were surface-sterilised using 0.1 % NaOCl (Sigma-Aldrich, Belgium). Plant cultures were set up using seeds of WT, GSH deficient *cad2-1* mutant and ethylene-insensitive *ein2-1* mutant *A. thaliana* plants (ecotype Colombia (Col-0)). Plant growth was performed according to a validated hydroponic setup described by Smeets *et al*. (2008) (64). Plants were grown under controlled conditions (12 h photoperiod, 170 µmol $m^{-2}s^{-1}$ photosynthetic active radiation (PAR) at rosette level), 22 °C / 18 °C day/night, 65 % humidity, modified Hoagland solution) in a climate chamber using LED lights. After 19 days, plants were exposed to 5 µM $CdSO_4$ via the roots or continued to grow under control conditions dependent on the condition. At two time points, after 24 and 72 h of exposure, roots and leaves (rosettes) were harvested and the fresh weight was determined before samples were snap-frozen in liquid nitrogen and stored at -70 °C for further analyses.

2.2 Gene expression analysis

Frozen root and leaf samples were ground using two stainless steel beads and the Retsch Mixer Mill MM400 (30 s^{-1}, 2 min; Retsch, Belgium). To extract RNA, the RNAqueous® Kit (Ambion, Applied Biosystems) was used according to the manufacturer's protocol. Once the RNA was obtained, its concentration and purity was evaluated using the NanoDrop® ND-1000 spectrophotometer (ThermoScientific, USA). In addition, the integrity of the extracted RNA was verified using the Experion™ RNA StdSens Analysis Kit (Bio-Rad, Hercules, CA, USA).

Via the TURBO DNA-*free*™ Kit (Life Technologies, Waltham, MA, USA), a DNase treatment was performed to eliminate possible genomic DNA contamination. Single-stranded cDNA was obtained by converting 1 µg of the treated RNA using the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa Bio Inc., the Netherlands) according to manufacturer's instructions. The obtained cDNA was 10-fold

diluted in 1/10 diluted TE buffer (1 mM Tris–HCl, 0.1 mM Na2-EDTA, pH 8.0; Sigma–Aldrich, Belgium) and subsequently stored at -20 °C.

After selecting genes of interest, quantitative real-time PCR (qPCR) was performed using the Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's protocol. For this, Master Mix and samples were added to a 96-well plate ran into the 7500 Fast Real-Time PCR System (Applied Biosystems). To measure the chosen gene of interest, forward and reverse primers were used in gene-specific concentrations (300 nM for *ACS2*, 900 nM for *ACS6* and 600 nM for *ERF1*). To check amplicon specificity, amplification was followed by a dissociation curve. Cycling conditions can be found in Supplemental Table A1, primer sequences in Supplemental Table A2 and an overview of our adherence to the MIQE guidelines in Supplemental Table A3.

2.3 Measurements of GSH and PC concentrations

The determination of GSH and PC contents was performed using High Performance Liquid Chromatography (HPLC), as described by Wójcik & Tukiendorf *et al*. 2005 (65) and in association with Małgorzata Wójcik (Maria Curie-Skłodowska University, Lublin, Poland). To ground the frozen root and leaf samples, a double volume of 0.1 M HCl was added in an ice-cooled mortar. After three centrifugation steps (14 000 rpm, 4 °C, 5 min), the supernatant was obtained and used in a Beckman chromatograph (126/166 model) with a Supelco precolumn (4.6 mm x 10 mm) and column (4.6 mm x 250 mm), which were filled with Ultrasphere C18. Next, peptides (100 µl) were separated in a linear gradient (0–20 %) using acetonitrile in 0.05 % trifluoroacetic acid which afterwards were processed by a post-column reaction with 200 µM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 0.05 M potassium-phosphate buffer (pH 7.6). The absorbance at 405 nm was measured by the Beckman detector (model 166) and the chromatograms were analysed using Karat 7.0 software from Beckman.

2.4 Long-term phenotypical analysis

Plants were sown and hydroponically grown as described in section 2.1. After 20 days, 15 WT and *ein2-1* mutant plants were either exposed to 5 µM Cd or further grown under control conditions. Starting from day 20, day-by-day follow-up of plant growth was performed until day 55. Starting with vegetative growth, the number of leaves and rosette diameter were measured until a plateau was reached. Furthermore, regenerative growth was monitored by determining the percentages of plants with an emerged inflorescence, as well as inflorescence height and silique formation at later stages. Throughout the entire experiment, plants were continuously exposed to 5 µM Cd or grown under control conditions. Different from section 2.1, Hoagland solution with or without Cd was refreshed once a week to ensure nutrient availability.

2.5 Analysis of nuclear ploidy levels

Nuclear DNA content was determined via flow cytometry. This translates to the ploidy level in the cell and thus the extent of endoreduplication. Nuclear DNA content was analysed in separate leaves, since ploidy levels are age-dependent. Preparation of separate leaf samples prior to flow cytometric measurements cover the extraction and staining of nuclei using CyStain PI Absolute P kit (Sysmex Partec, Germany). Specifically, leaves were added to a petri dish containing 500 µl extraction buffer, chopped and incubated for 2 minutes. Filtration through a 50 µm nylon Celltrics filter (Sysmex Partec) ensured separation of the nuclei. Next, staining solution containing 2 ml staining buffer, 12 µl propidium iodide and 6 µl RNase, was added to the flow cytometric tube. After an incubation time of at least one hour at 4 °C in the dark, nuclear DNA content of approximately 5000 to 10 000 nuclei was determined using the CyFlow® Cube 8 (Sysmex Partec, Belgium). Nuclei were excited with a 488 nm, after which PI fluorescence was detected in the FL-2 channel (580/30 nm) and plotted against the forward scatter. For data analysis, the software "FCS Express 4 Flow Cytometry software" (De Novo Software) was used. The endoreduplication factor (EF) was compiled of data obtained from all the measurements made (in %) of the different amounts of nuclear ploidy levels (2C-32C) within a specific sample. For this, the following calculation was used:

[0 x 2C (%) + 1 x 4C (%) + 2 x 8C (%)+ 3 x 16C (%) + 4 x 32C (%)]/100.

2.6 Statistical analyses

All statistics were performed in R (R Development Core Team, 2016) and were tested for normality and homoscedasticity. Normality was tested by the Shapiro test and homoscedasticity using the Bartlett test. If both tests resulted in p-values higher than 0.05, it is allowed to use a parametrical statistical test. Data obtained from experiments using both WT and mutant plants (*cad2-1* or *ein2-1*) were analysed for genotype, treatment and interaction effects by two-way ANOVA. Detection of outliers was performed using the studentised deviation method (GraphPad Software, San Diego, CA, USA). For gene expression data, RQs were log transformed before the statistical analysis was carried out. If statistical differences were significant, multiple comparisons using a post-hoc Tukey-Kramer test was performed. If normality and homoscedasticity assumptions were not met (not even after transformation of the data), the non-parametric Kruskal-Wallis test was used, followed by the Wilcoxon Rank sum test for pairwise data comparisons.

3 Results

This section is divided into two subparts, namely (1) the effects of short-term, moderate Cd exposure on ethylene-related responses in *A. thaliana* and (2) the effects after long-term moderate Cd exposure. Dependent on the experiment, either GSH deficient *cad2-1* or ethylene insensitive *ein2-1* mutants are compared to WT plants.

3.1 Ethylene- and GSH-related responses to short-term Cd exposure in *A. thaliana* plants

In the first part of this study, effects of short-term (24-72h) Cd exposure were investigated in relation to the potential link between ethylene signalling and GSH. For this, transcript levels of ethylene biosynthesis and signalling genes were measured in Cd-exposed GSH deficient *cad2-1* mutants as compared to WT *A. thaliana* plants. Next, the synthesis of PCs was studied in WT versus ethylene insensitive *ein2-1* mutants to further explore the effects of ethylene on GSH biosynthesis.

3.1.1 Ethylene biosynthesis and signalling is elevated in cad2-1 mutants

In Table 1, transcript levels of ethylene biosynthesis (*ACS2* and *ACS6*) as well as signalling (*ERF1*) genes are shown for roots and leaves of WT versus *cad2-1* mutant *A. thaliana* plants after 72 h exposure to 5 µM Cd. In roots, there was an overall significant increase in gene expression for all three genes upon Cd exposure in both genotypes. However, this increase was much more pronounced in roots of Cd-exposed *cad2-1* mutants as compared to WT plants (Table 1). In the leaves, similar observations were made, although the increases in gene expression were only significant for *ACS2* and *ERF1* in Cd-exposed *cad2-1* mutants (Table 1).

Table 1: Transcript levels of ethylene biosynthesis (*ACS2* and *ACS6*) and signalling (*ERF1*) genes in roots and leaves of WT and *cad2-1* mutant *A. thaliana* plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ during 72 h, starting 19 days after sowing. Data are given as the mean ± S.E. of at least four biological replicates relative to the control set at 1.00 for each genotype. Significant differences (Tukey's test: $p < 0.05$) between control and exposed plants are indicated in green, while differences between both genotypes are highlighted using asterisks.
Abbreviations: *ACS*: 1-aminocyclopropane-1-carboxylic acid synthase; *ERF1*: ethylene response factor 1.

Roots

Gene	$CdSO_4$	WT			*cad2-1*			
ACS2	Control	1.00	±	0.13	1.00	±	0.18	
	5 µM	2.12	±	0.17	17.71	±	0.73	*
ACS6	Control	1.00	±	0.13	1.00	±	0.08	
	5 µM	3.62	±	0.25	5.59	±	0.28	*
ERF1	Control	1.00	±	0.14	1.00	±	0.07	
	5 µM	7.06	±	0.58	35.48	±	8.15	*

Leaves

Gene	$CdSO_4$	WT			*cad2-1*			
ACS2	Control	1.00	±	0.38	1.00	±	0.53	
	5 µM	2.35	±	0.82	38.15	±	6.65	*
ACS6	Control	1.00	±	0.26	1.00	±	0.41	
	5 µM	1.90	±	0.42	3.18	±	1.18	
ERF1	Control	1.00	±	0.27	1.00	±	0.47	
	5 µM	2.40	±	0.61	47.21	±	22.49	*

3.1.2 Phytochelatins as a potential link between ethylene and GSH

The effect of short-term moderate (5 µM) Cd exposure on plant growth was investigated by determining the fresh weight of roots and leaves at the point of harvest after 19 days of growth and 72 h of exposure (Figure 4). For both genotypes, Cd exposure caused a significant decrease in root fresh weight (Figure 4A). However, only the fresh weight of WT leaves was affected by Cd after 72 h (Figure 4B).

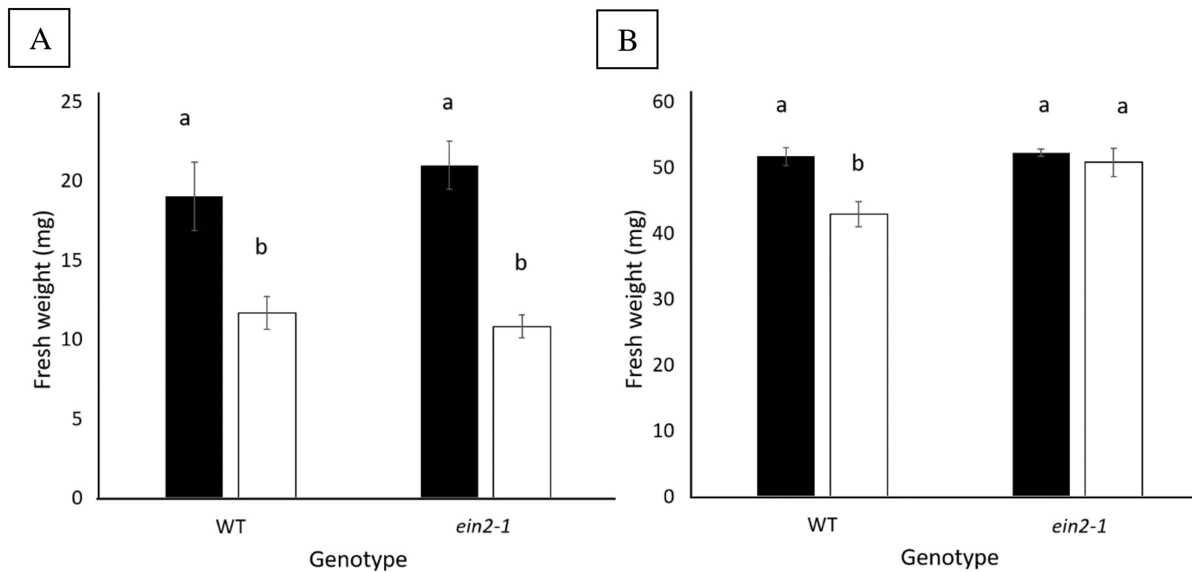


Figure 4: Absolute root (A) and leaf (B) fresh weight (mg) of WT and *ein2-1* mutant *A. thaliana* plants grown under control conditions (0 µM $CdSO_4$, black) or exposed to 5 µM $CdSO_4$ (white) for 72 h, starting 19 days after sowing. For each time point, data represent the mean ± S.E. of eight biological replicates. Significant differences (Tukey's test: $p < 0.05$) comparing all conditions per organ are indicated using different letters (a, b).

Related to the potential interaction between ethylene and GSH, levels of total GSH and PCs were determined and compared between leaves of WT and *ein2-1* mutant plants under control and 5 µM Cd conditions (Figure 5). After 24 h (Figure 5A), Cd exposure did increase PC production but no significant differences were noted between genotypes. After 72 h (Figure 5B), total GSH concentrations increased after Cd exposure in leaves of WT plants. Nonetheless, a similar trend could be observed for *ein2-1* mutants. In addition, total PC concentrations were similar for both genotypes (Figure 5B).

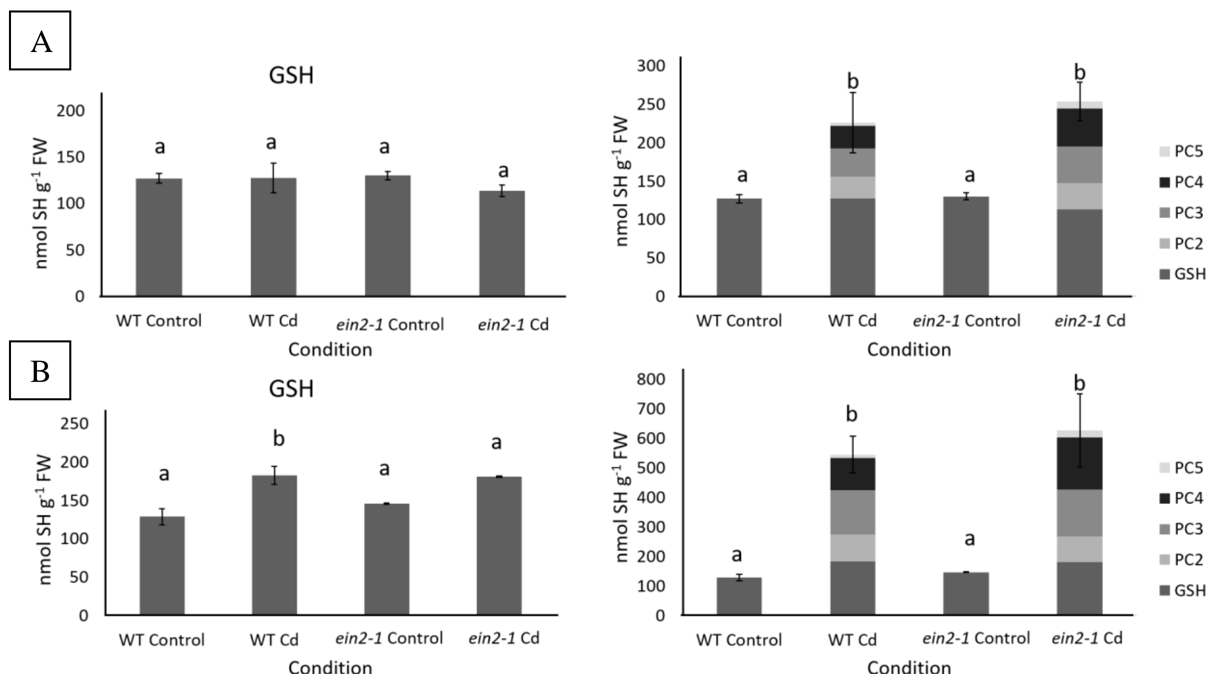


Figure 5: Profiling total GSH and phytochelatin (PC2-5) content in leaves of WT and *ein2-1* mutant *A. thaliana* plants exposed to 5 µM $CdSO_4$ for (A) 24 and (B) 72 h. Thiol content is expressed in SH equivalents (nmol SH g^{-1} fresh weight). Data represent the mean ± S.E. of four biological replicates. Significant differences (Tukey's test: $p < 0.05$) comparing all conditions per organ are indicated using different letters (a, b).

3.2 Cadmium-induced responses after long-term exposure in WT and *ein2-1* mutant *A. thaliana* plants

This part covers the results related to the effects of long-term exposure to 5 µM Cd in WT and *ein2-1* mutant plants. Firstly, results of the phenotypical follow-up experiment are shown with the distinction between vegetative growth (*i.e.* rosette diameter and leaf count) and reproductive growth (*i.e.* inflorescence emergence, inflorescence height and silique formation). These results are subsequently coupled to the extent of endoreduplication after 72 h and 12 days of Cd exposure. In this way, ethylene is linked to plant growth and development focussing on DNA ploidy levels under control and Cd exposure conditions in a prolonged time frame.

3.2.1 Vegetative and reproductive growth stages compared between WT and ein2-1 mutant plants

<u>*Vegetative growth phase*</u>

Rosette diameter (Figure 6A) and leaf count (Figure 6B) were monitored daily starting from the day of Cd exposure (day 20) until a plateau phase was reached (day 47 for rosette diameter, day 40 for leaf count). Cadmium had a clear negative impact on the rosette diameter of both WT and *ein2-1* mutant plants (Figure 6A). However, there were some morphological differences between the leaves of both genotypes. Under Cd stress, leaves of *ein2-1* mutants were thicker and curled as compared to WT leaves (data not shown). Comparing the amount of leaves grown over time (Figure 6B), no clear differences between both genotypes could be observed. Although *ein2-1* mutants had slightly more leaves than WT plants, especially under control conditions, the overall difference between control and Cd exposure in both genotypes remained the same (Figure 6B). In conclusion, both WT and *ein2-1* mutant plants seem to react similarly to long-term moderate Cd exposure, at least in the vegetative growth phase.

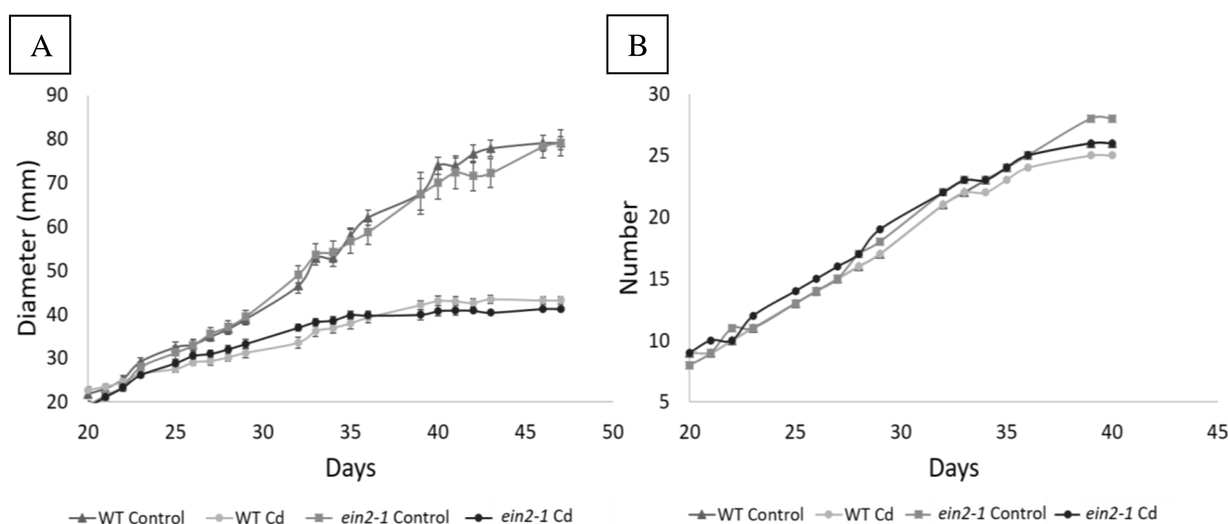


Figure 6: Rosette diameter and leaf count of WT and *ein2-1* mutant *A. thaliana* plants continuously exposed to 5 µM $CdSO_4$ or grown under control conditions (0 µM $CdSO_4$). (A) The average rosette diameter (mm) over time. (B) The average number of rosette leaves over time. Data are given as the average ± S.E. of 15 biological replicates; days are counted starting from Cd exposure (day 20 after sowing).

Reproductive growth phase

Reproductive growth of WT and *ein2-1* mutant plants under control and Cd exposure conditions was compared with the focus on inflorescence emergence (Figure 7A) and height (Figure 7B) as well as silique formation (Figure 8).

Related to the inflorescence emergence (Figure 7A), WT plants under control and Cd exposure conditions already showed a head start as compared to the *ein2-1* mutant, which was followed by a delay in inflorescence height for the latter (Figure 7B). When comparing the effect of Cd, however, both genotypes showed the same trend which could indicate that the delay seen for *ein2-1* mutants is probably due to the genotype itself (blocked ethylene signalling) and not to Cd phytotoxicity. Next, this was also supported by a delay in silique (and coherent flower) formation in *ein2-1* mutants as compared to WT plants (Figure 8).

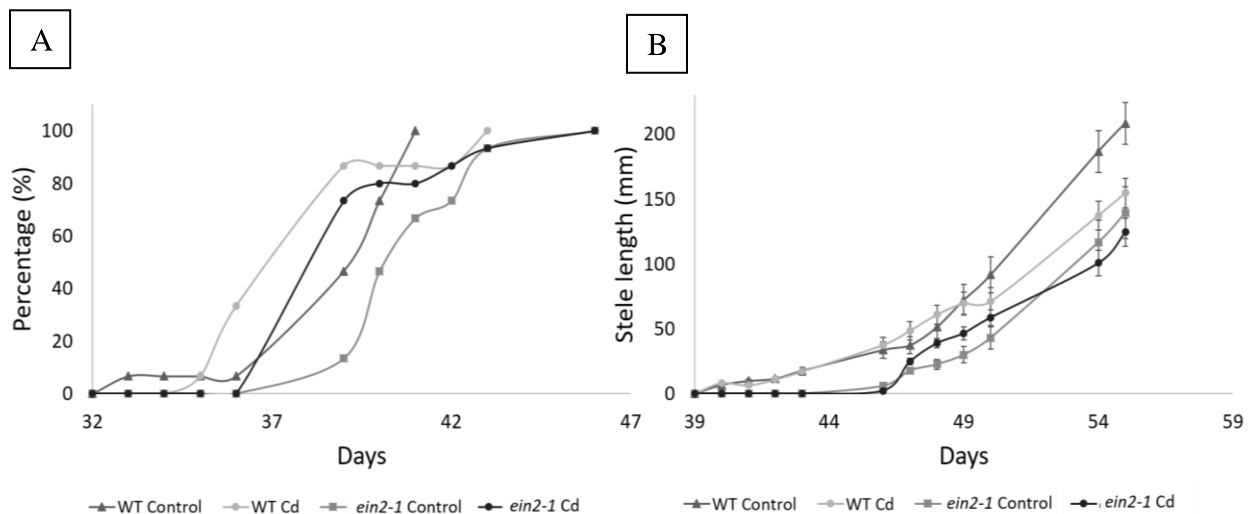


Figure 7: Inflorescence emergence and height of WT and *ein2-1* mutant *A. thaliana* plants continuously exposed to 5 µM $CdSO_4$ or grown under control conditions (0 µM Cd). (A) The percentage of plants with inflorescence meristems over time. (B) The average inflorescence height (mm) over time. Data are given as the average ± S.E. of 15 biological replicates; days are counted starting from Cd exposure (day 20 after sowing).

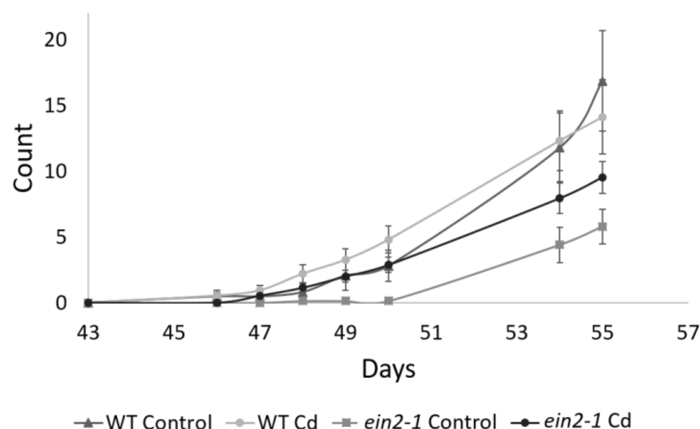


Figure 8: Silique formation in WT and *ein2-1* mutant *A. thaliana* plants continuously exposed to 5 µM $CdSO_4$ or grown under control conditions (0 µM $CdSO_4$). The average number of siliques over time are shown. Data is given as the average ± S.E. of 15 biological replicates; days are counted starting from Cd exposure (day 20 after sowing).

3.2.2 Endoreduplication linked to growth responses of WT versus ein2-1 mutant A. thaliana plants under control and Cd exposure **conditions**

Ploidy levels of cellular DNA shed a new dimension to plant growth, taken into account the importance of endoreduplication. Figure 9 provides an overview of the setup as well as the results of the experiment conducted to reveal the impact of ethylene insensitivity upon growth under control and Cd exposure conditions. Both WT and *ein2-1* mutant plants were exposed to 5 µM Cd on day 20, after which ploidy levels were measured at two time points: 72 h as short-term exposure and 12 days as long-term exposure (Figure 9). Since leaf 11 was not present yet after 72 h, the extent of endoreduplication was determined for leaves 1 and 4, a mature and young leaf respectively. On day 12 of Cd exposure, ploidy levels were measured in leaves 4 and 11. In this way, it was possible to compare the results for leaf 4 after 72 h and 12 days. In addition, the effects of Cd exposure could also be determined in a newly developed leaf after the exposure started (leaf 11) (Figure 9). The extent of endoreduplication is indicated as the endoreduplication factor (EF), giving a relative idea about the amount of endoreduplication taking place in a certain leaf.

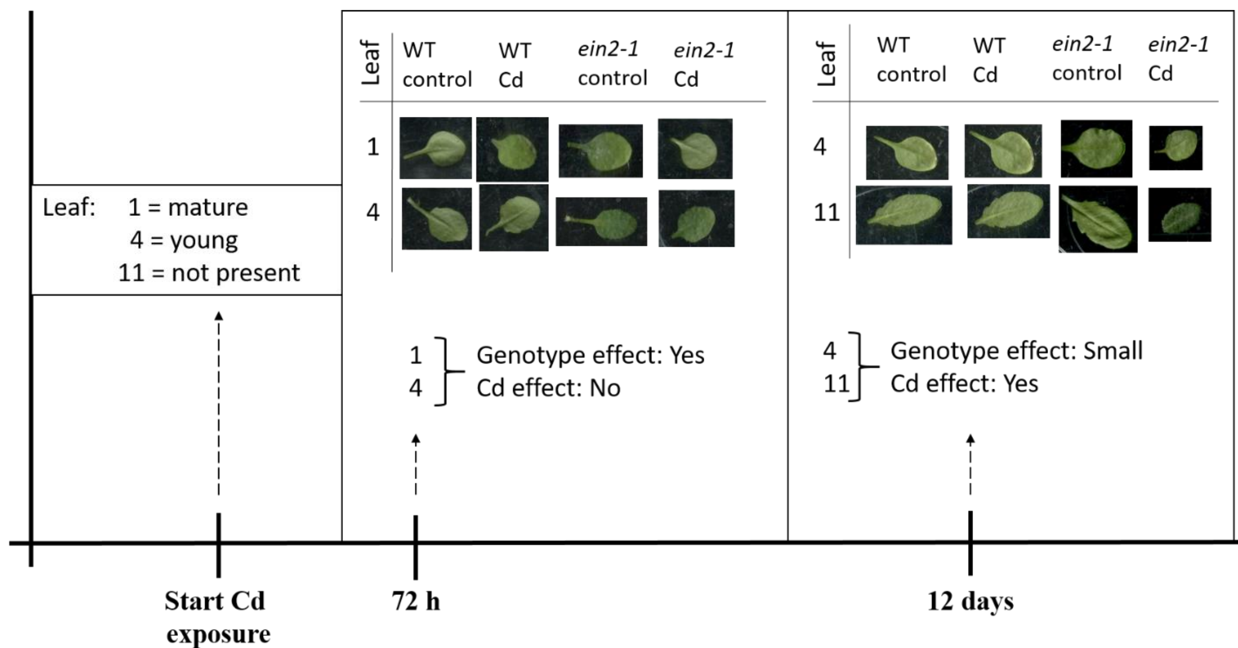


Figure 9: Overview of the endoreduplication experiment conducted at two time points: 72 h (short-term) and 12 days (long-term) exposure of WT and *ein2-1* mutant *A. thaliana* plants. Leaves 1 and 4 were measured at 72 h, while leaves 4 and 11 were measured at 12 days. Plants were exposed to 5 µM Cd after 20 days of growth or further grown under control conditions. "Genotype effect" indicates that a difference in the amount of endoreduplication is observed between *ein2-1* mutant as compared to WT plants, independent of Cd exposure. "Cadmium effect" indicates a difference in the amount of endoreduplication between plants exposed to 5 µM Cd as compared to the control condition (0 µM Cd).

After 72 h, Cd exposure did not significantly affect the extent of endoreduplication in the mature leaf 1 of WT plants (Figure 10). However, ploidy level 16C was significantly increased in leaf 1 of *ein2-1* mutants both under control and Cd exposure conditions (Figure 10A). In addition, 32C followed the same trend albeit only significant under Cd exposure, while the percentage of ploidy level 8C was significantly decreased in both conditions (Figure 10A). This resulted in a significantly higher extent of endoreduplication in *ein2-1* mutants as compared to WT plants (Figure 10B). In conlusion, ethylene insensitivity affects the extent of endoreduplication independently from Cd exposure in leaf 1 after 72 h.

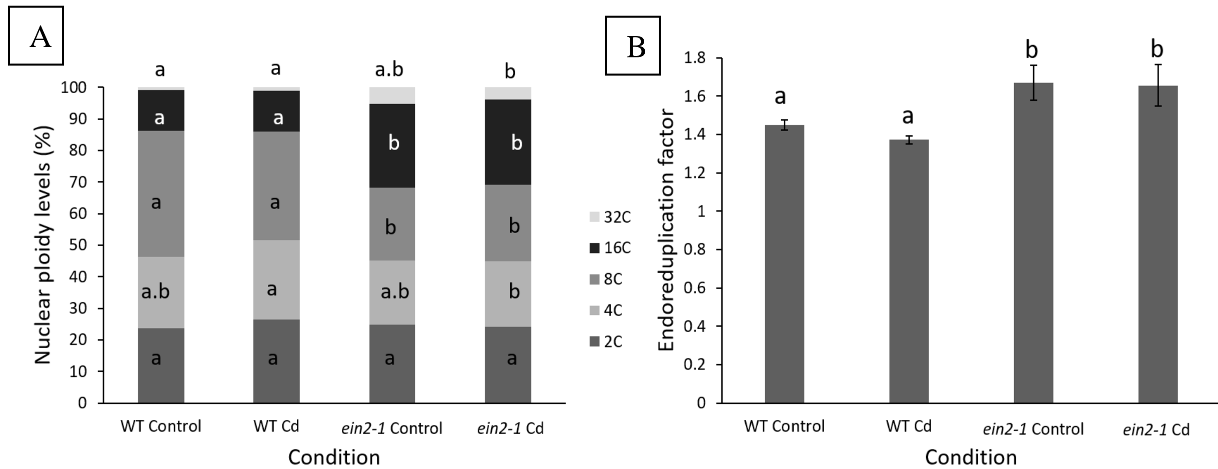


Figure 10: Percentage of nuclear ploidy levels (A) and the endoreduplication factor (B) in the first leaf of WT and *ein2-1* mutant *A. thaliana* plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ for 72 h, starting at day 20 after sowing. Data represent the mean ± S.E. of four biological replicates. Significant differences (Wilcoxon rank sum test: $p < 0.05$) obtained when comparing all four conditions are indicated using a different letter (a, b). For the nuclear ploidy levels, significant differences are indicated for each ploidy level separately using different letters within each ploidy level.

When looking at the effects of 72 h Cd exposure on the extent of endoreduplication in the younger leaf 4 of WT and *ein2-1* mutant plants, no significant differences were found between between genotypes and/or conditions (Figure 11A). Accordingly, the same was observed when calculating the EF (Figure 11B). However, when looking at the different ploidy levels separately, a trend of genotype and Cd effect could be observed for the 16C and 32C levels. Under control conditions, these levels were higher in the *ein2-1* mutant, but again dropped under Cd exposure (Figure 11A, Appendix Figure A1).

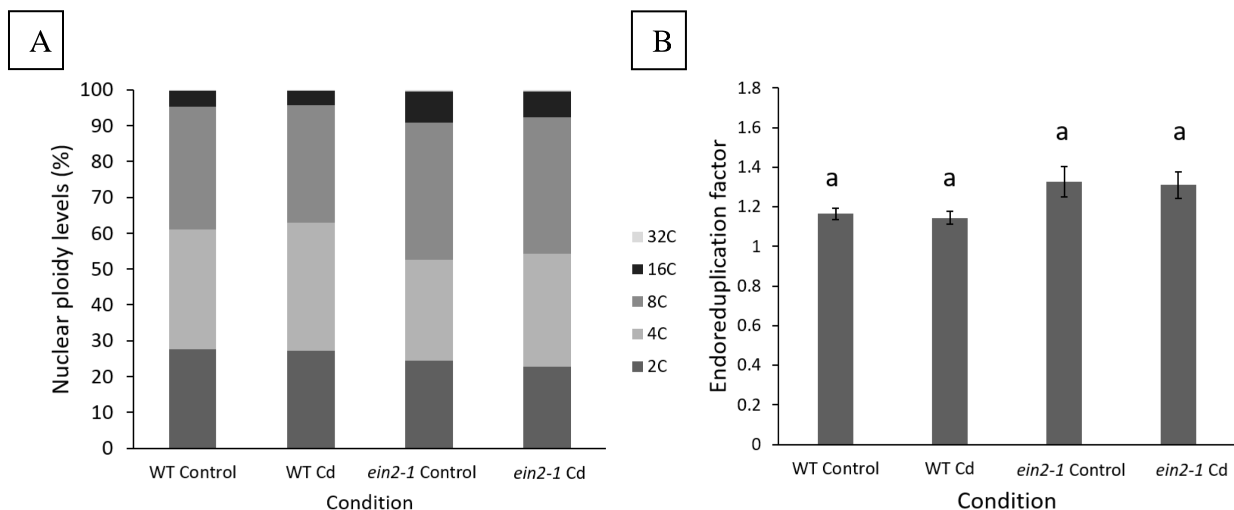


Figure 11: Percentage of nuclear ploidy levels (A) and the endoreduplication factor (B) in leaf 4 of WT and ein2-1 mutant A. thaliana plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ for 72 h, starting on day 20 after sowing. Data represent the mean ± S.E. of four biological replicates. Significant differences (Wilcoxon rank sum test: $p < 0.05$) obtained when comparing all four conditions are indicated using a different letter (a, b). For the nuclear ploidy levels, significant differences are indicated for each ploidy level separately using different letters within each ploidy level.

After 12 days of Cd exposure, the extent of endoreduplication decreased in leaf 4 of both WT and *ein2-1* mutant plants (Figure 12). However, as seen before in leaf 4 after 72 h (Figure 11), a different trend in ploidy level 16C occurred under control conditions when comparing the two genotypes. Even though not significant, the 16C ploidy level was higher under control conditons in leaf 4 of *ein2-1* mutant as compared to WT plants (Figure 12A, Appendix Figure A2).

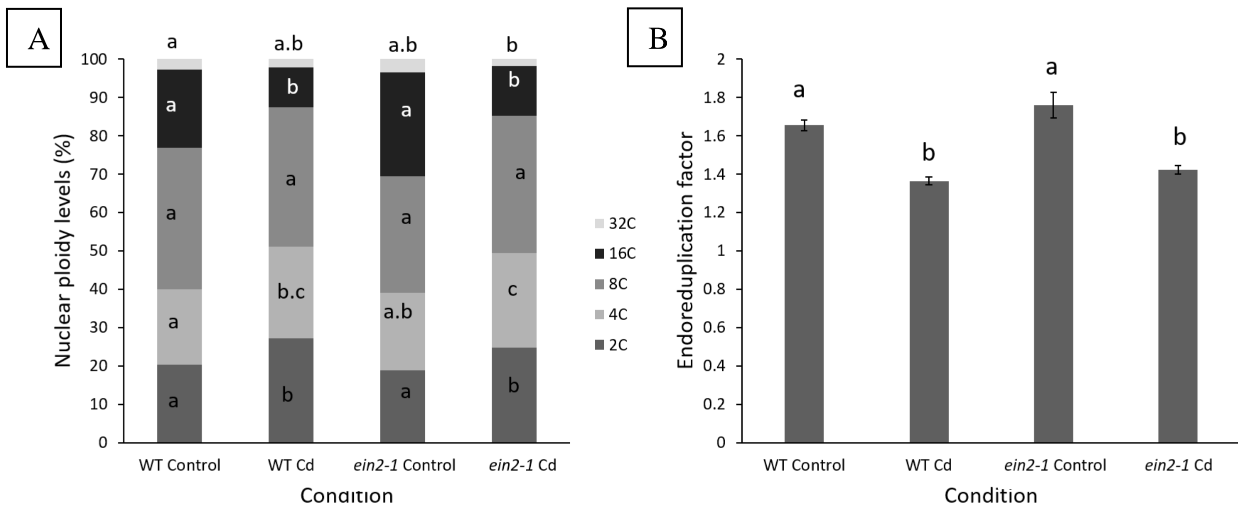
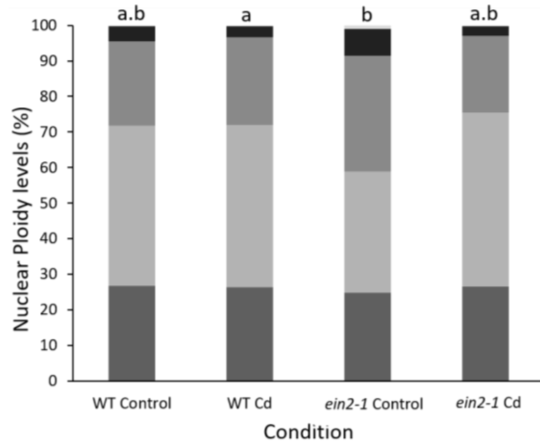


Figure 12: Percentage of nuclear ploidy levels (A) and the endoreduplication factor (B) in leaf 4 of WT and ein2-1 mutant A. thaliana plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ for 12 days, starting on day 20 after sowing. Data represent the mean ± S.E. of four biological replicates. Significant differences (Wilcoxon rank sum test: $p < 0.05$) obtained when comparing all four conditions are indicated using a different letter (a, b). For the nuclear ploidy levels, significant differences are indicated for each ploidy level separately using different letters within each ploidy level.

Finally, the extent of endoreduplication under control and Cd exposure conditions was determined in leaf 11, which was not present at the start of Cd exposure at day 20 (Figure 9). In *ein2-1* mutants, the EF significantly decreased in this leaf after 12 days of Cd exposure (Figure 13B), similarly to the results obtained in leaf 4 after 12 days (Figure 12B). This effect was mostly noticed for the higher ploidy levels (16C and 32C, Figure 13A and Appendix Figure A3). This effect however was not seen for WT plants (Figure 13). Also, leaf 11 of *ein2-1* mutants seemed to have a higher level of endoreduplication under control conditions as compared to WT plants (Figure 13B). When looking for these effects in the higher nuclear ploidy levels 16C and 32C (Appendix Figure A3), there was indeed an increasing trend for both levels in unexposed *ein2-1* mutant versus WT plants, as well as an aggravated decrease due to the Cd effect in *ein2-1* mutants for this leaf (Appendix Figure A3).

A



B

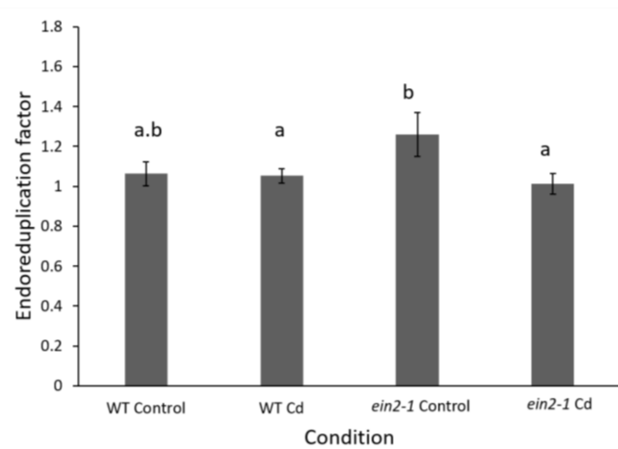


Figure 13: Percentage of nuclear ploidy levels (A) and the endoreduplication factor (B) in leaf 11 of WT and *ein2-1* mutant *A. thaliana* plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ for 12 days, starting on day 20 after sowing. Data represent the mean ± S.E. of four biological replicates. Significant differences (Wilcoxon rank sum test: $p < 0.05$) obtained when comparing all four conditions are indicated using a different letter (a, b). For the nuclear ploidy levels, significant differences are indicated for each ploidy level separately using different letters within each ploidy level.

4 Discussion

The ultimate goal of the current study is to increase our insight into the modulating role of the stress hormone ethylene under short- as well as long-term Cd exposure. Most of the experiments performed within this study were based on results obtained in previous research carried out by Schellingen *et al.* (2015a, 2015b) (29,50) and Jozefczak *et al.* (2014) (66). Related to short-term Cd exposure, the emerging link between ethylene and GSH is further unravelled. In addition, the effects of long-term Cd exposure on vegetative and reproductive growth of WT versus ethylene insensitive *ein2-1* mutants are based on the methods used by Keunen *et al.* (2011) (67) and coupled to the extent of endoreduplication.

4.1 Ethylene as a modulator of Cd-induced responses during short-term exposure

Plants grown in Cd-polluted soils have a coordinated way to cope with stress immediately. When Cd is taken up by plant roots, the occurrence of oxidative stress stimulates the induction of signalling cascades involving antioxidative defence mechanisms necessary to limit the extent of cellular damage. To limit the concentrations of free Cd, PCs are efficiently and immediately produced by the incorporation of their precursor, GSH. Next, sensing the use of large amounts of GSH in the roots, signals go out from root to leaf hereby setting in motion the transportation of leaf-synthesised GSH back to the roots (66,68). Via its thiol group obtained by the incorporation of cysteine in its biosynthesis process, GSH can directly chelate Cd or scavenge ROS, it can be used as a substrate for GSTs or can be incorporated in PCs by PCS. Either what mechanism is used, GSH transportation, signalling and replenishment is necessary to protect the plant against Cd exposure (25,50,69).

After short-term Cd exposure, research has indicated that ethylene is a crucial regulator of this Cd detoxification process (Figure 14) (50). It is hypothesised that ethylene biosynthesis and signalling is upregulated to stimulate the replenishment of the GSH pool in the leaves of Cd-exposed plants. As such, the loop is continued and plants are able to cope with Cd stress within this time frame.

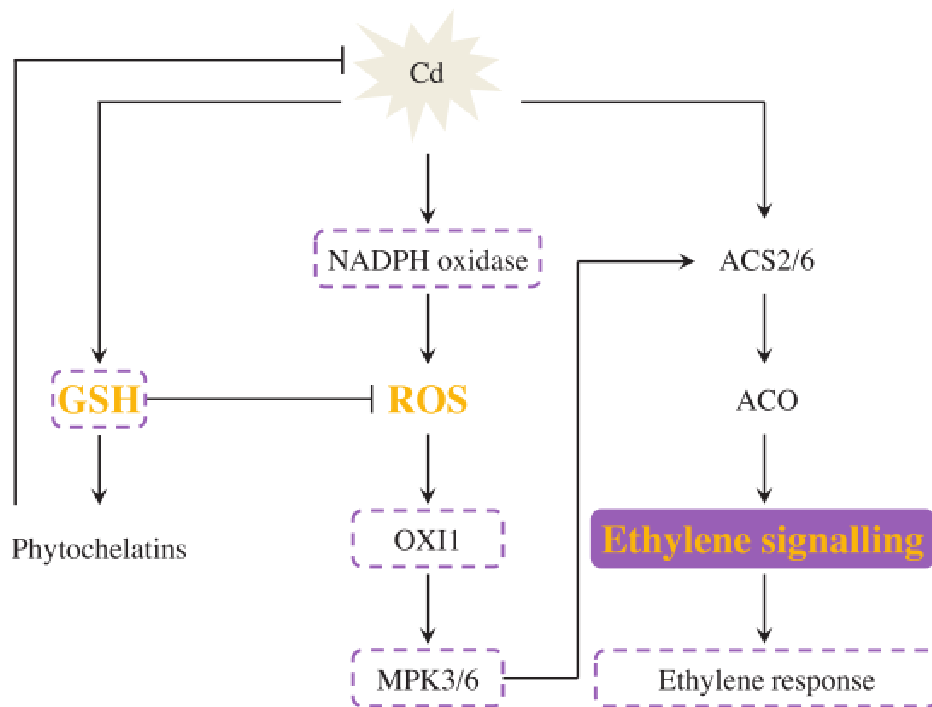


Figure 14: Model that gives an overview of the initiating responses after short-term, moderate (5 µM) Cd exposure: Cd-induced oxidative stress, ethylene biosynthesis (ACS2, ACS6 and ACO) and ethylene signalling. Due to Cd phytotoxicity, GSH production is stimulated leading to direct and indirect mechanisms of defence against ROS production, amongst others caused by NADPH oxidases under Cd stress. Through multiple activations of kinases (OXI1 and MPK3/6) or Cd stress itself, ethylene production is upregulated resulting in higher levels of ethylene signalling. This in its turn results in ethylene responses (purple boxes) coupling back to GSH metabolism, NADPH oxidases and subsequently downstream components. Abbreviations: Cd (cadmium), OXI (oxidative signal-inducible kinase 1), MPK (mitogen-activated protein kinase), ACO (ACC oxidase), ACS (ACC synthase). Adapted from Schellingen *et al.* (50).

4.1.1 *Under Cd exposure, increased ethylene signalling stimulates GSH replenishment*

Results covering gene expression analysis of ethylene biosynthesis and signalling genes in the GSH deficient *cad2-1* mutant confirmed the emerging link between ethylene and GSH as indicated by Schellingen *et al*. (2015a, 2015b) (29,50). Here, the expression of GSH biosynthesis genes was investigated in mutants that are defective in ethylene biosynthesis (*acs2-1acs6-1,* 2015a*)* and insensitive to ethylene signalling (*ein2-1*, 2015b*)*, showing a general delay in the GSH metabolism in the leaves of these mutants as compared to WT plants. In the current study, genes of interest were selectively chosen. Since the rate-limiting step in ethylene biosynthesis is the conversion of SAM to ACC by the action of ACS, genes encoding isoforms 2 and 6 known to be important to increase ethylene production under Cd stress (49) were chosen to study the effects of GSH deficiency on ethylene biosynthesis. On the other hand, ethylene signalling was monitored by the transcript levels of *ERF1*, which is one of the ethylene-responsive products of the ethylene signalling pathway (Figure 3B) inducing the expression of other ethylene-responsive genes (49). Expression levels of all three genes were more upregulated in roots and leaves of GSH deficient *cad2-1* mutants as compared to WT plants after 72 h Cd exposure (Table 1), supporting previous results. Due to the absence of normal GSH

amounts in the *cad2-1* mutant, it could be hypothesised that increasing ethylene biosynthesis and signalling might be a mechanism to cope with GSH depletion and a decrease of antioxidative defence. In this way, the plant tries to alleviate the lack of GSH transportation from leaf to root by increasing ethylene biosynthesis and thereby signalling, known to positively affect GSH biosynthesis in the leaves (25,50).

4.1.2 Bypass mechanisms intercept the loss of ethylene signalling under Cd exposure

The production of PCs as a defence mechanism to cope with phytotoxicity is dependent on (1) Cd to activate PCS and (2) the supply of their precursor GSH (24). Ethylene was shown to be important for the initiation and maintenance of immediate antioxidative defence coupled to GSH replenishment, but it is currently unknown how this affects PC production during Cd exposure. Under Cd stress, PCS immediately reacts in the roots, thereby using large amounts of GSH, which couples back to ethylene synthesis and signalling as presumed before. It was pointed out by Jozefczak *et al.* (2014) that the production of PCs is known to rise steadily, even though GSH concentrations in roots are known to drop between 2 and 24 h of Cd exposure due to its incorporation into PCs. After root-to-shoot signalling to promote GSH biosynthesis, a higher amount of GSH is produced to boost PC production in roots and leaves (28,66,69).

Since previous and current research has indicated that ethylene signalling plays a role in the feedback to GSH production, loss or decreased signalling of ethylene is therefore expected to result in a limitation of this feedback. The signal to the leaves demanding GSH pool replenishment would fall back and the entire detoxification mechanism that is based on GSH transportation to roots would drop with a decrease of available GSH and eventually PC production. To study the link between PC production and ethylene signalling, PC levels were monitored in leaves of WT as compared to *ein2-1* mutant plants under control and Cd exposure conditions. In general, no significant differences could be observed between both genotypes (Figure 5). This indicates the possible existence of bypass mechanisms that compensate for the loss of ethylene signalling in *ein2-1* mutant plants. Even though ethylene indeed seems to be the initiating signal mediating the replenishment of GSH from leaves to roots, loss of its signalling does not cause a complete failure but rather a delay of this feedback mechanism in the leaves (50). Therefore, it would be interesting to couple leaf data of this study to GSH and PC content measurements in the roots, since it is known that the GSH content drops in the roots before it is replenished under Cd exposure (25). This would extend the information about the interplay between root and leaf GSH signalling coupled to PC production under Cd exposure.

Research has indicated the possible role of other phytohormones that might be able to replace (or bypass) ethylene signalling. One of these is jasmonic acid (JA) which is known to play an important role in plant defence to pathogens (70). An overlap exists between defences against pathogens and abiotic stresses such as Cd toxicity, with plant hormones as important mediators of the stress signalling network. This hypothesis is mainly focussed on the overlap of gene expression analysis data, coupling the different stresses to similarities in transcript levels (71). As such, Cd toxicity increases the production of JA as well as ethylene (47,49). Mainly, interactions between JA and ethylene and their signalling cascades have been mentioned, thereby paving the way to new research questions (72,73). Generally, *ein2-1* mutant plants seem to cope well with short-term moderate Cd stress, showing resilience against its phytotoxic effects considering leaf growth (29). Although a loss of ethylene signalling under Cd exposure causes a delay in GSH biosynthesis in leaves, the immediate response of PC production is still present. Due to a possible bypass mechanism, GSH production and transportation from leaf to root is still accompanied by a normal increase of PC availability after 24 and 72 h of Cd exposure, at least in leaves. However, it is still unknown whether a lack of ethylene signalling affects plant responses to chronic Cd exposure.

4.2 Ethylene insensitivity and its effect under long-term Cd exposure

Experiments covering the modulating role of ethylene in short-term effects of moderate Cd exposure have indicated a possible bypass mechanism covering the loss of ethylene signalling and its role in activation of GSH-dependent Cd detoxification. To elucidate the effects of ethylene signalling in plants chronically exposed to Cd, a phenotypic experiment was carried out. Plants were grown under control or sublethal (5 µM) Cd exposure conditions in a controlled environment over a period of time, comparing morphological responses of WT with and *ein2-1* mutant plants related to vegetative and regenerative plant growth. Finally, these morphological responses were linked to the extent of endoreduplication occurring in separate plant leaves of a different age.

4.2.1 Ethylene signalling regulates reproductive plant growth

Growth of *A. thaliana* plants is divided into two growth phases (67). Firstly, vegetative growth takes place where seedlings gain more and more leaves while their rosette diameter expands. Results covering the rosette diameter over time (Figure 6A) showed that Cd toxicity had a clear negative effect on the rosette diameter of both WT and *ein2-1* mutant plants. Also, when looking at the amount of leaves developed over time (Figure 6B), no clear genotype effect was observed. In the end however, unexposed *ein2-1* mutant plants seemed to have a small lead on control WT plants, although the Cd effect stayed the same in both genotypes. Concluding from these results, *ein2-1* mutants do not show a higher resistance against long-term Cd toxicity. Also, ethylene does not seem to play a major role in the vegetative growth phase in *A. thaliana* in this experiment, which is contradictory to other findings

(74–76). Vandenbussche *et al.* (2012) described the importance of ethylene in its general physiological role, with the focus on vegetative plant growth and the cooperation between ethylene and auxin in *A. thaliana* root development, germination and day-and-night rhythm (74). Reasons behind the deviating findings in our study could be differences in culture set-up and growth conditions. Therefore, further research and repetition of experiments is of high importance.

Next, reproductive growth is set off with the emergence of the inflorescence meristem. Multiple studies have shown that ethylene is involved in ripening abscission and plant senescence, taking place in the later stages of plant growth (77). Therefore, not only inflorescence emergence and height were measured (Figure 7A and B respectively). Also, the formation of seed-containing siliques was monitored (Figure 8) to investigate the role of ethylene in the reproductive growth stage under control and Cd exposure conditions in our setup. Starting with the inflorescence emergence, there already seems to be a delay in emergence seen in the *ein2-1* mutant under both growth conditions. This can be extended to the following events, as the inflorescence height and silique formation in *ein2-1* mutants are delayed as compared to WT plants. This is in contradiction with results of Schellingen *et al.* (2015a), since in this study no genotype differences were observed for WT versus ethylene biosynthesis mutants (29). Here, after long-term exposure to moderate (5 µM) and severe (10 µM) Cd concentrations, there was a limitation in the reproductive capacity of both *acs2-1/acs6-1* mutants as WT plants. The long-term Cd effect showed that both genotypes were equal in their sensitivity for Cd. However, our results indicate the important role of ethylene signalling in the initiation of reproductive growth, hence the late start of *ein2-1* mutants as compared to WT plants. When comparing Cd effects between genotypes, it seems that in the reproductive growth phase, Cd stimulates and speeds the process up for both genotypes. This can already be noted when observing inflorescence emergence and continues up to silique formation (Figures 6, 7 and 8). This could logically be caused by sensing stress and focussing on the most important goal of plant growth, which is reproduction. However, as stated by Keunen et al (2011), silique formation should be negatively affected by Cd exposure in the long run which might indicate that due to the early termination of measurements in this experiment, not all Cd effects on silique formation were observed (67). Again, the existence of bypass mechanisms to overcome the lack of ethylene signalling is suggested, since all plants form an inflorescence meristem and the Cd effect (stimulating reproductive growth) is similar in both genotypes, and as such ethylene does not seem to play a major role in Cd-induced differences observed in this study.

4.2.2 Ethylene signalling affects the extent of endoreduplication in separate leaves

Research has indicated that endoreduplication could be a protective mechanism under stress conditions (56,57). Ethylene has been linked to plant developmental processes and, as pointed out in section 4.2.1, it seems to play a role in the initiation of reproductive growth. However, its role in the process of endoreduplication needs more elucidation. Therefore, ploidy levels of WT and *ein2-1* mutant plants were compared under control and 5 µM Cd exposure. Tactically, different leaf ages were chosen on two time points (Figure 9). A general conclusion made, was a different response over time, and this for both genotype and Cd effects.

A potential explanation for this shift from genotype to Cd effect under Cd exposure between leaf ages, is the amount of divisions taking place in the developing meristem. Leaf 1, being fully mature at the time of Cd exposure, has already ended its cell cycle activity at the meristem, while leaf 4 is still proliferating due to its ongoing development. Cadmium can therefore still affect cell developmental processes and cause DNA damage that subsequently is passed along to daughter cells and thereby increase the induction of endoreduplication. Even though DNA damage caused by Cd phytotoxicity is also occuring in mature leaves, this will have no effect on the cell cycle and subsequently endoreduplication, since it is no longer active. The same as mentioned for leaf 4 can be said for leaf 11, that only starts its meristemal divisions under continuous Cd exposure. Endoreduplication, being thought to react as a protective mechanism against DNA damage, should normally increase under these circumstances. However, the EF remained unchanged or decreased under Cd exposure in the current study. This might indicate a general inhibitory effect of Cd on the process of endoreduplication and/or the cell cycle, thereby causing cells to be stuck in certain ploidy levels. This could explain the decrease in progression from ploidy level 8 to 16C (Figure 12A and Appendix Figure A2). Another hypothesis is that under Cd stress, DNA damage activates endoreduplication as a protective mechanism but only works as a coping mechanism up to a certain treshold, which might explain the Cd effect seen under long-term exposure (EF, Figures 12B and 13B), where persistent damage surpasses the treshold. In the absence of ethylene, the effect of a decreased EF under long-term Cd exposure is aggravated, indicating that the previously mentioned treshold might be lowered and endoreduplication fails earlier than before. It is also possible that endoreduplication is immediately activated under moderate stress as to prevent DNA damage to occur but still promote cells to grow. When severity of stress increases, the cell cycle and therefore the process of endoreduplication is shut down, allowing DNA repair systems to set in effectively. Also, ethylene has been reported to stimulate the process of endoreduplication in hypocotyls (60,61), while results of the current study rather

indicate the opposite: ethylene insensitivity in the *ein2-1* mutant still results in a higher EF under control conditions (Figure 11B, 12B and 13B). However, this study focussed on the extent of endoreduplication in leaf samples as opposed to hypocotyls, which might explain the difference in responses. This might be an indication that due to the lack of ethylene signalling necessary for plant growth responses under normal physiological conditions, endoreduplication might try to compensate for this loss of signalling leading to a limitation of cell division (60). Important in the observation concerning the data of this experiment, is that endoreduplication was only measured in four replicates due to technical problems. Therefore, the use of more replicates is advised when performing experiments determining ploidy levels in *A. thaliana* leaves of a different age.

5 Conclusion and future perspectives

It is clear that the phytohormone ethylene modulates Cd-induced responses after short- as well as long-term moderate exposure. Under short-term Cd exposure, gene expression analysis confirmed the link between GSH production, necessary as one of the most important antioxidative defence and detoxification agents, and ethylene signalling. Even though there is a clear link between ethylene signalling and GSH production, there are indications for the existence of bypass mechanisms to cope with a loss of ethylene signalling. This could be concluded by the similar production of GSH-based PCs in leaves of *ein2-1* mutant and WT plants. Furthermore, the known crucial role of ethylene signalling in plant reproductive growth was supported by our experiments. Nevertheless, since there is a delay but no loss of reproductive growth characteristics (inflorescence emergence, growth and silique formation), bypass mechanisms with a focus on other phytohormones might be involved. Finally, the experiment investigating the extent of endoreduplication and its link to ethylene signalling under moderate Cd exposure gave contradictory results as compared to previous studies. However, since this study focussed on leaves rather than hypocotyls, different outcomes could be expected. Therefore, endoreduplication and ethylene signalling might cooperate under stress to alleviate Cd-induced DNA damage. However, different hypotheses around the subject need to be further unravelled in future experiments.

Concerning future prospects, much is yet to be discovered about the phytohormone ethylene and its role in plant responses to Cd stress. Since the biosynthesis of GSH is mainly stimulated due to the demand for sulphur, the incorporation of cysteine in both ethylene and GSH might be an interesting link to study. Therefore, mapping and comparing sulphur metabolites in general between WT and *ein2-1* mutant plants could elucidate possible mechanisms of interaction between ethylene and GSH production and maintenance. As PC levels were only measured in leaves, it would be interesting to also measure them in roots, since this will increase our understanding on root-to-shoot signalling as well. As for the long-term phenotypic experiment, measurements were made for the same plants at different time points. Therefore, statistical modelling approaches for repeated data should be performed to verify the significance of Cd-induced effects on vegetative and regenerative plant growth. It is also possible to measure plant growth characteristics until seed formation and germination, comparing WT and mutants under control and Cd exposure conditions. Also, follow-up should be extended up to the end of the plant's lifecycle to optimise the obtained results. Finally, the possibility of bypass mechanisms covering the loss of ethylene signalling opens the window to study the link between different phytohormones like JA and ethylene in more detail. Fundamental research will therefore step by step help in the discovery of the possibilities of plasticity that plant science has to offer in the optimisation of human life quality with a focus on soil rehabilitation.

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7 Appendix

Table A1: Cycling conditions for qPCR amplification and dissociation stage.

	Temperature	Time
Initial denaturation	95 °C	10 min
40 cycles:		
1. Denaturation	95 °C	15 s
2. Annealing and elongation	60 °C	60 s
Dissociation curve stage	95 °C	15 s
	60 °C	60 s
	95 °C	15 s
	60 °C	15 s

Table A2: Forward and reverse primers used to obtain gene expression levels via qPCR. Abbreviations: UBC: ubiquitin conjugating enzyme; TIP: TIP-family; ACS: ACC synthase; ERF: ethylene response factor. Protocol adaptations: *ACS6 primer concentrations were increased to 900 nM. **ERF1 primer concentrations were increased to 600 nM.

AGI	*Annotation*	*Forward primer sequences (5' - 3')*	*Reverse primer sequences (5' - 3')*
		Reference genes used to normalise data for the leaves	
AT5G25760	*UBC: ubiquitin conjugating enzyme*	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
AT4G34270	*TIP41-like*	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
AT5G08290	*mitosis protein YLS8*	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
AT4G26410	*expressed*	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
		Reference genes used to normalise data for the roots	
AT5G25760	*Ubiquitin conjugating enzyme*	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
AT4G34270	*TIP41-like*	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
AT4G05320	*Ubiquitin*	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
AGI	***Annotation***	***Forward primer sequences (5' - 3')***	***Reverse primer sequences (5' - 3')***
		Ethylene biosynthesis and signalling genes	
AT3G23240	*ACS2*	CATGTTCTGCCTTGCGGATC	ACCTGTCCGCCACCTCAAGT
AT1G01480	*ACS6**	TTAGCTAATCCCGGCGATGG	ACAAGATTCACTCCGGTTCTCCA
AT4G11280	*ERF1***	TCCTCGGCGATTCTCAATTTT	CAACCGGAGAACAACCATCCT

Table A3: Quantitative real-time PCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin et al. (2009) (78). * All procedures were performed according to manufacturer's protocols.

Sample/Template	
Source	Roots & leaves of *Arabidopsis thaliana* plants cultivated in hydroponics
Method of preservation	Liquid nitrogen
Storage time	Two weeks at – 70 °C
Handling	Frozen
Extraction method	Phenol-free Total RNA isolation: RNAqueous® Total RNA Isolation Kit* (Ambion, Life Technologies, Belgium)
RNA: DNA-free	TURBO DNA-*free*™ Kit* (Ambion, Life Technologies, Belgium) Design of intron-spanning primers whenever possible
Concentration	NanoDrop®: ND-1000 Spectrophotometer (ThermoScientific, USA)
Assay optimisation and validation	
Accession number	Supplemental table A2
Primer sequences	Supplemental table A2
In silico	Primers were blasted using the BLAST tool at http://arabidopsis.org/
Empirical	A primer concentration of 300 nM was used for *ACS2*, 900 nM for *ACS6 and* 600 nM for *ERF1* Annealing temperature: 60 °C
Priming conditions	Combination of oligodT-primers and random hexamers
Linear dynamic range	Samples are situated within the range of the efficiency curve
Reverse transcription – PCR	
Protocols	As stated in the Materials and Methods section
Reagents	As stated in the Materials and Methods section
No template control (NTC)	Cq and dissociation curve verification
Data analysis	
Specialist software	7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) Software v2.0.1
Statistical justification	At least four biological replicates Outliers were eliminated after statistical validation using the extreme studentised deviate analysis (GraphPad Software, Inc.) at significance level 0.05 Log transformation of the data Two-way ANOVA and the Tukey-Kramer post-hoc test to correct for multiple comparisons using R version 3.2.3 (x64)
Normalisation	Three reference genes were selected for root samples, while four reference genes were selected for leaf samples (Supplemental table A2)

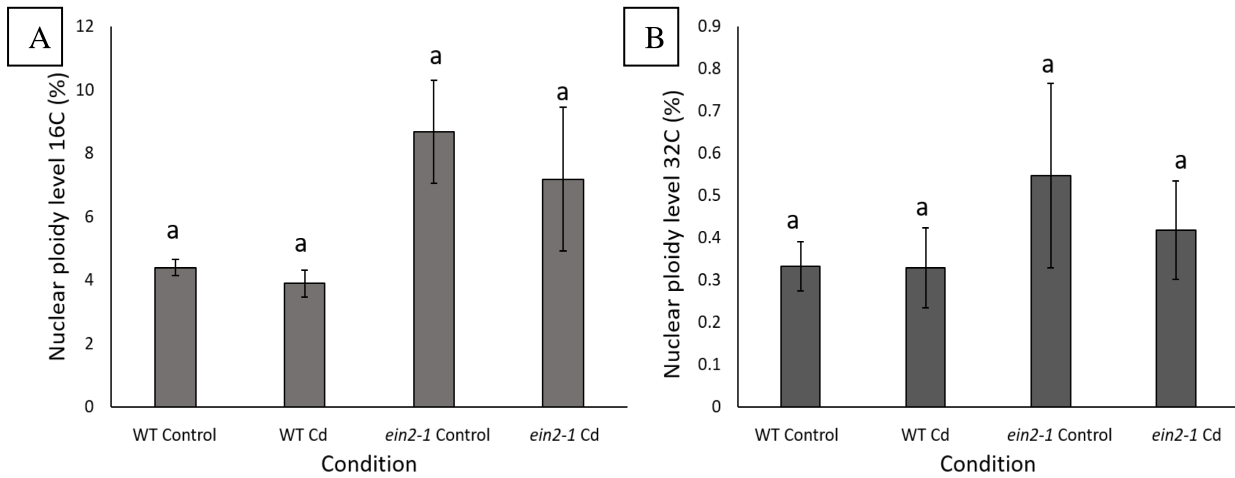

Figure A1: Percentage of nuclear ploidy level 16C (A) and 32C (B) in the fourth leaf of WT and *ein2-1* mutant *A. thaliana* plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ for 72 h, starting on day 20 after sowing. Data represent the mean ± S.E. of four biological replicates. Significant differences (Wilcoxon rank sum test: $p < 0.05$) obtained when comparing all four conditions are indicated using a different letter (a, b).

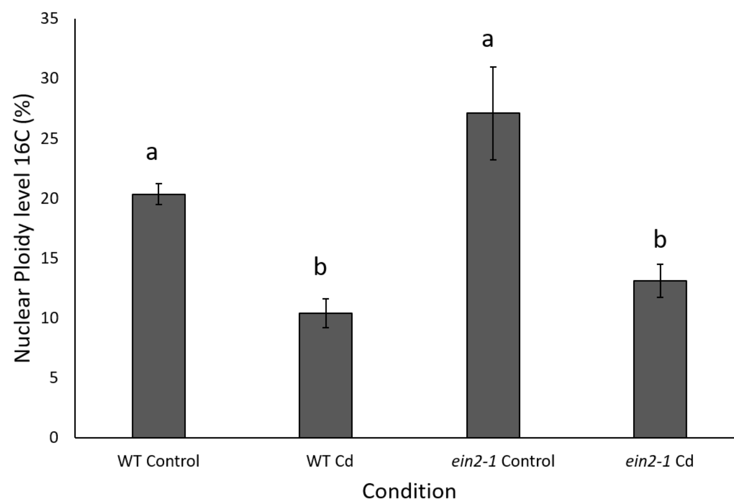

Figure A2: Percentage of nuclear ploidy level 16C in the fourth leaf of WT and *ein2-1* mutant *A. thaliana* plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ for 12 days, starting on day 20 after sowing. Data represent the mean ± S.E. of four biological replicates. Significant differences (Wilcoxon rank sum test: $p < 0.05$) obtained when comparing all four conditions are indicated using a different letter (a, b).

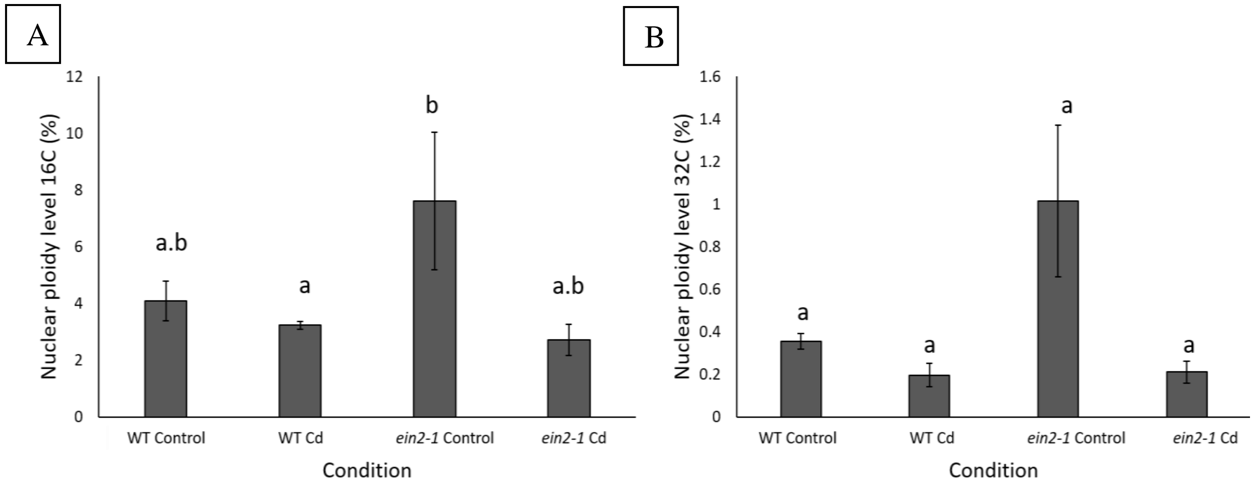

Figure A3: Percentage of nuclear ploidy level 16C (A) and 32C (B) in leaf 11 of WT and *ein2-1* mutant *A. thaliana* plants grown under control conditions (0 µM $CdSO_4$) or exposed to 5 µM $CdSO_4$ for 12 days, starting on day 20 after sowing. Data represent the mean ± S.E. of four biological replicates. Significant differences (Wilcoxon rank sum test: $p < 0.05$) obtained when comparing all four conditions are indicated using a different letter (a, b).

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Jaar: **2017**

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Jeuris, Elien

Datum: **8/06/2017**