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Maastricht University

Faculteit Geneeskunde en Levenswetenschappen
School voor Levenswetenschappen
master in de biomedische wetenschappen

Masterthesis

The effects of ethylene signaling in the cadmium-induced stress response in Arabidopsis thaliana

Kris Kunnen

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen, afstudeerrichting milieu en gezondheid

PROMOTOR :

Prof. dr. Ann CUYPERS

BEGELEIDER :

Mevrouw Sophie HENDRIX

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www.uhasselt.be
Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

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Inhoudsopgave

List of abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
APX	Ascorbate peroxidase
AsA	Ascorbate
A. thaliana	*Arabidopsis thaliana*
bZIP	basic leucine zipper
cad2-1	Cadmium sensitive
CAT	Catalase
Cd	Cadmium
ein2-1	Ethylene insensitive
ER	Endoplasmic reticulum
ERF	Ethylene response factor
FW	Fresh weight
GO	Gene ontology
GR	Glutathione reductase
GSH	Glutathione
GSH1	Glutamate cysteine ligase
GSH2	GSH synthetase
GSL	Glucosinolate
H_2O_2	Hydrogen peroxide
JA	Jasmonic acid
LOX	Lipoxygenase
MPK	Mitogen activated protein kinase
$O_2^{\bullet-}$	Superoxide
$^{\bullet}OH$	Hydroxyl radical
PC	Phytochelatins
PCD	Programmed cell death
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBArm	Thiobarbituric acid-reactive metabolites

Acknowledgements

As I still vividly remember my first days at Hasselt University almost five years ago, it comes to me as a bit of a shock that I will defend my master's thesis soon. Luckily, I got to spend the past 8 months doing my internship on a very interesting topic, in a very welcoming research group, and under outstanding supervision. Since the internship went as smoothly as it did, I would like to thank some people.

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Lastly, I would like to thank my parents for giving me the opportunity to study, and for supporting me in all the decisions I have made past 5 years. Without them I would not be writing these last words.

Abstract

Environmental pollution by cadmium (Cd) is a widespread problem, causing significant decreases in crop yield. Cadmium indirectly increases the formation of reactive oxygen species (ROS) in plants, thereby causing oxidative stress. To prevent oxidative damage, plant cells possess an extensive antioxidative defense system consisting of antioxidative enzymes and non-enzymatic antioxidants such as glutathione (GSH). Furthermore, Cd exposure is known to increase the production of ethylene, an important phytohormone. The aim of this project was to further investigate the importance of ethylene signaling and its interplay with GSH in Cd-induced stress responses in *Arabidopsis thaliana* plants.

A full-transcriptome analysis was performed on roots and leaves of wild-type (WT) and ethylene insensitive (*ein2-1*) mutant *A. thaliana* plants exposed to 5 µM Cd for 24 h. The obtained results indicate that ethylene signaling plays an important role in plant functioning under control as well as Cd exposure conditions. The RNA-seq data pointed out responses to Cd-induced ROS production in roots and leaves, and responses to oxidative damage and endoplasmic reticulum (ER) stress in leaves. In general, Cd-induced increases in the expression of genes involved in these processes were observed in WT plants, whereas these increases were less pronounced in *ein2-1* mutants. These responses were further investigated through molecular analyses of hydrogen peroxide (H_2O_2) concentrations and the extent of lipid peroxidation in plants exposed to Cd for 24 h, 72 h and 7 days. From the results, it became apparent that the role ethylene signaling depends on both the plant organ and the duration of exposure. Increases in H_2O_2 concentrations and lipid peroxidation levels upon short-term Cd exposure were generally more pronounced in WT plants as compared to *ein2-1* mutants. Upon prolonged exposure, differences between genotypes disappeared, indicating that ethylene signaling is mainly involved in early responses to Cd stress. When investigating the extent *bZIP60* splicing (*i.e.* a marker for ER stress) after 24 h and 72 h of Cd exposure, opposite responses were observed between roots and leaves. While Cd-induced ER stress was most pronounced in leaves after 24 h, roots displayed a larger extent of ER stress after 72 h of Cd exposure. Also, the extent of ER stress upon Cd exposure was lower in *ein2-1* leaves, while it was higher in *ein2-1* roots as compared to their WT counterparts.

Furthermore, the RNA sequencing data confirmed the interaction between GSH and ethylene signaling, which was hypothesized in previous research. Hydrogen peroxide concentrations, TBArm concentrations and the extent of ER stress were also determined in the GSH-deficient cadmium-sensitive *cad2-1* mutant and generally, Cd-induced effects on these parameters were more pronounced in the *cad2-1* mutant as compared to the WT. These data confirm the increased Cd sensitivity of the GSH-deficient mutant.

In conclusion, results of this study indicate that ethylene signaling mediates early responses to Cd exposure, regulating a wide array of processes, including responses to ROS, lipid peroxidation and ER stress. Furthermore, an interplay between ethylene and GSH in Cd-exposed plants was confirmed.

Samenvatting

Milieuvervuiling door cadmium (Cd) is een wijdverspreid probleem, waardoor de opbrengst van gewassen aanzienlijk daalt. Cadmium verhoogt indirect de vorming van reactieve zuurstofvormen in planten, waardoor oxidatieve stress wordt veroorzaakt. Om oxidatieve schade te voorkomen, beschikken plantencellen over een uitgebreid antioxidatief verdedigingssysteem dat bestaat uit antioxidatieve enzymen en niet-enzymatische antioxidanten zoals glutathion (GSH). Bovendien is bekend dat Cd-blootstelling de productie van ethyleen, een belangrijk fytohormoon, verhoogt. Het doel van dit project was om het belang van ethyleensignalering en zijn interactie met GSH in Cd-geïnduceerde stressresponsen in *Arabidopsis thaliana* planten verder te onderzoeken.

Een volledige transcriptoomanalyse werd uitgevoerd in wortels en bladeren van wildtype (WT) en ethyleen ongevoelige (*ein2-1*) mutante *A. thaliana* planten, blootgesteld aan 5 µM Cd gedurende 24 uur. De verkregen resultaten geven aan dat ethyleensignalering een belangrijke rol speelt in het functioneren van de plant onder zowel controlecondities als Cd-blootgestelde condities. De RNA-seq data toonden responsen op Cd-geïnduceerde ROS-productie in wortels en bladeren, en reacties op oxidatieve schade en endoplasmatisch reticulum (ER) stress in bladeren. Over het algemeen werden Cd-geïnduceerde verhogingen in expressie van genen betrokken in deze processen waargenomen in WT planten, terwijl deze verhogingen minder uitgesproken waren in *ein2-1* mutanten. Deze responsen werden verder onderzocht door middel van moleculaire analyses van waterstofperoxide (H_2O_2) concentraties en de mate van lipideperoxidatie (TBArm) in planten blootgesteld aan Cd gedurende 24 uur, 72 uur en 7 dagen. Uit de resultaten bleek dat de rol van ethyleensignalering afhangt van zowel het plantenorgaan als de duur van de blootstelling. Toenames in H_2O_2 en TBArm concentraties door korte termijn Cd-blootstelling waren over het algemeen meer uitgesproken in WT planten dan in *ein2-1* mutanten, terwijl verschillen tussen genotypes verdwenen na langdurige blootstelling, wat aangeeft dat ethyleen voornamelijk betrokken is bij vroege reacties op Cd-stress. Bij het onderzoeken van de mate van *bZIP* splicing (een merker voor ER-stress) na 24 uur en 72 uur blootstelling aan Cd, werden tegengestelde responsen waargenomen tussen wortels en bladeren. Terwijl Cd-geïnduceerde ER-stress meer uitgesproken was in bladeren na 24 uur, vertoonden wortels een grotere mate van ER-stress na 72 uur blootstelling aan Cd. Ook leek de mate van ER-stress na Cd-blootstelling lager in de *ein2-1* bladeren, terwijl deze parameters in *ein2-1* wortels hoger lag in vergelijking met hun WT tegenhangers.

Bovendien bevestigden de RNA-seq data de interactie tussen GSH en ethyleensignalering, die gesuggereerd werd in voorgaand onderzoek. Waterstofperoxide concentraties, TBArm-concentraties en de mate van ER-stress werden ook bepaald in de GSH-deficiënte cadmium-gevoelige *cad2-1* mutant. Over het algemeen waren de effecten van Cd meer uitgesproken in de *cad2-1* mutant vergeleken met het WT, hetgeen de verhoogde Cd-gevoeligheid van dit genotype bevestigt.

In conclusie wijzen de resultaten van dit onderzoek erop dat ethyleensignalering vroege reacties op blootstelling aan Cd medieert, en een breed scala aan processen reguleert, waaronder reacties op ROS, lipideperoxidatie en ER-stress. Bovendien werd de wisselwerking tussen ethyleen en GSH in Cd-blootgestelde planten bevestigd.

1. Introduction

If the exponential population growth continues as it does today, we will exceed 9 billion people on earth by 2050. As plants are our most important food source, improving crop yield is crucial. However, it will become more and more frequent that plants need to grow under sub-optimal conditions. Therefore, fundamental knowledge is needed to unravel how plants develop, react and adapt to different environmental stressors [1, 2]. Environmental pollution by cadmium (Cd) is a widespread problem caused by natural and mainly anthropogenic activities [3]. It enters the environment through industry, agriculture and mining. Electroplating, the production of nickel-Cd batteries and (in)organic phosphate fertilizers are great examples of anthropogenic contributions to Cd pollution [3, 4]. Even in Belgium, Cd pollution is found around Liège and the northern part of the Campine region, mainly due to historic industrial pollution [5]. Beside possible occupational exposure, the major source of Cd exposure in humans is cigarette smoking, whereas non-smokers get exposed through food contamination. It is proven to be a carcinogen and induces osteoporosis, bone fractures, renal and cardiovascular diseases [4, 6].

When present in the environment, Cd can be taken up by plants, thereby entering the food chain and posing a risk to human health. Although it is a non-essential element, Cd can be taken up through transporters for essential elements such as Fe^{2+}, Ca^{2+} and Zn^{2+}, as it has similar physicochemical properties. Once taken up in the roots, Cd is transported into the xylem through heavy metal ATPases (HMAs), enabling its transport to other plant organs [7]. Once taken up in plants, Cd induces growth inhibition, root browning, chlorosis, disturbances of the nutrient balance, photosynthetic inhibition, and possibly necrosis [8, 9].

Although it is non-redoxactive, Cd indirectly causes oxidative stress. This process is defined as an imbalance between pro- and antioxidants in the direction of the former. A major cause of this imbalance is the increased production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). Under natural conditions, ROS are mainly generated in organelles containing electron transport chains and organelles with a highly oxidizing metabolic rate, such as mitochondria or peroxisomes [10]. Cadmium-induced indirect ROS production occurs through various mechanisms (Fig. 1). Firstly, Cd binds with high affinity to thiol groups, and thereby inhibits the function of antioxidative metabolites and enzymes [11]. It can also displace essential cations from specific binding sites, for example the replacement of Ca^{2+} by Cd^{2+} causing the inhibition of PSII photoactivition [12]. However, these replacements can also cause increases in levels of redox-active metals such as Fe^{2+} or Cu^{2+}, which in their turn can generate ROS through Fenton and Haber-Weiss reactions [13]. Furthermore, Cd stimulates the biogenesis of peroxisomes, increasing organellar ROS production [13]. Additionally, it induces ROS production by increasing the activity of pro-oxidative enzymes such as NADPH oxidases [14].

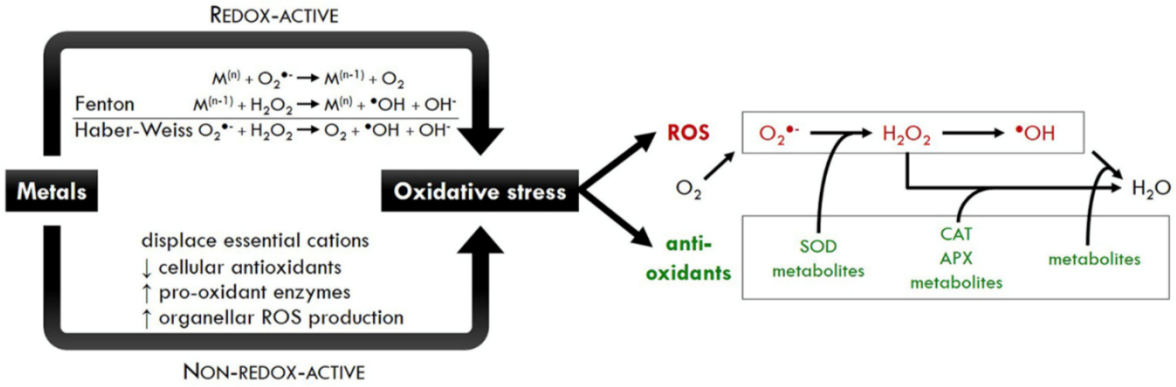

Fig. 1. An overview on the effect of metals on the cellular redox balance. Redox-active metals generate ROS through the Fenton and Haber-Weiss reactions, leading to the production of the highly toxic $^{\bullet}OH$ radicals from H_2O_2. Non-redox-active metals indirectly produce ROS by displacing essential cations, depleting antioxidants and increasing the activity of ROS producing enzymes and enhancing ROS production in organelles. Abbreviations: $M^{(n)}$, oxidized metal; $M^{(n-1)}$, reduced metal; $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radicals; OH^-, hydroxide ion; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase. Derived from Cuypers *et al.* 2016.

Despite their reactive nature, ROS play important roles in signal transduction in response to developmental and environmental stimuli [15]. Hydrogen peroxide is particularly suited as a signaling molecule, as it is relatively stable and can move through aquaporins, allowing intercellular transport. Through oxidation reactions, ROS can activate regulatory target molecules [11]. When present in elevated concentrations, however, ROS can damage cellular macromolecules such as DNA, proteins and membrane lipids. To prevent oxidative damage and maintain cellular function, plant cells attempt to keep ROS levels in balance using an array of enzymatic and non-enzymatic antioxidative mechanisms (Fig. 1). For example, highly reactive $O_2^{\bullet-}$ molecules are reduced to the more stable H_2O_2 by antioxidative enzyme superoxide dismutase (SOD). Depending on their metal cofactor, SODs are classified in three groups: iron SODs (FeSODs), copper/zinc SODs (CuZnSODs) and manganese SODs (MnSODs) [16]. The antioxidative enzymes catalase (CAT) and ascorbate peroxidase (APX) both neutralize H_2O_2 to H_2O. Whereas APX has a high affinity for H_2O_2, CAT displays a lower affinity but higher reaction rate. This renders CAT more appropriate for H_2O_2 detoxification, while APX is involved in fine-tuning H_2O_2 levels and therefore an ideal regulator of H_2O_2 signaling [17].

In addition to enzymatic antioxidants, plant cells also possess an array of antioxidative metabolites such as ascorbate (AsA) and glutathione (GSH), both participating in the AsA-GSH cycle. In this cycle, H_2O_2 is reduced to H_2O by ascorbate peroxidase (APX), using electrons derived from AsA, which is thereby oxidized to dehydroascorbate (DHA). This molecule is again reduced to AsA by dehydroascorbate reductase (DHAR), which oxidizes GSH to glutathione disulfide (GSSG). The latter is reduced back to GSH by the activity of glutathione reductase (GR), using the reducing power of NADPH [13].

In addition to its involvement in the AsA-GSH cycle, GSH executes its antioxidative role through other mechanisms, using the electron donating capacity of the thiol group of its cysteine

residue [18]. Glutathione biosynthesis occurs through two ATP-dependent steps. First, γ-glutamylcysteine (γ-EC) is formed from cysteine and γ-glutamate. This reaction forms the rate-limiting step in GSH biosynthesis, performed by glutamate cysteine ligase, also referred to as γ-EC synthetase or GSH1. Glutathione is subsequently formed through the addition of glycine to γ-EC, catalyzed by GSH synthetase or GSH2 (Fig. 2) [19]. Through the action of GSH transferases (GST), GSH can be conjugated to xenobiotics and products of oxidative damage, such as lipid peroxides, increasing their solubility and aiding their detoxification [18].

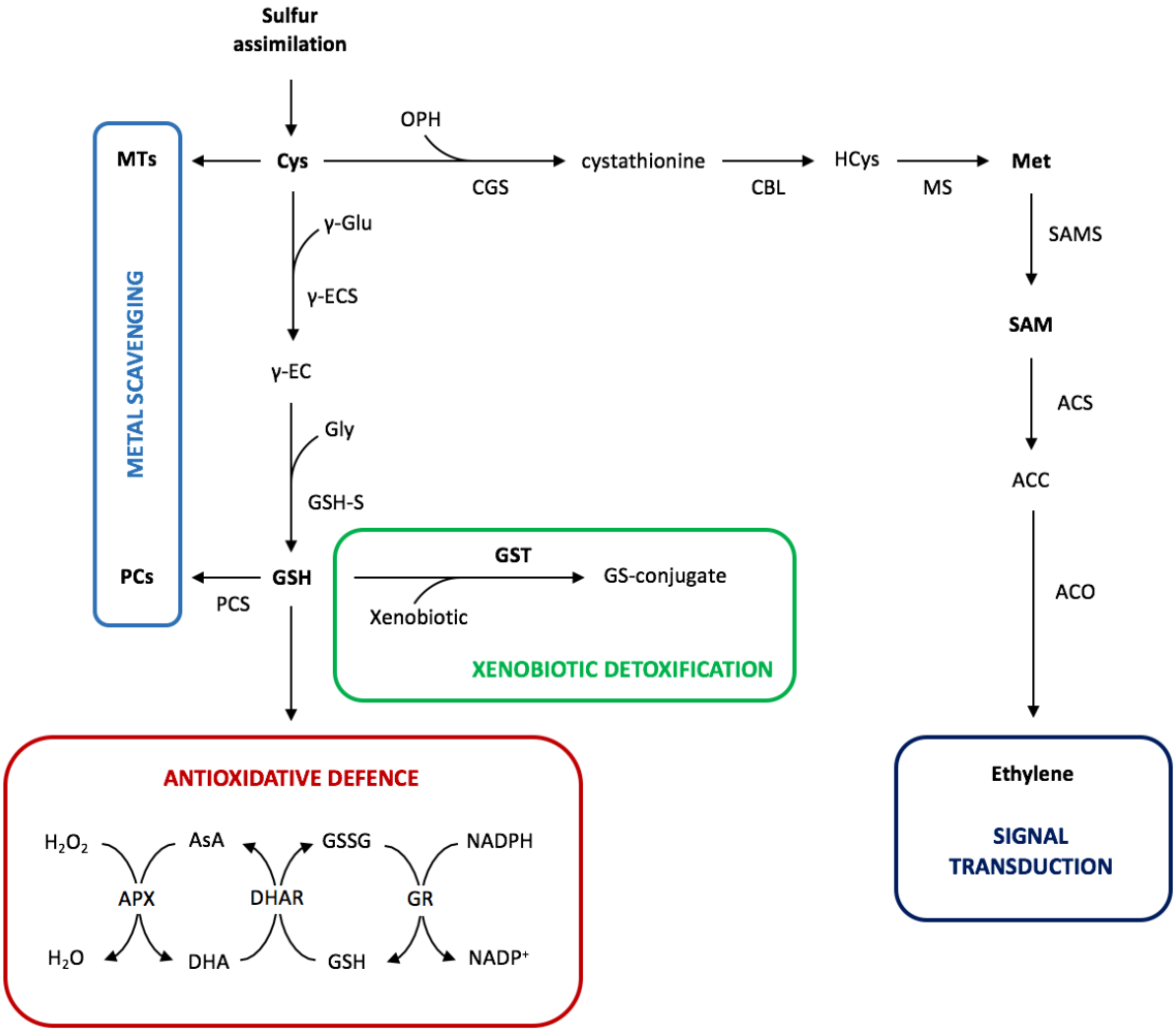

Fig. 2. Schematic overview of the synthesis pathways of sulfur-containing cellular compounds. Abbreviations: γ-EC, γ-glutamylcysteine; γ-ECS, γ-EC synthetase; γ-Glu, γ-glutamate; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; APX, ascorbate peroxidase; AsA, ascorbate; CBL, cystathionine b-lyase; CGS, cystathionine γ-synthase; Cys, cysteine; DHA, dehydroascorbate; DHAR, DHA reductase; Gly, glycine; GR, glutathione reductase; GS-conjugate, glutathione S-conjugate; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; H_2O, water; MT, metallothionein; PC, phytochelatin; PCS, phytochelatin synthase; SAM, S-adenosyl methionine; SAMS, S-adenosyl methionine synthetase. Derived from Hendrix et al. (2017).

Next to its role in antioxidative defense, GSH also functions as a chelator of Cd, both directly and through the formation of metal-chelating phytochelatins (PC), binding Cd through its high affinity towards thiol groups. Synthesized in a two-step reaction by PC synthase (PCS), PCs consist of a $(\gamma\text{-Glu-Cys})_n$-Gly structure, with n ranging from 2 to 11. Once PCs have scavenged Cd from the cytosol, PC-Cd complexes can be transported into the vacuole through ATP binding cassette (ABC) transporters. As shown by Jozefczak *et al.* (2014), Cd exposure in *A. thaliana* induces PC synthesis in roots, rapidly depleting GSH levels available for antioxidative defense [20].

In addition to GSH, also ethylene biosynthesis depends on the plant's cysteine pool (Fig. 2). Ethylene is an important hormone, functioning as a regulator of most developmental and physiological processes in plants. It plays a role in cell division, root and shoot growth, senescence, and the coordination of plant stress responses [21]. In the ethylene biosynthesis pathway, cysteine-derived methionine is conjugated with ATP by S-adenosylmethionine (SAM) synthetase (SAMS), forming SAM. This molecule is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). In a final step, ACC is oxidized to ethylene by ACC oxidase (ACO) in the presence of oxygen [18]. Ethylene biosynthesis can be regulated by an array of internal and external factors, frequently with ACS as the target [1]. For example, Cd is known to strongly stimulate ethylene biosynthesis through the induction of *ACS* gene expression [22].

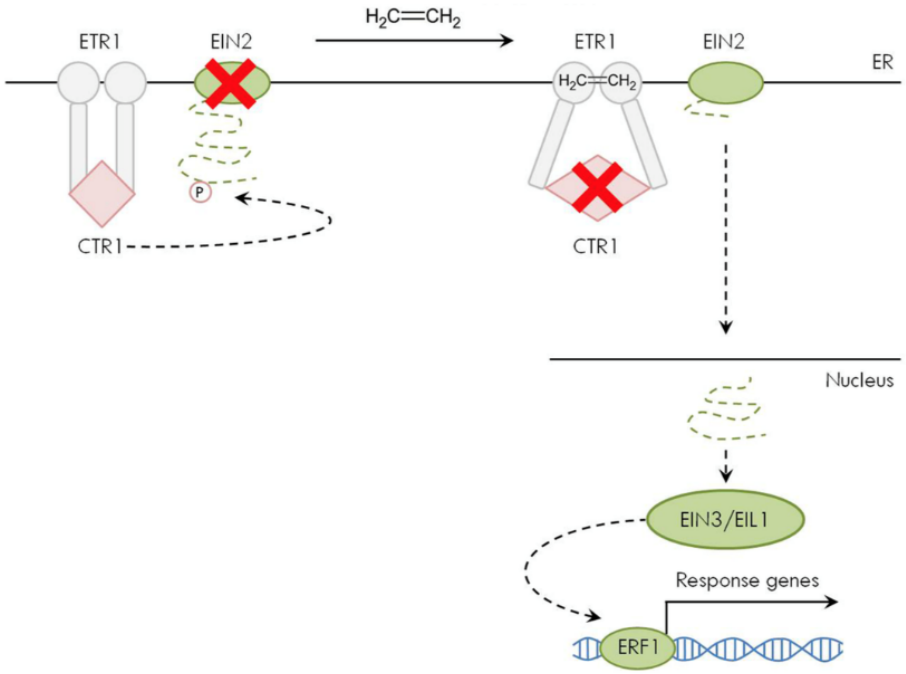

Fig. 3. The ethylene signal transduction pathway. When ethylene binds the endoplasmic reticulum (ER)-bound receptor ETR1 (ethylene resistant 1), CTR1 (constitutive triple receptor 1) is deactivated and the C-terminal domain of EIN2 (ethylene insensitive 2) is translocated to the nucleus and activates ethylene response genes through EIN3 (ethylene insensitive 3)/EIL1 (EIN3-like 1) and ERF1 (ethylene response factor 1). Derived from Keunen et al. (2016).

Ethylene signaling starts when ethylene binds the high affinity ethylene receptor ETHYLENE RESISTANT 1 (ETR1), which is located in the endoplasmic reticulum (ER) membrane [23]. When not bound to ethylene, ETR1 activates the Raf like protein kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), and CTR1 phosphorylates and inactivates the downstream molecule ETHYLENE INSENSITIVE 2 (EIN2) [24]. When ethylene binds the ETR1 receptor, CTR1 is inactivated, releasing EIN2 from its repression. The EIN2 protein's C-terminal end is cleaved, and used to transduce the signal from the ER membrane to the nucleus, activating ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1). As short-term positive regulators of the ethylene response, EIN3/EIL1 activate the expression of transcription factors such as *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), which in their turn activate the expression of ethylene response genes (Fig. 3) [23].

Being a key regulator in the ethylene signal transduction pathway, a mutation resulting in the loss of EIN2 function leads to complete ethylene insensitivity [25]. Recently, the involvement of ethylene signaling in metal-induced stress responses was reviewed by Keunen et al. (2016), discussing interactions between ethylene, the cellular redox balance, GSH and other phytohormones such as jasmonic acid (JA) [1]. When comparing WT and *ein2-1 A. thaliana* plants, differences in the Cd-induced oxidative stress responses were already observed. The *ein2-1* mutants displayed lower expression levels of oxidative stress marker genes, NADPH oxidase gene *RBOHC* and several GSH genes involved in GSH metabolism, indicating that ethylene signaling plays an important role in the Cd-induced stress response in plants. The aim of the current study is to further unravel processes regulated through ethylene signaling in Cd-exposed plants. For this purpose, wild-type (WT) plants and ethylene insensitive (*ein2-1*) mutants of *Arabidopsis thaliana* are grown under control and Cd-exposed conditions. To investigate and compare differences in Cd-induced effects on gene expression between both genotypes, a full transcriptome analysis is performed using RNA sequencing.

2. Materials and methods

2.1. Hydroculture and cadmium exposure

Wild-type, *ein2-1* and *cad2-1 A. thaliana* seeds (ecotype Columbia) were surface-sterilized in 10% sodium hypochlorite (Sigma-Aldrich) and kept at 4°C in the dark for 2 nights to achieve consistent germination. Subsequently, plants were grown in hydroculture using a modified Hoagland solution as described by Keunen *et al.* (2016) [26]. The plants were grown in a climate chamber set to a humidity of 65%, a 12 h photoperiod and day and night temperatures of 22°C and 18°C, respectively. Photosynthetic active radiation (PAR) of sunlight was simulated using blue, red and far-red LED lights with an intensity of 170 µmol m^{-2} s^{-1} at the rosette level. After 18 days of growing under control conditions, plants were exposed to 5 µM Cd by addition of $CdSO_4$ to the nutrient solution. After exposure, roots and leaf rosettes were harvested, weighed and snap-frozen in liquid nitrogen. Until further analysis, the samples were stored at -80°C.

2.2. Gene expression analysis

2.2.1. RNA isolation

Frozen samples were ground using two stainless steel beads in the Mixer Mill MM400 (Retsch). Next, total RNA was extracted using the RNAqueous™ Phenol-free total RNA Isolation kit (Ambion, Thermo Fisher Scientific) according to manufacturer's instructions. Concentrations and purity of the RNA were determined using the NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies), whereas RNA integrity was verified using the RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent).

2.2.3. Real-time PCR

For qPCR reactions, 2 µL of the 1/10 diluted cDNA sample was added to 8 µL master mix in 96-well plates. This master mix contained 5 µL QuantiNova SYBR® Green, 0.05 µL QN ROX reference dye (Qiagen), 0.3 µL forward and reverse primer and 2.35 µL RNase-free water. Next, the plates were run in the 7500 Fast Real-Time PCR System (Applied Biosystems), using 1 cycle of 2 min at 95°C, 40 cycles of 5 s at 95°C followed by 10 s at 60°C. A dissociation curve was added to verify product specificity. Relative gene expression levels were calculated using the $2^{-\Delta Cq}$ method and were normalized against the expression of 3 selected reference genes, selected through the GrayNorm algorithm described by Remans *et al.* (2014) [27]. *YLS8*, *MON1* and *TIP41* were used as reference genes for roots, whereas *MON1*, *TIP41* and *EF1a* were used for leaves. Supplemental Table S1 shows the qPCR parameters according to the Minimum Information for publication of qPCR Experiments (MIQE) guidelines [28], and Supplemental Table S2 contains the primer sequences.

2.3. Hydrogen peroxide measurements

Hydrogen peroxide concentrations were measured in leaf rosettes and roots using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) after shredding the samples as described before. 500 µL 1X reaction buffer was added to the ground samples, after which they were incubated at room temperature while mixing (450 rpm) in the dark. After centrifugation (12,000 g; 5 min; 20°C), supernatant was transferred to a clean tube and centrifuged again (12,000 g; 2 min; 20°C). Subsequently, 5 µL sample was added to 95 µL master mix, consisting of 93.57 µL 1X Reaction buffer, 0.95 µL horseradish peroxidase and 0.48 µL Amplex® Red reagent. Fluorescence at 590 nm was measured using the FLUOstar Omega microplate reader (BMG Labtech).

2.4. Thiobarbituric acid-reactive metabolite measurements

To determine the extent of lipid peroxidation, frozen root and leaf samples were placed into an ice-cooled mortar and homogenized in 1 mL of 0.1% trichloroacetic acid (TCA). After centrifugation (20,412 g; 10 min; 4°C), 400 µL of supernatant was added to 1 mL 0.5% TBA in 20% TCA and incubated at 95°C for 30 min. After a second centrifugation step, the absorbance of the supernatant at was determined at 532 nm and corrected for aspecific absorbance at 600 nm. All samples were analysed *in duplo.*

2.5. Statistical analyses

Outliers were determined using the extreme studentized deviate analysis (GraphPad Sofware, USA) at significance level 0.05. Statistical analyses were performed in R version 3.3.3 (R Core Team (2017), R foundation for Statistical Computing, Vienna, Austria). A two-way ANOVA was performed, followed by Tukey's post hoc test. Normality and homoscedasticity of the data were verified using the Shapiro-Wilk test and Bartlett's test, respectively. When the assumptions were not met, the data were transformed (square root, inverse, exponential, logarithmic). Gene expression data were standard log transformed. When the assumptions were not met after transformation, the non-parametrical Kruskal-Wallis and post hoc Wilcoxon rank sum test were used. P-values lower than 0.05 were considered significant.

2.6. RNA sequencing of Cd-exposed WT and *ein2-1* mutant

2.6.1. Library synthesis and sequencing

After RNA isolation, mRNA was purified from RNA samples using the Magnosphere™ UltraPure mRNA Purification Kit (Takara Bio Inc.), according to the manufacturer's instructions. After purification, the integrity and quantity of the mRNA was confirmed using the RNA 6000 Pico Kit and the Agilent 2100 Bioanalyzer (Agilent). To prepare the cDNA library, the SMARTer® Stranded RNA-Seq Kit (Takara Bio Inc.) was used. Each sample received a unique combination of dual-indexed primers using the SMARTer® RNA Unique Dual Index Kit (Takara Bio Inc.). All steps were completed according to manufacturer's

instructions. Correct fragment length and integrity of the library were determined using the Agilent High Sensitivity DNA Kit (Agilent). Pooled libraries were sequenced with Illumina NextSeq500 using the Illumina NextSeq 500 v2.5 reagent kit for 150 cycles to generate 75 base-pairs paired-end reads.

2.6.2. Data analysis

FASTQ files were imported in the CLC genomics workbench v11.0.1 and failed reads were discarded. For each library, reads were trimmed using the following criteria: QC below 0.01 (< 1 error / 1000 bases), no base ambiguity, minimal read length of 35 nucleotides. Illumina adapter residues were trimmed using the adapter sequences. Finally, hard trims were performed of 10 nucleotides at 5' ends and 2 nucleotides at 3' ends. The remaining reads were mapped to the TAIR 10 draft *A. thaliana* genome and annotated using the Araport 11 annotation [29] using following criteria: mismatch set at 2, insertion/deletion cost set at 3 (stringent), maximum number of hits for a read lower than 10, 80% identity and 80% coverage with the reference genome. Expression values were calculated using the RPKM (reads per kilobase transcript per million transcripts) method [30]. Differentially expressed genes were statistically determined using a two-way ANOVA, Benjamini-Hotchberg corrected p-values (false discovery rate) were set at 0.05, an absolute fold-change cut-off at 2 and RPKM differences of minimum 2. Complete linkage hierarchical clustering was performed using Cluster 3.0 software with uncentered Pearson correlation, defining clusters using a threshold of 0.70 correlation. Gene ontology (GO) enrichment analysis was performed on the significantly differentially expressed genes in Cytoscape (v3.7.1) with the ClueGO (v2.5.4) and CluePedia (v1.5.4) plugins [31, 32] using following criteria: Benjamini-Hotchberg corrected p-value < 0.05, gene ontology from level 3 to level 8, kappa score set at 0.4.

3. Results and discussion

Previous research indicates that ethylene signaling is an important factor in Cd-induced stress responses in plants [1]. To further expand the knowledge on this topic, an RNA sequencing analysis was performed, comparing transcript levels between wild-type and *ein2-1* knockout mutants of *A. thaliana* upon exposure to 5 µM Cd for 24 h.

3.1. Early Cd-induced effects on plant growth are mediated by ethylene signaling

After 24 h, 72 h and 7 days of exposure to 5 µM Cd, leaves and roots were harvested separately and weighted immediately (Fig. 4). After 24 h, the fresh weight of *ein2-1* leaves was already significantly lower than that of WT leaves under control conditions. However, Cd exposure did not affect fresh weight of leaves and roots of either genotype at this time point. Decreases in fresh weight observed in WT roots and leaves after 72 h of Cd exposure were less pronounced in *ein2-1* plants, but after 7 days of Cd exposure, roots and leaves of *ein2-1* mutant plants displayed similar decreases in fresh weight as compared to those of the WT upon Cd exposure. Generally, *ein2-1* plants displayed lower fresh weights under control conditions, which suggests that ethylene signaling is necessary for normal plant growth. Also, both genotypes seem similarly sensitive to prolonged Cd exposure, suggesting that ethylene signaling is mainly important in acute response to Cd stress. Schellingen *et al.* (2015) observed similar effects on root and leaf fresh weight after 24 h and 72 h of Cd exposure, and in addition determined Cd concentrations in leaf rosettes. The decreases in fresh weight were less pronounced in *ein2-1* after 72 h of Cd exposure, although Cd concentrations did not differ between genotypes after 24 h and 72 h of Cd exposure. This indicates that *ein2-1* leaves are less sensitive upon acute Cd exposure as compared to WT leaves [14].

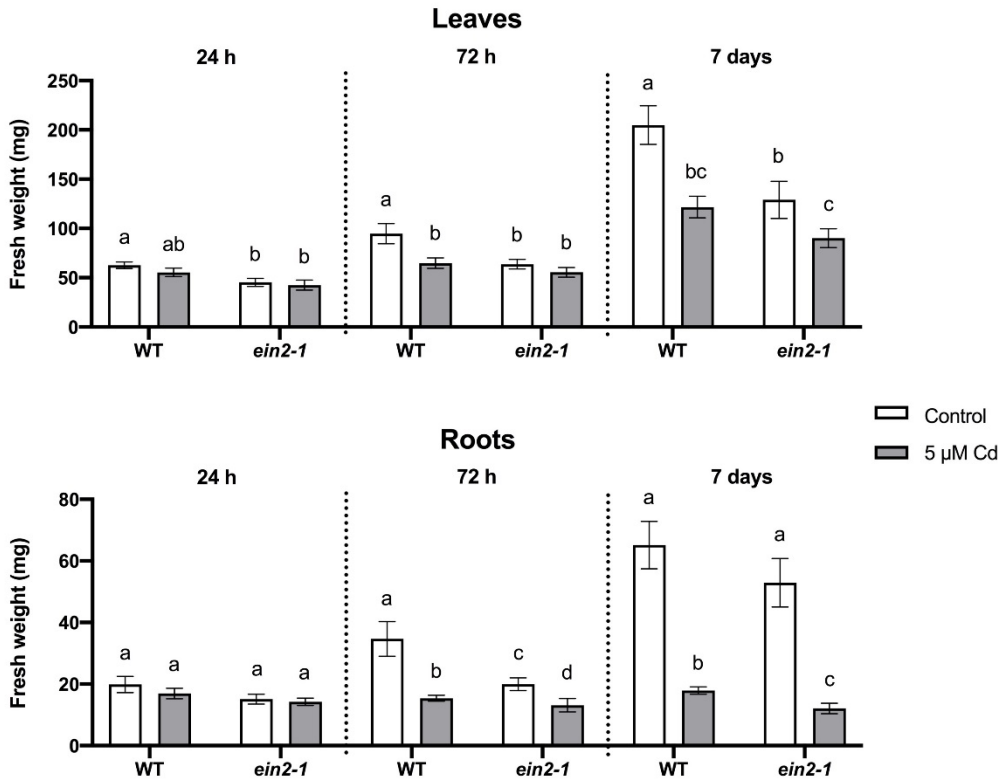

Fig. 4. Fresh weight (mg) of leaves and roots of WT and *ein2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 h, 72 h and 7 days. Fresh weight measurements were performed directly after harvesting. Values represent the mean ± S.E. of at least 10 biological independent replicates. Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

3.2. RNA sequencing data

In order to uncover these processes regulated by ethylene in Cd-exposed plants, 24 cDNA libraries were built from mRNA samples originating from roots and leaves (3 samples per experimental group). From the NextSeq run, a total of 589,691,476 75 bp reads was generated with libraries ranging from 20,412,818 to 28,468,330 reads each. After the filtering process, as described in the methods section, 63 to 83 % of the total number of reads was mapped against the reference genome, with a specificity of 83 to 91 %, indicating the number of reads that were only mapped against one gene (Supplemental Table S3). Expression levels of a set of 10 genes in leaves and 13 genes in roots (Supplemental Table S2) were validated using qPCR. A strong correlation ($R^2 = 0.977$) was obtained between the data obtained via qPCR and RNA-seq (Fig. 5).

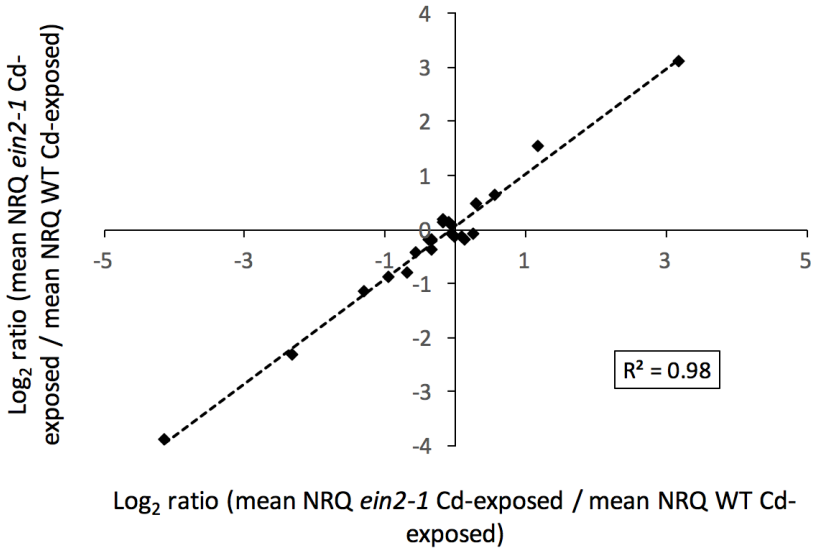

Fig. 5. Correlation between qPCR and RNA-seq data. X-axis: qPCR data expressed as Log_2 ratio (mean NRQ *ein2-1* Cd-exposed / mean NRQ WT Cd-exposed). Y-axis: RNA-seq data expressed as Log_2 ratio (mean RPKM *ein2-1* Cd-exposed / mean RPKM WT Cd-exposed). Each data point represents the expression of one gene in either roots or leaves.

Using a two-way ANOVA, all experimental conditions were compared within each organ. In total, 4422 and 1526 differentially expressed genes were found in leaves and roots, respectively. To determine the genes and biological processes affected by ethylene signaling in Cd-exposed plants, hierarchical clustering and GO enrichment analysis were performed, resulting in 7 clusters in leaves and 9 clusters in roots (Fig. 6). The relative gene expression profiles for each cluster are depicted in Fig. 7 for leaves and Fig. 8 for roots. Certain clusters only consisted of a small number of genes and did not contain significantly enriched biological processes according to the GO enrichment analysis. These were leaf clusters 2, 5, 6 (Fig. 7) and root clusters 5, 6 and 9 (Fig. 8).

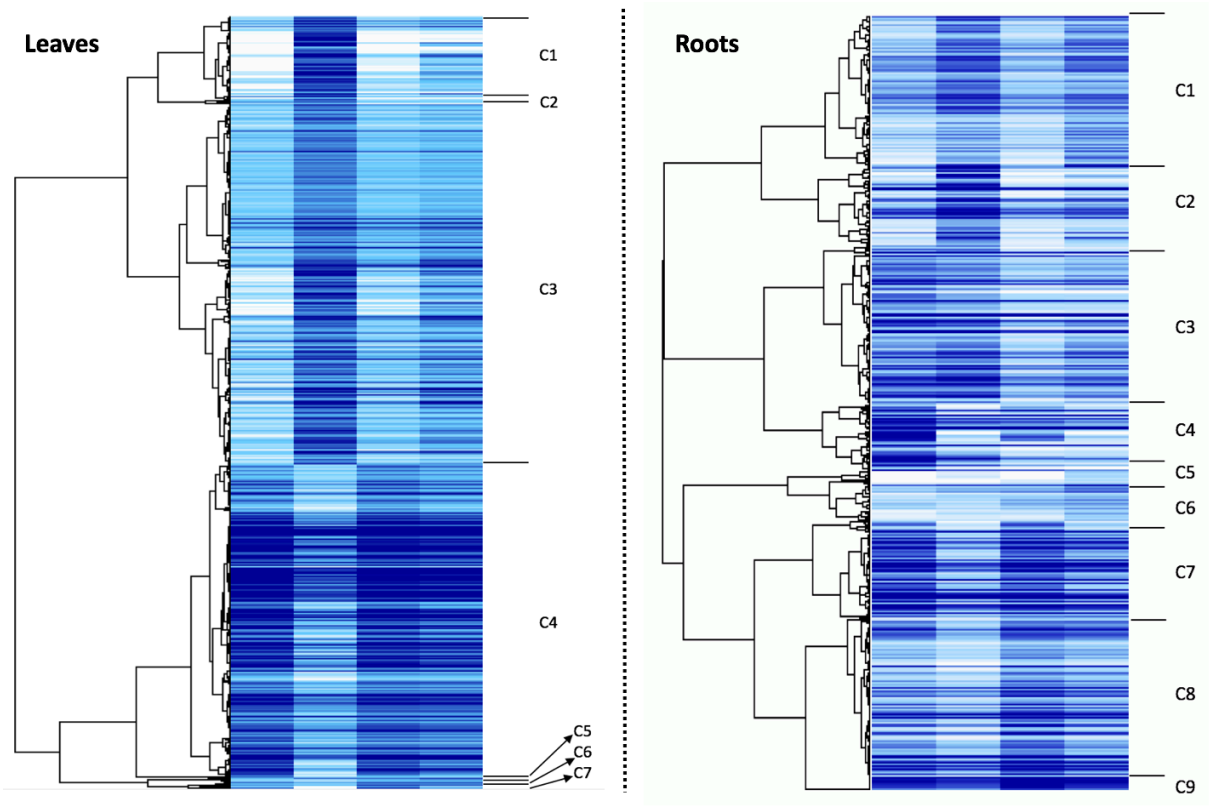

Fig. 6. Hierarchical clustering performed on RNA sequencing differential gene expression results. Complete linkage hierarchical clustering was performed using Cluster 3.0 software with uncentered Pearson correlation, defining clusters using a correlation threshold of 0.70. White indicates low, blue indicates high expression values.

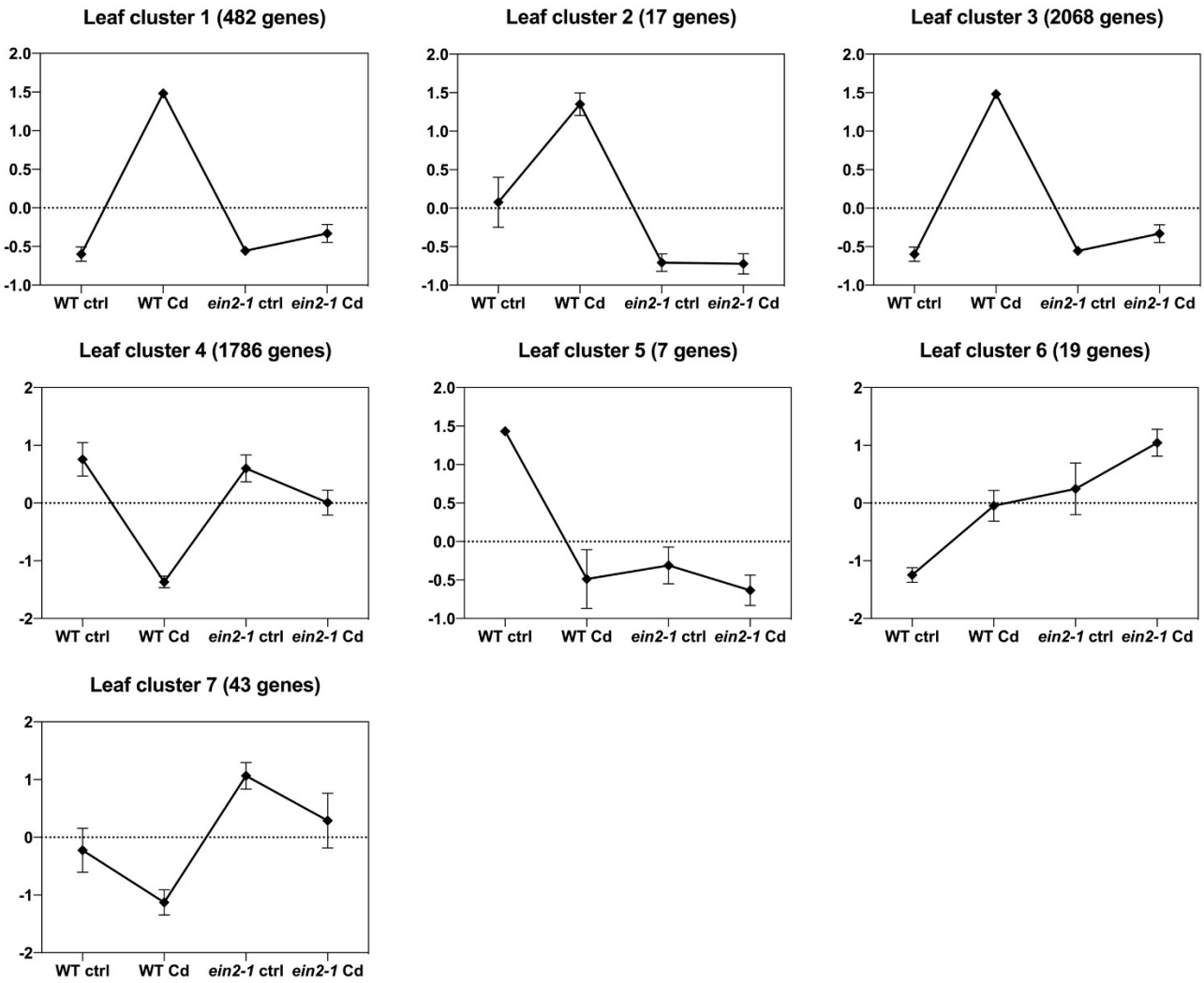

Fig. 7. Relative gene expression profiles of leaf clusters. Clusters are defined by hierarchical clustering. The number of genes included in each cluster is indicated between brackets. Each leaf cluster depicts the relative gene expression of genes within the cluster, calculated using a rescaling method: $y = (x - mean_i) / \sigma_i$). Y, rescaled value; $mean_i$, mean of all gene expression values within one the condition; σ_i, S.D. of all gene expression values within one condition.

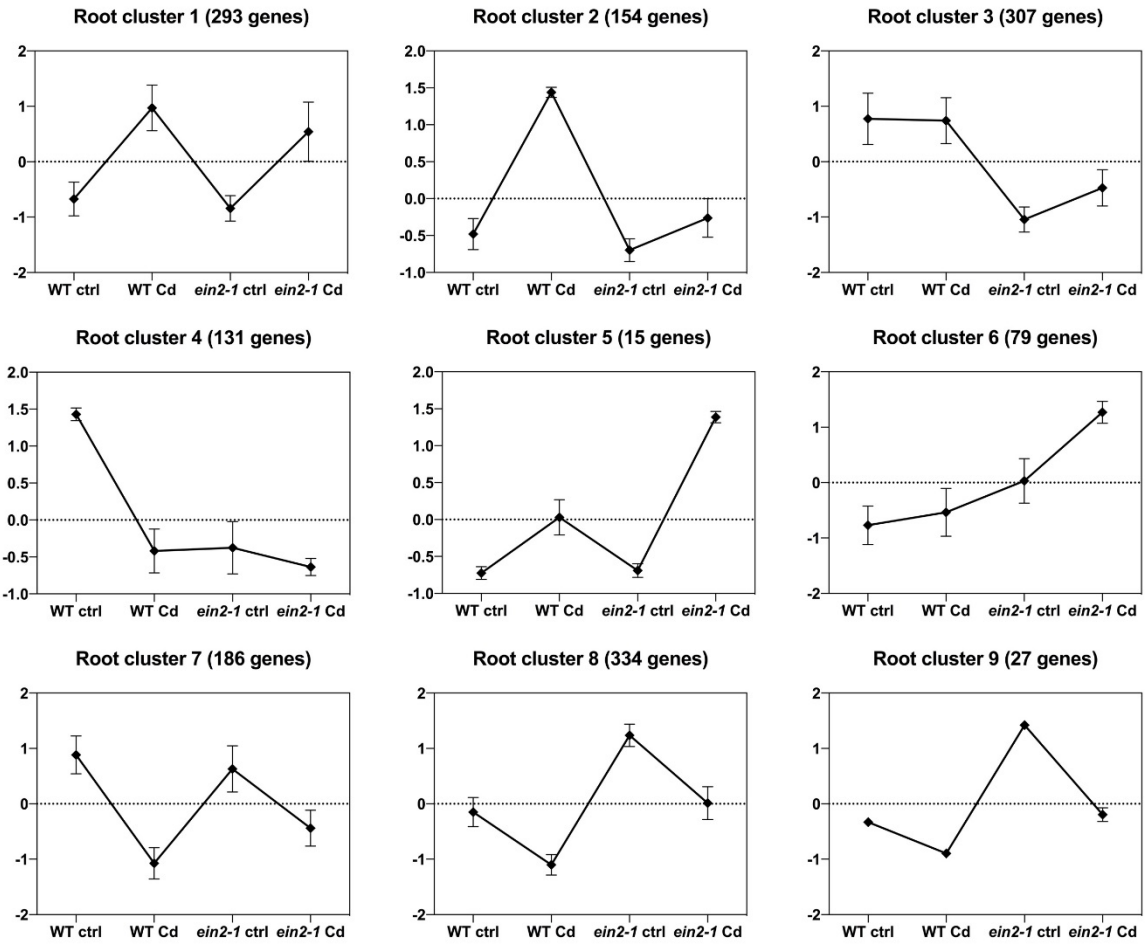

Fig. 8. Relative gene expression profiles of root clusters. Clusters are defined by hierarchical clustering. The number of genes included in each cluster is indicated between brackets. Each root cluster depicts the relative gene expression of genes within the cluster, calculated using a rescaling method: $y = (x - mean_i) / \sigma_i$). Y: rescaled value; $mean_i$: mean of all gene expression values within one the condition; σ_i, S.D. of all gene expression values within one condition.

3.3. Ethylene insensitivity causes differences in gene expression under control conditions

In total, 69 genes in leaves (clusters 5, 6 and 7, Fig. 7) and 878 genes in roots (clusters 3, 4, 6, 8 and 9, Fig. 8) were included in clusters where expression levels differed between both genotypes under control conditions. Whereas this number was small for leaves, more than half of the differentially expressed genes in roots showed a different expression level between WT and *ein2-1* mutant plants under control conditions. These data suggest that ethylene also plays important roles in roots of plants not exposed to Cd.

Leaf clusters 5, 6 and root clusters 6 and 9 did not contain any enriched biological processes. Leaf cluster 7 (Fig. 7) mostly contained genes related to sulfur-containing metabolites and was characterized by higher gene expression levels in *ein2*-1 mutant leaves as compared to WT leaves. Genes in root clusters 3 and 4 were more strongly expressed in WT roots as compared to *ein2-1* mutant roots under control conditions. Root cluster 3 (Fig. 8) was enriched in genes involved in processes related to inorganic ion transport and defense responses by cell wall thickening. Metal ion homeostasis and root hair development were enriched biological processes found in root cluster 4. In contrast, genes in root cluster 8 (Fig. 8) showed higher expression levels in *ein2-1* mutant roots as compared to WT roots, and contained genes involved in RNA processing and cytokinesis.

3.4. Cadmium-induced effects on gene expression levels in roots and leaves

Previous research already demonstrated that Cd exposure affects many physiological processes in plants, including photosynthesis [8]. Gene ontology enrichment analysis revealed that photosynthesis-related processes such as chlorophyll biosynthesis, photosystem I assembly and photosystem II repair were enriched in leaf cluster 4 (Fig. 7). This indicates that their expression was downregulated upon Cd exposure in WT leaves, whereas this effect was less pronounced in leaves of the *ein2-1* mutant. The downregulation of genes responsible for photosystem assembly or repair, as observed in Cd-exposed WT leaves, could contribute to damage to the photosynthetic apparatus, as previously reviewed by Parmar *et al.* (2013) [33]. Similarly, Liu *et al.* (2016) showed that the negative effect of low temperatures on photosynthetic activity was less pronounced in *ein2-1* mutants as compared to WT plants [34]. These data suggest that ethylene signaling is involved in the disturbance of photosynthetic processes under abiotic stress conditions.

Root hair development and elongation were GO terms enriched in root cluster 4 (Fig. 8). The genes in this cluster displayed a Cd-induced downregulation in WT roots that was less pronounced in *ein2-1* mutant roots. The downregulation of gene expression in WT roots exposed to Cd suggests a retardation in root and root hair development, which is in agreement with the negative effect of Cd on root growth, as reviewed by De Smet *et al.* (2015) [35]. Similarly, Fan *et al.* (2011) demonstrated a time- and dose-dependent delay in root hair development in Cd-exposed *A. thaliana* plants [36]. Tanimoto *et al.* (1995) demonstrated an increase in root hair cell number in *A. thaliana* plants exposed to the ethylene precursor ACC, whereas this parameter decreased upon exposure to the ethylene biosynthesis inhibitor aminovinylglycine (AVG) [37]. Likewise, Kong *et al.* (2018) demonstrated that Cd-induced

root growth inhibition was enhanced in seedlings simultaneously treated with ACC, while it was mitigated when seedlings were treated with AVG [38]. The lack of a Cd-induced decrease in gene expression levels in *ein2-1* mutant roots (cluster 4, Fig. 8) supports the possible role of ethylene as a regulator of Cd-induced root growth inhibition. De Smet *et al.* (2015) proposed that ethylene achieves this inhibitory role on root growth by regulating auxin transport throughout the root tip [35].

3.5. The role of ethylene signaling in the Cd-induced oxidative stress response

3.5.1. Ethylene signaling affects Cd-induced H_2O_2 production

The response to H_2O_2 was found as an enriched biological process in both leaf cluster 1 (Fig. 7) and root cluster 2 (Fig. 8). Both clusters displayed a similar gene expression profile, characterized by a Cd-induced upregulation in WT plants, which was less pronounced in *ein2-1* mutant plants. Cadmium is known to induce the production of ROS [11], and aiming on gaining a broader view on the effect of ethylene signaling on Cd-induced H_2O_2 production, H_2O_2 concentrations were determined after 24 h, 72 h and 7 days of Cd exposure (Fig. 9).

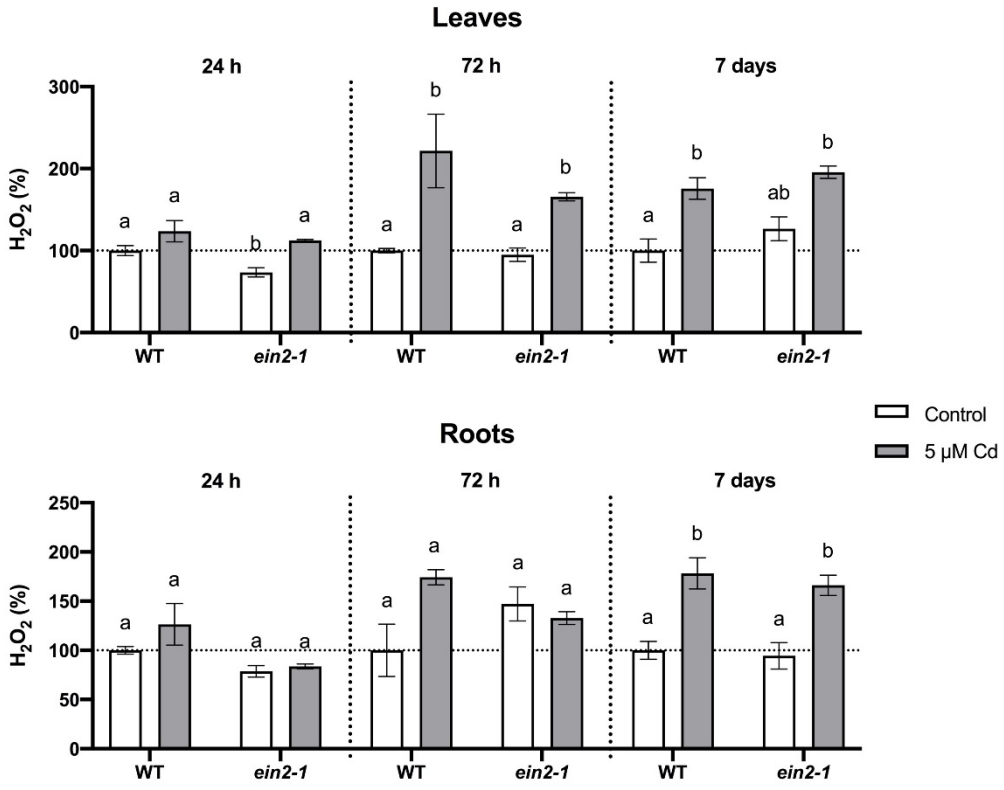

Fig. 9. Hydrogen peroxide (H_2O_2) concentrations in roots and leaves of WT and *ein2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 h, 72 h and 7 days. Hydrogen peroxide concentrations in leaves and roots are expressed relatively to the WT control (set to 100%: dashed line). Values are mean ± S.E. of 3 biological independent replicates. Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

After 24 h, H_2O_2 concentrations were significantly lower in *ein2-1* control leaves as compared to WT control leaves. When exposed to Cd, leaves of both genotypes showed an increase in H_2O_2 concentrations, although this increase was not significant in the WT leaves. After 72 h, both genotypes showed Cd-induced increases in H_2O_2 concentrations, apparently more pronounced in WT leaves. In roots, no effects on H_2O_2 concentrations were observed after 24 h and 72 h of Cd exposure. However, an increasing trend was observed in WT roots after 72 h, whereas no such effect was seen in *ein2*-1 roots. When plants were exposed to Cd for 7 days, roots and leaves of both genotypes showed similar increases in H_2O_2 concentrations (Fig. 9). To summarize, higher increases in Cd-induced H_2O_2 concentrations were observed in WT roots and leaves after 72 h, whereas these increases were very similar in both genotypes after 7 days of Cd exposure.

Previously, Cuypers *et al.* (2011) observed increases in H_2O_2 concentration in *A. thaliana* roots and leaves after 24 h of exposure to 5 and 10 µM Cd [17], which is in agreement with the Cd-induced effects on WT leaves and roots observed in this study (Fig. 9). These increases in H_2O_2 concentrations can be explained by the fact that Cd indirectly causes an increased ROS production through the activation of NADPH oxidases or the depletion of antioxidants (Fig. 1) [11].

The RNA-seq analysis indicated that the Cd-induced increase in expression levels of genes related to responses to H_2O_2 was much less pronounced in the *ein2-1* mutant leaves (cluster 1, Fig. 7) and roots (cluster 2, Fig. 8) as compared to that of WT plants. This is supported by the fact that Cd-induced increases in H_2O_2 concentrations in *ein2-1* mutant leaves and roots were either less pronounced or completely absent after 24 and 72 h of exposure (Fig. 9). Upon exposure to the herbicide paraquat, lower increases in H_2O_2 concentrations were also observed in *ein2-1* mutants as compared to WT plants. This response could be explained by higher SOD and CAT activities observed in *ein2-1* mutants as compared to WT plants under control conditions, which might counteract stress-induced increases in H_2O_2 levels [39].

Considering the time-dependence of the response, Schellingen *et al.* (2015) no longer observed phenotypic differences between WT plants and ethylene biosynthesis mutant (*asc2-1/asc6-1*) plants after long-term Cd exposure for 7 days, and suggested that ethylene is mainly involved in early responses to Cd exposure [40]. Similarly, our results indicated that differences in H_2O_2 production between WT plants and *ein2-1* mutants were mainly observed after 24 and 72 h of Cd exposure, whereas a similar response was seen in both genotypes upon prolonged exposure (Fig. 9). These data confirm the early and transient role of ethylene in the Cd-induced stress response.

3.5.2. Ethylene signaling affects Cd-induced lipid peroxidation

Leaf cluster 3 (Fig. 7) was significantly enriched in genes related to lipid peroxidation, characterized by an Cd-induced increase in gene expression in WT leaves, which was less pronounced in *ein2-1* leaves. In roots, no clusters were significantly enriched in processes related to lipid peroxidation.

As discussed in the introduction, Cd-induced ROS production can damage several cellular biomolecules such as DNA, proteins and lipids [13]. To gather more knowledge on the involvement of ethylene signaling in Cd-induced lipid peroxidation, TBArm measurements

were performed in roots and leaves of both genotypes exposed to Cd for 24 h, 72 h and 7 days (Fig. 10). After 72 h and 7 days, the Cd-induced increase in lipid peroxidation was more pronounced in WT leaves as compared to *ein2-1* leaves. In roots, no significant differences were observed when comparing control and Cd-exposed plants of either genotype. However, an increase in TBArm concentrations was observed in *ein2-1* roots after 72 h and 7 days of exposure, whereas in WT plants this increase was only observed at 7 days after exposure (Fig. 10).

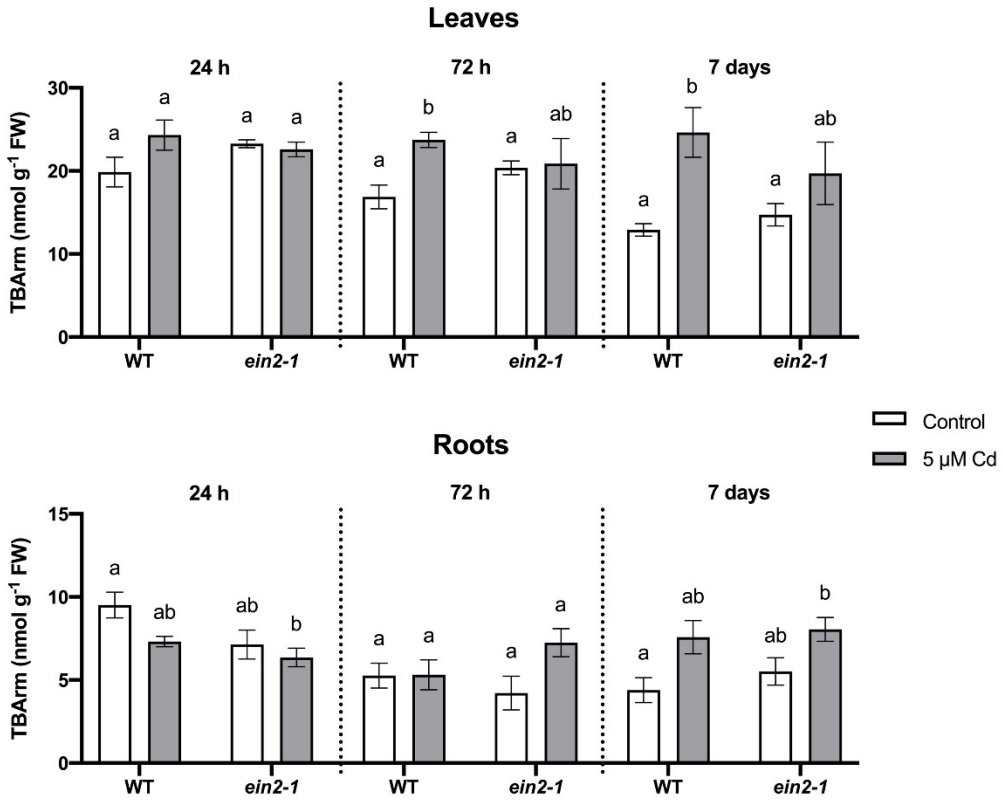

Fig. 10. Levels of TBArm in roots and leaves of WT and *ein2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$. Graphs display TBArm levels measured in leaves and roots after 24 h, 72 h and 7 days of exposure. Values represent mean ± S.E. of at least 4 biological independent replicates. Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

Skorzynska *et al.* (2010) observed an increase in both enzymatic and non-enzymatic lipid peroxidation in WT *A. thaliana* leaves after 7 days of Cd exposure [41]. In their study, non-enzymatic lipid peroxidation, responsible for the formation of TBArm, showed an increase after 7 days of Cd exposure (5 µM), similar to the observed increase in Cd-exposed WT leaves in our study (Fig. 10). In addition to non-enzymatic lipid oxidation, Skorzynska *et al.* (2010) also studied enzymatic lipid peroxidation, which is mediated by lipoxygenases (LOX). Their activities can be determined through analysis of lipid hydroperoxides, which increased in WT leaves after Cd exposure in a concentration-dependent matter [41].

Interestingly, many *LOX* genes were also found in leaf cluster 3 (Fig. 7) obtained through RNA-seq. The increase in their expression observed in leaves of Cd-exposed WT plants was less

pronounced in the *ein2-1* mutant, indicating that ethylene signaling affects their expression upon Cd exposure. A link between ethylene and lipid peroxidation was already established in Cd-exposed *never ripe* (*nr*) mutant tomato plants, characterized by disturbed ethylene signaling. The time-dependent Cd-induced increase in lipid peroxidation observed in WT tomato plants was significantly less pronounced in *nr* mutants [42]. The expression of *LOX* genes was also delayed in the *nr* mutant tomato plants [43], supporting the possible role of ethylene as an upstream regulator of *LOX* gene expression and lipid peroxidation. Interestingly, mitogen activated protein kinase (MPK) 3 and MPK6 were put forward as transcriptional activators of *LOX* genes under high temperature stress in *A. thaliana* [44]. Additionally, Li *et al*. (2012) suggested an interaction between the MPK cascade and ethylene biosynthesis. They studied gene expression levels of *ACS2* and *ACS6* in response to the ethylene-inducing pathogen *Botrytis cinerea*, and observed that in MPK3/6-deficient mutants *ACS2* and *ACS6* gene expression levels were much lower after inoculation with the pathogen [45]. Remarkably, the MPK cascade was also found as a biologically enriched process in leaf cluster 3 (Fig. 7), which is the same cluster in which genes related to lipid peroxidation were enriched. These data suggest that ethylene signaling affects *LOX* gene expression, possibly through the MPK cascade under Cd-exposed conditions. For roots, the MPK cascade was not found as an enriched process, again indicating that Cd-induced stress responses are organ-dependent. The interaction between ethylene signaling, the MPK cascade, *LOX* gene expression and lipid peroxidation required additional investigation in future studies.

3.5.3. Ethylene signaling affects the Cd-induced ER stress response

In addition to lipid peroxidation, genes related to ER stress were also significantly enriched in leaf cluster 3 (Fig. 7), whereas this process was not enriched in any of the root clusters. Expression levels of genes involved in ER stress and the unfolded protein response (UPR) were upregulated upon Cd exposure in WT leaves, while this effect was less pronounced in *ein2-1* leaves.

Under stress conditions, the demand of protein folding in the ER can exceed the capacity of folding and degradation mechanisms, setting off the UPR. The ER stress response signaling pathway can be subdivided in two important parts. One includes membrane bound transcription factors such as basic leucine zipper 28 (bZIP28), while the second involves the operation of inositol-requiring enzyme 1 (IRE1), which is a dual-functioning protein kinase/ribonuclease that splices the mRNA encoding bZIP60. Under stress conditions, unfolded proteins accumulate in the ER, activating IRE1 and resulting in the unconventional splicing of the *bZIP60* mRNA. Then, together with bZIP28, bZIP60 is translocated to the nucleus, where it functions as a transcription factor inducing the expression of ER stress-responsive genes [46]. Since unconventional splicing of *bZIP60* is an important factor in the unfolded protein response (UPR), the extent of *bZIP60* splicing was determined after 24 and 72 h of Cd exposure in roots and leaves of WT and *ein2-1* plants via qPCR (Fig. 11).

After 24 h of Cd exposure, spliced *bZIP60* levels showed a 15-fold increase in WT leaves, and only increased approximately 5-fold in *ein2-1* leaves, whereas spliced levels did not increase after 72 h in leaves of either genotype. Spliced *bZIP60* levels in roots significantly increased upon Cd exposure in the *ein2-1* mutant, whereas they were not affected in WT roots after 24 h

or 72 h of Cd exposure. These data suggest that in leaves, the extent of ER stress is largest after 24 h of Cd exposure and is less pronounced in *ein2-1* mutant leaves. However, the opposite is observed in roots, which display higher ER stress levels after 72 h of Cd exposure, a response which is more strongly pronounced in the *ein2-1* mutant (Fig. 11). This indicates that the association between ethylene signaling and the Cd-induced ER stress response is strongly organ-dependent.

The transcription levels of unspliced *bZIP60* were also studied, and mainly followed a similar trend as compared to the levels of spliced *bZIP60*, indicating that the increase in *bZIP60* splicing requires an increase in the expression of unspliced *bZIP60* (Supplemental Fig. 1).

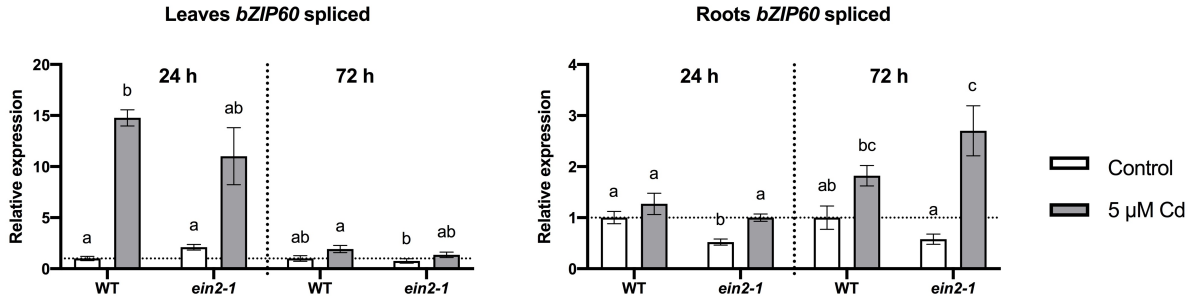

Fig. 11. Relative expression levels of spliced *bZIP60* in roots and leaves of WT and *ein2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 h, 72 h and 7 days. Levels of spliced *bZIP60* were quantified using qPCR in leaves and roots and relatively expressed to WT control 24 h (set to 1: dashed line). Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

Xu *et al.* (2013) demonstrated that Cd exposure induced both ER stress and programmed cell death (PCD) in BY-2 tobacco cells. When treated with the ER luminal binding protein BiP, ER stress was alleviated and PCD was delayed, suggesting that Cd-induced PCD is mediated by ER stress [47]. The Cd-induced ER stress response was also observed by Xi *et al.* (2016) in *A. thaliana* seedlings grown on agar medium [48], and Howell *et al.* (2013) proposed a link between ER stress and PCD in *A. thaliana* [46]. In the RNA-seq data, the regulation of PCD was found as an enriched biological process in the same cluster containing the ER stress response (leaf cluster 3, Fig. 7), suggesting that ethylene signaling is involved in mediating ER stress-induced PCD in leaves of Cd-exposed plants. To determine the effect of ethylene signaling on ER stress-related PCD, future research should aim to analyze the extent of PCD in Cd-exposed WT and *ein2-1* mutant plants. Also, the effect of ethylene signaling on the Cd-induced ER stress response itself requires more investigation. For example, the effect of ethylene signaling on the expression of target genes of the transcription factors bZIP28 and bZIP60 could be investigated, and the use of ER stress mutants could contribute to a broader understanding of the Cd-induced stress response.

3.6. Ethylene signaling and Cd exposure affect hormonal signaling

The RNA-seq analysis pointed out that ethylene signaling has an influence on the expression of hormone-related genes under Cd-exposed conditions. Leaf cluster 1 (Fig. 7) was significantly enriched in genes related to responses to ethylene stimuli, whereas responses to jasmonic acid (JA) stimuli were enriched in leaf cluster 3 (Fig. 7), and biological processes related to both hormonal stimuli were enriched in root cluster 2 (Fig. 8). Remarkably, all three clusters displayed a similar gene expression pattern, characterized by increases in expressions in Cd-exposed WT plants, which were less pronounced in *ein2-1* plants.

Cadmium-induced increases in ethylene signaling can be explained by the fact that Cd specifically induces the expression of ethylene biosynthesis genes *ACS2* and *ACS6* as the Cd-induced ethylene production was much less pronounced in the *acs2-1 acs6-1* double knockout mutant [22]. As it is a key protein in the ethylene signaling pathway [49], a knockout mutation of EIN2 prevents further ethylene signaling, explaining the absence of response to an ethylene stimulus in *ein2-1* mutant leaves (cluster 1, Fig. 7) and roots (cluster 2, Fig. 8) exposed to Cd.

Effects related to JA stimuli are possibly linked to differences in the extent of Cd-induced lipid peroxidation. As described in the previous section, Cd induces the enzymatic LOX-mediated generation of oxidized lipids, which can be precursors of jasmonates. Cadmium also induces the expression of genes involved in the synthesis and accumulation of JA [50, 51], explaining the increase in response to JA stimuli in Cd-exposed WT plants. Nonetheless, the lack of ethylene signaling in the *ein2-1* mutant plants resulted in less pronounced responses to JA stimuli. Lorenzo *et al.* (2003) suggested that the ERF1 transcription factor is a common component for both ethylene and JA signaling pathways, and that ERF1 needs both JA and ethylene signaling for the activation of defense-related gene expression [52]. As such, the fact that ethylene signaling, and thereby the induction of ERF1, are blocked in the *ein2-1* mutant plants could explain the lack of response to JA stimuli in the Cd-exposed *ein2-1* mutant.

The interaction between different hormonal pathways under both control and stress conditions remains a complex given and requires additional investigation. For example, the possible ethylene-induced *LOX* expression and subsequent production of JA precursors presents new perspectives for study in Cd-exposed plants.

3.7. Ethylene signaling affects the Cd-induced effects on the sulfur metabolism

According to the RNA-seq and GO enrichment analyses, processes related to the sulfur metabolism were enriched in both leaves (cluster 7, Fig. 7) and roots (cluster 7, Fig. 8), and noticeably, these processes were mainly related to glucosinolate (GSL) biosynthesis. Leaf cluster 7 (Fig. 7) was one of the clusters displaying a gene expression profile in which the WT and *ein2-1* control conditions differed. Both genotypes showed a similar decrease in expression upon Cd exposure. However, gene expression levels in *ein2-1* control leaves were higher than those in WT control leaves, indicating a suppressive role of ethylene signaling on the expression of genes related to GSL biosynthesis in leaves. The gene expression profile in root cluster 7 (Fig. 8) showed similar decreases in gene expression after Cd exposure in both genotypes. However, differences between WT and *ein2-1* leaves under control conditions were absent.

Glucosinolates are secondary metabolites synthesized from cysteine [53] and are found almost exclusively in Brassicaceae [54]. Sun *et al.* (2009) and Durenne *et al.* (2018) previously demonstrated that GSL levels decreased upon Cd exposure in both *A. thaliana* [55] and *Brassica napus* [53]. These data are in agreement with the results obtained via RNA-seq. However, the precise role of GSLs in the Cd-induced stress response remains unclear. Martínez-Ballesta *et al.* (2013) reviewed that, depending on the stressor, GSL levels either increased or decreased, and hypothesized that GSL participates in signal transduction [56]. Pangesti *et al.* (2016) introduced a model on the regulation of GSL under biotic stress, proposing antagonistic roles of ethylene and JA signaling in regulating GSL levels [57]. The stimulating role of JA signaling proposed in the model was also observed in Mewis *et al.* (2005), who demonstrated that GSL levels were significantly decreased in *A. thaliana coi-1* mutants, in which JA signaling is blocked [58]. The inhibitory role of ethylene signaling, on the contrary, is in agreement with our RNA-seq data which showed that expression levels of GSL biosynthesis genes increased in the ethylene insensitive *ein2-1* mutant leaves under both control as Cd-exposed conditions as compared to WT leaves (cluster 7, Fig. 7). These data suggest that the GSL response is similarly regulated under biotic stress and Cd stress. Yet, further research on GSL regulation is needed in both *ein2-1* and *coi-1* mutants to verify this model in Cd-exposed *A. thaliana* plants.

Interestingly, previous research indicated that ethylene signaling also affects concentrations of GSH – another cysteine-derived metabolite – in Cd-exposed plants [18]. This interaction between ethylene and GSH was shown to be reciprocal. Indeed, Schellingen *et al.* (2015) demonstrated a delay in Cd-induced GSH biosynthesis in ethylene insensitive *etr1-1* and *ein2-1 A. thaliana* mutants [14], whereas Han *et al.* (2013) showed that *ERF2* expression levels, indicative of ethylene signaling, were decreased in GSH-deficient *cad2-1* mutants under control conditions [59]. The gene encoding GSH2, involved in GSH biosynthesis, was found in leaf cluster 3 (Fig. 7) and root cluster 2 (Fig. 8), characterized by a Cd-induced upregulation in the WT which was less pronounced in *ein2-1* mutants, confirming an interaction between ethylene signaling and GSH.

3.8. The importance of glutathione in the Cd-induced stress response

Since ethylene signaling and GSH are intertwined in the Cd-induced stress response, parameters related to fresh weight, ROS production, oxidative damage and ER stress were also investigated in Cd-exposed *cad2-1* mutants, containing only 30% of WT GSH levels [16, 60].

3.8.1 Cadmium-induced effects on plant growth are mediated by GSH levels

Leaves and roots of WT and *cad2-1* mutant plants were harvested separately and weighted immediately after 24 h, 72 h and 7 days of exposure to 5 µM Cd (Fig. 12). After 24 h and 72 h, Cd-induced effects on root and leaf fresh weight were similar between both genotypes, yet after 72 h, the *cad2-1* leaves already displayed lower fresh weights as compared to WT leaves. After 7 days, the Cd-induced decrease in fresh weight was significantly more pronounced in *cad2-1* leaves and roots as compared to their WT counterparts, indicating an increased Cd sensitivity of the mutant. Jozefczak *et al.* (2015) studied the Cd uptake in WT and *cad2-1* roots and leaves after 72 h of exposure, but did not observe any differences between genotypes [16]. These results indicate that both WT and *cad2-1* genotypes respond similarly to short-term Cd exposure, but that GSH deficiency affects the phenotype after longer-term Cd exposure.

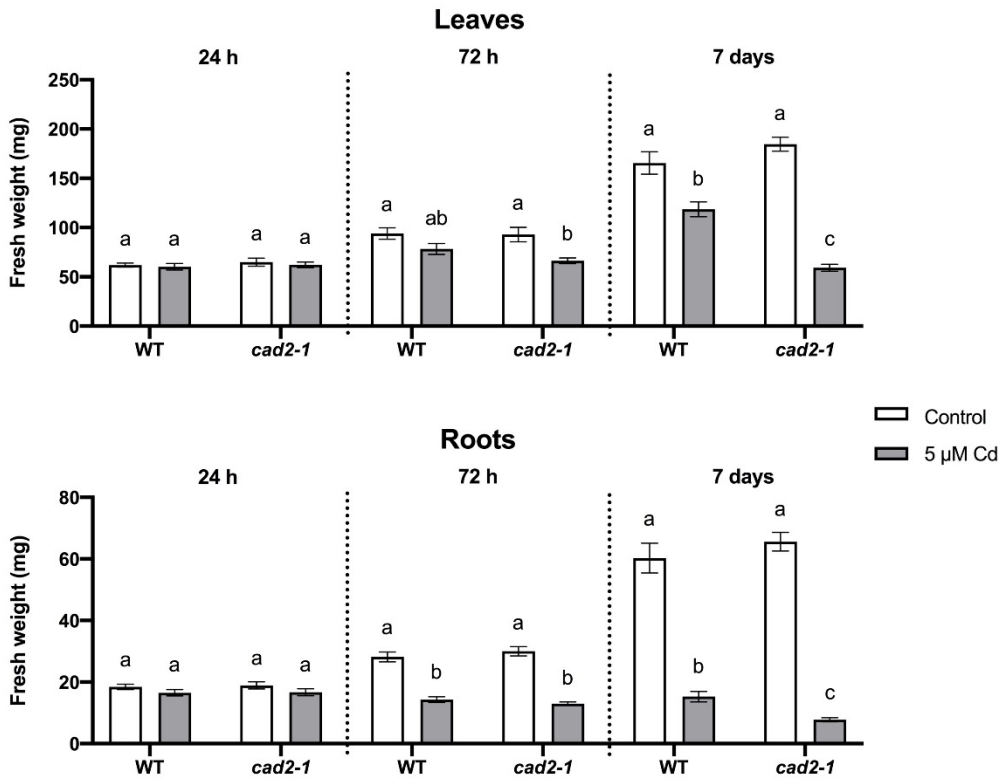

Fig. 12. Fresh weight (mg) of leaves and roots of WT and *cad2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 h, 72 h and 7 days. Fresh weight measurements were performed directly after harvesting. Values represent the mean ± S.E. of at least 10 biological independent replicates. Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

3.8.2. Glutathione levels influence the Cd-induced oxidative stress response

To investigate the influence of GSH levels on Cd-induced ROS production, H_2O_2 concentrations were determined in WT and *cad2-1* plants after 24 h, 72 h and 7 days of Cd exposure (Fig. 13).

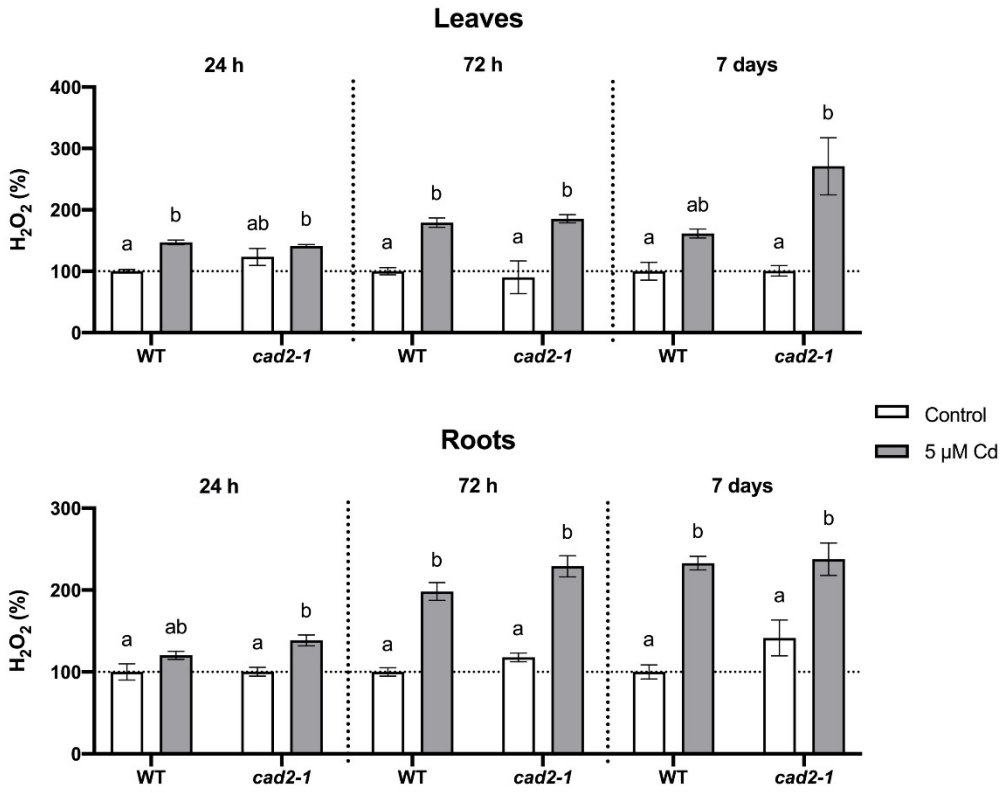

Fig. 13. Hydrogen peroxide (H_2O_2) concentrations in roots and leaves of WT and *cad2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 h, 72 h and 7 days. Hydrogen peroxide concentrations in leaves and roots are expressed relatively to the WT control (set to 100%: dashed line). Values are mean ± S.E. of 3 biological independent replicates. Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

In Cd-exposed *cad2-1* leaves, a time-dependent increase in H_2O_2 concentrations was observed, whereas this was not present in WT leaves. In WT roots, the increase in H_2O_2 concentrations after Cd exposure became significant after 72 h, while in *cad2-1* roots this Cd-induced increase was already significant after 24 h (Fig. 13). Generally, despite the increased Cd sensitivity in the *cad2-1* mutants, H_2O_2 concentrations remained relatively similar between both genotypes upon Cd exposure. This indicates that in addition to GSH, other antioxidative mechanisms are involved in counteracting the Cd-induced H_2O_2 production. In the *ein2-1* mutant, activities of antioxidative enzymes CAT and SOD were already upregulated under control conditions [39], possibly explaining the lack of Cd-induced increases in H_2O_2 concentrations in *ein2-1* roots (Fig. 9). Similarly, *cad2-1* mutants also exhibit higher activities of antioxidative enzymes such as SOD, CAT and APX under control conditions. Jozefczak *et al.* (2015) suggested that these

increases in antioxidative enzyme activities serve to compensate for the lower reduced GSH levels in the mutant [16]. While GSH deficiency affects these enzyme activities through a disturbance in the redox balance, their regulation by ethylene remains elusive.

To compare the extent of lipid peroxidation between both genotypes TBArm concentrations were determined in WT and *cad2-1* plants after 24 h, 72 h and 7 days of Cd exposure (Fig. 14). Generally, Cd-induced increases in lipid peroxidation were rather similar between both organs, especially after 24 h and 72 h of Cd exposure. After 72 h and 7 days, the extent of Cd-induced lipid peroxidation was more pronounced in roots and leaves of *cad2-1* mutants as compared to WT plants. These results suggest that GSH plays a key role in the defense against oxidative damage upon prolonged Cd exposure. For example, Yin *et al.* (2017) studied effects of aluminum exposure on lipid peroxidation in GR-overexpressing transgenic *A. thaliana* plants. In these overexpressors, levels of oxidized GSH levels were higher and levels of H_2O_2 and lipid oxidation were reduced as compared to the WT plants, confirming the role of GSH in the Cd-induced antioxidative stress response.

Since there is a link between ethylene signaling and the regulation of *LOX* genes, it would be interesting to also investigate *LOX* gene expression in Cd-exposed *cad2-1* mutant roots and leaves.

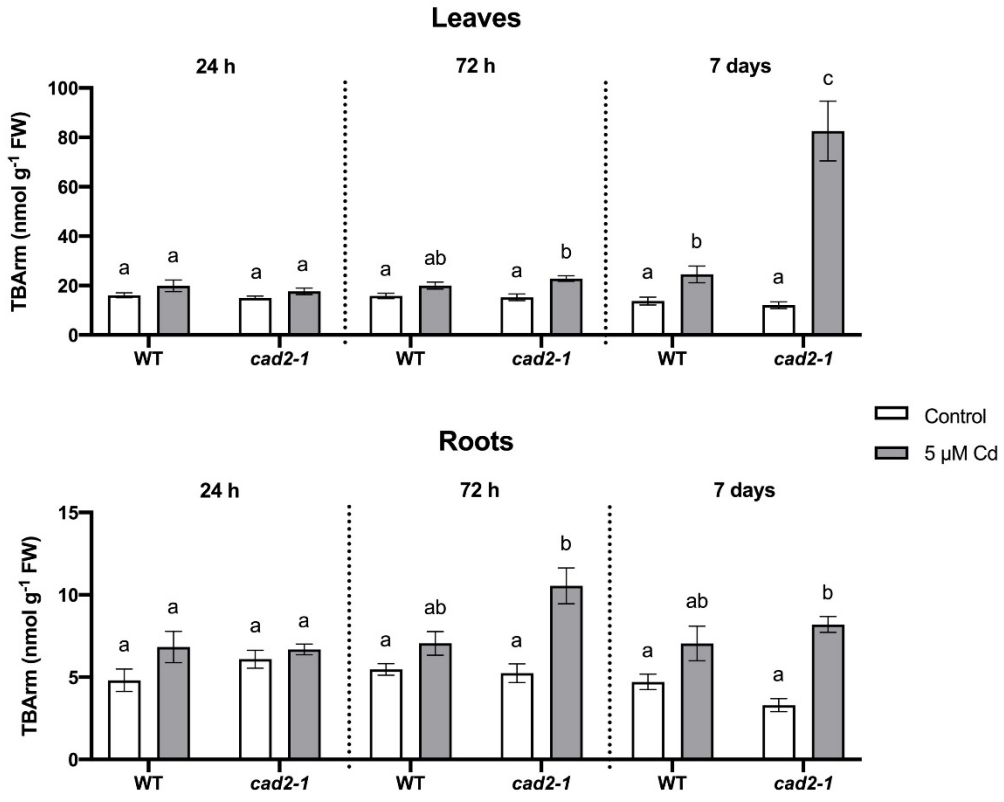

Fig. 14. Levels of TBArm in roots and leaves of WT and *cad2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$. Graphs display TBArm levels measured in leaves and roots after 24 h, 72 h and 7 days of exposure. Values represent mean ± S.E. of at least 4 biological independent replicates. Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

3.8.3. Glutathione levels influence the Cd-induced ER stress response

Since ethylene signaling affected *bZIP60* splicing levels in Cd-exposed plants (Fig. 11), the role of GSH in the Cd-induced ER stress response was also investigated. To this end, levels of spliced and unspliced *bZIP60* were determined using qPCR in WT and *cad2-1* plants exposed to Cd for 24 h and 72 h (Fig. 15).

Spliced *bZIP60* levels in leaves displayed similar responses in both genotypes upon Cd exposure. However, the extent of *bZIP60* splicing seemed to decrease over time in WT leaves, whereas it remained similar in *cad2-1* mutant leaves. In contrast to what was observed in leaves, *bZIP60* splicing was differentially affected by Cd exposure between roots of both genotypes. Roots of WT plants showed an increase in *bZIP60* splicing levels after 72 h, which was absent after 24 h of Cd exposure. The *cad2-1* roots showed an opposite trend as compared to WT roots, and displayed major increases of *bZIP60* splicing levels upon Cd exposure. After 24 h, a 50-fold increase was reached in *cad2-1* mutant roots, and after 72 h this increase was less pronounced but remained highly significant. These data indicate that, especially in roots, GSH plays an important role in mitigating the Cd-induced ER stress response. Again, unspliced *bZIP60* transcript levels showed a highly similar pattern to those of spliced *bZIP60* (Supplemental Fig. 2).

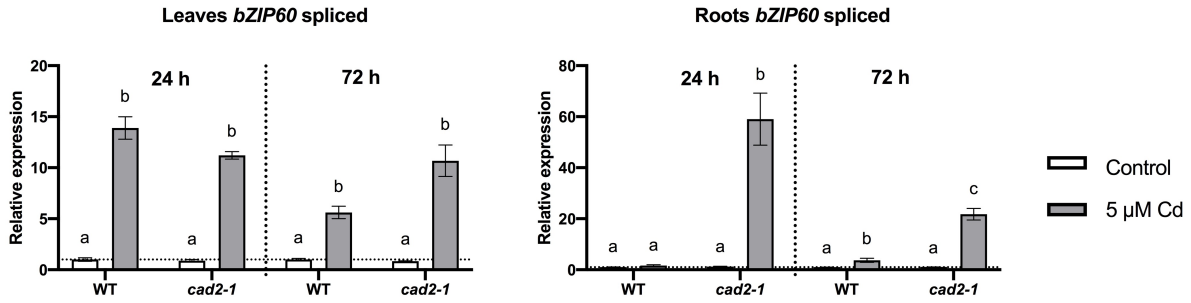

Fig. 15. Relative expression levels of spliced *bZIP60* in roots and leaves of WT and *cad2-1 A. thaliana* plants. Plants were grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 h, 72 h and 7 days. Levels of spliced *bZIP60* were quantified using qPCR in leaves and roots and relatively expressed to WT control 24 h (set to 1: dashed line). Different letters indicate significant differences between conditions (two-way ANOVA within each time-point; $p < 0.05$).

Previously, Chakravarthi *et al.* (2006) proposed a model in which misfolded proteins induce the synthesis of GSH [61]. This was recently confirmed by Uzilday *et al.* (2018), showing that tunicamycin-induced ER stress increases the activity of GR, and induces the expression of the GSH biosynthesis genes *GSH1* and *GSH2* [62]. Previously, it has been shown that GSH plays an important role in the formation of disulfide bonds in the ER and the maintenance of the ER redox state [61, 63]. Reduced GSH is transported from the cytosol to the ER lumen, as no GR is present in the ER [63]. The lower GSH concentrations in the *cad2-1* mutant might explain the stronger Cd-induced increase in ER stress in this genotype, since the oxidative imbalance in the ER lumen can possibly not be compensated by the usual supply of reduced GSH.

Thus, our results showed that the Cd-induced ER stress response was affected by both ethylene signaling and GSH, which reinforces the hypothesized interaction between ethylene and GSH in Cd-exposed plants. Also, these results suggest that ER stress is an important factor in Cd-induced stress responses, requiring more investigation. Remarkably, Cd-induced responses were shown to be strongly organ-dependent, emphasizing the need to perform analyses in both roots and leaves in future studies.

4. Conclusions

To further investigate the involvement of ethylene signaling in Cd-induced stress responses in *A. thaliana*, RNA sequencing was performed in roots and leaves of WT and *ein2-1* mutant plants exposed to 5 µM Cd for 24 h. The RNA-seq data suggested that ethylene signaling plays an important role in plant functioning under control as well as Cd exposure conditions.

The Cd-induced decrease in expression of genes related to photosynthesis and root growth, as observed in WT plants, was less pronounced in the *ein2-1* mutants, confirming the role of ethylene signaling in the regulation of these physiological processes. The sequencing data also pointed out that responses to Cd-induced ROS production and oxidative damage were less pronounced in *ein2-1* mutants as compared to WT plants. When further comparing H_2O_2 concentrations and the extent of lipid peroxidation between both genotypes upon Cd exposure, it became apparent that ethylene signaling plays an early and transient role in these processes. The fact that ethylene signaling is mainly involved in early responses to Cd, was also reflected in fresh weights of the plants, where genotype-dependent differences became less pronounced over time. Ethylene was also suggested as an upstream regulator of LOXs, possibly inducing enzymatic lipid peroxidation in Cd-exposed plants. Since LOXs produce oxidized lipids, which are precursors of jasmonates, this also puts new perspectives on the observed interaction between ethylene and JA.

Furthermore, the RNA-seq data indicated that genes related to the ER stress response displayed smaller upregulations in *ein2-1* leaves as compared to WT leaves. When further investigating the extent of ER stress by measuring the level of *bZIP60* splicing, it became apparent that the effect of ethylene signaling on this process differed between plant organs. While Cd-induced ER stress was more pronounced in leaves after 24 h, roots displayed a larger extent of ER stress after 72 h of Cd exposure. Also, the extent of ER stress induced by Cd exposure seemed lower in the *ein2-1* leaves, while it seemed higher in *ein2-1* roots as compared to their WT counterparts.

In addition, the RNA-seq data showed that Cd increased the expression of *GSH2* in WT plants, whereas no such effect was observed in the *ein2-1* plants. This further supported an interaction between ethylene and GSH. Therefore, the involvement of GSH in the cellular processes affected by ethylene signaling were also investigated in GSH-deficient *cad2-1* mutant plants. Lipid peroxidation levels pointed out that GSH is also important in the defense against oxidative damage upon prolonged Cd exposure, whereas ethylene signaling seemed to mainly play a role in the early Cd-induced stress response. The importance of GSH in the defense upon prolonged Cd exposure was also reflected in the stronger negative effect on fresh weight observed after 7 days of Cd exposure, whereas these differences were not as pronounced before. In addition, GSH seemed to also play an important role in the ER stress response. Especially *cad2-1* roots showed a tremendous increase in ER stress upon Cd exposure, whereas this effect was less pronounced in leaves. These data further emphasize that Cd-induced stress responses are organ-dependent.

Taken together, this study gained novel insight into the importance of ethylene signaling and its interaction with GSH in Cd-induced stress responses. However, additional research remains

to be done to fully comprehend the mechanisms underlying ethylene-mediated regulation of Cd-induced stress responses. The results of this study strongly emphasize the need to perform analyses in both roots and leaves, as responses are strongly organ-dependent.

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Supplemental information

Table S1. Reverse transcription quantitative PCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin *et al*. (2009).

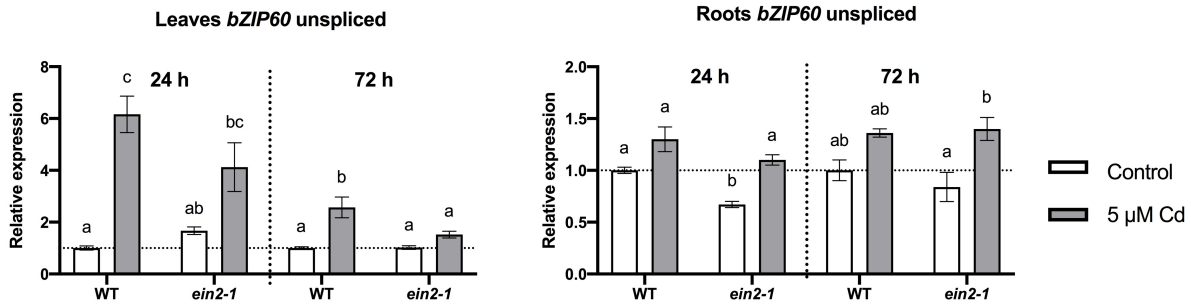
Sample/Template	
Source	*Arabidopsis thaliana* leaves and roots in a hydroponic culture
Method of preservation	Harvest in liquid nitrogen, storage at -80 °C
Storage time (if appropriate)	Maximum two weeks
Handling	Frozen
Extraction method	Columns: RNAqueous™ Kit
RNA: DNA-free	Turbo DNA-*free*™ Kit
	Use of intron-spanning primers whenever possible
	Verification of single peak on dissociation curves
RNA: concentration	NanoDrop® ND-1000 spectrophotometer
RNA: integrity	RNA 6000 Nano Assay, Agilent 2100 Bioanalyzer
Assay optimisation/validation	
Accession number	Table S2
Amplicon details	Exon location and amplicon size: Table S2
Primer sequence	Table S2
In silico	Primer-BLAST (http://www.arabidopsis.org/Blast/index.jsp)
Empirical	Primer concentrations of 300 nM unless stated otherwise (Table S1)
	Annealing temperature of 60 °C
Priming conditions	Combination of oligo-dT primers and random hexamers
PCR efficiency	Dilution curves (slope, deviation)
Linear dynamic range	Samples are within the range of the efficiency curve
RT and qPCR	
Protocols	Turbo DNA-*free*™ Kit
	PrimeScript™ RT Reagent Kit
	QuantiNova SYBR Green
	As described in the Materials and methods section
Reagents	As described in the materials and methods section
NTC	Cq and dissociation curve verification
Data analysis	
Specialist software	7500 Fast System Sequence Detection Software, version 1.4.0
Statistical justification	As described in the Materials and methods section and Table legends
Transparent, validated normalisation	Minimum three references genes selected using the GrayNorm algorithm
	As described in the Materials and methods section

Table S2. Primer sequences for RT-qPCR. Primer sequences are given in 5' to 3' direction. The gene name, locus and organ (L: leaves, R: roots) of investigation, primer exon locations and amplicon size are indicated. Abbreviations: bZIP: basic leucine zipper, CSD: Cu/Zn superoxide dismutase, FSD: Fe superoxide dismutase, COPT: copper transporter, MON: monesin sensitivity, TIP: tonoplast intrinsic protein, EF: elongation factor, YLS: yellow-leaf-specific, ETR: ethylene response, GST: glutathione-S-transferase, LOX: lipoxygenase.

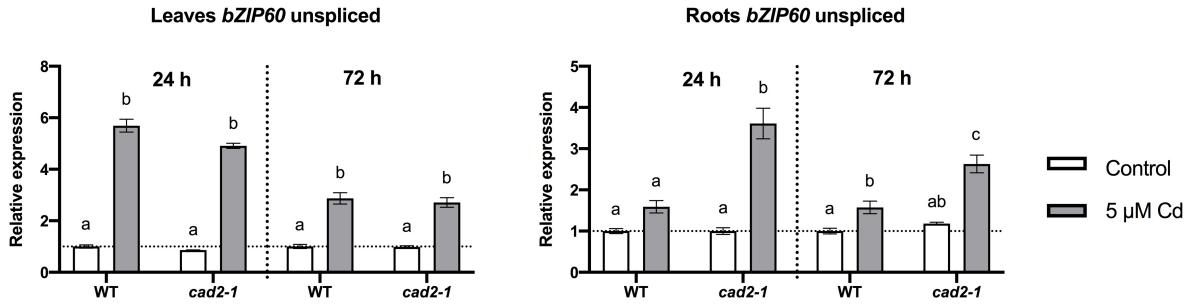
Gene	Locus	Organ	Forward primer	Reverse primer	Exon (E) location	Amplicon size
bZIP60	*AT1G42990*	L/R	TGATTCTGGTAGCGAGATTCATGA	CCGCATCTCTATTTCTTACTCTCCTTC	E1 and E2	274 bp
CSD1	*AT1G08830*	L/R	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC	E5 and E6-E7 junction	102 bp
CSD2	*AT2G28190*	L/R	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC	E6 and E7	101 bp
FSD1	*AT4G25100*	L/R	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC	E4 and E6	101 bp
COPT1	*AT5G59030*	L/R	GTTAATCCAAACCGCCGTGTATAC	GAGAAACACACCGGCGTTAAAC	E1	91 bp
COPT2	*AT3G46900*	L/R	TCCGGCATGTACGCTCTCTG	CACTGACACGTAGGATCGGTGAA	E1	91 bp
COPT6	*AT2G26975*	L/R	TGCTTATCTCGTGATGCTCGC	GAAGAGCATGAAACCCACGG	E1	144 bp
MON1	*AT2G28390*	L/R	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC	E13 and E14	61 bp
TIP41	*AT4G34270*	L/R	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA	E1-E2 junction and E2	61 bp
EF1α	*AT5G60390*	L	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA	E1 and E1-E2 junction	61 bp
YLS8	*AT5G08290*	R	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT	UTR	61 bp
ETR2	*AT3G23150*	R	TTCGAACCGGGCAGTTACAC	AATGGCGGTAAGGCAATCG	E2	91 bp
GST2	*AT4G02520*	R	ATCACCAGTTCGACCCAGTG	CTCCTCTTCTGCAACAACGG	E3	101 bp
LOX2	*AT3G45140*	R	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC	E3	102 bp

Table S3. Summary of the sequenced, filtered and mapped reads with their specificity. Sequenced reads were filtered based on quality (QC below 0.01, no ambiguity, minimal read length of 35 nucleotides), then sequencing adapter residues were trimmed, and then hard trims were performed. Filtered reads were mapped against the TAIR 10 draft *A. thaliana* genome using additional selection criteria (mismatch set at 2, maximum number of hits for a read lower than 10, 80% identity and 80% coverage with the reference genome). Specificity indicates the number of reads that were only mapped against one gene.

Condition	Total number reads	Filtered number reads	Mapped reads	% specificity
WT, leaves, control	23268594	20437784	19005331	89,13
	21693094	19470266	18146798	89,33
	26251746	23302495	21699571	89,35
WT, leaves, 5 µM Cd	21366770	19238421	17918490	89,93
	23280512	20797525	19214371	89,6
	21232516	18880462	17511196	90,44
ein2-1, leaves, control	25739488	22830784	21295677	88,86
	23451182	20598755	19082513	88,74
	22639692	19603166	18200240	88,49
ein2-1, leaves, 5 µM Cd	24963764	21640678	20102888	89,15
	26211380	23263427	21396699	87,99
	20412818	18047751	16809485	89,66
WT, roots, control	26533898	22340384	20486291	88,94
	27940358	22476550	20542061	86,73
	28254470	23950000	21993025	87,11
WT, roots, 5 µM Cd	25354960	17425911	15996182	87,43
	28468330	23571962	21608543	88,16
	25335098	20826562	19186762	88,38
ein2-1, roots, control	24104230	19846648	18346311	90,07
	23445550	20040665	16366274	82,75
	24336718	21547623	19885042	88,47
ein2-1, roots, 5 µM Cd	23118990	19425107	17998035	86,89
	26320862	20423054	19089284	88,24
	25966456	21348307	19909643	89,19



Supplemental Fig. 1. Relative expression levels of unspliced *bZIP60* in roots and leaves of WT and *ein2-1 A. thaliana* plants. Levels of unspliced *bZIP60* were quantified using qPCR in leaves and roots of WT and *ein2-1* mutant *A. thaliana* plants exposed to 5 µM Cd for 24 h and 72 h, and relatively expressed to WT control 24 h (set to 1: dashed line). A two-way ANOVA was performed within each time-point, different characters indicate significant differences between conditions ($p < 0.05$). White: control, grey: 5 µM Cd.



Supplemental Fig. 2. Relative expression levels of unspliced *bZIP60* in roots and leaves of WT and *cad2-1 A. thaliana* plants. Levels of unspliced *bZIP60* were quantified using qPCR in leaves and roots of WT and *ein2-1* mutant *A. thaliana* plants exposed to 5 µM Cd for 24 h and 72 h, and relatively expressed to WT control 24 h (set to 1: dashed line). A two-way ANOVA was performed within each time-point, different characters indicate significant differences between conditions ($p < 0.05$). White: control, grey: 5 µM Cd.